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Behavioral Changes in *Gammarus pulex* and Its Significance in the Toxicity Assessment of Very Low Levels of Environmental Pollutants

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Phenolic compounds are an important group of contaminants in tap water (Kraybill 1978). To prevent water-transmittable epidemic diseases, drinking water is often chemically chlorinated, which may cause chemical chlorination of these contaminants and the formation of stable and unstable chloro-derivatives. Although industrial and agricultural practice appear to be major contributors of organic water contamination, we were able to draw attention to the importance of plant-derived phenolic compounds as an alternative source of phenolic water contamination (Borlakoglu and Kickuth 1986). Model chlorination of 7 major plant-derived phenolic compounds produced > 65 stable chlorophenolic derivatives. The formation of such a large number of chlorophenolic derivatives mirrors the synthetic complexity of water chlorination and the presence of these contaminants impose uncertain biological and ecotoxicological implications. Studies on the bactericidal activity of the formed chlorophenolic mixtures using Eschericha coli as test species indicated a complex pattern of varying toxicity with inhibition of growth estimates ranging between 156 to 1250 mg/L (Borlakoglu and Kickuth 1987).

The objectives of the work presented were to assess the effects of these chemicals at very low levels without producing lethal effects. We chose the fresh water amphipod Gammarus pulex as a sensitive indicator organism for environmental pollution and exposed it under controlled conditions to sub-lethal levels of a previously identified major plant-derived chlorophenolic derivative. Swimming behavioral changes, escape reaction and measurements of activity of Gammarus pulex are carried out at levels $^{1}/20$th of the acute oral toxicity. With the aid of this screening model, it was hoped to predict possible ecological changes at sublethal concentrations. Sublethal perturbations are defined as changes in the expected behavior and biological response if exposed to the chemical and to changes in their natural environment.

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MATERIALS AND METHODS

The identification of major plant-derived phenolic water contaminants and the synthesis of chlorophenolic derivatives was recently described by us (Borlakoglu and Kickuth 1986). We also reported the bactericidal activity of the chloro-phenolic mixtures using Escherichia coli DSM 613 (Borlakoglu and Kickuth 1987). Gammarus pulex was collected from a small natural fresh water stream close to the University of Kassel (West Germany). After collection, they were transferred into 10-L aquaria which contained plant and soil material from their natural habitat. The temperature was controlled and ranged between 10-12^{o}C. Air was continuously supplied. The pH was adjusted to their natural environment and ranged between 6.5-6.6. For each experiment individuals were isolated and transferred to observation tubes. Each experiment was carried out with six test individuals and two control individuals. Exposure of the test individuals was obtained by transferring a thin-layer chromatographically (TLC) purified major chlorophenolic derivative of 4-hydroxycinnamic acid into the observation tube and adjusting the concentration to approximately 40 μg/mL. This compound was previously identified as 3,5-dichloro-4-hydroxyphenyl-α,β-dichloropropionic acid (Borlakoglu and Kickuth 1986). One of the controls was exposed to an equal amount of non-chemical containing thin-layer particles (silica gel) to account for possible behavioral changes due to the mere presence of non-chemical containing TLC particles. Within the first hour of each experiment observations were made at 15 min intervals followed by 1 h assessments. Each experiment was conducted over a period of up to 14 h.

The following measurements were taken:

1. Observation of an escape reaction, e.g., Gammarus pulex was permitted to move away from the chemical.

2. Changes in the swimming behavior and swimming position compared with the controls.

The swimming positions were defined as

a = lateral swimming position
b = dorsal swimming position
c = ventral swimming position
d = to curl up
e = crainio-caudal swimming position.

3. Effect of chemical exposure on copulation behavior.

4. Measurement of the activity of Gammarus pulex by evaluating its activity on a scale between (0 = no activity), (1 = hypoactive activity), (2 = normal activity), (3 = hyperactive).

5. Observation of macroscopical damage to the exoskeleton, extremities and antennae.

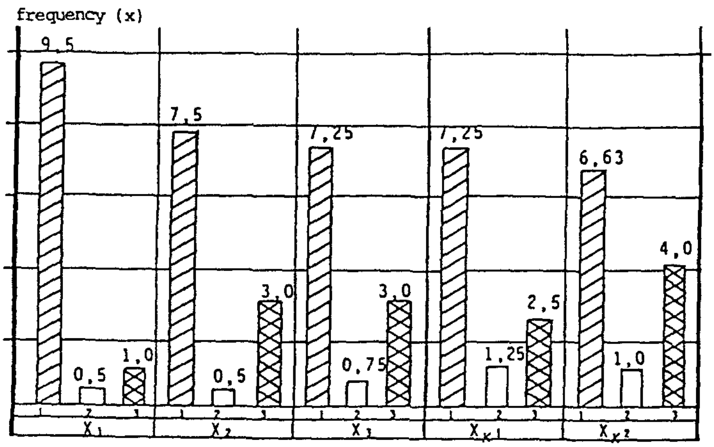
6. Estimation of acute lethal concentration LC_{50} (e.g., death of 50% of the population within 60 min of exposure) was carried out using the chlorophenolic mixture of 4-hydroxycinnamic acid.

Statistical analysis was performed using arithmetic mean, standard deviation, students-t-distribution and ^{2} goodness of fit test.

RESULTS AND DISCUSSION

The general distribution pattern of Gammarus pulex is illustrated in Figure 1. As expected, Gammarus pulex responded to its gravitational field with a preferential residence at the bottom of the observation vial. Comparison between the control group and the treatment group showed that the treatment group was more frequently on the bottom of the observation vial. This observation was interpreted as a sublethal toxic effect. Since Gammarus pulex was expected to avoid the phenol-contaminated thin-layer particle region the lack of an escape reaction was further evidenced in Table 1. Compared with the control group Gammarus pulex behavior was significantly altered ($P < 0.05$) (see Table 1) in the presence of the chlorophenolic compound. From the data obtained Gammarus pulex was more frequently on the bottom of the observation vial and moved less frequently to the water surface when exposed to the chemical. In addition, Figures 2 and 3 illustrate significant changes ($P < 0.05$) in the activity pattern of Gammarus pulex when exposed to the chlorophenolic compound, although the results contrast the reduced escape reaction described in Table 1. If Gammarus pulex was more active, it was assumed that it would respond with an ecape reaction. Nevertheless the statistical significance of these changes was only observed within the first 4 h (see Figure 3). The lack of an escape reaction could be explained as a sublethal toxic effect. Further, supporting evidence for a sublethal intoxication of Gammarus pulex is given in Figures 4 and Table 2. From this data it became apparent that Gammarus pulex changed its swimming behavior in an unusual manner with an unexpected frequent change of 'unusual' swimming positions. The swimming behavior was shown to be significantly changed ($P < 0.001$), when exposed to the chemical, as judged by statistical analysis using ^{2}-goodness of fit test (Table 2). This significant change and a more frequent use of unusual swimming positions provided strong indication of sublethal toxic effects observed in Gammarus pulex at levels $^1/20$th of the acute toxicity.

The interpretation of the data obtained at very low levels of intoxication is a difficult task. However, with sufficient numbers of replicates, it is possible to demonstrate the observed effects in Gammarus pulex.



1 : " Attraction" towards the chlorophenol contaminated TLC - material

2 : Movement away from the compound

3 : Attraction towards the air supply

X_1 to X_3 identifies test groups from three different collection dates for Gammarus

X_{k1} : control individuals in the absence of TLC particles

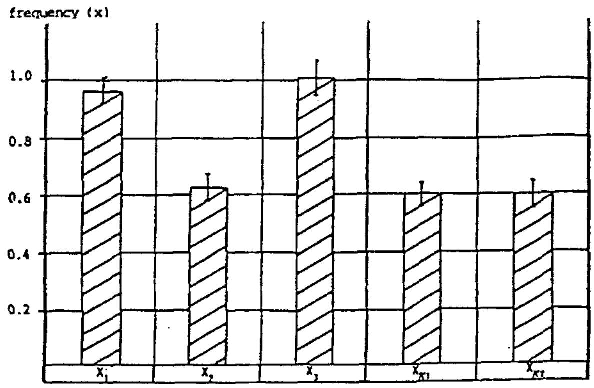
X_{k2} : control individuals in the presence of TLC particles

Figure 1. Distribution pattern of Gammarus in the presence of the main chlorophenolic derivative of 4 - hydroxycinnamic acid.

Table 1. Percentage distribution of Gammarus exposed to the main chlorophenolic derivative of 4 - hydroxycinnamic acid.(1)

	Attraction towards the main chlorophenol contaminated thin-layer material	Mowement away from the compound	Attraction towards the air supply
Test Group	73.45	5.30	21.25
Control Group	61.33	9.94	28.73

(1) The percentage distribution of the test group was significantly different at $p < 0.05$ using X^2 - goodness of fit test.

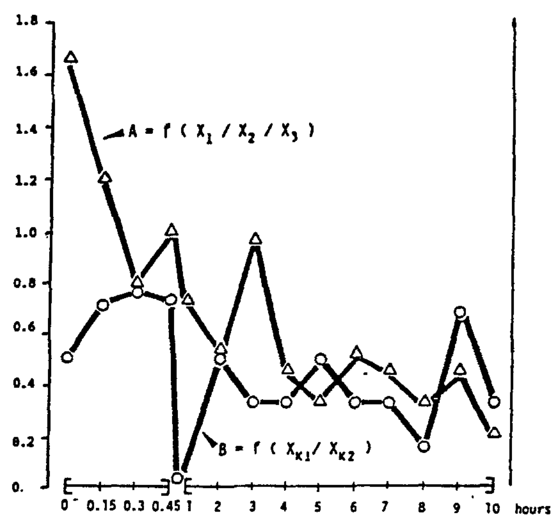


X_1 to X_3 identifies test groups from three different collection dates

X_{k1} to X_{k2} identifies the control group

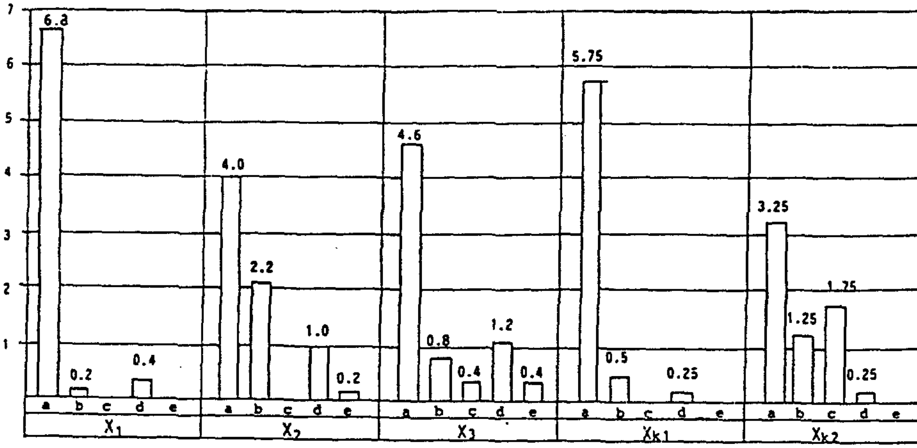
The activity of Gammarus was evaluated on a scale between 0 to 3; the treatment group being significantly different ($p < 0.05$) using the t - test.

Figure 2. Activity changes of Gammarus exposed to the main chlorophenolic derivative of 4 - hydroxycinnamic acid.



The activity of Gammarus was evaluated on a scale between 0 to 3 and all values are expressed as the mean of n = 150 independent measurements.

Figure 3. Time dependent activity changes of Gammarus exposed to the main derivative of 4 - hydroxycinnamic acid.



X_1, X_2, X_3 = test groups. X_{k1} and X_{k2} = control groups.
a = lateral swimming position, b = dorsal swimming position, c = ventral swimming position, d = to curl, e = cranio - caudal swimming position. (n = 150 independent measurements)

Figure 4. Average swimming behavior of Gammarus exposed to the main chlorophenolic derivative of 4 - hydroxycinnamic acid.

Table 2. Percentage change in the swimming behavior of Gammarus exposed to the main chlorophenolic derivative of 4 - hydroxycinnamic acid.

Swimming Position	Test Group (0)	Control Group (E)	0-E	$\frac{(0-E)^2}{E} = X^2$
lateral	69.36	69.23	0.13	0.0
dorsal	14.41	13.46	0.95	0.06
ventral	1.80	13.46	-11.66	10.10
to curl	11.71	3.85	7.87	16.02
cranio-caudal	2.72	0.0	2.72	0.0

Other workers studied behavioral changes in Gammarus pulex when exposed to low levels of intoxicants. Corta (1967) showed that Gammarus pulex responds to slight pH changes with a typical escape reaction at a pH below 6.2. In the present investigation, we noticed that at the beginning of each experiment the pH dropped from 6.6 to 6.0; however, the pH increased subsequently between the first hour to approximately 6.5 and for some individuals up to 6.8. It was not unexpected that the chlorophenolic acid decreased the pH. Brehm and Meijering (1980) could show that Gammarus pulex was able to respond to pH changes with an enhanced faecal excretion. Their faeces contain amino acids and ammonia which have proton accepting properties. Since they are acting as proton scavengers, it is not surprising that the pH in the test solution was readjusted to more physiological values. That would also explain why Gammarus pulex appeared to be more active in the presence of the chlorophenolic compound, since it suffered from an unphysiological pH environment. Brehm and Meijering (1982) also demonstrated that lowering the pH causes a disturbance in the respiration of Gammarus pulex, which collapses at levels < 4.0. At extremely low levels of pH (i.e., > 2.0) it is most likely that $CaCO_3$ from the exoskeleton may interact with the acid to form a salt. However, in such an unphysiological environment, the chances of survival for Gammarus pulex are poor indeed. Depending on the ambient pH, phenolic compounds have a high partition coefficient for lipids and hydrophobic compounds. The test compound in question lowered the pH and partitioned into lipophilic compartments of Gammarus pulex. Phenolic compounds may interact with membranes and have been identified as membrane poisons (Riley and Seal, 1974).

Lethal concentrations of the chlorophenolic mixture of 4-hydroxycinnamic acid were achieved at 700 μg/L. Light microscopical examination revealed indications of plasmolysis. Since the chemical is likely to interact with membranes, it is assumed that membrane leakage may be an important factor for causing acute toxicity. In addition, light microscopical examination revealed damage to the extremities and antennae, if exposed to lethal concentrations. Typical features were necrotic antennae and damage to the exoskeleton.

Block (1977) studied low levels of chlorine in marine amphipoda. Levels as low as 0.63 ppm caused severe damage to the osmoregulation, changes in the ratio of body fluid electrolytes (Na^+, K^+) and biochemical changes in the activities of carboanhydrase, glutamaldehydrogenase and glutamate pyruvate transaminase, factors very important for moulting of Gammarus pulex. The chlorination of tap water with an excess of chlorine may cause similar effects to Gammarus pulex. Unfortunately, during the course of the experiment, it was not possible to obtain significant replicates to study the copulation behavior in the presence of the chlorophenolic compound. However, preliminary data suggested that at $^1/20$th of the LC_{50} Gammarus pulex copulation behavior changed. Copulation was impaired when exposed to the chemical; however this effect was reversible. After 3 to 4 h of exposure *Gammarus pulex* restarted copulation.

In the present investigation a model compound of a group of important environmental water pollutants was studied. At levels $^{1}/20$th of the LC_{50} behavioral changes with *Gammarus pulex* were observed. Although the experimental procedures are time consuming and also necessitate frequent experimental replication, it was possible to observe significant changes. Behavioral studies are probably more sensitive than standard toxicological assessments. Comparing the lethal concentration of the chlorophenolic mixture of 4-hydroxycinnamic acid, it became evident that *Gammarus pulex* was 1.8 times more sensitive when compared with the inhibition of growth studies using *Escherichia coli* (Borlakoglu and Kickuth 1987). In addition, *Gammarus pulex* showed changes in its expected behavior at $^{1}/20$th of the acute LC_{50}. These changes are important alterations. For example, the lack of a sufficient escape reaction may lead to the death of a local *Gammarus pulex* population. Changes in the swimming behavior and activity of *Gammarus pulex* are important alterations, which could lead to an impaired food uptake and altered copulation behavior and a disturbed ecology. Ecological changes may not be acutely lethal; however, the effects of low levels of xenobiotic molecules, such as these important water contaminants may cause loss of important genetical pools. The results presented suggest that behavioral studies provide important and vital information in understanding the ecotoxicology of environmental pollutants and their ecological significance.

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