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Effects of Water Hardness, Water Temperature, and Size of the Test Organism on the Susceptibility of the Freshwater Shrimp, *Gammarus pulex* (L.), to Toxicants

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Information on the acute toxicity of substances to invertebrates is an essential part of the assessment of their hazard in aquatic environments. Daphnia spp. have become the principal invertebrate test species and there are well developed guidelines for tests with them (OECD 1982; ISO 1982). However, there will be occasions when data on the acute toxicity to species of invertebrate other than Daphnia will be required for a more complete hazard assessment.

Freshwater amphipods have been used as test organisms in aquatic toxicology for many years (JONES 1937), species of the genus Gammarus being particularly popular. This genus is widely distributed throughout the world and is common and abundant in many regions. Gammarus spp. are important components of freshwater food webs, are easily collected and maintained, and have proved to be susceptible to a wide variety of toxicants (ARTHUR & LEONARD 1970; MACEK & SANDERS 1970; MACEK et al. 1976). Despite the use of Gammarus in toxicity tests in both the USA and Europe (APHA 1975; EPA 1976; BESCH 1977) little work has been done on the effects of non-toxicant variables on the outcome of tests. Until such data are collected it will not be possible to develop guidelines for testing that will be accepted as having wide applicability.

In this study we examined the effects of water hardness, water temperature, size of test organism and duration of exposure on the acute toxicity of four substances to Gammarus pulex (L.), the freshwater shrimp.

MATERIALS AND METHODS

The four test substances were phenol, copper (as cupric chloride), γ-hexachlorocyclohexane (γ-HCH, lindane) and di-ethylhexyl phthalate (DEHP).

Stock solutions of each test substance were made up daily; those for phenol and copper in distilled water and those for γ-HCH and DEHP in acetone (Analar grade). The concentrations of the stock solutions were such that the quantities of glass-distilled water or acetone added to the test vessels did not exceed 1.0 ml l^{-1} and 0.66 ml l^{-1} respectively.

A factorial experimental design was used to look at the effects of each variable (water hardness, water temperature and organism

size), at two levels, on the toxic effects of the four test substances. The levels for each variable were as follows:

Total hardness (mg l^{-1} as $CaCO_3$)	250 (hard)
	100 (soft)
Temperature (°C)	15 (high)
	7 (low)
Size (length mm)	>8 (large)
	<5 (small)

A static water test was used with daily renewal of the test solutions. After 96 h exposure to the test substance the test animals were returned to toxicant-free water for 24 h.

Gammarus pulex (L.) were collected from a tributary of the River Len (total hardness approximately 250 mg l^{-1} as $CaCO_3$) at Hollingbourne, Kent, U.K. They were taken to the laboratory and acclimated to the test conditions for at least 7 days. During acclimation the G. pulex were held in filtered (8 µm), dechlorinated mains water of the appropriate hardness and temperature. They were fed fresh washed lettuce and small quantities of trout pellets. The concentration of dissolved oxygen in the acclimation tanks was maintained at or close to 100% of the air saturation value by vigorous aeration. Only those G. pulex that appeared healthy at the end of acclimation were used in the tests. Immediately prior to testing the G. pulex were sorted into two size categories; large (>8 mm), and small (<5 mm). A sample of 200 from each size category were weighed individually, and the mean wet weight for each category was determined.

A hard and a soft water were used for the tests. The hard water was filtered (8 µm) dechlorinated mains water with a nominal total hardness of 250 mg l^{-1} as $CaCO_3$. The soft water was the same mains water with its total hardness reduced to a nominal 100 mg l^{-1} as $CaCO_3$ by the addition of glass-distilled water. The total hardness of all batches of water used in the tests was determined prior to use, by titration against EDTA.

The temperature of the diluent waters was adjusted to 7 or 15°C for acclimation and testing. The tests were carried out in constant temperature rooms which maintained these water temperatures $\pm$ 1°C.

A static water test procedure with daily renewal of the test solutions was used. The tests were carried out in 350 ml Pyrex glass crystallising dishes containing 300 ml of test solution made up in water of the appropriate hardness and temperature. The test solutions were not aerated. For each test an approximately logarithmic series of concentrations of the test substances was prepared in duplicate. Duplicate control vessels were also prepared; where the test substance stock solution was in acetone, the controls received a dose of acetone equal to that in the test vessels containing the test substance. Immediately before and after renewal of the test solutions the concentration of dissolved oxygen (DO), the pH of a control, and a highest test concentration were determined.

Ten G. Pulex of the required size were placed in each test vessel. They were not fed during the experiments. Observations of the numbers of dead G. pulex in each dish were made 3, 8, 24, 48, 72 and 96 h after their introduction. They were considered dead if no appendage movement was visible during a 15 second period of observation. Dead animals were removed. After 96 h in the test solutions all the G. pulex (alive or dead) were transferred to similar dishes containing 300 ml of the appropriate diluent, free of test substance. Mortality during the following 24 h was recorded.

The LC_{50} values (those concentrations lethal to 50% of the animals exposed) were calculated by probit analysis using log-transformed concentration values (FINNEY 1971). In the text reference is made to "120 h LC_{50}" values, these values are based on mortalities after 96 h exposure to the toxicant and a further 24 h in toxicant-free water.

The LC_{50} values for each compound at 24, 48, 72, 96 and "120" h were subjected to a separate three-way analysis of variance with water hardness, temperature and size of organism, each at two levels. Initially all two-factor interactions were included and the three factor interactions were used to estimate error. Subsequently these interactions and main effects were dropped sequentially omitting the non-significant terms. The significance of the model terms was answered using the variance ratio, i.e performing an F test.

RESULTS AND DISCUSSION

The mean hardness of the batches of soft and hard water used as diluent in the experiments was $\pm$ 4% of the nominal values of 100 and 250 mg l^{-1} (Table 1). Water temperatures were controlled to within $\pm$ 1°C of the nominal values of 7 and 15°C. pH values and concentrations of dissolved oxygen measured during the tests were similar in all experiments and would not have stressed the G. pulex (Table 1).

Table 1. Water quality during the tests

	Mean	Standard deviation	Number of observations
pH	8.33	0.11	155
Concentration of DO (mg l^{-1})	10.9	0.4	155
Water hardness (mg l^{-1}) Hard	249	25	20
Soft	104	6	25

The mean wet weight of the small G. pulex was 1.3 mg (standard deviation 0.9 mg) and that of the large 6.4 mg (standard deviation 2.2 mg).

Control mortalities during the test were low, with only 25 out of 640 control animals dying (i.e. less than 4%). Of the 25 deaths only 4 occurred in tests carried out in hard water.

DEHP was not acutely lethal to G. pulex in any of the experiments, despite the maximum concentration in all tests being in excess of its solubility in water, 0.4 mg l^{-1} (WOLFE et al. 1980).

The other three test substances were acutely lethal to G. pulex. LC_{50} values, or best estimates, were obtained for 3, 8, 24, 48, 72, 96 and "120" h in most of the 24 experiments; of a possible 168 LC_{50} values only 10 were missing. The 10 missing values were all for the shorter periods of exposure, 3 and 8 h.

Analysis of variance was used to investigate the influence of water hardness, water temperature and size of organism on LC_{50} values. The analysis was limited to LC_{50} values for periods of exposure of 24 h or greater, because of the incompleteness of data at 3 and 8 h. Initially, analyses were made of LC_{50} values at 24, 48, 72, 96 and "120" h for each test substance with interaction terms included. These indicated no significant effects, but in all cases more than 50% of the variance was accounted for by the main effects. The analyses were therefore run with only main effects, and significant differences were found. Water hardness was the only variable to have significant effects, apart from a temperature effect with γ-HCH at 24 h. This temperature effect was not present at 48, 72, 96 or 120 h and is considered to be a false positive. The analysis was therefore run with water hardness as the only variable. Of the 15 pairs of mean LC_{50} values obtained from this last analysis, 8 were significantly different.

Figure 1 plots the toxicity curves for the three test substances that were acutely toxic. Grand means of LC_{50} values for each exposure time are plotted where no significant differences between hard and soft water were found. Where significant differences were found main effects means (i.e. means for hard and soft water) have been plotted.

In the tests with phenol it was only at 48 h that there were significant differences in the mean LC_{50} values for hard and soft water (Table 2). The lack of significant effects of hardness for other exposure periods suggests that this result may be a false positive. The LC_{50} values for phenol declined from approximately 160 mg l^{-1} at 3 h to 106 mg l^{-1} after 24 h and 45 mg l^{-1} after 96 h. From 96 to 120 h, when the animals were in toxicant-free water, there was only a small decrease in the LC_{50} value; from 45 to 40 mg l^{-1}.

The effects of water hardness on copper toxicity were more apparent with significant differences in LC_{50} values from 48 to "120" h (Table 2, Figure 1). The copper was 4 to 6 times more toxic in the soft water than in the hard. After 3 h exposure to copper the LC_{50} value was greater than 5 mg l^{-1} but by 24 h this had declined to 0.23 mg l^{-1} and by 96 h was 0.109 mg l^{-1} in hard water and 0.021 mg l^{-1} in soft water. As with phenol the decreases in LC_{50} values from 96 to "120" h were small; from 0.109 to 0.096 mg l^{-1} in hard water and from 0.021 to 0.017 mg l^{-1} in soft water.

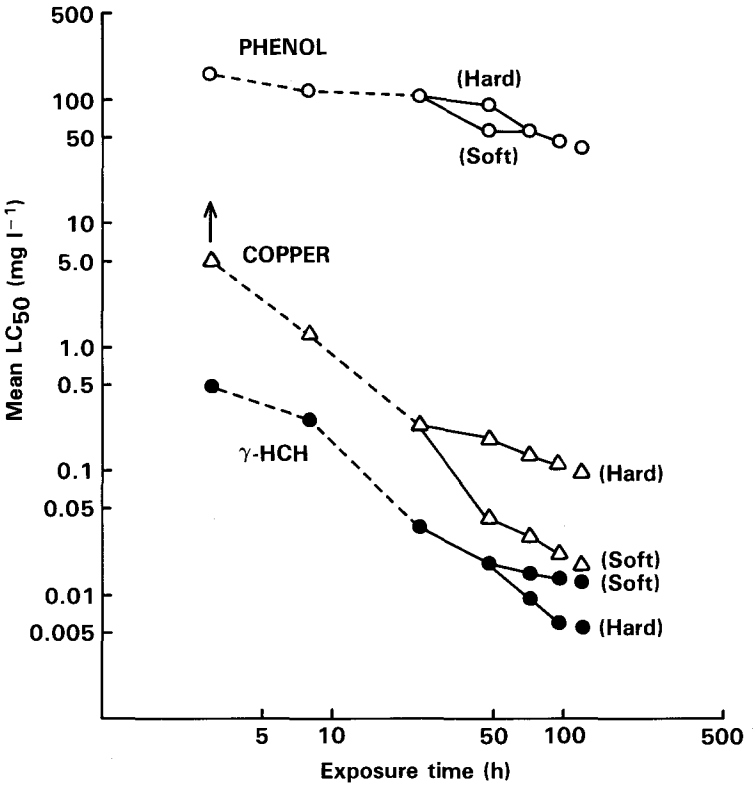

Fig 1 Relationship between exposure time and the acute toxicity (LC_{50}) of phenol, copp and γ-HCH to G.pulex. Separate curves are plotted where there were significant differences in LC_{50} values for hard and soft water. Values for 3 and 8 h are best estimates, in some tests the data were inadequate for the calculation of LC_{50} values, and the means plotted are therefore based on less than 8 values.

The toxicity of γ-HCH was also influenced by water hardness (Table 2, Figure 1), although the effects did not appear until 72 h after exposure began. From 72 h onwards LC_{50} values in soft water were consistently and significantly greater than those in hard water. From 3 to 24 h the LC_{50} values decreased from approximately 0.5 to 0.036 mg l^{-1}, and by 96 h the LC_{50} in soft water was 0.013 mg l^{-1} and in hard water 0.0059 mg l^{-1}. Again there was a slight decrease in LC_{50} values from 96 h to 120 h; from 0.0136 to 0.0129 mg l^{-1} in soft water and from 0.0059 to 0.0055 mg l^{-1} in hard water.

Table 2. Mean LC_{50} values obtained in hard and soft water and significant differences between them

Test substance	Period of exposure (h)	LC_{50} (mg l^{-1}) Hard water	Soft water	Variance ratio	Degrees of freedom
Phenol	24	100	112	0.15	1, 6
	48	89	55	7.14**	1, 6
	72	67	43	4.09	1, 6
	96	51	40	0.89	1, 6
	(120)*	45	34	1.29	1, 6
Copper	24	0.375	0.086	3.49	1, 6
	48	0.183	0.041	8.45**	1, 6
	72	0.127	0.030	7.99**	1, 6
	96	0.109	0.021	9.76**	1, 6
	(120)*	0.096	0.017	8.06**	1, 6
γ-HCH	24	0.0485	0.0236	5.53	1, 6
	48	0.0195	0.0173	0.63	1, 6
	72	0.0095	0.0151	6.31**	1, 6
	96	0.0059	0.0136	15.72**	1, 6
	(120)*	0.0055	0.0129	11.13**	1, 6

* Exposure to the toxicant was for 96 h with a further 24 h period in toxicant-free water.

** Mean LC_{50} value for hard and soft water are significantly different, $P < 0.05$.

Table 3. Published data on the acute toxicity of the four test substances to species of Gammarus

Test substance	Species	96 h LC_{50} mg l^{-1}	Source
Phenol	G. fasciatus	7-35*	Emery, 1970
Copper	G. pseudolimnaeus	0.02	Arthur and Leonard, 1970
γ-HCH	G. lacustris	0.048	Sanders, 1969
	G. fasciatus	0.010-0.011	Sanders, 1972
	G. fasciatus	0.039*	Macek et al., 1976
	G. pulex	0.034	Abel, 1980
DEHP	G. pseudolimnaeus	10	Mayer and Sanders, 1973

*48 h LC_{50} values.

The LC_{50} values obtained for the four test substances are in general agreement with those reported previously in the literature for G. pulex and other species of the genus (Table 3).

The influence of the three variables, water hardness, water temperature and size of organism, on the acute lethal toxicity of the four test substances to G. pulex was generally small. Only water hardness had statistically significant effects on LC_{50} values, and then only for copper and γ-HCH when exposure periods were 48 h or greater. The magnitude and direction of the change in toxic effects seen were different for the two substances. Copper was some 4 to 6 times more toxic in soft water than in hard, whereas γ-HCH was 1.5 to 2.5 times more toxic in hard water than in soft. The lower toxicity of copper in hard water was expected. Many authors have reported this to be the case for both fish and invertebrates (ANDREW et al. 1977; EIFAC 1976; EPA 1976). The lower toxicity of copper in hard water is caused by the greater capacity of the anions present for complexing the toxic cupric ions (STIFF 1971). No clear explanation of the greater toxicity of γ-HCH in hard water is apparent, though if the G. pulex were less stressed in this water than in the soft (control mortalities indicate this to have been the case) it is possible that moulting frequency was higher in the hard water. And there is evidence that the moult period in crustaceans is a time of increased susceptibility to toxicants (LEE & BUIKEMA 1979).

HANSEN & KAWATSKI (1976) recommended that a post-exposure observation period of 24 h be included in acute toxicity tests with aquatic invertebrates. Their reasons were twofold. They felt that the criterion of death based on immobility was suspect and that immobilised animals might recover. Secondly, organisms not dead at the end of the period of exposure might subsequently die as a result of exposure. In the experiments with G. pulex reported here there was no indication that the definition of death used was inadequate; no animals counted as dead after 96 h exposure were found to be alive during the 24 h period in toxicant-free water. And whilst some mortalities did occur in the post-exposure period, the "120" h LC_{50} values were only some 12% lower than the 96 h LC_{50} values.

In the light of the results obtained in the tests reported here it is suggested that guidelines for acute lethality tests with G. pulex recommend that tests be carried out in hard water, at 15°C, with large specimens and that the exposure period should be 96 h without a period of post-exposure observation. Hard water is recommended as water hardness had relatively small effects on toxicity and soft water gave higher control mortalities. The use of large specimens is preferred because size did not influence toxicity and large specimens are more easily observed and handled than smaller ones. A test temperature of 15°C is suggested because it is relatively easy to achieve and maintain and no significant temperature effects were observed.

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