SETAC – Brazil

J. Braz. Soc. Ecotoxicol., v. 2, n. 1, 2007, 53-60

JBSE

Biochemical Biomarkers in Individual Larvae of *Chironomus xanthus* (Rempel, 1939) (Diptera, Chironomidae)

L. B. Printes,[1*] E. L. G. Espíndola[1] & M. N. Fernandes[2]

[1]Escola de Engenharia de São Carlos, Departamento de Hidráulica e Saneamento,
Centro de Recursos Hídricos e Ecologia Aplicada, Universidade de São Paulo,
Av. Trabalhador São-carlense, 400, Centro, C.P. 292, CEP 13560-900, São Carlos, SP, Brasil

[2]Universidade Federal de São Carlos, Departamento de Ciências Fisiológicas, Via Washington Luís km 235, C.P. 676,
CEP 13565-905, São Carlos, SP, Brasil, e-mail: printes_liane@yahoo.com.br

(Received July 6, 2006; Accepted December 7, 2006)

ABSTRACT

Biochemical biomarkers have shown to be useful toxicity tools in environmental assessment programmes. However, few studies involving freshwater invertebrate species have been performed and even fewer on tropical species. The aim of this paper was to evaluate cholinesterase (ChE) and glutathione-S-transferase (GST) activities in larvae of *Chironomus xanthus* (Chironomidae, Diptera) exposed to the organophosphate parathion (ethyl) and the metals copper and cadmium. Considering ChE, significant reduction in activity was observed following *C. xanthus* exposure to parathion and cadmium (48 and 96 h, respectively). Inhibition of ChE activity (IC50) related to parathion seemed to be a more consistent and sensitive effect criteria than the LC50. For GST, there was significant reduction in activity following parathion exposure (48 h). Copper did not elicit any change in ChE or GST activities in *C. xanthus*. The findings of this paper show the two biomarkers as promising tools for assessing exposure to contaminats in tropical regions.

Key words: *Chironomus xanthus*, glutathione-*S*-transferase, cholinesterase, pesticides, metals.

RESUMO

Biomarcadores bioquímicos em larvas individuais de *Chironomus xanthus* (Rempel, 1939) (Diptera, Chironomidae)

Diversos biomarcadores bioquímicos têm demonstrado importante papel como ferramentas de toxicidade em programas de avaliação ambiental. Entretanto, poucos estudos envolvem espécies de invertebrados de água doce; menor ainda é o número de trabalhos que incluem análises de espécies tropicais. O objetivo deste artigo foi avaliar as atividades de colinesterase (ChE) e glutationa-S-transferase (GST) em larvas de *Chironomus xanthus* (Chironomidae, Diptera) expostas ao organofosfato paration (etil) e aos metais cobre e cádmio. Em relação a ChE, foi observada redução significativa na atividade dessa enzima em *C. xanthus* expostos ao paration e ao cádmio (48 e 96 h, respectivamente). Além disso, a inibição de ChE (CI50) relacionada ao paration foi mais consistente e sensível que a CL50. Foi também encontrada redução significativa da atividade de GST em *C. xanthus* após exposição ao paration (48 h). A exposição ao cobre não resultou em alteração nas atividades de colinesterase e glutationa-S-transferase. Os resultados obtidos demonstram o potencial dos dois biomarcadores como ferramentas de avaliação da exposição a poluentes em regiões tropicais.

Palavras-chave: *Chironomus xanthus*, glutationa-*S*-transferase, colinesterase, pesticidas, metais.

---

*Corresponding author: Liane Biehl Printes, e-mail: printes_liane@yahoo.com.br.

INTRODUCTION

A biomarker can be defined as a biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status (Walker *et al.*, 2001). Among the biological responses that have been commonly evaluated as biomarkers are the biochemical responses, which include measurements of activities of various enzymes (Huggett *et al.*, 1992).

Cholinesterases (ChEs) are important enzymes of the nervous system, and are readily inhibited by organophosphate and carbamate pesticides (Habig & Di Giulio, 1991). There are also evidences for the effect of metals on ChE activity (Diamantino *et al.*, 2003). They are of two types: (1) acetylcholinesterases (AChE) which are highly specific to acetylcholine and hydrolyze acetylcholine at the synaptic cleft; and (2) pseudocholinesterases (PChE), which are relatively non-specific esterases and hydrolyze a number of cholinesteres (e.g. butyrylcholine) in addition to acetylcholine itself. The physiological role of PChE is not yet well understood (Walker *et al.*, 2001). The two types are usually measured together in biochemical assays (Habig & Di Giulio, 1991).

Glutathione-S-transferases (GSTs) are involved in the phase II of the biotransformation of organic contaminants in living organisms. They catalyse the conjugation of a variety of electrophilic compounds with the tripetide glutathione. They are also involved in the transport of xenobiotics through the cytoplasm to sites of phase I metabolism. In addition, they are capable of forming covalent bonds with electrophilic compounds produced by phase I enzymes, preventing binding of the activated species to DNA and other cellular macromolecules (Stegeman *et al.*, 1992). GST biomarkers may be sensitive indicators of exposure to organic contaminants and metals (McLoughlin *et al.*, 2000).

Although biomarker measurements are equally feasible in invertebrate samples, its employment within this group has been considered disproportionately small (Sturm & Hansen, 1999; Fulton & Key, 2001). However, considering aquatic insects, including midges, significant advances have been made in recent years (Callaghan *et al.*, 2001; Moreira-Santos *et al.*, 2005). And both ChE and GST activities have been successfully measured in the correlated species *C. riparius* (Sturm & Hansen, 1999; Fisher *et al.*, 2000; Callaghan *et al*., 2001). In relation to tropical species, not many studies have been performed. Moreira-Santos *et al.* (2005) found that the measurement of AChE activity in *C. xanthus* heads might be useful tool for ecosystem quality assessment in tropical regions.

Chironomidae are predominant species in the benthic communities in most freshwater ecosystems (Sixtrino & Sixtrino, 1982). Among the tropical species *C. xanthus* (synonym of *Chironomus sancticaroli* (Sixtrino & Sixtrino, *op. cit.),* are well spread in the Neotropical Region. Larvae of *C. xanthus* have been successfully employed as test organisms in sediment toxicity tests in Brazil (Fonseca, 1997; Dornfeld, 2006).

The aim of this paper was to evaluate cholinesterase (ChE) and glutathione-S-transferase (GST) activities in IV instar larvae of *Chironomus xanthus* (Chironomidae, Diptera) exposed to the organophosphate parathion (ethyl) and the metals copper and cadmium. There was also the intention of comparing effects in terms of enzyme activity and lethality (LC50) to verify possible relationships between the two endpoints. This work intends to contribute towards the establishment of a basis for the use of these biomarkers as toxicological tools in environmental assessment programmes.

MATERIAL AND METHODS

Test organism

Test organisms were cultured in the Laboratory of Ecotoxicology, School of Engineering of São Carlos, University of São Paulo. Cultures were kept under static conditions at 20 ± 2ºC and 12:12 h light: dark regime. *C. xanthus* were maintained in reconstituted soft water prepared according to American Society for Testing and Materials (ASTM, 1980) and natural, acid-washed and organic matter-free, sediment. They were fed every other day with distilled water suspensions of Tetramin® fish food (0.04 mg mL^{-1}). Culturing procedures followed those proposed by Fonseca & Rocha (2004).

Chemicals

The pesticide tested was parathion (ethyl) (99%, PST-761, Ultra Scientific). As for metals copper sulphate (100%, 4844, Mallinckodt) and cadmium chloride (99.3%, 1212-04, J.T. Baker) were used.

Parathion stock solutions were made fresh on the day of the experiment in pure acetone and the test solutions were obtained by adding 1.0 μL of each stock solution per mL of ASTM water to give the required concentrations in the test vessels. The sole addition of 1 μL of acetone per mL of ASTM did not cause any reduction in survival following 48 h of exposure and did not affect AChE and GST activities (see results section). The ranges of concentrations tested were: 1, 2, 5, 10, 20, 50 and 100 μg L^{-1} of parathion.

Metal stock solutions were prepared in ASTM water and dilutions were made directly in the test vessel. The concentrations used were: 0.10, 0.20, 0.40, 0.80 and 1.60 mg L^{-1} of Cu and 0.25, 0.50, 0.75, 1.50 and 3.0 mg L^{-1} of Cd.

Toxicity tests

The method for determining acute effects of toxicants on *C. xanthus* were derived from Fonseca (1997). Six IV instar larvae were placed in 200 mL acid-washed beakers filled with test solutions or culture water for controls. For the pesticide exposures, 1 μL of pure acetone was added to an extra set of controls. Three replicates were set up for each treatment. No previous information on the toxicity of parathion to *C. xanthus* was available, so a series of tests was performed for calculating the LC50 (48 h). On the other hand, for Cu and Cd the LC50

(96 h) had been previously determined (Dornfeld, 2006). Hence, one test with copper sulphate and two tests with cadmium chloride were then performed in 96 h to verify possible effects on ChE and GST activities. All animals were exposed under the same conditions described for culturing. Mortality was recorded at the end of the exposure periods. In all metal exposures, animals were fed (0.04 mg mL^{-1}) on the first day. Considering parathion, half of the toxicity tests were performed with the addition of food on the first day and the other half without it (as indicated). Food can have a substantial influence on the outcome of toxicity tests. It has been shown, for instance, that food can interact with contaminants during toxicity tests (Pery *et al.*, 2002). Thus, the effect of feeding on the LC50 of parathion to *C. xanthus* was also evaluated.

Surviving animals from the parathion tests 4 to 10 and from all tests performed with metals were snap-frozen individually in 1.5 mL micro-centrifuge tubes dropped into liquid nitrogen. Considering ChE activity, organisms from tests 4 to 8 were assayed whereas for GST activity only organisms from tests 8 and 10 were analysed. ChE and GST activities were measured in three replicates per treatment (one from each test replicate). The larvae were stored for up to 3 months at –80ºC for ChE and GST analyses.

Biochemical assays

Cholinesterase activity was determined according to Ellman *et al.* (1961), adapted for use in microplates with *C. riparius* by Fisher *et al.* (2000). The homogenates were obtained from a single larva. Whole animals were homogenized in 1.5 mL eppendorf tubes with ice-cold 0.02 M sodium phosphate buffer (PB), pH 8.0, with 1% Triton X-100 (Sigma). The homogenization was manual, using a microcentrifuge tube pestle (50 cycles, 10 s). The initial homogenate was made up to 350 µL mixed and centrifuged at 14,000 × g and 2-4°C for 4 min. Supernatants (315 µL) were transferred to a clean pre-cooled tube, mixed using a whirlimixer and assayed immediately. Additions to the microplate were made in the following order: 100 µL of 8 mM 5,5'-dithio bis-2-nitrobenzoate (D-8130, Sigma Chemical) in PB supplemented with 0.75 mg mL^{-1} sodium hydrogen carbonate; 50 µL of assay blank (PB containing 0.1% triton-x-100) or supernatant; and 50 µL of 16 mM acetylthiocholine iodide (ATCI) in PB (A-5751 Sigma). Absorbances (405 nm) were read using a microtiter plate reader at 30°C, after 5 min incubation, over 10 min with intermittent shaking. A Dynex MRX microtiter plate reader (Intercientífica, São José dos Campos, SP) was used.

Glutathione S-transferase assay was based on the method described by Habig *et al.* (1974), modified for use in microplates with *C. riparius* (Callaghan *et al.*, 2001). The homogenates were obtained as described for ChE assay. However, ice-cold 0.02 M sodium phosphate buffer (PB), pH 6.5 was used instead. The microplate was loaded on ice with three replicates of 50 µL of assay blank (PB containing 0.1% Triton X-100 and 0.1% phenylmethylsulfonylfloride) or supernatant. Subsequently, the microplate was incubated for 5 min at 30ºC in the plate reader. Meanwhile the substrate mixture was prepared by adding 7 mL of 20 mM reduced glutathione (Sigma G-4251) in 0.1 M potassium dihydrogen phosphate (Sigma P5379), 1 mM ethylene-diamine tetracetic acid (Sigma ED4SS) to 12.5 mL of 0.02 M PB, pH 6.5. Following 5 min incubation of the solution at 30ºC, 1.4 mL of 40 mM 1-chloro-2,4-dinitrobenzene (Sigma C-6369) in 95% ethanol was added. Thus, 150 µL of the substrate mixture was added to the microplate, and the activity rate was measured as change in OD/min at 340 nm over 10 min with intermittent shaking.

The activities of both GST and ChE were expressed as activity per unit of protein (µM/L/min/g protein). Protein concentration in homogenate supernatants was determined by using a modification of the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) (Smith *et al.*, 1985). The protein standard curve was prepared with a series of bovine serum albumin (BSA) standards diluted in ChE blank buffer (BB).

Statistics

Median lethal concentrations (LC50) were calculated using the Spearman-Karber method with 95% confidence limits (Hamilton *et al*., 1977). Inhibition median concentrations for ChE activity (IC50) were estimated by non-linear regression analysis applying the exponential model. The approximated confidence intervals were obtained by the delta method (Sen & Singer, 1993). Comparative analyses were performed using 2 sample t-test or one-way ANOVA in Minitab (Minitab for Windows, version 13.1).

RESULTS

Acute toxicity

LC50 (48 h) values obtained from the toxicity tests with *C. xanthus* exposed to parathion are depicted in Table 1; there was no difference between the tests performed with the addition of food and without it (t-test, $p = 0.497$). Therefore, all data were treated together. Results obtained with copper sulphate and cadmium chloride (LC50; 96 h) are given in Table 2.

A total of nine tests performed with parathion were considered valid (i.e. less than 10% mortality in the controls). The mean value for LC50 (48 h) was 15.03 µg L^{-1} with a coefficient of variation (CV) of 48%. Considering the tests performed with metals, the mean LC50 (96 h) for copper sulphate was 0.34 mg L^{-1} of Cu; whereas the LC50 (96 h) value obtained for the test carried out with cadmium chloride was 0.52 mg L^{-1} of Cd.

Table 1 – Acute toxicity of parathion (ethyl) to IV instar larvae of *C. xanthus* and effect on cholinesterase (ChE) activity. *Lethal median concentration (LC50; 48 h) (lower and upper 95% confidence limits); **inhibition median concentration (IC50; 48 h) (lower and upper 95% confidence limits); test number in bold = tests performed without food; n.c. = not calculable; – ChE assays not performed.

Insecticide	Test n.	LC50; 48 h (μg L^{-1})*	IC50 48 h (μg L^{-1})**	IC50/LC50
Parathion	**2**	n.c.	–	–
Parathion	**3**	15.49 (10.49-22.88)	–	–
Parathion	**4**	n.c.	n.c.	n.c.
Parathion	5	6.93 (5.68-8.46)	n.c.	n.c.
Parathion	**6**	9.44 (3.56-25.03)	0.95 (0.74-1.16)	0.10
Parathion	7	17.78 (12.98-24.36)	0.64 (0.31-0.98)	0.04
Parathion	8	7.89 (6.03-10.32)	0.89 (0.52-1.26)	0.11
Parathion	9	25.60 (19.47-33.67)	–	–
Parathion	10	22.06 (15.88-30.64)	–	–
Average ± SD		15.03 ± 6.73	0,83 ± 0.13	

Table 2 – Acute toxicity of copper and cadmium to IV instar larvae of *C. xanthus*. *Lethal median concentration (LC50; 96 h) (lower and upper 95% confidence limits).

Metal	Test n.	LC50; 96 h (mg L^{-1})*
Cu	1	0.35 (0.13-0.95)
Cu	2	0.33 (0.26-0.41)
Average for Cu ± SD	1 and 2	0.34 ± 0.014
Cd	unique	0.52 (0.42-0.64)

Cholinesterase activity

The sensitivity of the ChE assay when IV instar larvae of *C. xanthus* were exposed to different concentrations of the organophosphate parathion is demonstrated in Figure 1. Significant decrease in ChE activity levels were seen in all assays performed: $F_{3,4} = 31.70$, $p = 0.03$; $F_{2,8} = 57.48$, $p = 0.000$; $F_{5,6} = 7.59$; $p = 0.014$; $F_{5,6} = 73.09$; $p = 0.000$ and $F_{4,10} = 13.94$; $p = 0.000$, for toxicity tests 4 to 8, respectively.

The IC50 values estimated for ChE activity are presented in Table 1. The mean value for IC50 (48 h) was 0.83 μg L^{-1} with a CV of 16.24%.

There was a tendency for a reduction in ChE activity in *C. xanthus* exposed to the higher concentrations of copper sulphate however the differences were not statistically significant ($F_{3,5} = 3.03$; $p = 0.132$ and $F_{3,8} = 3.52$; $p = 0.069$ for tests 1 and 2, respectively). Cadmium chloride has effectively reduced ChE activity in *C. xanthus* ($F_{2,6} = 13.84$; $p = 0.006$) (Figure 2).

Glutathione-S-transferase activity

GST activities of IV instar larvae of *C. xanthus* exposed to different concentrations of parathion are presented in Figure 3. Significant reductions in GST activities were obtained in animals from the higher test concentrations ($F_{4,10} = 2.91$; $p = 0.078$ and $F_{6,14} = 23.50$; $p = 0.000$, for tests 1 and 2, respectively).

The GST assay was not sensitive to copper sulphate ($F_{2,6} = 1.43$; $p = 0.310$) or cadmium chloride ($F_{2,6} = 2.33$; $p = 0.179$).

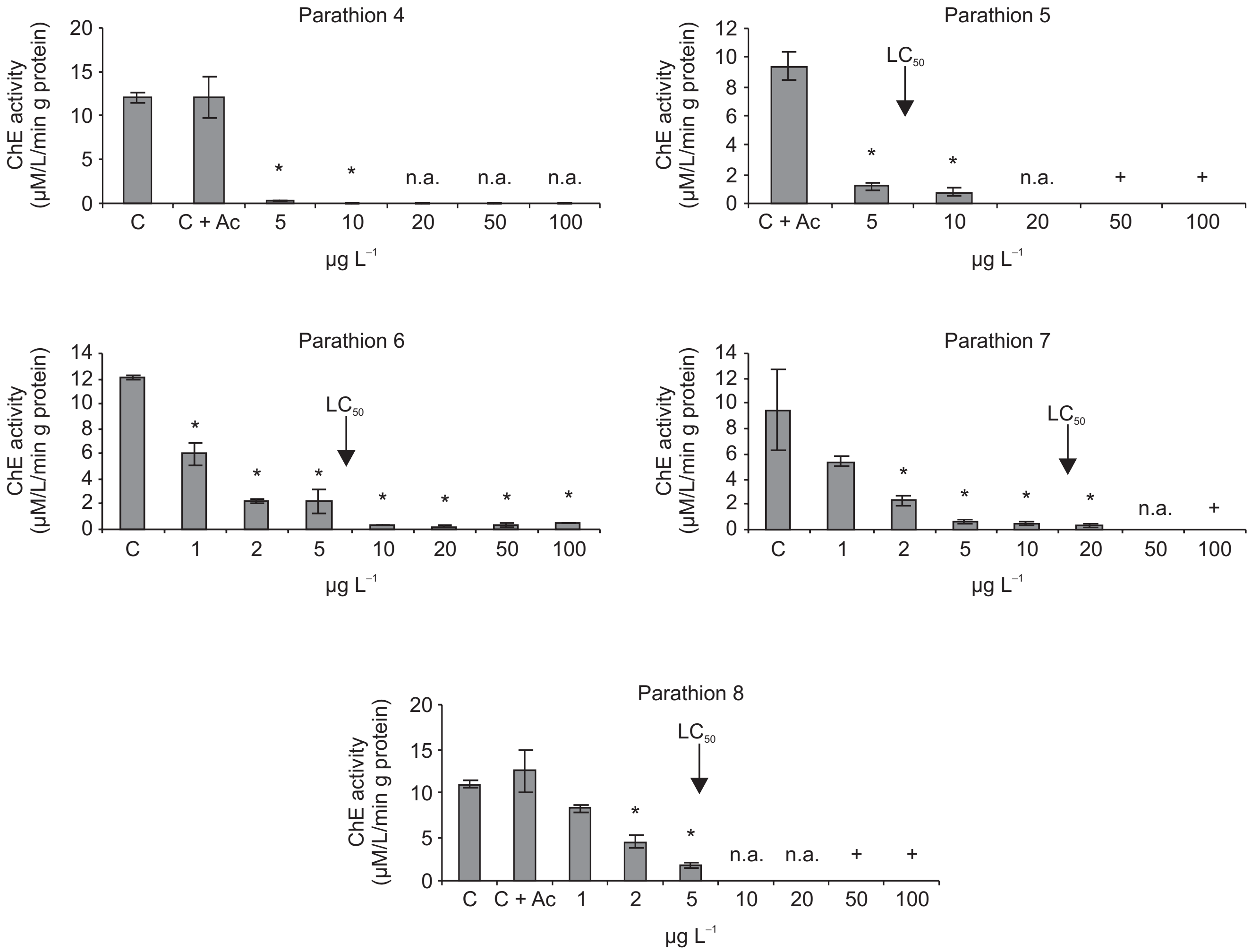

Figure 1 – Cholinesterase activity in IV instar larvae of *C. xanthus* following 48 h exposure to different concentrations of parathion (tests 4 to 8) (average ± SEM). C: water control group; C + Ac = acetone control; *: significant differences ($p < 0.05$); n.a.: not analysed; +: no survivals.

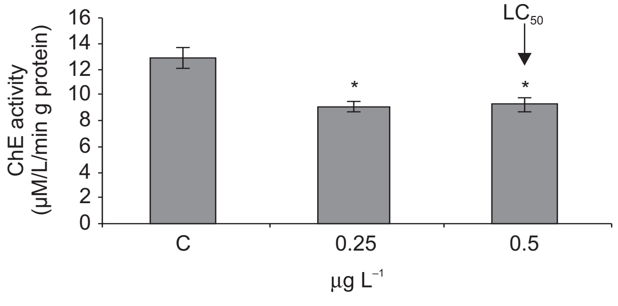

Figure 2 – Cholinesterase activity in IV instar larvae of *C. xanthus* following 96 h exposure to different concentrations of cadmium chloride (average ± SEM). C: water control group; *: significant difference ($p < 0.05$).

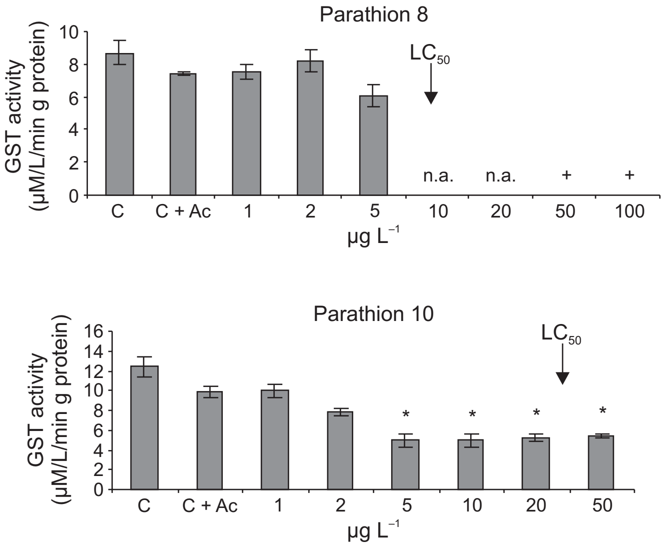

Figure 3 – Glutathione-S-transferase activity in IV instar larvae of *C. xanthus* following 48 h exposure to different concentrations of parathion (average ± SEM). C + Ac = acetone control; * significant difference ($p < 0.05$); n.a. not analyzed; + no survivors.

DISCUSSION

Biochemical biomarkers can give measures of exposure, and sometimes also of toxic effects (Walker *et al.*, 2001). They are very sensitive responses and should, in theory, anticipate more ecologically relevant effects at higher levels of biological organization (Van Gestel & Van Brummelen, 1996). The findings of this study have demonstrated the sensitivity of both ChE and GST activity in IV instar larvae of *C. xanthus*. For ChE activity, a clear dose-response relationship was observed in all tests performed with parathion. This effect was even more evident when organisms were exposed to very low concentrations (between 1 and 5 μg L^{-1}) (Figure 1). A significant effect of cadmium on *C. xanthus* ChE activity was also verified, although the dose-response relationship was not so clear. Considering GST, a significant reduction in activity was demonstrated in larvae that had been exposed to parathion. Hence, the findings of this work point to the usefulness of these two biomarkers as indicators of exposure.

The baseline reaction rates obtained with the methods used for measuring ChE and GST activities in untreated IV instar larvae of *C. xanthus* are in agreement with the optimum rates obtained for IV instar *C. riparius* assayed under very similar conditions (i.e. buffer pH, substrate concentration and reaction time) (Fisher & Callaghan, 1999; Fisher *et al.*, 2000). Fisher & Callaghan (1999) define the range of kinetic readings between 60-80 mOD/min for the ChE assay; whereas for the GST assay this range is between 20-30 mOD/min. The same values were obtained with *C. xanthus* in both assays (results not shown). Moreover, the ChE activity in terms of protein in untreated IV instar larvae of *C. xanthus* is the same found for *C. riparius* (around 15 μM/L/min/g protein) (Fisher *et al.*, 2000). These evidences, together with the consistent results that were obtained with *C. xanthus* exposed to a specific ChE inhibitor in the present study, support the suitability of the methodologies employed.

Results in terms of ChE activity in larvae of *C. xanthus* exposed to parathion seem to be more consistent than the data obtained for LC50 (48 h) (Table 1 and Figure 1). A much lower coefficient of variation (CV) was obtained in terms of IC50 (48 h) in contrast with the CV derived from the average LC50 (48 h). These findings support the idea that the IC50 could be a better index to evaluate parathion toxicity to *C. xanthus* than the LC50. Difficulty in determining death of the organisms or genetic variability among the different batches of *C. xanthus* evaluated might have contributed to this high variability in the LC50 (48 h). Both factors have previously been pointed as likely causes for intraspecific variability in LC50 values (Baird *et al.*, 1989; Barata *et al.*, 2000). Considering the correlated species *C. riparius*, concentrations up to 2 μg L^{-1} have been considered highly toxic (Detra & Collins, 1991). Sturm & Hansen (1999) found a median effect concentration (EC50; 24 h) of 7.2 μg L^{-1} of parathion (ethyl) for *C. riparius*.

For the sensitivity of the responses following parathion exposure it appears that ChE and GST reduction in activities came earlier than the effect in terms of mortality (Figures 1 and 3). Considering ChE activity, the ratios IC50/LC50 (Table 1) were below 0.10. Sturm & Hansen (1999) have also demonstrated that the effect in terms of ChE inhibition in *C. riparius* exposed to parathion anticipates EC50 (24 h). The calculated IC50/EC50 was 0.40. Inhibition of postmitochondrial and cytosolic GSTs was a more sensitive parameter than growth and reproduction in the freshwater mollusc *Lymnea palustris* treated with atrazine (Baturo & Lagadic, 1996).

In relation to the metals, the LC50 (96 h) values obtained for copper are also in agreement with what has been previously obtained for *C. xanthus* (0.30 mg L^{-1}). However for cadmium, our values are a bit lower than previous findings (0.70 mg L^{-1}) (Dornfeld, 2006). Metals have sometimes been associated with a direct effect on ChE activity. Among those related with ChE inhibition in invertebrates are cadmium, mercury, chrome, copper and zinc (Lagadic *et al.*, 1994; Guilhermino *et al.*, 1998; Diamantino *et al.*, 2003). Metals can interfere with the enzyme molecular structure by forming covalent bonds; making the enzyme unavailable for the substrate (Walker *et al.*, 2001). Our findings have shown that differently from what was demonstrated with parathion, inhibition of ChE activity due to cadmium exposure does not appear to be a more sensitive parameter than LC50 (96 h) (Figure 2). However, more studies should be performed for a better understanding of the relationship between these two parameters.

The findings of this work have shown the value of measuring ChE and GST activity in *C. xanthus*. The ChE biomarker was highly sensitive to parathion and cadmium; whereas the GST biomarker was sensitive to parathion. Inhibition of ChE activity related to parathion seemed to be a more consistent and sensitive effect criteria than LC50. Additional studies must be performed to evaluate relationships between inhibition of both ChE and GST activities and effects on life history and population parameters. Also, field studies involving the two biomarkers are needed to understand their relationship with more ecologically relevant responses. Nevertheless, ChE and GST activities in IV instar larvae of *C. xanthus* appear as promising ecotoxicological tools for assessing exposure to contaminats in tropical regions.

Acknowledgements — This work was supported by FAPESP. The authors wish to thank Professor Dulcinei Garcia, Physics Department, Federal University of São Carlos and Professor Mário de Castro, Mathematics and Computer Science Institute, School of Engineering of São Carlos, University of São Paulo.

REFERENCES

ASTM – AMERICAN SOCIETY FOR TESTING AND MATERIALS, 1980, *Standard practice for conducting acute toxicity test with fishes, microinvertebrates and amphibians.* Philadelphia, PA, USA, Report E-790-80.

BAIRD, D. J., BARBER, I., BRADLEY, M., CALOW, P. & SOARES, A. M. V. M., 1989, The *Daphnia* bioassay: a critique. *Hydrobiologia*, 188/189: 403-406.

BARATA, C., BAIRD, D. J., AMAT, F. & SOARES, A. M. V. M., 2000, Comparing population response to contaminants between laboratory and field: an approach using *Daphnia magna* ephippial egg banks. *Funct. Ecology*, *14*: 513-523.

BATURO, W. & LAGADIC, L., 1996, Benzo[a]pyrene hydroxilase and glutathione *S*-transferase activities as biomarkers in *Limnea palustris* (mollusca, gastropoda) exposed to atrazine and hexachlorobenzene in freshwater mesocosms. *Environ. Toxicol. Chem*., *15*: 771-781.

CALLAGHAN, A., HIRTTHE, G., FISHER, T. & CRANE, M., 2001, Effect of short-term exposure to chlorpyrifos on developmental parameters and biochemical biomarkers in *Chironomus riparius* Meigen. *Ecotoxicol. Environ. Saf*., *50*: 19-24.

DETRA, R. L. & COLLINS, W. J., 1991, The relationship of parathion concentration, exposure time, cholinesterase inhibition and symptoms of toxicity in midge larvae (Chironomidae: Diptera). *Environ. Toxicol. Chem*., *10*: 1089-1095.

DIAMANTINO, T. C., ALMEIDA, E., SOARES, A. M. V. M. & GUILHERMINO, L., 2003, Characterization of cholinesterases from *Daphnia magna* Straus and its inhibition by zinc. *B. Environ. Contam. Tox*., *71*: 219-225.

DORNFELD, C. B., 2006, *Utilização de Chironomus sp. (Díptera, Chironomidae) para avaliação da qualidade de sedimentos e contaminação por metais*. PhD Thesis, Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos, 211p.

ELLMAN, G. L., COURTNEY, K. D., ANDRES JR., V. A. & FEATHERSTONE, R. M., 1961, A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol*., *7*: 88-95.

FISHER, T. C. & CALLAGHAN, A., 1999, *Standard operating procedures of enzyme activity assays and protein assay for use with III and IV instar larvae of C. riparius*. Ecotoxicology Research Group, University of Reading.

FISHER, T. C., CRANE, M. & CALLAGHAN, A., 2000, An optimized microtitreplate assay to detect acetylcholinesterase activity in individual *Chironomus riparius* meigen. *Environ. Toxicol. Chem*., *19*: 1749-1752.

FONSECA, A. L., 1997, *Avaliação da qualidade da água do rio Piracicaba/SP através de testes de toxicidade com invertebrados.* PhD Thesis, Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos, 211p.

FONSECA, A. L. & ROCHA, O., 2004, Laboratory cultures of the native species of *Chironomus xanthus*, Rempel, 1939 (Diptera, Chironomidae). *Acta Limnol. Bras.*, *16*: 153-161.

FULTON, M. H. & KEY, P. B., 2001, Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ. Toxicol. Chem*., *20*: 37-45.

GUILHERMINO, L., BARROS, P., SILVA, M. C. & SOARES, A. M. V. M., 1998, Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Biomarkers*, *3*: 157-163.

HABIG, W. H., PABST, M. J. & JAKOBY, W. B., 1974, Glutathione-S-transferases: the first step in mercapturic acid formation. *J. Biol. Chem*., *249*: 7130-7139.

HABIG, C. & DI GIULIO, R. T., 1991, Biochemical characteristics of cholinesterases in aquatic organisms. *In*: P. Mineau (ed.). *Cholinesterase-inhibiting Insecticides – Their Impact on Wildlife and the Environment.* Elsevier, London, UK.

HAMILTON, M. A., RUSSO, R. C. & THURTON, R. V., 1977, Trimed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.*, *11*: 714-719.

HUGGETT, R. J., KIMERLE, R. A., MEHRLE JR., P. M. & BERGMAN, H. L. (eds.), 1992, *Biomarkers. Biochemical, physiological, and histological markers of anthropogenic stress.* Lewis Publishers, Boca Raton, FL, USA.

LAGADIC, L., CAQUET, T. & RAMADE, F., 1994, The role of biomarkers in environmental risk assessment (5). Invertebrate populations and communities. *Ecotoxicology*, *3*: 193-208.

MCLOUGHLIN, N., YIN, D., MALTBY, L., WOOD, R. M. & YU, H., 2000, Evaluation of sensitivity and specificity of two crustacean biochemical biomarkers. *Environ. Toxicol. Chem*., *19*: 2085-2092.

MOREIRA-SANTOS, M., FONSECA, A. L., MOREIRA, M. S., *et al.*, 2005, Short-term sublethal (sediment and aquatic roots of floating macrophytes) assays with a tropical chironomid based on postexposure feeding and biomarkers. *Environ. Toxicol. Chem*., *24*: 2234-2242.

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. & KLENK, D. C., 1985, Measurement of protein using bicinchoninic acid. *Anal. Biochem*., *150*: 76-85.

STEGEMAN, J. J., BROUWER, M., DI GIULIO, R. T., FÖRLIN, L., FOWLER, B. A., SANDERS, B. M. & VAN VELD, P. A., 1992, Enzyme and protein synthesis as indicators of contaminant exposure and effect. *In*: R. J. Huggett, R. A. Kimerle, P. M. Mehrle Jr. and H. L Bergman (eds.). *Biomarkers. Biochemical, physiological, and histological markers of anthropogenic stress*. Lewis Publishers, Boca Raton, FL, USA.

SEN, P. K. & SINGER, J. M., 1993, *Large sample methods in statistics: an introduction with applications*. Chapman & Hall, New York.

STRIXINO, S. T. & STRIXINO, G., 1982, Ciclo de vida de *Chironomus sancticaroli* Strixino & Strixino (Díptera, Chironomidae). *Rev. Bras. Ent., 26*: 183-189.

STURM, A. & HANSEN, P. D., 1999, Altered cholinesterase and monooxygenase levels in *Daphnia magna* and *Chironomus riparius* exposed to environmental pollutants. *Ecotoxicol. Environ. Saf*., *42*: 9-15.

VAN GESTEL, C. A. M. & VAN BRUMMELEN, T. C., 1996, Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology*, *5*: 217-225.

WALKER, C. H., HOPKIN, S. P., SIBLY, R. M. & PEAKALL, D. B., 2001, *Principles of ecotoxicology*. 2. ed. Taylor & Francis, London, UK, 309p.