Bull Environ Contam Toxicol (2008) 80:63–67
DOI 10.1007/s00128-007-9317-1

The Effect of Multi-generational Exposure to Metals and Resultant Change in Median Lethal Toxicity Tests Values Over Subsequent Generations

V. J. Vedamanikam · N. A. M. Shazilli

Received: 15 November 2006 / Accepted: 16 March 2007 / Published online: 6 December 2007

Abstract A study was conducted on the long term effects of nine heavy metals on the *Chironomus plumosus* and *Culicoides furens* larvae. This study tested the effect of the heavy metals on several generations of the larvae to observe the formation of increased hardiness against pollutants present within the aquatic habitat. From this study it was observed that susceptibility or sensitivity to heavy metals decreased with LC50 values becoming larger indicating a decreased toxicity level. Significant variations ($p < 0.05$) were observed between first generation and third generation culicoides for all metals and at all concentrations. Variations between third and fourth generation culicoides were also significantly different ($p < 0.05$) with the exception of chromium at 25°C and nickel and lead at every temperature range group. The variation between all generations 4, 5 and 6 was found to be insignificant ($p > 0.05$). This would indicate that metal tolerance would have occurred in these generations and the effect of metals was less toxic to the culicoides. Generation 9 was found to have LC50 values ($p > 0.05$) the same as the LC50 values obtained in third generation culicoides. Thus it would appear that heavy metal resistance was developed when the organisms were exposed to prolonged exposure of the heavy metals but was lost when the organisms were bred in non-contaminated water.

Keywords Chironomids · Culicoides · Heavy metals · LC50 · Toxicity · Multi-generation

V. J. Vedamanikam (✉) · N. A. M. Shazilli
University College of Science and Technology (KUSTEM), Mengabang Telipot, 21030 Kuala Terengganu, Malaysia
e-mail: vyk_diamond@yahoo.com

Members of the order Diptera, or true flies, are especially good bioindicators of aquatic environmental conditions because, in addition to the attributes of other aquatic insects, they occupy the full spectrum of habitats and. Aquatic dipterians are among the most prolific animals on earth, but are highly specialized and represent less than 1% of the total animal diversity (Pennak 1978). Aquatic insects are excellent overall indicators of both recent and long-term environmental conditions (Patrick and Palavage 1994). The immature stages of aquatic insects have short life cycles, often several generations a year, and remain in the general area of propagation. Thus, when environmental changes occur, the species must endure the disturbance, adapt quickly, or die and be replaced by more tolerant species. These changes often result in an overabundance of a few tolerant species, and the communities become destabilized or "unbalanced." Studies have shown that macroinvertebrates from contaminated sites are more resistant to metals then those that were obtained from clean sites (Klerks and Weis 1987). Studies have shown that an increased tolerance to metals can occur within a few generations. This study was conducted to determine the effects of long term exposure to sub-lethal concentrations of heavy metals on two dipterian larvae and the development of metal tolerance in the two test organisms.

Methodology

A range-finding experiment was conducted to determine the appropriate concentrations of the nine different heavy metals for further toxicity studies on *Chironomus plumosus* and *Culicoides 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 the 96 h LC50 experiments. All tests were conducted with three replicates and with control groups. Tests were carried out in 600 mL plastic beakers. 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 chironomids 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, one control mortality out of 30 individuals was noted, indicating the HNO_3–NaOH interactions had no effect on the toxicity of these two metals. This was also conducted for the *Culicoides 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 LC50 values for the different metals selected. Nine metals were selected for this investigation – zinc, chromium, silver, nickel, mercury, lead, copper, manganese, and cadmium. All metal salts were of analytical grade supplied by MERCK, Germany. About 1,000 ppm stock solutions were prepared for each metal. 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 tap water. To each test container, a single larvae 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 LC50 values were calculated using the trimmed Spearman–Karber toxicity program. Animals were considered dead when all movement had ceased and the organisms exhibited no 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 Table 1. Concentrations are nominal values.

Insects are extremely adaptable and develop resistance to chemicals when exposed to toxicants over a long period of time. Median lethal toxicity tests were conducted using different consecutive generations of the two test organisms to study the development of resistance to metals. A series of nine generations of *C. plumosus* and *C. furens* were reared in culture chambers containing metal contaminated water. The level of contamination was equal to 0.3 of the 96 h LC50 value, or 0.3 toxic units of the 96 h LC50 value. The two dipterian larvae were then allowed to breed and produce second generation larvae. A selection of second generation larvae were then removed from the culture tank and a 96 h LC50 experiment was conducted using the concentrations and methodology used in the median lethal toxicity test described above. The 96 h LC50 value was calculated from the mortality data generated from this experiment. From the breeding tank the second generation was bred to produce the third generation and other subsequent generations and from each generation a sample was taken and a median lethal toxicity test was conducted. In total nine generations were used in this experiment. However, the offspring of the sixth generation were removed from the contaminated culture tanks and bred in clean water. This was done to observe if resistance gained was lost when the organism was placed in a clean environment. No 96 h LC50 experiments were conducted on the seventh and eighth generations and they were permitted to just breed and grow in the clean water. The nineth generation was again tested in a median lethal toxicity test to observe how the toxicity of the metals was affected. Statistical analysis of the data was conducted following the methods followed by Sánchez et al. (2004). Data from the different generations studied of *C. plumosus* and *C. furens* were analyzed using analysis of variance (ANOVA) to detect significant differences between treated groups and control, followed by Duncan test ($p < 0.05$) with a SPSS

Table 1 Concentrations of the different metals used in the two bioassays conducted in this investigation

Metal	Concentrations utilized in bioassay (ppm) – *C. plumosus*				
Zn	3.2	5.6	10	32	56
Cr	0.32	0.56	1	3.2	10
Ag	1	3.2	5.6	10	32
Ni	0.1	0.32	0.56	1	3.2
Hg	0.1	0.32	0.56	1	3.2
Pb	3.2	5.6	10	32	56
Cu	10	32	56	100	132
Mn	1	3.2	5.6	10	32
Cd	0.1	0.32	0.56	1	3.2
	Concentrations utilized in bioassay (ppm) – *C. furens*				
Zn	1.0	3.2	5.6	10	32
Cr	0.1	0.32	0.56	1	3.2
Ag	0.1	0.32	0.56	1	3.2
Ni	0.1	0.32	0.56	1	3.2
Hg	0.01	0.032	0.056	0.1	0.32
Pb	0.1	0.32	0.56	1	3.2
Cu	10	32	56	100	132
Mn	1	3.2	5.6	10	32
Cd	0.1	0.32	0.56	1	3.2

Concentrations followed the geometric breakdown method

computer program (Hull and Nie 1981). Results among generations were compared using Student's t test ($p < 0.05$).

Results and Discussion

From the mortality data obtained from the different experiments conducted in this study the 96 h LC50 values were determined. Mortalities in the different treatments were observed to occur at the higher concentrations in both test organisms. Culicoides exposed to zinc were observed to have an 96 h LC50 value of 4.54 mg/L in the first generation, in the second generation the 96 h LC50 value had become lower (3.84 mg/L) and decreased in toxicity to 6.11 mg/L in the third generation and subsequent generations had 96 h LC50 values of 9.56 mg/L of zinc. When the organisms was reared in clean water for two generations (generation 7 and 8) and introduced to spiked water again in the nineth generation it was observed that the 96 h LC50 value had increased to 6.11 mg/L, a value observed in third generation Culicoides. This would indicate that the resistance to the metals was lost when the organism was permitted to grow and breed in clean water for two generations (Tables 2, 3). This trend was observed for all metals with the exception that in the second generation the toxicity of the metals became less toxic to the organisms unlike the case observed with zinc. This trend was also observed in *C. plumosus* larvae that were exposed to the nine metals with the exception that with zinc there was no decrease in the 96 h LC50 value, but the value increased steadily from one generation to another without becoming more toxic to the larvae as was observed in the Culicoides.

An observation of the two test organisms over the course of the nine generations indicated their behavior was similar to those observed in *C. plumosus* and *C. furens* in the 96 h LC50 experiment. However it should be noted that the long term exposure to the metals produced organisms that were smaller in size compared to the parents, while the parents, in the case of the chironomids, were approximately 1 cm (±0.05 cm) in length the offspring in the third and fourth generation were 0.8 cm (±0.05 cm) and the seventh generation were 0.5 cm (±0.05 cm) in length. Statistical analysis of the body length data indicated that the changes in body length between the different generations was statistically significant ($p < 0.05$). It was also observed that the long exposure period altered the blood red colour of the chironomids to a dull brown, but when the chironomids were bred in clean water they regained their blood red coloration. In the case of the Culicoides, it was observed that no pigmentation change occurred and size was not altered. However the breeding cycle increased from 46.6 h (at 28°C) to 98.4 h a change that was observed to be statistically significant ($p < 0.05$). Mortality responses and behavior of both organisms when exposed to the test chamber and the test solutions remained the same.

Long term, multigenerational exposure of the chironomids and culicoides showed that the LC50 value increased indicating decreased toxicity and development of tolerance. Significant variations ($p < 0.05$) were observed between first generation and third generation culicoides for all metals and at all concentrations with the exceptions caused by temperature interactions as mentioned in earlier in this chapter. Variations between third and fourth generation culicoides were also significantly different ($p < 0.05$) with the exception of chromium at 25°C and nickel and lead at every temperature range group. The variation between all generations 4, 5 and 6 was found to be insignificant ($p > 0.05$). This would indicate that metal tolerance would have occurred in these generations and the effect of metals was less toxic to the culicoides. Generation 9 was found to have LC50 values ($p > 0.05$) the same as the LC50 values obtained in third generation culicoides. This trend was observed in all nine metals. This was probably due to loss of tolerance to the metals when the organisms were bred in clean water. Studies by Postma and Davids (1995) agree with the findings of this study.

Among the chironomids, there was no significant variation in LC50 value among generation 1 and 2 chironomids, significant variations ($p < 0.05$) were observed between generation 2 and 3 chironomids and also with generation 4 chironomids. Generations 4–6 was not significant ($p > 0.05$) and indicated the evolution of a tolerance threshold. It was also noted that generation 9 chironomids produced LC50 values which were significantly different ($p < 0.05$) to all other generations, it was noted to fall between the LC50 values observed among generation 2 and 3 LC50 values. This may be due to loss of tolerance to the metals when the organisms were bred in an environment without metals. Similar results were observed by Postma and Davids (1995) who reared *C. riparius* obtained from metal polluted sites in clean water and then tested their tolerance to metals. He observed that the median lethal toxicity values obtained were lower then values obtained from organisms taken directly from the polluted sites in the first generation of exposure. Tolerance to the metals was observed to evolve in the second generation and found to reach a peak in the third an subsequent generations when LC50 values no longer fluctuated significantly. Multigenerational studies on other aquatic microfauna like *Daphnia magna*, *Limnodrilus hoffmeisteri* and *Tisbe holothuriae* showed a similar development of tolerance to metals (Münzinger and Monicelli 1992).

As the *C. plumosus* and *C. furens* were reared in spiked water, it is comparable to studies conducted on metal tolerance from organisms obtained from polluted and non-polluted sources. This study would indicate that

Table 2 Summary of 96 h LC50 data for *C. furens* exposed to different metals for nine generations

Metal	Cd	Mn	Cu	Pb	Hg	Ni	Ag	Cr	Zn
G1	0.3	3.8	52.8	0.3	0.03	0.5	0.6	0.3	4.5
	0.5 ± 0.2	5.9 ± 1.7	55 ± 50.7	0.5 ± 0.2	0.1 ± 0.02	0.6 ± 0.4	0.7 ± 0.5	0.5 ± 0.2	6.6 ± 2.4
G2	0.3	3.8	38.4	0.4	0.04	0.6	0.6	0.3	3.8
	0.5 ± 0.2	5.9 ± 1.7	40.5 ± 36	0.5 ± 0.2	0.1 ± 0.03	0.7 ± 0.4	0.7 ± 0.4	0.5 ± 0.2	5.9 ± 1.7
G3	0.6	6.1	55.3	0.6	0.06	0.6	0.6	0.6	6.1
	0.7 ± 0.48	8.2 ± 3.9	57.5 ± 53	0.7 ± 0.4	0.1 ± 0.05	0.7 ± 0.4	0.7 ± 0.4	0.7 ± 0.4	8.2 ± 3.97
G4	0.9	9.5	55.3	0.9	0.10	0.9	0.9	0.9	9.5
	1.1 ± 0.8	11.7 ± 7.4	57.5 ± 53	1.1 ± 0.8	0.1 ± 0.09	1.1 ± 0.8	1.1 ± 0.8	1.1 ± 0.8	11.7 ± 7.4
G5	1.0	9.5	55.3	1.0	0.11	1.0	1.0	1	9.5
	1.2 ± 0.9	11.7 ± 7.4	57.5 ± 53	1.1 ± 0.9	0.12 ± 0.1	1.1 ± 0.9	1.1 ± 0.9	1.1 ± 0.9	11.7 ± 7.4
G6	1.0	9.5	55.3	1.0	0.11	1.0	1.0	1	9.5
	1.2 ± 0.9	11.7 ± 7.4	57.5 ± 53	1.1 ± 0.9	0.12 ± 0.1	1.1 ± 0.9	1.1 ± 0.2	1.1 ± 0.9	11.7 ± 7.4
G9	0.5	6.1	48.7	0.4	0.10	0.5	0.4	0.5	6.1
	0.6 ± 0.3	8.2 ± 3.9	50.9 ± 46	0.6 ± 0.3	0.11 ± 0.1	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	8.2 ± 3.9
Statistical comparison between generations	$R^2 = 0.3$	$R^2 = 0.4$	$R^2 = 0.06$	$R^2 = 0.2$	$R^2 = 0.8$	$R^2 = 0.1$	$R^2 = 0.3$	$R^2 = 0.03$	$R^2 = 0.3$
	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Generation 7 and 8 were placed in clean water while generation 9 was again exposed to metals

Table 3 Summary of 96 h LC50 data for *C. plumosus* exposed to different metals for nine generations

Metal	Cd	Mn	Cu	Pb	Hg	Ni	Ag	Cr	Zn
G1	0.4	4.5	42.6	8.2	0.3	0.4	4.5	1	9.5
	0.5 ± 0.3	6.6 ± 2.4	44.7 ± 40	8.4 ± 8.1	0.4 ± 0.3	0.5 ± 0.2	3.9 ± 3.7	0.9 ± 0.7	11.7 ± 7.4
G2	0.4	3.8	38.4	9.5	0.3	0.3	3.8	0.8	9.5
	0.5 ± 0.3	5.9 ± 1.7	40.5 ± 36	9.7 ± 9.4	0.4 ± 0.3	0.5 ± 0.2	3.9 ± 3.7	0.9 ± 0.7	11.7 ± 7.4
G3	0.9	6.1	55.3	13.5	0.6	0.6	6.1	1.4	13.5
	1.1 ± 0.8	8.2 ± 3.9	57.5 ± 53	13.7 ± 13	0.6 ± 0.5	0.7 ± 0.4	6.2 ± 5.9	1.5 ± 1.3	15.7 ± 11
G4	0.9	9.5	55.3	20.5	0.9	0.9	9.5	2.3	20.5
	1.1 ± 0.8	9.7 ± 9.4	57.5 ± 53	20.7 ± 20	0.9 ± 0.9	1 ± 0.8	9.7 ± 9.4	2.5 ± 2.2	22.7 ± 18
G5	1	9.5	55.3	22	1.05	1	9.5	2.7	22
	1.1 ± 0.9	9.7 ± 9.4	57.5 ± 53	22.1 ± 21	1.06 ± 1	1.1 ± 0.9	9.7 ± 9.4	2.8 ± 2.6	24.1 ± 19
G6	1.2	12.4	99.6	27.5	1.2	1.2	12.4	3.6	27.5
	1.3 ± 1.1	12.5 ± 12	101 ± 97	27.6 ± 27	1.3 ± 1.2	1.3 ± 1.1	12.5 ± 12	3.8 ± 3.5	29.6 ± 25
G9	0.4	5.6	48.7	10.5	0.4	0.4	5.6	1	10.5
	0.6 ± 0.3	7.7 ± 3.5	50.9 ± 46	10.6 ± 10	0.5 ± 0.4	0.6 ± 0.3	5.7 ± 5.5	1.1 ± 0.9	12.6 ± 8.3
Statistical comparison between generations	$R^2 = 0.1$	$R^2 = 0.3$	$R^2 = 0.2$	$R^2 = 0.2$	$R^2 = 0.2$	$R^2 = 0.2$	$R^2 = 0.3$	$R^2 = 0.2$	$R^2 = 0.2$
	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Generation 7 and 8 were placed in clean water while generation 9 was again exposed to metals

dipterian larvae reared in contaminated water would be less affected by pollution than organisms originating in clean waters. This would be due to the development of tolerance to the pollutants from the breeding site. Studies by Wentsel et al. (1978) found similar trends that support this finding. In contrast, studies on the *Tanypus neopunctipennis* (Klerks and Levinton 1993) found no differences in tolerance between larvae obtained from contaminated sources and those obtained from clean sources. Tolerance development may be a result of physiological changes resulting in lowered metabolic rates, or may be due to the development of metallothionein-like proteins (Krantzburg and Stokes 1989). Studies also indicate that exposure to pollutants may alter the life history of the organism and this in turn could have an adaptive importance. Life history changes would require the expenditure of more metabolic energy and may cause a

slowing down of other activities, which would result in changes or reductions in the population growth rate and reduction in the size of individuals (Maltby 1991; Posthuma et al. 1993). Very little information is available on the effects of life history responses to tolerance development to pollutants. However observations from this study indicated that organisms reared in contaminated water produced larval populations comparable to those reared in clear water. It must however be noted that that the time duration for the organisms to complete the life cycle increased from 6 to 13 days in the case of the *C. plumosus* and from 10 to 17 days in the case of the *C. furens*. These changes were observed to occur slowly over several generations. Studies by Postma and Davids (1995) were found to agree with the findings in this study, and the exact mechanism by which this happens is not understood, but may be due to a slowing down of the metabolic rate.

References

Klerks PL, Levinton JS (1993) Evolution of resistance and changes in community composition in metal-polluted environments: a case study on Foundry Cove. In: Dallinger D, Rainbow PS (eds) Ecotoxicology of metals in invertebrates. Lewis Publishers, Boca Raton, pp 223–241

Klerks PL, Weis JS (1987) Genetic adaptation to metals in aquatic organisms. A review. Environ Pollut 45:173–204

Krantzberg G, Stokes PM (1989) Metal regulation, tolerance and body burdens in the larvae of the genus Chironomus. Can J Fish Aquat Sci 46:389–398

Maltby L (1991) Responses of *Gammarus pulex* (Amphipoda, Crustacea) to metalliferous effluents identification of toxic components and the importance of interpopulation variation. Environ Pollut 84:45–52

Münzinger A, Monicelli F (1992) Metal co-tolerance in a chromium tolerant strain of *Daphnia magna*. Aquat Toxicol 23:203–216

Hull CH, Nie NH (1981) SPSS update 7-9. McGraw Hill Book Co., New York, pp 237–239

Patrick R, Palavage DM (1994) The value of species as indicators of water quality. Proc Acad Nat Sci Philadelphia 145:55–92

Pennak RW (1978) Fresh-water invertebrates of the United States, 2nd edn. Wiley, New York, p 803

Posthuma L, Hogervorst RF, Joose ENG, van Straalan NM (1993) Genetic variation and covariation for characteristics associated with cadmium tolerance in natural populations of the springtail *Orchesella cincta* (L). Evolution 47:619–631

Postma JF, Davids C (1995) Tolerance induction and life cycle changes in cadmium-exposed *Chironomus riparius* (Diptera) during consecutive generations. Ecotoxicol Environ Saf 30:195–202

Sánchez M, Andreu-Moliner E, Ferrando MD (2004). Laboratory investigation into the development of resistance of *Daphnia magna* to the herbicide molinate. Ecotoxicol Environ Saf 59:316–323

Wentsel R, McIntosh A, Anderson V (1978) Sediment contamination and benthic macroinvertebrate distribution in a metal-impacted lake. Environ Pollut 14:187–193