Bull. Environ. Contam. Toxicol. (1998) 60:923-930

Bulletin of Environmental Contamination and Toxicology

Evaluation of the Acute Toxicity to Juvenile Pacific Coho Salmon and Rainbow Trout of Some Plant Essential Oils, a Formulated Product, and the Carrier

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Received: 26 January 1998/Accepted: 7 April 1998

Thyme oil, α-terpineol, and eugenol are major constituents of some plant essential oils potentially useful as active ingredients in the formulation of natural insecticides. To date, toxicity of these materials has not been established for salmonids. The proposed use of these active ingredients in a formulated product for agricultural insect pest management programs in coastal British Columbia may result in its accidental introduction into waterbodies inhabited by anadromous fish. The objective of this study was to evaluate the acute toxicity of these essential oil active ingredients, a blend thereof, and the product carrier to juvenile salmonids of the Pacific Northwest.

MATERIALS AND METHODS

The active ingredients and identification numbers are summarized in Table 1. All test materials were supplied by Dr. M. B. Isman of the University of British Columbia and EcoSmart Technologies, Inc., Roswell, Georgia, USA. The RSB/181/4 is an emulsifiable concentrate (EC) containing equal amounts of thyme oil, α-terpineol and eugenol.

Table 1. Test materials

Active Ingredient (a.i.)	Identification number	Concentration
Thyme oil	RSB/181/1	90% a.i., 10% emulsifier
α-Terpineol	RSB/181/2	90% a.i., 10% emulsifier
Eugenol	RSB/181/3	90% a.i., 10% emulsifier
Blended Product	RSB/181/4	90%; thyme oil : α-terpineol : eugenol (1:1:1)
Emulsifier	RSB/181/5	

Both thyme oil and eugenol are on the US EPA List of Exempted Least Toxic Pesticide Active Ingredients. Although α-terpineol is not on the List, it is a volatile component of essential oils found in nectarines, grapes and

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cranberry juice. As well, it is widely used in commercial corn fields for luring northern (*Diabrotica barberi*) and western (*D. virgifera virgifera*) corn rootworm beetles (Hammack, 1996). The emulsifier RSB/181/4 contains a proprietary blend of anionic and nonionic emulsifiers.

A series of 96-h static acute toxicity tests were conducted in moderately hard fresh water using juvenile (~2-3 months old) coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) obtained respectively from Inch Creek Hatchery and Spring Valley Trout Farm, British Columbia. The toxicity tests using Juvenile coho salmon were conducted in July, 1997, while the tests using rainbow trout were carried out in September, 1997. The tests were undertaken in accordance with the protocol outlined by Wan et al. (1990; 1991), and by Environment Canada (1990a; 1990b). Oil-free air was delivered to the test concentrations at a rate of 6.5 ± 1 mL/min/L by means of disposable borosilicate glass Pasteur pipettes. Testing was carried out at 15 ± 1 °C, and 16:8 LD with 15 minute dawn/dusk simulation. The average fish loading density was 0.4 (0.3 - 0.5) g/L, within a bioassay test volume of 20 L. Conditions for holding and acclimating salmonids such as source of fish, water, temperature, oxygen/aeration, pH, water quality, lighting, feeding, cleaning and disease are performed as recommended by Environment Canada (1990a). Toxicant testing on fish used in study indicated their response was well within the control chart range (Environment Canada 1990a).

After preliminary tests were carried out to determine the exposure concentration ranges, 5 nominal concentrations below 100 ppm were selected for each test chemical using a Logarithmic Series of Concentrations. (Environment Canada, Appendix D, 1990a; 1990b). Each test concentration was replicated three times. Conductivity, pH, dissolved oxygen and temperature measurements were determined for each test vessel prior to test fish introduction and at the end of the 96-h test period. Ten fish were gently placed in each test vessel. Cumulative fish mortality was recorded at 24, 48, 72, and 96-h time intervals and the LC_{50} were calculated using the “Lethal” computer program developed by Stephen (1977, revised in 1983).

Fresh ground water with a mean hardness of 95 mg/L $CaCO_3$ from the Pacific Environmental Science Centre (PESC) well in North Vancouver, British Columbia, Canada was used as dilution water. Five water samples were taken in July and September, 1997 for water quality and pesticide residue analyses. Analyses of water samples for alkalinity, chemical element/ionic contents, conductivity, hardness, pH and organochlorine,

organophosphorus and nitrogen containing pesticides was carried out at the PESC.

To evaluate chemical loss in the test materials, water samples were taken from control and treatment vessels (with and without fish) containing 18 ppm (nominal) RSB/181/4 after preaeration and at 0, 24, 48, 72 and 96-h intervals. A volume of 100 mL of acetone was used to rinse each test vessel at the end of the 96-h bioassay. The acetone rinse was used to determine if the active ingredients were adsorbed on the glass surfaces of the test vessel.

The water samples (100 mL / sample) were collected as follows: a 10 mL pipette was used to collect 10 x 10 mL sub-samples to make up the 100 mL sample at each sampling time. These sub-samples were drawn randomly from various locations and depths of the test vessel. Water samples collected were stored in 200 mL amber heat treated glass bottles with aluminum foil lined caps and kept at 4°C. Samples were shipped at 4-5°C to EcoSmart Technologies Inc. on July 12, 1997, and they were analyzed on September 9 and 10, 1997. Gas chromatography-mass spectrometry (GC-MS) was used to analyze for eugenol content by MAS Inc., Atlanta, GA., using their standard protocols.

RESULTS AND DISCUSSION

Chemical characteristics of each dilution water type during the study period of July and September, 1997 are summarized in Table 2. The characteristics of dilution water differed significantly ($p<0.01$) in terms of hardness, pH and Na between July, and September. Alkalinity, Ca, Cl, SO_4 remained the same for both months. As well, the characteristics of C(total Inorg), K, Mg and conductivity varied slightly for the months of July and September.

No residues of organochlorine, organophosphorus or nitrogen containing pesticides (Table 3) were detected in the water samples used for diluting test materials.

The GC-MS results (Table 4) show that eugenol was observed only in the water samples at 0 and 24 hours from the test vessel (with fish) containing the nominal dose of RSB/181/4. Eugenol however, was not found in the water sample from the test vessel (without fish) containing RSB/181/4. As expected, eugenol was found in the acetone rinse samples taken at the end of 96 hours from vessels (with and without fish) containing the test chemical RSB/181/4.

Table 2. Average Characteristics* of dilution water

Parameter analyzed	1997 (n = 5) July	September
Alkalinity	57.1 ± 0.1	56.1 ± 0.1
Chemical elements / ions		
C (total)	13.36 ± 0.04	12.9 ± 0.1
Ca	32.2 ± 0.1	35.2 ± 0.3
K	4.42 ± 0.04	4.9 ± 0.2
Mg	3.4 ± 0.0	3.88 ± 0.04
Na	39.32 ± 0.07	40.8 ± 0.3
Cl	100 ± 1	96.3 ± 0.4
SO_4	8.58 ± 0.02	8.4 ± 0.1
Conductivity (μmhos/cm)	465 ± 3	479 ± 1
Hardness (total)	94.6 ± 0.2	104 ± 1
pH (rel. U.)	7.71 ± 0.01	7.79 ± 0.01

* - mg/L (mean ± S.E., n=5); detection limits <0.001 - 0.01 mg/L

There was a chemical loss in all vessels with and without fish. This loss of test chemicals in water samples may be attributed to volatilization during the initial aeration process (Doudoroff et al., 1951; Wan et al., 1990) and glass adsorption (Sharom and Solomon, 1981; Wan et al., 1991). Based on the expected ± 15% error of chemical residue recovery, there appears to be a substantial loss of eugenol occurring via volatilization and possibly glass adsorption during the initial aeration process. It seems that the total loss resulting. from both volatilization and glass adsorption phenomenon/process may be more than 90% of the nominal concentration. This explains why residues of eugenol were not detected at 48-h, 72-h, and 96-h in the treated water tank containing fish. Some of the lost test chemicals may have been retained inside the fish themselves. However, no fish tissue analyses were conducted in this bioassay experiment.

The 24, 48, 72, and 96-h LC_{50} values for thyme oil, α-terpineol, eugenol, the formulated product RSB/181/4 and the emulsifier were calculated based on nominal concentrations and are shown in Table 5.

It appears that the toxic effects of all materials tested occurred within the first 24h, as there was no change in toxicity from then on. Based on the 96-h LC_{50} data, α-terpineol is the most toxic ingredient to salmonids (coho salmon, 6.3 ppm; rainbow trout, 6.6 ppm) among the materials tested. RSB/181/4 is also quite toxic to both coho salmon (13.2 ppm) and rainbow trout (11.5 ppm) while toxicity of thyme oil, emulsifier, and eugenol to juvenile salmonids are less toxic to slight ranging from 16.1 ppm to 66.1

ppm. The order of toxicity to both coho salmon and rainbow trout is α-terpineol > RSB/181/4 > thyme oil > Emulsifier > eugenol.

Table 3. Pesticide residues looked for in dilution water

Organochlorine pesticides (detection limit = 0.05 mg/L)
dachtal, dichlobenil, dichloran, dicofol, folpet, oxyflurfen, tetradifon, trifluralin, captan, α-BHC, β-BHC, γ-BHC, δ-BHC, lindane, heptachlor, aldrin, heptachlor epoxide, endosulfan1, endosulfan2, endosulfan sulfate, p,p'-DDD, p,p'-DDE, p,p'-DDT, dieldrin, endrin aldehyde, endrin, methoxychlor
Organophosphorus Compounds (detection limit = 0.05 - 0.10 mg/L)
mevinphos, demeton s, diazinon, chlorpyrifos, malathion, ethion, parathion, methidathion, naled, dimethoate
Nitrogen Containing Compounds (detection limit = 0.05 - 0.50 mg/L)
carbaryl, metalaxyl, hexazinone, anilizine, atrazine, propazine, simazine

Table 4. Residues of Eugenol in test water samples (ppm)

Test chemicals	0-h	24-h	48-h	72-h	96-h	Acetone rinse
Control (with fish)	nd*	-	-	-	-	nd
RSB/181/4 (with fish)	9	12	nd	nd	nd	8
Control (without fish)	nd	-	-	-	-	nd
RSB/181/4 (without fish)	nd	nd	nd	nd	nd	5

* - nd = not detected

Table 5 compares 24, 48, 72 and 96-h toxicity of test materials to coho salmon and rainbow trout. Results of statistical F-tests indicate that variances for thyme oil and the emulsifier differed. Therefore, a log, transformation was carried out on all LC_{50} values. Tukey's test (Montgomery, 1984; Sokal et al, 1969) shows that thyme oil and the emulsifier are significantly ($p<0.05$) more toxic to rainbow trout than to coho salmon. It also indicates that α-terpineol, eugenol, and RSB/181/4 are equitoxic to the test species.

Of the five materials tested, the emulsifier appeared to have the least stability during the 96-h test period. The LC_{50} values for both coho salmon and rainbow trout differed ($p<0.05$) during the test period. The emulsifier is more toxic to rainbow trout than coho salmon.

The blended product RSB/181/4 appeared to be less toxic to young salmon (Table 6) than azadirachtin, the a.i. in neem, a new botanical insecticide, and some of the more common synthetic insecticides such as the carbamate carbofuran, the organochlorine endosulfan, and the

organo-phosphates azinphosmethyl and malathion. It has a slightly better margin of safety to fish than the neem product Azatin, but has an even greater margin of safety to salmonids when compared with some of the

Table 5. Toxicity[1] of test materials to juvenile salmonids

Test chemicals / Bioassay Time	Toxicity LC_{50} coho salmon / rainbow trout Mean (mg/ML)	Log_e(Mean)	Statistical tests and comparison T_α[2] (diff in Log_e(Mean))	Tukey's test[3] ($p < 0.05$)
Thyme oil				
24-h	21.1 / 16.1	3.05 / 2.78	0.27	S*
48-h	21.1 / 16.1	3.05 / 2.78	0.27	S
72-h	21.1 / 16.1	3.05 / 2.78	0.27	S
96-h	20.5 / 16.1	3.02 / 2.78	0.24	S
Alpha-terpineol				
24-h	6.8 / 6.7	1.92 / 1.90	0.02	NS
48-h	6.5 / 6.7	1.87 / 1.90	0.03	NS
72-h	6.5 / 6.6	1.87 / 1.89	0.02	NS
96-h	6.3 / 6.6	1.84 / 1.89	0.05	NS
Eugenol				
24-h	67.6 / 61.5	4.21 / 4.12	0.09	NS
48-h	67.6 / 60.8	4.21 / 4.11	0.10	NS
72-h	67.6 / 60.8	4.21 / 4.11	0.10	NS
96-h	66.1 / 60.8	4.19 / 4.11	0.08	NS
RSB/181/4				
24-h	13.2 / 11.6	2.58 / 2.45	0.125	NS
48-h	13.2 / 11.5	2.58 / 2.44	0.133	NS
72-h	13.2 / 11.5	2.58 / 2.44	0.133	NS
96-h	13.2 / 11.5	2.58 / 2.44	0.133	NS
Emulsifier				
24-h	27.4 / 21.5	3.31 / 3.07	0.241	S
48-h	26.6 / 18.9	3.28 / 2.94	0.344	S
72-h	24.2 / 18.3	3.19 / 2.91	0.285	S
96-h	22.9 / 18.2	3.13 / 2.90	0.232	S

[1]- Mean LC_{50}±S.E. (n = 3)

[2]- Tukey's Test Statistics, $T_\alpha = [q_\alpha (a,f)]S_{\bar{Y}i}$ =(Percentage Points of the Studentized Range Statistic) x (Standard error of each average), with 2 fish species (a=2) and 4 error degree of freedom (f=4)

[3]- Null hypothesis: no difference in Log_e(Mean LC_{so}) between coho salmon and rainbow trout

*- S = significant; NS = non-significant

common synthetic insecticides and botanical neurotoxic insecticides such as pyrethrum and rotenone.

Table 6. Comparison of Acute Toxicity to Juvenile Salmonids of RSB/181/4 with Diesel oil[1], azadirachtin, and some common insecticides

Pesticide[2]	Acute Toxicity[3] 96-h LC_{50} (mg/L)	
	Coho Salmon	Rainbow Trout
Azadirachtin (3% as Azatin)	5	4
Azadirachtin (23% as neem extract)	13	3
Azinphosmethyl (94%)	0.006	0.004
Carbofuran (99%)	0.5	0.4
Endosulfan (96%)	N/A	0.001
Malathion (95%)	0.2	0.2
Pyrethrum (20%)	0.03	0.04
Rotenone (44%)	N/A	0.03
RSB/181/4 (thymeoil:α-terpineol:eugenol=1:1:1)	13.2	11.5

[1] - a mosquito larvicide
[2] - % active ingredient
[3] -Johnston and Finley (1980), Wan et al (1996)
N/A - not available

Under field conditions, the concentration of RSB/181/4 in a waterbody unintentionally oversprayed at the manufacturer's highest recommended rate of 5 Kg product/ha (or 4.4 lb/A) approximates 3.5 mg/L in 15 cm water. Although this concentration does not exceed the 96-h LC_{50} of juvenile salmonids (coho salmon, 13.2 mg/L; rainbow trout, 11.5 mg/L), the potential sub-lethal effects to fish of RSB/181/4 is presently unknown. When used at the labeled rate of application, the product may, in the event of misapplications, generate sub-lethal concentrations.

To summarize, our study indicates that RSB/181/4 is moderately toxic to juvenile salmonids. The GC-MS analytical results for eugenol suggest that a substantial loss of chemical occurred via both aeration and glass adsorption. At the manufacturer's recommended rate of application (5 Kg product/ha), the risk of generating lethal concentrations to juvenile salmonds of RSB/181/4 is low in small fish streams unintentionally oversprayed. Overall, RSB/181/4 has a greater margin of safety to young salmon than some common synthetic and botanical insecticides.

Acknowledgments. We thank Dr. Leslie Churchland, Mr. Bryan Kelso and Mr. Ron Watts (Environment Canada) for their comments; G. Van Aggelen, Celia Wong, Brad McPherson of the Pacific Environmental

Science Centre, Environment Canada, for technical support; and University of British Columbia for partial funding of this study.

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