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Studies on Sediment Toxicity Bioassays Using *Chironomus thummi* K., 1911 Larvae

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Abstract: The acute toxicity of zinc, copper and lead to freshwater dipteran *Chironomus thummi* larvae was evaluated by static bioassays, calculating the LC_{50} (lethality concentration for 50%). Mortality increased with increasing concentrations of zinc, copper and lead. The LC_{50}s of Zn, Cu and Pb for survival were 11.2, 19.1 and 14.3 μg g^{-1}, respectively. The results indicated that Zn had the greatest toxicity, followed by Pb and Cu. Individual weight increased with increasing Cu, Zn and Pb concentrations. The results are discussed and compared with those of other studies.

Key Words: Zinc, copper, lead, lethality concentration, *Chironomus thummi,* larvae, bioassay

Chironomus thummi K., 1911 Larvalarının Sediman Toksisite Biyolojik Deneylerinde Kullanılması Üzerine Çalışmalar

Özet: Tatlısu dipteralarından *Chironomus thummi* larvaları kullanılarak çinko, bakır ve kurşunun akut toksisitesi statik biyolojik deneylerle ölçülmüş ve öldürücü konsantrasyon (LC_{50}) hesaplanmıştır. Çinko, bakır ve kurşun konsantrasyonlarının artmasıyla ölüm oranı da artmıştır. Çinko, bakır ve kurşun LC_{50} değerleri sırasıyla 11,2, 19,1 ve 14,3 μg g^{-1}olarak bulunmuştur. Bu tür için en toksik metal çinko olmuş ve bunu kurşun ve bakır izlemiştir. Organizmaların bireysel ağırlığı çinko, bakır ve kurşun konsantrasyonlarının artmasıyla artmıştır. Bu sonuçlar tartışılmış ve diğer çalışmalarla karşılaştırılmıştır.

Anahtar Sözcükler: Çinko, bakır, kurşun, öldürücü konsantrasyon, *Chironomus thummi*, larva, biyolojik deney

1. Introduction

Many discharges to the aquatic environment contain substances which are sparingly water-soluble. These substances bind preferentially to suspended particle material and bottom sediments in lakes, rivers, estuaries and coastal waters. As a result, some very toxic and persistent chemicals, such as heavy metals, can be found at high concentrations in the sediment. This can lead to deleterious effects on benthic communities, fisheries and potentially on human health, through direct contact of organisms or resuspension into the overlying water. Thus, it is important that sediment quality should be considered in conjunction with monitoring of the water column in order to ensure effective protection of aquatic ecosystems. The protection of aquatic habitat from damage due to contaminants requires an understanding of both the sensitivity of invertebrates to contaminants and their ecological requirements. Toxicity tests are a convenient and appropriate way of assessing this sensitivity and also have the advantage of reflecting the bioavailable fraction of a contaminant, which can be very different from the total amount determined by chemical analysis (1).

Sediment toxicity testing began in the late 1970s (2), but the science of sediment toxicity is still very young (3,4) and there were no standard methods for conducting sediment toxicity tests until the early 1990s (3). Even so, no completely standardised methodology has been published (5), despite the advantages of these techniques for providing information on the ecological impact of contaminated sediment (6-9).

Sediment toxicity may be defined as 'the ecological and biological changes that are caused by contaminated sediments' or 'an adverse response observed in a test organism exposed to a contaminated sediment' (4,5,7).

According to Chapman (10), sediment bioassays can be used in two separate ways in order to develop sediment quality criteria: (a) sediment bioassays and chemical analyses can be conducted with sediments collected from contaminated and reference areas. The bioassay responses can be compared quantitatively to identify whether problems exist and the levels of contaminants in sediments can be related to the bioassay responses (10,11); (b) dose-response relationships can be developed in the laboratory by spiking sediments with individual and mixed contaminants and then carrying out bioassays on these sediments (12). In this study, the latter approach was adopted. There have been few studies to date on this. A variety of test methods have been developed by the American Public Health Association (APHA), the American Society for Testing and Materials (ASTM), the U.S. Environmental Protection Agency (U.S. EPA), the U.S. Army Corps of Engineers of Materials, the Society of Environmental Toxicology and Chemistry (SETAC) and the Water Research Centre (WRc) and these form the basis of the tests described in this study.

In developing a marine estuarine sediment bioassay protocol a number of properties are desirable (4,13,14): (a) broad salinity tolerance; (b) high sensitivity to common sediment contaminants; (c) a high survival rate under control conditions; (d) occupation of microhabitat(s) at or preferably below the sediment-water interface to ensure maximum and consistent exposure to sediment contaminants; (e) low sensitivity to natural sediment variables, such as particle size and organic content, to allow a wide variety of sediment types to be tested; (f) a broad geographic range to enhance the breadth of the application as a test species; (g) ease of collection, handling and maintenance in the laboratory; (h) ecological importance in estuarine systems; and (i) the ability to be cultured or have year-round availability in the field. Ideally, a sediment toxicity test should also be rapid, simple and inexpensive (4,5).

Relatively few species have been extensively used for toxicity testing (14,15) and there is no single biological response or test species that can meet all environmental and legislative requirements for effective toxicity testing (4,16,17). Nevertheless, benthic invertebrates have great potential for sediment toxicity tests (18), because they are intimately associated with sediments either through their burrowing activity or by ingestion of sediment particles (4,5,19-23).

Ecologically, Chironomids form an important group of organisms. The greatest part of their life cycle, the larval stage, is spent in sediment. During the egg stage and the adult midge stage they live in the water and in the air respectively. They are part of different food chains. The eggs, larvae and pupae comprise an important source of food for fish (24-28), while *Gammarus* sp. (personal observations) and midges serve as food for terrestrial organisms (e.g., birds) and probably play important roles in sediment community organisation. Chironomids are widespread and abundant in European (24) and Turkish (25-28) freshwater systems.

Clearly, *Chironomus* spp. larvae have potential as a test species for sediment bioassays in Turkish waters. Not only do they respond to contaminated sediment, but they also fulfil many of the criteria listed above (4,13,14). Because these organisms spend the majority of their life in sediment, they are continuously exposed to contaminants and they ingest sediment (and contaminants) when feeding. They are usually available all year round, often occur in high densities, tolerate a wide range of particle sizes and have a broad geographic range.

Specimens of *Chironomus thummi* are found in rivers, lakes and shallow pools in Sinop. They are deposit feeders, the larvae feeding on small silt particles and/or on detritus (24-28). There are four distinct phases in their life cycle: egg, larva, pupa and adult. Larval growth occurs in four instars. The first instar is approximately 1 mm long, the second instar is 1-3 mm long, the third instar is 3-6 mm long and the fourth instar is 6-20 mm long. The fourth instar increases in weight as the larva prepares to pupate.

In this study, the freshwater dipteran *Chironomus thummi* was evaluated as a test organism for use in sediment toxicity bioassays by adapting the standard protocols developed by the ASTM and WRc for conducting sediment toxicity tests. Although *Chironomus* spp. have been used (29-32) to assess the toxicity of freshwater sediments, detailed ecotoxicologies of these animals have not been documented. In particular, the effects of specific contaminants of known concentrations on this bioassay are not known. In this study, several experiments were carried out using clean sediment contaminated with the heavy metals copper, zinc and lead and employing the *Chironomus* bioassay protocol.

2. Materials and Methods

All the animals used in these experiments were collected from the sediment of shallow pools located along the Sinop to Ayancık road by sieving fine sediment through a 250 µm mesh. The area does not have any significant history of contamination by metals (personal observations). Large pieces of debris and other macrofauna (e.g. *Gammarus*) were discarded.The animals were kept in tapwater in a tank which was continually aerated.

The sediments were collected to a depth of 1-2 cm from an area known to be clean and to support a healthy population of *Chironomus thummi* (personal observations). The body length and headcapsule width of the larval instars of *Chironomus thummi* were 8-9 mm and 450-500 µm, respectively. The colour of the animals was red.

The sediment was washed through a 250 µm mesh sieve into a tank in order to remove any macrofauna and larger sediment particles, then washed again through a 250 µm mesh to ensure a standard particle size for the sediment in all the experiments. The sediments were stirred and rinsed three times with filtered tapwater, and then allowed to stand for 24 hours. After that time the overlying water was poured off and the sediment placed in test containers. These sediments were then treated by shaking with solutions of copper (prepared from $CuSO_4$), zinc (prepared from $ZnCl_2$), or lead (prepared from $Pb(NO_3)_2$) (volume ratio 1:4 w/v). Sediments with different concentrations of metals were obtained through serial dilutions of the metal stock solution. The concentrations of zinc, copper and lead were 0.1, 1, 10, 20 and 50 mg l^{-1}. The mixing time was limited to 3-4 hours. The sediments used as the controls were treated as described above with uncontaminated tapwater.

Treated and control sediment supernatants were decanted and the test and control sediments were placed in the bioassay containers. Clean tapwater was added to the containers to 0.5 cm from the top to allow the sediment and water to equilibrate to test conditions and to allow suspended sediment to settle before the addition of the test animals (13).

Each set-up consisted of three replicate containers for each of the 6 concentrations, plus 6 controls. All the containers were aerated in order to maintain the dissolved oxygen levels above 60% of the air saturation value and covered with black material to exclude direct light except from directly above. The larval instars were separated from the stock tank on the basis of body length and headcapsule width measured using a light microscope. The containers were examined daily and any dead organisms were removed. The LC_{50} was calculated by probit analysis (33). All glassware was acid-washed in 10% HCl for 24 h and then thoroughly rinsed in distilled water prior to use.

The sensitivity of the fourth-instar larvae was determined by an acute test in water with potassium dichromate ($K_2Cr_2O_7$) as the reference compound (29,30).

Ten fourth-instar larvae were placed in each container. After 1 hour any *Chironomus* that were dead or showed abnormal behaviour were removed and replaced and the numbers of individuals that had not burrowed or were dead were noted. The mean dry weight was 0.26 mg (4-5 mg wet wt). Dead larval instars were removed but not replaced. At 96 h, the individuals that had not burrowed were removed, and those remaining in the sediment were counted (13).

Following the standard ASTM and WRc protocols (29-31, 34-36), three response criteria (survival, avoidance of sediment and dry weight) were examined for all three metal bioassays. The criteria for death were immobility and/or lack of reaction to a mechanical stimulus. During the course of the bioassays the number of *Chironomus* that had avoided the sediment, either floating on the water surface or lying on top of the sediment, was also recorded daily.

All the bioassays were static and the test organisms were not fed during the test period. The dry weight of the surviving fourth-instar larvae per test container was measured. For each contaminant a preliminary range-finding test was conducted using the static 4-day tests with survivorship as the end point.

2.1. Organic content

Samples for total sediment organic carbon analysis were dried at 60°C in an oven for 48 h. A 5-gram sample was then treated with 1 *N* HCl to remove carbonates (32). The weighed, dried samples were then placed in a muffle furnace at 600°C for four hours and the loss on ignition was taken to be the organic carbon content of the sediment (37).

2.2. Statistical analyses

The data were used in the calculation of both the LC_{50} and EC_{50} values. LC_{50} is the lethal concentration at which 50% of the animals did not survive. EC_{50} is the effective concentration at which 50% of the animals were not able to nest in self-made tubes in the sediment by the end of the bioassay. Both LC_{50} and EC_{50} were calculated by probit analysis according to the method of Finney (33).

Bioassay data were generated as the proportions F1/N and F2/F1, where N=total number of animals, F1=total number of survivors (number able to nest plus number unable to nest) and F2=number unable to nest. These fractions were then converted to percentages to obtain a normal distribution of the variances (38).

2.3. Dry weight

On the 4th day of the experiment, the survivors within each replicate container were counted and placed together in separate vials which had previously been numbered, dried and weighed so that each vial corresponded to a specific replicate. Vials containing Chironomids were dried immediately (no depuration was permitted) at 105°C for 24 h and the dry weight of the animals was calculated for each container. Variations within each set of replicates were analysed using analysis of variance (ANOVA). In order to make a multiple comparison between the results at individual concentrations and those for the controls, Dunnett's method was used (38).

3. Results and Discussion

For the reference compound $K_2Cr_2O_7$, the 96h LC_{50} for survival was 35 mg / l. The ranges of the reference compound potassium dichromate ($K_2Cr_2O_7$) for acceptable sensitivity of *Chironomus* are 20< x <65 mg/l for 96 h test duration (29,30).

The primary criterion of toxicity tests is survival after 4 days of exposure to test and control sediments (13,39-46). There was no mortality in any of the controls, demonstrating that the holding facilities, water, control sediment and handling techniques were acceptable for conducting the 4-day sediment toxicity test, as required in the standard EPA/COE protocol (34), where the mean survival should be ≥90%. Survival is also possible in conditions where there is wet sediment with no overlying water (personal observations).

The mean temperature for the 96h experimental period in all the bioassays was 15°C±1, the dissolved oxygen content was 71%±4 and the pH was 7.65±0.23. The organic content of the sediment was 1.92% ±0.32.

Chironomus survival declined with increasing sediment metal concentration, as did burrowing activity, whilst in the control sediment there was no mortality and all the animals burrowed.

More than 50% of the animals survived at the end of the exposure to concentrations of 30 µg g^{-1} Cu or less in the sediment. Only 65% of the animals survived at the end of the exposure to concentrations of 40 µg g^{-1} Zn and Pb in sediment. The results of LC_{50} and EC_{50} analyses show that zinc was much more toxic than copper or lead (Table).

Table The 96-hour LC_{50} and EC_{50} values with 95% fiducial limits (FL) for *Chironomus thummi* larvae exposed to copper, zinc and lead (mg l-1).

Metals	LC_{50} (95% FL)	EC_{50} (95% FL)
Cu	19.1 (17-21)	15.8 (13-18)
Zn	11.2 (9.8-12.3)	8.1 (6.9-10.1)
Pb	14.3 (12.1-15.8)	11.2 (9.3-12.9)

No *Chironomus* avoided clean (control) sediment under bioassay conditions, whereas many animals avoided contaminated sediment. In the sediments with the highest concentration of copper, zinc and lead (100 µg g^{-1}), avoidance of sediment was not significantly different ($P>0.05$) from that in the control. No animals avoided sediment after 4 days and all of them died during the bioassay. The avoidance of sediment differed greatly for zinc, copper and lead.

Published guidelines for conducting toxicity bioassays with *Chironomus* spp. recommend that first-to-second instar larvae be used (29-31). Fourth-instar larvae were used in this study due to the ease of identifying and handling the organisms.

Of the 97% surviving in the control sediment, all were able to nest in self-made tubes in the sediment. Overall, 600 survivors in the bioassay tests 492 (82%) were able to nest in self-made tubes in the sediment at the end of the 4-day period, whereas *Chironomus* survival and

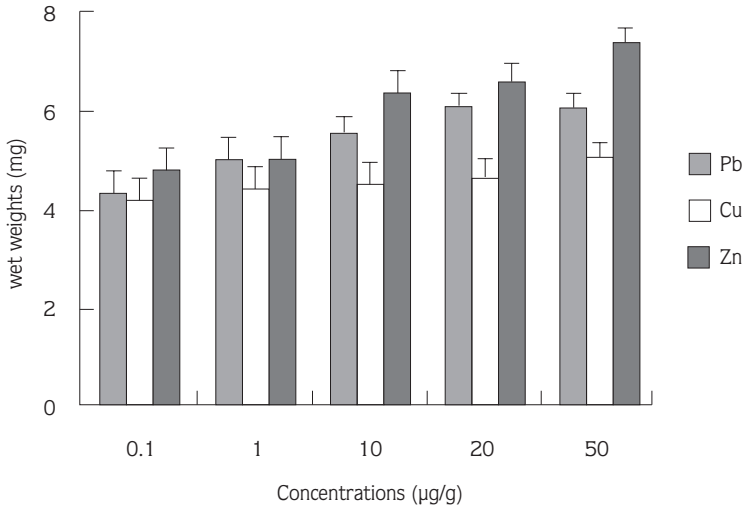

Figure Mean wet weight of surviving *Chironomus thummi* larvae at the end of 4 days in sediment treated with Zn, Cu and Pb. Each treatment included 3 replicates. (Error bars = SE).

burrowing success decreased with increasing sediment metal concentration for all metals, indicating that *Chironomus* was able to detect the metals in the sediment and avoided them.

The larvae of the chironomid *Chironomus thummi* had a greater resistance to the contaminated sediment. This was based on survival and growth responses. This may be due to the fact that some individuals can tolerate or adapt themselves to slight contamination quickly or perhaps low concentrations of metals in sediment were oxidised due to constant aeration and this reduced their toxicity.

The behaviour of *Chironomus* varied between the different treatments. The avoidance of sediment increased with increasing Zn, Cu and Pb concentrations and was significantly different ($P<0.05$) from the control, indicating that the contaminants had a sublethal effect. Even on the first day, concentrations of 30 µg Cu g^{-1}, 15 µg Zn g^{-1} and 20 µg Pb g^{-1} induced marked changes in burrowing behaviour. The avoidance by *Chironomus* of contaminated sediment may reduce exposure to metal in the sediment, but, once emerged, individuals become vulnerable to predation by birds, fish and epibenthic crustaceans. This makes the ability of surviving animals to rebury in clean sediments an ecologically relevant sublethal test end point (12,13,47-51).

This study shows that *Chironomus* is a very robust organism, capable of tolerating quite high levels of heavy metals. Moreover, if heavy metals were removed from the environment, *Chironomus* would be able to burrow and behave normally. This burrowing behaviour is adaptive and allows *Chironomus* to escape predation.

At the end of the experiment, all the sediments were sieved through a 250 µm mesh and survivors only were dried without allowing depuration. Sediment was probably present in the gut, affecting the dry weight, but all the treatments were the same in this respect. Individual weight increased with increasing Zn, Cu and Pb concentrations, this becoming significant ($P<0.05$) at higher concentrations (Figure 1), somewhat surprisingly since stressed animals might be expected to grow less or even lose weight. The same pattern was seen in the amphipod *Corophium volutator* in sediment bioassays (13,50,51). The results were also very similar in both permethrin and lindane inter-laboratory comparisons using *Chironomus* riparius (29,30). The increased mean weight with increasing concentration was stated as being due to decreased competition for food (30), but this explanation seems highly improbable. The density of *Chironomus* was much lower in the containers than in the field (personal observations). Bat and Raffaelli (50) suspected that smaller individuals are more susceptible at higher concentrations so that only heavier individuals remain alive at high concentrations. However, the low organic carbon content of the sediment (approx. 1.92%) appears to have had little effect on the growth of the *Chironomus* larvae.

This highlights a problem with the use of dry weight as a sublethal end point in bioassays. Only by recording the change in weight of known individuals and/or by standardising more tightly on initial sizes should the mean weight of individuals be used in this respect. Unless this is done, a difference in mean dry weight between treatments could reflect changes in the growth of individuals, size-selective mortality, or both, making interpretation of the results very difficult (51). However, this study shows that *Chironomus* is a very robust organism, capable of tolerating quite high levels of heavy metals.

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