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Comparative Toxicity of Nine Metals to Two Malaysian Aquatic Dipterian Larvae with Reference to Temperature Variation

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Abstract A study was conducted to determine the suitability of using selected aquatic dipterian larvae for biomonitoring bioassays. The organisms included a member of the biting midge family that was identified as *Culicoides furens* and a member of the non-biting midge family, identified as *Chironomus plumosus*. Median lethal toxicity tests were conducted to observe the variation between metal sensitivities between the two larval forms and how variations in temperature could affect the experimental setup. Nine heavy metals were used in the study. It was observed that the 96 h LC_{50} (in mg/L) for the different metals was found to be Zn-16.21 (18.55 ± 13.87); Cr-0.96 (1.08 ± 0.84); Ag-4.22 (6.87 ± 1.57); Ni-0.42 (0.59 ± 0.25); Hg-0.42 (0.59 ± 0.25); Pb-16.21 (18.31 ± 14.11); Cu-42.24 (45.18 ± 39.30); Mn-4.22 (7.19 ± 1.25); Cd-0.42 (0.59 ± 0.25) for the *Chironomus plumosus* and Zn-4.22 (6.56 ± 1.88); Cr-0.42 (0.54 ± 0.30); Ag-0.42 (0.54 ± 0.30); Ni-0.42 (0.54 ± 0.30); Hg-0.04 (0.07 ± 0.01); Pb-0.42 (0.54 ± 0.30); Cu-42.24 (45.18 ± 39.30); Mn-4.22 (6.56 ± 1.88); Cd-0.42 (0.54 ± 0.30) in the case of the *Culicoides furens*. With temperature as a variable the LC_{50} values were observed to increase from 2.51 mg/L at 10°C to 4.22 ppm at 30°C and to reduce slightly to 3.72 mg/L at 35°C as seen in the case of Zn. It was also observed that at 40°C thermal toxicity and chemical toxicity overlapped as 100% mortality was observed in the controls. This trend was observed in all metals for both *C. plumosus* and *C.* furens. Thus indicating temperature played an important role in determining LC_{50} values of toxicants.

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Studies on the Chironomus larvae have been conducted for the last 50 years (Rehwoldt et al. 1973; Anderson et al. 1980; Rao and Sexana 1981; Khangarot and Ray 1989; Griffiths 1991; Halpern et al. 2002). However in Malaysia very little information exists on the effects of metals or any toxicant for that matter on this genus. There is a dearth of information on the *Culicoides furens*, a sister form belonging to the biting midge family. The effects of metals on aquatic organisms have been the subject of numerous investigations (Khangarot et al. 1987; Leland and Kuwabara 1984; De Nicola et al. 1992). Studies have also shown that environmental factors play an important role in modifying the toxicity of metals (Cairns and Scheier 1958; Macinnes and Calabrese 1978, 1979; McLusky et al. 1986). To determine the necessary temperature regimes needed to conduct metal toxicity tests with reference to the Malaysian environment and laboratory conditions, extensive studies were conducted on the effects of temperature on the toxicity of nine metals to two dipterian larvae.

Materials and Methods

To study the effects of different metals on *Chironomous plumosus* and *Culicoides furens* larvae two definitive bioassays were conducted. The fist bioassay was a 96-h median lethal concentration test to determine the 96-h LC_{50} values for the different metals selected. The second bioassay again consisted of a 96 h LC_{50} but in this case,

temperature was a variable and tests were conducted at different fixed temperature values. Nine metal salts were selected for this investigation – Zinc Chloride, Chromium(III) chloride, Silver chloride, Nickel(II) chloride, Mercuric chloride, Lead(II) chloride, Copper(II) chloride, Manganese(II) chloride, and Cadmium(II) chloride. All metal salts were of analytical grade supplied by MERCK, Germany. 1,000 mg/L stock solutions were prepared for each metal.

A range-finding experiment was conducted to determine the appropriate concentrations of the nine different metals for further toxicity studies on *C. plumosus* and *C. furens*. The range-finding test consisted of a series of seven concentrations that differed by a factor of 10%. This was observed to be adequate to delineate the concentration range needed to establish test concentrations for 96 h LC_{50} experiments. All tests were conducted with three replicates and with control groups. In the case of lead chloride and silver chloride, the metals had to be dissolved in nitric acid, which was then normalized using sodium hydroxide. To observe the effect of HNO_3–NaOH interactions on the Chironomus larvae a 96-h run was conducted using these two chemicals in combination and run against a control solution of de-chlorinated tap water. No significant mortalities were observed in this test, indicating the HNO_3–NaOH interactions had no effect on the toxicity of these two metals. This was also conducted for *C. furens* bioassays. To study the effects of different metals on *C. plumosus* and *C. furens* larvae two definitive bioassays were conducted. The fist bioassay was a 96-h median lethal concentration test to determine the 96-h LC_{50} values for the different metals selected. The second bioassay again consisted of a 96 h LC_{50} but in this case, temperature was a variable and tests were conducted at different fixed temperature values. Test containers with volumes of 600 mL were selected for the execution of the different bioassays. Each chamber was filled with 400 mL of de-chlorinated water. To each test container, a single larva was randomly added, moving from one chamber to another, till each chamber had 10 larvae, each randomly selected to prevent any bias. For each metal six test containers were allocated, of which five were for the metal concentrations and one a control. Each bioassay had three replicates. Environmental parameters were monitored throughout the 96-h period. At the termination of each test, the mortality data was compiled and the 96-h LC_{50} values were calculated using the trimmed Spearman–Karber toxicity program. Animals were considered dead when all movement had ceased and the organisms exhibited nil response to gentle stimulation. This was carried out by gently touching the larvae with a glass rod and observing the effect of the stimulation on the organism. The concentrations tested in for *C. plumosus* and *C. furens* for the different metals are listed in Tables 1 and 2. Concentrations listed are the nominal values and measured values.

The effect of temperature on the 96-h LC_{50} of the nine different metals on the two dipterans was explored. This

Table 1 Concentration of the different metals used in the two bioassays conducted in this investigation using *C. plumosus*. The measured concentrations are in parenthesis

Metal	Concentrations utilized in bioassay (mg/L)				
$ZnCl_2$	3.2 (2.9 ± 0.5)	5.6 (5.4 ± 0.3)	10 (8.2 ± 0.5)	32 (30.0 ± 0.5)	56 (53 ± 2)
$CrCl_3$	0.32 (0.29 ± 0.9)	0.56 (0.49 ± 0.02)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)	10 (8.2 ± 0.5)
$AgCl_2$	1 (0.8 ± 0.2)	3.2 (3.0 ± 0.1)	5.6 (5.2 ± 0.3)	10 (8.3 ± 0.4)	32 (3.0 ± 1)
$NiCl_2$	0.1 (0.08 ± 0.01)	0.32 (0.29 ± 0.01)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
$HgCl_2$	0.1 (0.07 ± 0.01)	0.32 (0.29 ± 0.01)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
$PbCl_2$	3.2 (3.0 ± 0.2)	5.6 (5.4 ± 0.01)	10 (8.2 ± 0.5)	32 (3.0 ± 1)	56 (54.2 ± 0.2)
$CuCl_2$	10 (8.2 ± 0.5)	3.2 (2.9 ± 0.5)	56 (54.2 ± 0.2)	100 (98 ± 1)	132 (130 ± 1.5)
MnCl	1 (0.8 ± 0.1)	0.32 (0.29 ± 0.9)	5.6 (5.4 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
$CdCl_2$	0.1 (0.07 ± 0.0)	3.2 (2.8 ± 0.8)	5.6 (5.4 ± 0.01)	10 (8.2 ± 0.5)	32 (3.0 ± 1)

Table 2 Concentration of the different metals used in the two bioassays conducted in this investigation using *C. furens*. The measured concentrations are in parenthesis

Metal	Concentrations utilized in bioassay (mg/L)				
$ZnCl_2$	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)	5.6 (5.4 ± 0.01)	10 (8.2 ± 0.5)	32 (3.0 ± 0.5)
$CrCl_3$	0.1 (0.07 ± 0.0)	0.32 (0.29 ± 0.9)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
AgCl2	0.1 (0.07 ± 0.0)	0.32 (0.29 ± 0.9)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
$NiCl_2$	0.1 (0.06 ± 0.0)	0.32 (0.29 ± 0.9)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
$HgCl_2$	0.01 (0.009 ± 0)	0.032 (.028 ± 0)	0.056 (0.051 ± 0)	0.1 (0.07 ± 0)	0.32 (0.29 ± 0)
$PbCl_2$	0.1 (0.08 ± 0)	0.32 (0.29 ± 0.9)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)
$CuCl_2$	10 (8.2 ± 0.5)	32 (3.0 ± 0.5)	56 (54.2 ± 0.2)	100 (98 ± 1)	132 (129 ± 2)
MnCl	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)	5.6 (5.4 ± 0.01)	10 (8.2 ± 0.5)	32 (3.0 ± 0.5)
$CdCl_2$	0.1 (0.07 ± 0.0)	0.32 (0.29 ± 0.9)	0.56 (0.51 ± 0.01)	1 (0.8 ± 0.1)	3.2 (2.8 ± 0.8)

was to determine the optimal temperature at which these organisms can be studied under laboratory conditions and also to determine the effects of extreme temperature changes, due to failure of heaters or coolers, or if there is electricity failure resulting in elevated temperatures. The first experiment in this series, the 96-h LC_{50} experiments were conducted in an enclosed test room with the air-conditioners switched off. Studies on the temperature fluctuation of the room revealed that the room had a maximum temperature of 30°C and a minimum temperature of 24°C. The temperature of the water in the test containers fluctuated from 28°C to 25°C. This was to observe the effects of a total electrical failure in the laboratory during the execution of a median lethal toxicity test using the either *C. plumosus* or *C. furens* larvae.

The second experiment in this series, the median lethal toxicity experiments were again conducted in an enclosed test room, but in this case the air-conditioners were left on at setting of 22°C, which is the average setting of air-conditioners in labs in KUSTEM (personal observation). An observation of the diurnal variation in temperature of the room revealed that the maximum temperature reached in the course of a 24 h period was 28°C and the lowest temperature obtained was 18°C, the maximum temperature observed in the test chamber revealed that the water reached a temperature of 26°C and a minimum temperature of 20°C. This test was conducted to observe the 96 h LC_{50} values obtained from tests conducted in the absence of specific temperature control devises like aquarium heaters, cooling chambers or temperature control cabinets.

The third and final experiment in this series was the execution of 96-h LC_{50} experiments under conditions of fixed temperature. Nine temperatures were selected (10°C, 15°C, 20°C, 23°C, 25°C, 28°C, 30°C, 35°C, and 40°C). Temperature of 10°C, 15°C, and 20°C were maintained with the help of a temperature control cabinet, while the higher temperatures were maintained with the help of heating elements (Askol-IP68). Temperature fluctuations were observed to be ±0.5°C. For each temperature, a median lethal toxicity test was conducted for each metal for the two dipterian larvae. The tests conducted at the lower temperatures of 10°C, 15°C and 20°C were conducted to observe if thermal mortality would outweigh mortalities caused by metal toxicity. The second reason these low temperatures were selected was to observe how the organisms would respond if the test was conducted in cooler environments within Malaysia, or if the test room inadvertently became cooler due to the combined effect of the air-conditioner and low environmental temperatures caused by monsoons.

Higher temperature of 30°C, 35°C and 40°C were conducted again to observe the relationship between thermal mortality and metal mortality and also to study how the organisms would respond to high temperatures. When a median lethal toxicity test were conducted in the month of July, with the air-conditioners turned off, it was observed that the temperature in room had risen to 40°C by midday, the temperature in the water was 36°C. As this research is aimed at developing test protocols for the two dipterian larvae, it became necessary to conduct controlled tests at high temperatures to observe the effects on the organism and the toxicities of the metals. The static tests were monitored at test initiation for pH, hardness, alkalinity, conductivity, and dissolved oxygen, and every hour thereafter. Monitoring was also conducted hourly for mortality. Temperature was monitored continuously throughout the test periods. The response measured for the *C. plumosus* and *C. furens* larvae was mortality over the 96-h exposure period. The median lethal response (LC_{50}) was calculated using the Trimmed Spearman–Karber toxicity program.

Results and Discussion

The LC_{50} values of the nine metals to *C. plumosus* and *C. furens* were generated from the mortality data (Table 3) Exposure to different metals produced a varying LC_{50}

Table 3 Summery of 96 h LC_{50} values for different metals to which the *C. plumosus* and *C. furens* were exposed

Metal	C. plumosus			C. furens		
	28°C–25°C9	26°C–20°C	25°C	28°C–25°C	26°C–20°C	25°C
$ZnCl_2$	16.2 (17.5–14.9)	9.5 (10.9–8.3)	9.6 (10.8–8.2)	4.2 (5.3–3.1)	4.5 (5.6–3.4)	4.2 (5.3–3.1)
$CrCl_3$	0.9 (1.1–0.8)	1.0 (1.1–0.8)	0.9 (1–0.8)	0.4 (0.6–0.3)	0.3 (0.5–0.2)	0.4 (0.5–0.2)
$AgCl_2$	4.2 (5.4–3)	4.5 (5.7–3.3)	4.2 (5.4–3)	0.4 (0.6–0.3)	0.6 (0.7–0.5)	0.4 (0.5–0.2)
$NiCl_2$	0.4 (0.5–0.3)	0.4 (0.5–0.3)	0.4 (0.5–0.2)	0.4 (0.6–0.3)	0.5 (0.6–0.3)	0.4 (0.5–0.2)
$HgCl_2$	0.4 (0.6–0.2)	0.4 (0.5–0.2)	0.4 (0.6–0.2)	0.04 (0.05–0.03)	0.03 (0.04–0.02)	0.04 (0.05–0.03)
$PbCl_2$	16.2 (17.4–14.9)	8.3 (9.5–7)	9.5 (10.8–8.3)	0.4 (0.5–0.3)	0.3 (0.5–0.2)	0.4 (0.5–0.2)
$CuCl_2$	42.2 (47.7–36.7)	42.6 (48.1–37.1)	42.2 (47.7–36.7)	42.2 (47.7–36.7)	52.8 (58.3–47.3)	42.2 (47.3–36.7)
MnCl	4.2 (5.3–3.0)	4.5 (5.7–3.4)	4.2 (5.4–3)	4.2 (5.4–3.1)	3.8 (4.9–2.7)	4.2 (5.3–3.1)
$CdCl_2$	0.4 (0.6–0.2)	0.4 (0.6–0.2)	0.4 (0.6–0.2)	0.4 (0.6–0.2)	0.3 (0.6–0.2)	0.6 (0.8–0.4)

levels in the two organisms exposed, *C. furens* and *C. plumosus* (table 3). Comparing the effects of the nine metals on *C. furens* and *C. plumosus*, it was observed that *C. furens* was more sensitive to the metals then *C. plumosus* (Table 3). With regard to temperature variations, it was observed that higher and lower temperatures brought about increased toxicities (graph 1). When temperatures (Table 3) were allowed to fluctuate it was observed that temperature fluctuations entering the lower temperature levels (24–25) produced higher mortalities then those tested with a temperature fluctuation of (28–25) (graph 1), although with copper this trend was not observed. Exposure to a temperature of 40°C produced 90% mortality in the controls and 100% mortality in the spiked test containers within 36 h showing the threshold between metal toxicity and thermal toxicity. LC_{50} values could not be calculated from the organisms exposed to 40°C. Control concentrations showed no mortalities at other temperatures. Exposing the two dipterans to different temperatures ranging from 10°C to 40°C showed the effect of temperature on the lethal concentration of metals. The LC_{50} values of the different metals was observed to generally increase between the temperatures of 10°C and 25°C, decreases in LC_{50} values were observed in the higher temperature ranges of 28–40°C (Tables 4 and 5).

The results obtained from this investigation provide LC_{50} values, which differed from the values obtained by other researchers. Studies by Milani et al. (2003), found that the mean LC_{50} of copper was 0.043 mg/L to *C. riparius*. The values obtained in this study were lower then the toxicity values obtained by Khangarot and Ray (1989) in their investigation of the sensitivity of *Chironomus tentans*, indicating that the test organisms used in this investigation were more sensitive to metals. However in the case of silver, copper and zinc, the *C. plumosus* demonstrated a greater sensitivity to these metals. One possible reason for the increased sensitivity of the test organisms on this study and other similar studies, may be due to the observation that larvae without tubes are more sensitive to metals than are those living in sand or silt tubes (Halpern et al. 2002; Milani et al. 2003). The effect of temperature on the metal toxicity can be attributed towards the changes in the metabolism of the organism with the thermal changes, resulting in lower quantities of metals being absorbed into the tissues. Temperature changes would also cause changes to the water chemistry, which in turn would affect the toxicity of the metals (Eisler and Honnekey 1977). The *C. furens* demonstrated a greater sensitivity to metals, with an exception of copper, when compared to the *C. plumosus* larvae. A review of the literature indicates a dearth of data on the effects of metals on the Culicoides making comparison difficult. The increased sensitivity of the Culicoides larvae may correspond to the lower body

Table 4 Summary of 96 h LC_{50} data for *Culicoides furens* exposed to different heavy metals at different temperatures. LC_{50} values at 40°C could not be obtained due to thermal toxicity

Metal	Temp°C							
	35	30	28	25	23	20	15	10
$ZnCl_2$	3.7 (5.2–2.2)	4.2 (5.7–2.7)	4.2 (5.7–2.7)	4.2 (5.7–2.7)	3.7 (5.2–2.2)	3.4 (4.9–1.9)	2.5 (4.1–1.1)	2.5 (4–1)
$CrCl_3$	3.8 (5.3–2.3)	4.3 (5.8–2.8)	4.3 (5.8–2.8)	4.3 (5.8–2.8)	3.8 (5.3–2.3)	3.5 (5–2)	2.6 (4.2–1.2)	2.6 (4.1–1.1)
$AgCl_2$	34.1 (44.6–23.7)	42.2 (52.7–31.7)	42.2 (52.7–31.7)	42.2 (52.7–31.7)	37.1 (47.6–26.7)	34 (44.5–23.5)	34.1 (44.6–23.7)	25.6 (36.5–15.1)
$NiCl_2$	0.04 (0.05–0.03)	0.04 (0.05–0.03)	0.04 (0.05–0.03)	0.04 (0.05–0.03)	0.03 (0.04–0.02)	0.03 (0.04–0.02)	0.03 (0.04–0.02)	0.03 (0.04–0.02)
$HgCl_2$	0.4 (0.550.2)	0.4 (0.550.2)	0.4 (0.550.2)	0.4 (0.550.2)	0.6 (0.8–0.5)	0.3 (0.5–0.2)	0.3 (0.5–0.2)	0.3 (0.4–0.1)
$PbCl_2$	0.5 (0.6–0.3)	0.5 (0.6–0.3)	0.5 (0.6–0.3)	0.5 (0.6–0.3)	0.7 (0.9–0.6)	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.357 (0.51–0.21)
$CuCl_2$	0.7 (0.8–0.5)	0.7 (0.8–0.5)	0.7 (0.8–0.5)	0.7 (0.8–0.5)	0.9 (1.1–0.8)	0.6 (0.8–0.5)	0.6 (0.8–0.5)	0.5 (0.7–0.4)
MnCl	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.7 (0.8–0.5)	0.4 (0.5–0.2)	0.3 (0.5–0.2)	0.307 (0.45–0.16)
$CdCl_2$	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.4 (0.6–0.3)	0.7 (0.8–0.5)	0.4 (0.6–0.3)	0.4 (0.5–0.2)	0.3 (0.5–0.2)

Table 5 Summary of 96 h LC_{50} data for *Chironomus plumosus* exposed to different heavy metals at different temperatures. LC_{50} values at 40°C could not be obtained due to thermal toxicity

Metal	Temp°C							
	35	30	28	25	23	20	15	10
$ZnCl_2$	7.5 (9–6)	7.5 (9–6)	9.5 (11–8)	9.5 (11–8)	7.5 (9–6)	4.5 (6–3)	3.6 (5.1–2.1)	2.8 (4.3–1.3)
$CrCl_3$	3.7 (5.2–2.2)	3.7 (5.2–2.2)	4.2 (5.7–2.7)	4.2 (5.7–2.7)	3.7 (5.2–2.2)	3.4 (4.1–9.9)	3.4 (4.9–1.9)	2.5 (4–1)
$AgCl_2$	7.8 (9.4–6.2)	7.8 (9.4–6.2)	9.8 (11.4–8.1)	9.8 (11.4–8.1)	7.8 (9.4–6.2)	4.8 (6.4–3.2)	3.9 (5.5–2.3)	3.1 (4.7–1.5)
$NiCl_2$	4.1 (5.2–3)	4.1 (5.2–3)	4.6 (5.7–3.5)	4.6 (5.7–3.5)	4.1 (5.2–3)	3.8 (4.9–2.7)	3.8 (4.9–2.7)	2.9 (4.1–1.8)
$HgCl_2$	34.1 (37.6–29.1)	34 (37.5–30.5)	42.2 (45.7–38.9)	42.2 (45.7–38.9)	37.1 (40.6–33.9)	34 (37.5–30.5)	34.1 (37.63–306)	25.6 (29.5–22.7)
$PbCl_2$	0.7 (0.9–0.6)	0.7 (0.9–0.6)	0.9 (1.10–0.81)	0.9 (1.10–0.81)	0.7 (0.9–0.6)	0.6 (0.8–0.5)	0.6 (0.7–0.4)	0.5 (0.6–0.3)
$CuCl_2$	0.7 (0.9–0.6)	0.7 (0.9–0.6)	0.5 (0.6–0.3)	0.5 (0.6–0.3)	0.7 (0.9–0.6)	0.4 (0.5–0.2)	0.4 (0.5–0.2)	0.3 (0.5–0.2)
MnCl	0.6 (0.8–0.5)	0.6 (0.8–0.5)	0.4 (0.5–0.2)	0.4 (0.5–0.2)	0.6 (0.8–0.5)	0.3 (0.4–0.1)	0.3 (0.4–0.1)	0.2 (0.4–0.1)
CdC_l	0.6 (0.8–0.5)	0.6 (0.8–0.5)	0.4 (0.1–0.2)	0.4 (0.1–0.2)	0.6 (0.8–0.5)	0.3 (0.5–0.2)	0.3 (0.5–02)	0.3 (0.4–0.1)

mass of these larval worms which are on average less then 2 mm in length compared to the *C. plumosus* which average 5 mm in length. Studies on different aquatic organisms shows that size plays an important role in determining the sensitivity of metals to the test organism (Bandouin and Scoppa 1974; Rao and Saxana 1981; Eisler and Hennkey 1977).

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