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Comparative Tolerance of Three Populations of the Freshwater Shrimp *(Paratya australiensis)* to the Organophosphate Pesticide, Chlorpyrifos

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Toxicity testing is an important tool in hazard assessment and environmental management (Parrish 1984). Laboratory cultured and field collected animals are often used in toxicity testing. The source of these animals is important because previous exposure to toxicants over an extended period can give rise to resistance as is often seen in populations of insect pests (Hemmingway *et al.* 1985, Bisset *et al.* 1990). Insecticide resistance has been observed in several nontarget organisms such as copepods, fish, frogs and crayfish (Naqvi and Ferguson 1968; Mowbray 1979). Populations of nontarget organisms in agricultural and urban areas are frequently exposed to pesticides as a result of spray drift from agricultural areas and pest control activities (Naqvi and Ferguson 1968). With such exposure, more resistant genotypes are selected for, while less resistant ones are eliminated (Chuiko and Slynko 1995). The responses of such individuals to various toxicants may therefore not be representative of the general response of the species and threshold values determined on such populations may not adequately protect the species.

In Australia, chlorpyrifos is an important organophosphate pesticide (OP) used for termite and cockroach control in urban areas and in the pest management program in cotton and sugarcane industries (Hughes *et al.* 1991). This pesticide achieves its effect by inhibiting activity of acetylcholinesterase (AChE), an enzyme important in neuro transmission (Abdullah *et al.* 1993). This paper discusses the toxicity of chlorpyrifos to three populations of the freshwater shrimp *(Paratya australiensis)* to determine whether the populations exhibit differential sensitivities to the pesticide as a result of previous exposure to pollutants. In addition, this paper attempts to relate AChE inhibition to the development of tolerance.

MATERIALS AND METHODS

Shrimps were collected from the Upper Colo River, South Creek and Parramatta Lake, all within 65 km NW of Sydney, NSW, Australia. The Upper Colo River and South Creek are part of the Hawkesbury-Nepean River system while Parramatta Lake is part of the Parramatta River which drains into Sydney harbour. The catchment of the Upper Colo River is mainly state forest and is regarded as an 'unpolluted site'. South Creek, on the other hand, is impacted as a result of urbanization and industrial activity in the Hornsby catchment, while Parramatta Lake catchment is principally urbanized. These two sites are regarded as 'polluted

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sites'. Animals collected from these sites were acclimated for at least 10 days in 50 L tanks containing filtered Sydney mains water before subjected to chlorpyrifos treatment. They were provided with stones to give shelter and fed with fish flakes twice a day.

Toxicity testing was carried out in a Labec environmental cabinet set at 24±1.0°C. Animals were exposed to a light regime of 16 hr light and 8 hr dark during the tests. In all tests, to prevent cannibalism, shrimps were isolated in 20 cm glass tubes (3 cm in diameter) and acclimated for 3 hr before they were transferred to 2 L beakers containing the test solution. Each experiment consisted of three replicates of five to six concentrations of chlorpyrifos. Five animals were used in each replicate and toxicity testing for each population was carried out in triplicate. All test solutions were gently aerated to maintain oxygen levels and test solutions were renewed every 24 hr. Acetone solvent controls were also carried out; the volume of solvent used never exceeded 0.05 % in the highest concentration of the test solutions. Mortality and water quality parameters were recorded every 24 hr. Temperature and pH of the test solutions were measured daily before and after solution renewal using a microcomputer pH-VISION 6007 pH meter, dissolved oxygen using a WTW OXI 92 DO meter and conductivity using an FE 280 conductivity meter. Shrimps were exposed to chlorpyrifos for 96 hr.

Chlorpyrifos concentrations used in the toxicity tests to determine 24,48, 72 and 96 hr LC50 values varied between 0.04 to 0.5 ppb. The LC50 value was determined using the trimmed Spearman-Karber method (Hamilton *et al.* 1977, 1978). Statistical estimations of NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values were determined using the Bonferroni test in the TOXSTAT 3.3 package (Gulley *et al.* 1991).

Toxicity tests for which AChE analyses on the shrimps were carried out after exposure, used chlorpyrifos concentrations ranging from 0.005 to 0.5 ppb. Comparisons across the three shrimp populations were made by using same concentration intervals but highest concentrations used were 0.2, 0.25 and 0.5 ppb for shrimps from the Upper Colo River, South Creek and Parramatta Lake, respectively. Three surviving animals from each test solution were pooled and immediately analyzed for AChE. Analysis of AChE was done using the method of Ellman (Ellman *et al.* 1961) and enzyme activity was expressed relative to the amount of protein present which was determined using the Lowry method (Lowry and Rosebrough 1951). All spectrophotometric measurements were duplicated and the average change in absorbance per minute determined. AChE activity is presented as microunits per gram protein µU/g protein). A unit is regarded as the conversion of one mole of substrate to products in one minute. Percent inhibition was calculated relative to the mean value for control shrimps assayed.

The amounts of chlorpyrifos in the test solutions were measured before and after solution renewal to determine losses of the pesticide over 24 hr. For concentrations below 0.1 ppb, 700 mL of the aqueous solution was used for extraction while for samples above 0.1 ppb, 500 mL was used. Water samples were extracted twice using pesticide grade dichloromethane (100 mL), dried over anhydrous Na_2SO_4 and evaporated to 1 mL under N_2 atmosphere. Chlorpyrifos was quantified using a Hewlett-Packard 5890 gas chromatograph, with an HP 7673 auto sampler, splitless injector and an electron capture detector (ECD). One pL of the sample was injected into an HP-5 capillary column (30m long x 0.25mm x 0.32mm i.d.) using hydrogen as the mobile phase with a flow rate of 2 mL/min. The detector

temperature was 250°C. Oven temperature programming was used, initially at 100°C maintained for 1 minute, then raised at the rate of 3°C/min to 275°C where it remained for 0.5 min. Recovery of chlorpyrifos-spiked water samples was above 80%.

RESULTS AND DISCUSSION

The water quality parameters did not vary greatly during the test. The median pH was 7.35 (range 6.70-7.50) and the measured conductivity was 170±5 µS/cm. Dissolved oxygen remained above 75% (median 80%). The mean temperature of the test solutions was 23.0±1°C. Average measured initial concentrations of chlorpyrifos were close to nominal values (Table l), while average measured values after 24 hr were between 40 to 80% of the nominal values. Losses of chlorpyrifos can be attributed to absorption by the animals and degradation in the water as a result of hydrolysis, as well as adsorption onto glass surfaces of both test containers and isolation tubes used.

Triplicate LC50 values were determined for the different populations of the shrimp (Table 2). Shrimps collected from the Upper Colo River were more sensitive than shrimps from either South Creek or Parramatta Lake. The degree of sensitivity of the three populations of shrimps decreased in the order: Upper Colo River > South Creek > Parramatta Lake. No data on the acute LC50 of chlorpyrifos to *P. australiensis* is available in the literature. However, Krassoi *(pers. comm.)* obtained an LC50 value of 0.2 ppb for Parramatta Lake shrimps which was slightly lower than the values we obtained. The most sensitive species of shrimp recorded so far is the mysid *Mysidopsis bahia* which has a 96 hr LC50 value of 0.035 ppb (Marshall & Roberts 1978). The NOEC and LOEC values for the three populations were also significantly different from each other ($p < 0.001$) with shrimps from the Upper Colo River being the most sensitive population followed by populations from South Creek and then from Parramatta Lake. In the absence of chronic data, these NOEC and LOEC values can be useful in deriving water quality criteria.

Table 1. Nominal and average measured concentrations of chlorpyrifos.

Nominal	Concentration (ppb)			
	Measured			
	Initial	% Nominal	After 24 hr	% Nominal
0.005	0.005±0.002	100	0.004 ±0.002	80
0.01	0.011±0.001	110	0.006±0.001	60
0.02	0.020±0.014	100	0.012±0.001	60
0.04	0.037±0.018	93	0.023±0.004	58
0.07	0.063±0.004	90	0.042±0.015	60
0.09	0.075±0.007	84	0.048±0.005	53
0.12	0.115±0.013	97	0.060±0.001	50
0.15	0.145±0.021	97	0.067±0.008	45
0.20	0.165±0.021	83	0.085±0.004	43
0.25	0.252±0.030	101	0.120±0.034	48
0.35	0.311±0.020	89	0.14±0.010	40
0.50	0.421±0.020	84	0.200±0.004	40

Table 2. LC50, NOEC and LOEC values for *P. australiensis* from three sites exposed to chlorpyrifos for 96 hr.

Site	Replicate no.	LC50 (95% confidence limits) (ppb)	NOEC (ppb)	LOEC (ppb)
Colo River	1	0.08 (0.07-0.09)	0.04	0.07
	2	0.08 (0.07-0.09)	0.04	0.07
	3	0.10 (0.08-0.13)	0.04	0.07
South Creek	1	0.14 (0.13-0.16)	0.09	0.12
	2	0.15 (0.13-0.16)	0.09	0.12
	3	0.15 (0.12-0.18)	0.09	0.12
Parramatta Lake	1	0.28 (0.25-0.32)	0.20	0.25
	2	0.28 (0.26-0.31)	0.20	0.27
	3	0.25 (0.20-0.29)	0.20	0.15

Mortality was recorded every 24 hr and LC50 values were computed at 24, 48, 72 and 96 hr intervals. For shrimps from South Creek and Parramatta Lake, only 72 and 96 hr LC50 values could be calculated as significant death occurred only after 72 hr (Figure 1), while LC50 values for shrimps from the Upper Colo River were calculated over all the time intervals. These results suggest that in shrimps from the Upper Colo River, the toxicant achieved its effect faster than in the other two populations. Figure 2 shows the probit mortality in the three populations. The regression lines for the populations from Parramatta Lake and South Creek are shifted to the right of the line for the population from the Upper Colo River indicating the possibility of the development of tolerance.

One possible reason for the increased tolerance to chlorpyrifos of shrimps from Parramatta Lake and South Creek relative to shrimps from the Upper Colo River was that the shrimps from the Upper Colo River were more prone to environmental stress than the other two populations. The greater tolerance of the two former populations was presumed to be due to previous exposure to pollutants including pesticides. Since South Creek is polluted as a result of industrialization and urbanization in its catchment, it is presumed that contamination of the aquatic system could have resulted, causing the shrimp population to evolve some kind of tolerance to a variety of chemicals including pesticides. The area around Parramatta Lake, on the other hand, is one of the areas of early European settlement and has been impacted by human activities for a long time. Since the duration of possible contamination is longer in Parramatta Lake compared with South Creek, it is possible that the differences in the LC50 values of populations between South Creek and Parramatta Lake could be due to differences in duration of exposure to various pollutants. Differences in tolerance of mosquito fish *Gambusia affinis* from Mississippi and the Namoi valley to several pesticides was attributed to a shorter history of exposure in the latter (Mowbray 1979). Or perhaps some additional unknown mechanism is at work to contribute to the observed differences in LC50 values between Parramatta Lake and South Creek.

AChE activity in control and exposed shrimps was measured and the results are given in Table 3. One way analysis of variance (ANOVA) showed significant differences in AChE activity at different concentrations for all populations of shrimp. For shrimps from the Upper Colo River, Bonferroni's t test showed

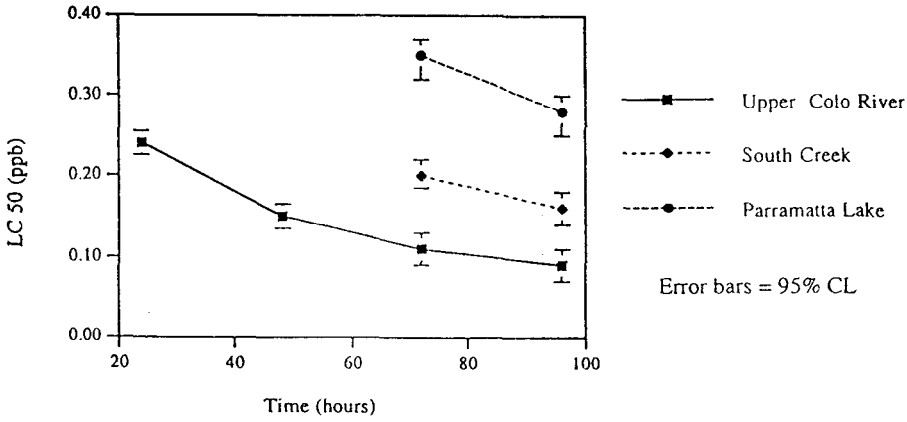

Figure 1. LC50 values for the three different populations of the shrimp *P. australiensis* exposed to chlorpyrifos.

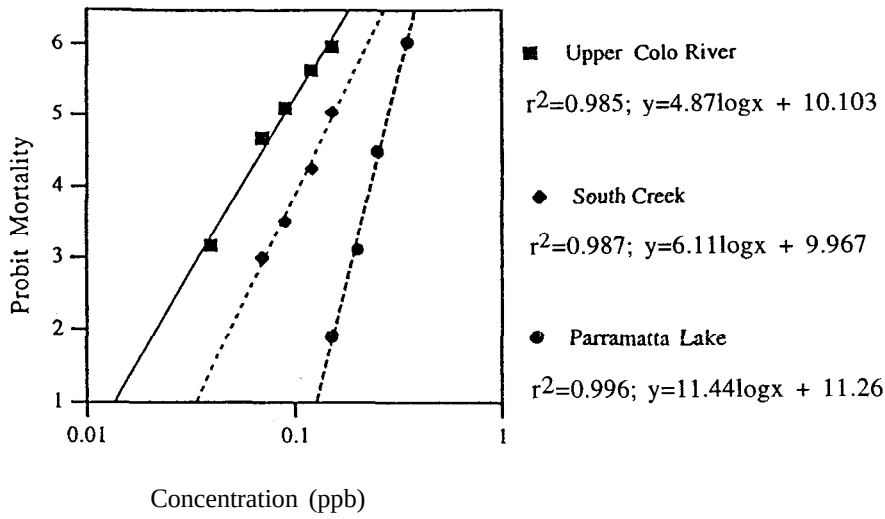

Figure 2. Toxicity of chlorpyrifos to three populations of the shrimp *P. australiensis* exposed to chlorpyrifos.

significant differences between AChE activity at 0.01 ppb chlorpyrifos compared with the controls ($p<0.01$), while activity at 0.005 ppb did not show significant difference with control ($p>0.01$). These gave NOEC and LOEC values of 0.005 and 0.01 ppb, respectively, and corresponding to 17% and 25% inhibition of AChE activity. Shrimps from the Upper Colo River had the highest inhibition level with

Table 3. Mean AChE activity in control and chlorpyrifos exposed shrimps from the three sites

Chlorpyrifos	Acetylcholinesterase activity (mmol/min/g protein)					
conc (ppb)	Colo River	% inhibition	South Creek	% inhibition	Parramatta Lake	% inhibition
Control	21.14±1.67	-	30.91±1.97	-	30.77±0.38	-
0.005	17.59±1.30	17	28.48±3.02	8	29.39±0.54	5
0.010	15.80±2.71	25	28.80±3.68	7	28.84±1.50	6
0.020	14.74±3.65	30	28.78±0.92	7	28.42±0.79	8
0.040	13.01±2.77	39	25.11±0.68	19	28.73±0.51	7
0.070	9.54±0.81	55	24.50±4.41	21	27.39±0.90	11
0.090	8.35±0.63	61	21.02±1.75	32	23.92±1.60	22
0.120	5.84±0.98	72	20.20 ±2.80	35	17.45±1.28	43
0.150	4.90±1.95	77	11.91±1.78	62	14.97±1.52	51
0.200	-	-	10.89±2.19	65	11.95±3.13	61
0.250	-	-	-	-	10.51±1.56	66
0.350	-	-	-	-	8.57±0.22	72

surviving shrimps having up to 77% inhibition after exposure to 0.15 ppb of chlorpyrifos. AChE activity in these shrimps was significantly reduced at very low concentrations even though there was no mortality at these concentrations.

AChE activity in shrimps from South Creek was significantly higher than activity in shrimps from the Upper Colo River ($p<0.01$), but not significantly different from the activity in shrimps from Parramatta Lake ($p>0.01$). Bonferroni's t test showed a significant difference in AChE activity of South Creek shrimps between the control and 0.09 ppb chlorpyrifos treatment ($p>0.01$) but no significant differences between the control and concentrations of 0.005 to 0.07 ppb. These give NOEC and LOEC values of 0.07 and 0.09 ppb, respectively, corresponding to a reduction in AChE activity of 21% and 32%. Shrimps that had survived after exposure to 0.2 ppb of chlorpyrifos had an AChE inhibition of 65%.

AChE activity in shrimps from Parramatta Lake was significantly higher than the activity in shrimps from the Upper Colo River ($p<0.01$). Bonferroni's t test showed a significant difference in AChE activity between the control and 0.09 ppb ($p<0.01$) but no significant differences between the control and concentrations below 0.09 ppb ($p>0.01$). These give NOEC and LOEC values of 0.07 and 0.09 ppb chlorpyrifos, respectively, corresponding to 11% and 22% AChE inhibition. Shrimps that had survived exposure to 0.35 ppb chlorpyrifos had an inhibition of 72%. Exposure at low concentrations (0.005-0.07 ppb) did not inhibit AChE activity significantly. Milatovic and Dettbarn (1996) postulated that in cases where AChE is reduced by low concentrations of toxicants, the rate of inhibition may be balanced by the rate of protein synthesis and unless the concentration of the inhibitor is increased significantly, AChE activity tends to remain at a constant level.

Shrimps from the Upper Colo River had lower AChE activity compared with either Parramatta Lake or South Creek shrimps prior to exposure to chlorpyrifos. Karnak and Collin (1974) related the increased susceptibility of midge larvae to OPs to low amounts of the target enzyme suggesting that low enzyme activities would require

proportionately smaller amounts of inhibitor to achieve an effective inhibition. This could explain the greater sensitivity of shrimps from the Upper Colo River to chlorpyrifos compared with the sensitivity of shrimps from South Creek .and Parramatta Lake. It is possible that previous exposure to OPs could have caused resistant animals to produce more enzymes to cope with the increase in substrate. However, extensive enzyme kinetics on the inhibitory process in the different populations should be carried out before making conclusions on the degree of sensitivity of AChE to inhibition.

It is generally accepted that an inhibition less than 20% is not regarded as significant due to natural variations in AChE activity. However, reductions greater than 20% are indicative of possible exposure to acetylcholinesterase inhibiting agents (Zinkl *et al.* 1987). There is controversy in the literature on the levels of inhibition that could lead to death. Coppage and Matthews (1974) found a mean inhibition of 75% in the shrimp *Penaeus duorarum* exposed to malathion at concentrations where 40-60% of the shrimps had died. Reddy and Rao (1988) noted survival of the shrimps *Metapenaeus monoceros* that had AChE inhibitions up to 64% after exposure to OPs. There is also controversy on the mechanisms of the toxic action of OPs. While most authors argue that AChE inhibition is the major toxic effect of OPs and that the response is dose dependent (Reddy and Rao 1988; Abdullah *et al.* 1993), Schoor and Brausch (1980) argue that AChE is not dose dependent and therefore a direct dose vs enzyme activity curve cannot be used to predict the levels of decline of an organism. Results from this study show that a dose-response relationship between chlorpyrifos concentration and enzyme inhibition exists, with animals exposed to higher concentrations having higher levels of inhibition. However, AChE inhibition may not be the only toxic action of OPs as indicated by the relatively lower inhibition in shrimps from South Creek and Parramatta Lake exposed to relatively high chlorpyrifos concentrations.

In conclusion, this study shows that shrimps inhabiting polluted waters are more tolerant to chlorpyrifos compared with those from nonpolluted waters. However, there is need to investigate the nature and mechanism of this tolerance. Consequently, toxicity testing should be done using field collected animals from unpolluted sites in order to obtain toxicity data that would effectively protect most individuals of a particular species. In addition, acetylcholinesterase activity in aquatic organisms should be incorporated in hazard assessment for OPs. It is also necessary to determine what levels of inhibition significantly reduce the survival of organisms and whether statistical significance in tolerance of the shrimp to chlorpyrifos is biologically significant.

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