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Acute Toxicity of Chlorpyrifos to Fish, a Newt, and Aquatic Invertebrates

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In current procedures, ecotoxicogical risk assessment of pesticides for regulatory purposes in the Netherlands is essentially based on information from physico-chemical properties and a set of standard single-species toxicity tests. It has been debated however, whether extrapolation of effects on individual species was inadequate for predicting effects in the complexity of ecosystems (e.g., Cairns 1983; Kimball and Levin 1985).

To evaluate the value of laboratory ecotoxicological research for the extrapolation to the natural environment, the effects of Dursban® 4E are being studied in the laboratory, in indoor and outdoor microcosms, and in mesocosms. In this paper, we report on sixteen single-species toxicity tests which we performed with invertebrates, fish and a newt (all indigenous) (Table 1). The single-species toxicity tests represent the simplest level of biological complexity in our evaluation program. The presented $L(E)C_{10}$ and $L(E)C_{50}$-values from these tests form the reference for evaluation or interpretation of ecotoxicological effects in our microcosm and mesocosm studies.

MATERIALS AND METHODS

All tests were performed with the organo-phosphorus insecticide chlorpyrifos (purity: 99.8%) or with its emulsifiable formulation Dursban® 4E (a.i. chlorpyrifos, 480 g/L). Stock solutions were made by diluting chlorpyrifos in acetone or Dursban® 4E in distilled water. Test media were prepared by diluting stock solutions in tapwater (total hardness: 1.07 m*M*/L (0.11 g/L as $CaCO_3$), pH 8) or in standard water of distilled water, which contained $CaCl_2.6H_2O$ (0.4 g/L), NaCl (0.04), $NaN0_3$ (0.01), $MgSO_4.H_2O$ (0.19), Na_2SO_4 (0.1), $NaHCO_3$ (0.34); total hardness 2.57 m*M*/L, pH 8 (Table 2).

Using stock solutions with chlorpyrifos, the volume fraction of acetone added in the test medium never exceeded 0.1 mL/L. For Dursban® 4E stock solutions, the mass concentration of Dursban® 4E adjuvants in the test media depended on the dose. It never exceeded 35 µg/L, being equivalent to 35 µg chlorpyrifos/L.

Besides the sixteen toxicity tests with active ingredient, a separate experiment was done to test the toxicity of adjuvants. Therefore, *Asellus aquaticus*, *Cloeon dipterum*, and *Gammarus pulex* were exposed (96 hr, semi-static, tapwater) to 35 µg adjuvants/L (as Dursban® 4E Blank, nominal concentration). In the toxicity tests with *Limnodrilus hoffmeisteri*, *Gam. pulex*, *Gasterosteus aculeatus*, and *Pungitius pungitius* controls with Dursban® 4E Blank were added in the same adjuvant concentrations as in the treatments with the highest concentrations of pesticide. Since neither an effect of the adjuvants was seen in the separate experiment nor in the above

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Table 1. Taxonomic group, origin and stage of tested organisms.

CLASS, order, *genus*, and *species*	Source	Size/Stage
OLIGOCHAETA		
Limnicola		
Limnodrilus hoffmeisteri	lab. culture	adult
MOLLUSCA		
Gastropoda		
Anisus vortex	pond, Schaffelse bos,	7.2±0.7 mm
Bithynia tentaculata	Barneveld	10.5±1.3 mm
Lymnaea stagnalis	(all three spp.)	22.4±7.5 mm
CRUSTACEA		
Cladocera		
Daphnia longispina	experimental ditches, Renkum	(sub)adult
Simocephalus vetulus	experimental pond, SC-DLO, Wageningen	juvenile-adult
Isopoda		
Asellus aquaticus	pond, Schaffelse bos, Barneveld	(sub)adult
Proasellus coxalis	ditch, Bennekom	(sub)adult
Amphipoda		
Gammarus pulex	pond, Wijchen	6.5-16.8 mm
INSECTA		
Heteroptera		
Corixa punctata	experimental ditches, Renkum	adult
Ephemeroptera		
Cloeon dipterum	experimental ditches,	naiads
Caenis horaria	Renkum	naiads
	(both spp.)	
Diptera		
Chaoborus obscuripes	experimental ditches, Renkum	larvae
PISCES		
Gasterosteiformes		
Gastrosteus aculeatus	lab. culture RIVM, Bilthoven	1-2 yr old
Pungitius pungitius	ditches, Wageningen	adult
AMPHIBIA		
Urodela		
Triturus vulgaris	pond, Delft	aquatic adult

SC-DLO= DLO the Winand Staring Centre
RIVM = National Institute of Public Health and Environmental Sciences

mentioned toxicity tests, we did not include controls with adjuvants in the other toxicity tests.

Table 2 (part A). Testing conditions and L(E)C for tested species. exp.set= experimental set-up, where "static" indicates a semi-static test. On the second line the test medium replacement interval is given; con.interv.= concentration interval; ± mean SD= ± mean relative SD; 95% conf.limits= 95% confidence limits; * highest tested concentration

species	test medium	exp. set-up	temp. °C	test range ± mean SD µg/L	conc. interv.	criteria for EC-values	ECx (µg/L) (95% conf. limits) x	48 hr	96 hr	LCx (µg/L) (95% conf. limits) x	48 hr	96 hr
Limnodrilus hoffmeisteri	Dursban4E tapwater	static 24 hr	19±0.5	5.0-36 ± 18%	3.2	movement and breakage	10:		>36*			
Anisus vortex, *Bithynia tentaculata* & *Lymnaea stagnalis*	chlorpyr. tapwater	discont. flow 0.46 L/hr	20±0.5	5.0-94 ± 8%	2	no movement > 24 hr	10:		>94*			
Daphnia longispina	Dursban4E tapwater	static 48 hr	18±0.5	0.05-2.64 ± 9%	2	ability to maintain in suspension	10: 50:	0.2(0.2-0.3) 0.3(0.3-0.3)	0.2(x - x) 0.3(x - x)	10: 50:	0.2(0.2-0.7) 0.8(0.6-1.0)	0.2(x - x) 0.3(x - x)
Simocephalus vetulus	Dursban4E tapwater	static 48 hr	18±0.5	0.07-2.81 ± 19%	2	ability to maintain in suspension	10: 50:	0.3(0.2-0.4) 0.4(0.4-0.5)	0.3(0.2-0.4) 0.4(0.3-0.5)	10: 50:	0.4(0.3-0.7) 0.8(0.7-0.9)	0.3(0.2-0.5) 0.5(0.4-0.6)
Asellus aquaticus	chlorpyr. std water	static 24 hr	20±0.5	1.7-53.1 ± 2%	2	response to tactile stimulus	10: 50:	2.0(1.2-4.3) 4.3(3.3-5.6)	1.4(1.8-3.0) 2.7(2.1-3.6)			
Proasellus coxalis	Dursban4E tapwater	discont. flow 2.5 L/hr	18±0.5	1.0-20.3 ± 11%	2	response to tactile stimulus	10:		> 20*			
Gammarus pulex	Dursban4E tapwater	discont. flow 5 L/hr	19±0.5	0.02-1.55 ± 2%	3.2					10: 50:	0.03(0.01-0.07) 0.08(0.05-0.14)	0.02(0.01-0.05) 0.07(0.04-0.11)

Table 2 (part B). Testing conditions and L(E)C for tested species. exp.set= experimental set-up, where "static" indicates a semi-static test. On the second line the test medium replacement interval is given; con.interv.= concentration interval; ± mean SD= ± mean relative SD; 95% conf.limits= 95% confidence limits; * highest tested concentration

species	test medium	exp. set-up	temp. °C	test range ± mean SD μg/L	conc. interv.	criteria for EC-values	ECx (μg/L) (95% conf. limits) x	48 hr	96 hr	LCx (μg/L) (95% conf. limits) x	48 hr	96 hr
Corixa punctata	Dursban4E tapwater	discont. flow 0.33 L/hr	20±0.5	0.35-31.3 ± 9%	3.2	trembling of extremities	10:	2.2(1.6-3.3)	1.0(0.6-2.0)	10:	2.2(1.3-3.8)	1.0(0.7-1.6)
							50:	3.2(2.4-4.3)	1.7(1.1-2.5)	50:	6.0(4.2-8.5)	2.0(1.5-2.6)
Cloeon dipterum	Dursban4E tapwater	discont. flow 2.5 L/hr	18±0.5	0.2-4.7 ± 8%	2	response to tactile stimulus	10:	0.3(0.2-0.4)	0.1(0.1-0.2)	10:	0.3(0.2-0.9)	0.1(0.1-0.3)
							50:	0.4(0.3-0.4)	0.2(0.2-0.2)	50:	1.0(0.8-1.4)	0.3(0.2-0.3)
Caenis horaria	Dursban4E tapwater	discont. flow 2.5 L/hr	18±0.5	0.2-3.6 ± 10%	2	response to tactile stimulus	10:	0.6(0.3-0.9)	0.3(0.3-0.6)	10:		> 3*
							50:	0.7(0.6-0.8)	0.5(0.4-0.6)			
Chaoborus obscuripes	Dursban4E tapwater	discont. flow 2.5 L/hr	18±0.5	0.2-3.7 ± 10%	2	response to tactile stimulus	10:	0.6(0.4-1.2)	0.3(0.2-0.6)	10:		2.5(1.7-7.5)
							50:	1.4(1.1-1.7)	0.7(0.6-0.8)	50:		6.6(3.0-14.6)
Gasterosteus aculeatus	Dursban4E tapwater	discont. flow 1.85 L/hr	21±0.5	0.2-28.9 ± 23%	3.2					10:	4.5(2.3-13.7)	3.8(2.0-9.2)
										50:	13.4(9.0-19.9)	8.5(6.2-11.9)
Pungitius pungitius	chorpyr. tapwater	discont. flow 1.85 L/hr	19±0.8	0.6-13.9 ± 15%	2					10:	2.3(1.3-5.5)	2.1(1.3-4.6)
										50:	5.7(4.4-7.5)	4.7(3.6-6.0)
Triturus vulgaris	Dursban4E tapwater	static 24 hr	18±1.0	96		locomotional behaviour	10:		> 96*			

In the sixteen toxicity tests, 5 to 6 concentrations were used. Test media without chlorpyrifos or Dursban® 4E were used as controls. Concentration intervals in the test ranges differed by a factor 2 to 3.2 (Table 2). All tests were done in duplicate, except for the one with the newt *Triturus vulgaris*. This species was only tested in a range-finding trial at a concentration of 96 μg chlorpyrifos/L; no replication was made.

Ten specimens of *L.hoffmeisteri* were individually tested for each concentration. *T. vulgaris* was also individually tested (4 specimens). In the other tests, 6 *Anisus vortex*, *Bithynia tentaculata*, *Lymnaea stagnalis*; 8 *A. aquaticus*; 9 *Corixa punctata*; 10 *Gam. pulex*, *Proasellus coxalis*, *Cl. dipterum*, *Caenis horaria*, *Gas. aculeatus*, *Pu. pungitius*; 15 *Chaoborus obscuripes*; 25 *Daphnia longispina* and *Simocephalus vetulus* were used for each testing vessel. The specimens were randomly placed in the test media. The stage of the life-cycle or the size of the individuals tested is given in Table 1. *Li. hoffmeisteri* and *Gas. aculeatus* were obtained from laboratory cultures. The other species were collected in the field (Table 1). The invertebrates were checked or approved for species identity using a stereomicroscope. The invertebrates were not fed during acclimatization and testing. The newts were fed every 48 hr (*Artemia* and *Tubifex*) and the fish were fed daily (dry food and guppies less than 2 wk old).

The tests were done in a temperature-controlled room; light regime: 14 hr light, 10 hr dark. The temperature in the test aquaria remained within the limits given in Table 2. All species were acclimatized in laboratory conditions for at least 48 hr.

Of the sixteen toxictity tests, five were set up as semi-static tests and eleven were done in a flow-through system. The acute toxicity of chlorpyrifos to *D. longispina*, *S. vetulus* (both Cladocera), *Li. hoffmeisteri* (Limnicola), *A. aquaticus* (Isopoda) and *T. vulgaris* (Urodela) was estimated in 96-hr semi-static tests. *Li. hoffmeisteri* was placed in open 100-mL glass tubes filled with 25 mL of test medium. For the other species in the semi-static tests, 2-L glass jars were filled with 1 L of test medium (1.5 L for *A. aquaticus*) and covered with a glass lid. For type of medium and treatment concentrations, see Table 2. The test media were not aerated during the tests. The test media were renewed every 24 hr for *A. aquaticus* and *Li. hoffmeisteri*, or 48 hr for *D. longispina*, *S. vetulus*, and *T. vulgaris*. Concentrations of dissolved oxygen (Yellow Springs Instruments Co., YSI model 58) and pH (Schott Geräte, CG817) in the controls and treatments with the highest concentrations were measured at the beginning and end of the 24 or 48 hr exposure. The O_2 concentration was $\geq$ 7.9 mg/L. The pH was 7.6 to 9.1.

Acute toxicity of chlorpyrifos to *A. vortex*, *B. tentaculata*, *Ly. stagnalis* (all three Gastropoda), *Pr. coxalis* (Isopoda), *Gam. pulex* (Amphipoda), *Co. punctata* (Heteroptera), *Cl. dipterum*, *Cae. horaria* (both Ephemeroptera), *Ch. obscuripes* (Diptera), *Gas. aculeatus* and *Pu. pungitius* (both Gasterosteiformes) was determined using 96-hr discontinuous flow-through tests. In the flow-through system test concentrations were prepared by diluting stock solutions with tapwater. Open glass vessels of 3.8 L were used for invertebrates and vessels of 18.7 L for fish. The concentrations desired were maintained by releasing an amount of test solution at certain time intervals into the vessels. Flow rates for the tests are given in Table 2. The tapwater was extensively aerated before use, and also its temperature and that of the stock solutions were adjusted to the test temperature before use. Oxygen concentrations and pH were daily measured in the controls and in vessels with the highest concentrations. For invertebrates, the O_2 concentration was > 6.0 mg/L, and the pH was 7.5 to 8.7. In one of the control vessels of *Gas. aculeatus* an O_2 concentration of 2.8 mg/L was once measured; otherwise the concentration was 4.8 to 7.6 mg/L. For *Pu. pungitius*, in a control vessel an O_2-concentration of 1.1 mg/L was once measured; otherwise the concentration was 3.6 to 7.2 mg/L. Such low oxygen concentrations did not observably affect the fish in any adverse way. The pH for both fish species was 6.6 to 8.2.

Toxic effects monitored were immobility or mortality. Details of immobility can be found in Table 2. Since mortality is the ultimate phase of immobility, scores on mortality were incorporated in that of immobility. For the arthropods (the crustaceans and insects), effects were scored as mortality when no response of any kind was observed for about 10 sec under a stereomicroscope after repeated tactile stimulation. The fish were considered dead when no gill movement was observed. During the tests, the animals were examined daily and dead specimens were removed.

In the semi-static tests, samples of the solutions were taken from the vessels for the analysis for chlorpyrifos at the beginning and end of 24 hr (*A. aquaticus, Li. hoffmeisteri*) or 48 hr (*D. longispina, S. vetulus*) exposure. In the vessels of the flow-through tests, water samples were taken daily. The water was extracted with hexane and analysed by gas chromatography (HP 5890A Gas Chromatograph equiped with a HP 7672 autosampler and NP-detector. Brock et al. (1992) have described the analytical procedures. Chlorpyrifos recovery from water was 99.4 ± 1.5% (mean ± SD, n = 12).

The $L(E)C_{10}$ and $L(E)C_{50}$ and their 95% confidence limits were calculated by a log concentration-logit effect regression method. No confidence limits could be calculated when the effect was partial only in one test concentration. Within the regression, calculated L(E)Cs were adapted for immobility or mortality in the controls. Tests were rejected when immobility or mortality in the controls was > 10%. In the static tests, concentrations used for estimation of L(E)C were based on the geometric means over time calculated from the measured concentrations just before and after refreshing the test solution. For the flow-through tests, the concentrations used in the estimation of L(E)C were the means of the daily measurements of chlorpyrifos in the test solutions. Results from duplicate tests were combined in one regression analysis.

By combining the 48 hr- and 96 hr-data from a toxicity test in one logistic regression model, we tested whether 96hr-L(E)C were significantly different. For this, we tested whether the model "log concentration + time" gave a significantly ($p = 0.05$) better fit than the model "log concentration" by comparing the deviances of both models (McCullagh and Nelder 1989). Both the calculations for the toxicity parameters and the statistical analysis were programmed in GENSTAT (Payne and Lane 1987).

RESULTS AND DISCUSSION

Dursban 4E Blank did not show observable effects on the species tested. Also, Van der Hoeve and Oldersma (1989) did not find effects on *Daphnia pulex* at their highest concentration of adjuvant, which was 455 times as high as the 48h-EC_{50} for chlorpyrifos. Because of these results, we did not expect any effects of the adjuvants.

The ranges of the geometric mean concentrations in the semi-static tests are given in Table 2. For *A. aquaticus*, the mean relative decrease (± abolute SD) of chlorpyrifos concentrations in 24 hr was 2 ± 1% of the initial concentrations. The mean decrease was 18 ± 11% in 24 hr for *Li. hoffmeisteri*. The mean decrease over 48 hr was 9 ± 7% and 19 ± 14% for *D. longispina* and *S. vetulus*, respectively (Table 2).

The ranges of mean concentrations and mean SD in the flow-through tests are also given in Table 2. Fluctuations around mean concentrations, expressed as mean SD, remained between 2 and 11% for the invertebrates. In the tests with *Gas. aculeatus* and *Pu. pungitius*, these fluctuations were 23 and 15%, respectively. In the tests with fish, the lowest concentrations were measured in the first 24 hr. This could be explained by the rate of uptake of chlorpyrifos being much higher than its depuration rate in these first hours. In the tests with fish, this phenomenon came to expression much more clearly because of the greater mass of the fish than of the invertebrates.

When 48hr- and 96hr-ECs (Table 2) within toxicity tests were statistically compared, those of *S. vetulus*, *Gam. pulex*, *Gas. aculeatus* and *Pu. pungitius* did not differ significantly ($p > 0.05$).

Comparing the results of the tests within the group of invertebrates (Table 2), they showed that acute toxicity within this group can be widely different (gastropods against *Gam. pulex*). Further, the oligochaet species *Li. hoffmeisteri*, the gastropods *A. vortex*, *B. tentaculata*, *L. stagnalis*, and the amphibian *T. vulgaris* were relative insusceptible (96hr-LC_{10} > 35 µg/L). Within the arthropods, the acute toxicity generally was < 10 µg/L. When classified for susceptibility, the most susceptible species was the amphipod *G. pulex* (96hr-LC_{10} 0.02 µg/L), followed by the cladocerans *D. longispina*, *S. vetulus*; next the ephemeropterans *Cl. dipterum*, *Cae. horaria*; next a group of more or less equally susceptible species with the isopod *A. aquaticus*, the heteropteran *Co. punctata*, and the dipteran *Ch. obscuripes*; and as most insusceptible species, the isopod *Pr. coxalis* (96hr-LC_{10} > 20 µg/L). The data show that within the crustaceans the most susceptible (*Gam. pulex*) and the most insusceptible species (*Pr. coxalis*) was found (differing by a factor 10^3). Furthermore it was shown that closely related taxa (*A. aquaticus* and *Pr. coxalis*) can differ at least an order of magnitude. The fish *Gas. aculeatus* and *Pu. pungitius* were almost equally susceptible to the aquatic insects.

Literature data show that our results are comparable with those in other studies. The insusceptibility of *Li. hoffmeisteri* was in accordance with observations on other Oligochaeta in field studies (review of Marshall and Roberts 1978; Siefert et al. 1987; Brazner et al. 1989; Van Wijngaarden and Leeuwangh 1989).

Like *A. vortex*, *B. tentaculata*, and *Ly. stagnalis*, the gastropods *Helisoma trivolis*, *Lanistes carinatus*, and *Planorbis* sp. were not affected by chlorpyrifos. For the latter three species (review of Odenkirchen and Eisler 1988; DowElanco 1990), the LC_{50} amounts to around or even above the water solubility (0.4 to 2 mg/L; Marshall and Roberts 1987) of the pesticide.

For *D.longispina* and *S.vetulus* the 48hr-EC_{50} (0.3 and 0.4 µg/L, respectively) lie well in the range of that of other cladocerans (*Daphnia pulex* 0.2 µg/L; *D.magna*: 1.0 µg/L; van der Hoeven and Oldersma 1989; Kersting and Van Wijngaarden 1992).

The 96hr-LC_{50} of 0.07 µg/L for *Gam. pulex* is comparable with that for other amphipods. For *Gam. lacustris* the 96hr-LC_{50} was 0.11 µg/L (Sanders 1969). For *Gam. pseudolimnaeus* and *Hyalella azteca* these values were 0.18 and 0.14 µg/L, respectively (Siefert et al. 1987).

Siefert et al. (1987) found a 72hr-L(E)C_{50} of 0.33 µg/L for the ephemeropteran *Ephemerella* sp., and we found a 96hr-EC_{50} of 0.2 and 0.5 µg/L respectively for *Cl. dipterum* and *Cae. horaria.*

The susceptibility of the dipterans *Ch. obscuripes* (96hr-EC_{50}: 0.7 µg/L) and *Ch. americanus* (114hr-EC_{50}: 0.85 µg/L; Siefert et al. 1987) are comparable.
The review of Odenkirchen and Eisler (1988) shows that the 96hr-LC_{50} values for fish can differ by four orders of magnitude (0.1 to 520 µg/L). The values for *Gas. aculeatus* and *Pu. pungitius* (96hr-LC_{50}: 10.7 and 4.7 µg/L, respectively) had median values in this range.

The similarity of the susceptibility on species level within the Oligochaeta, Mollusca, Cladocera, and Amphipoda might suggest that toxicity for one or a few species is indicative for groups at a higher taxonomic order. But the difference in interspecies susceptibility between fish, and between the isopods *A. aquaticus* and *Pr. coxalis* (Table 2) indicates that, even to closely related species, extrapolation of toxicological data of tested species for ecotoxicological risk cannot as yet be assessed on a scientific basis. Furthermore, our laboratory tests only gave data on acute toxicity for the organisms we tested. Aspects such as technique of application, patterns of use, weather, and fate characteristics determine whether such concentrations are reached and maintained in the field.

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