Low malathion concentrations influence metabolism in *Chironomus sancticaroli* (Diptera, Chironomidae) in acute and chronic toxicity tests

Débora Rebechi¹, Vinicius S. Richardi¹, Maiara Vicentini¹, Izonete C. Guiloski², Helena C. Silva de Assis³ & Mário A. Navarro-Silva¹,⁴

¹Laboratório de Entomologia Médica e Veterinária, Departamento de Zoologia, Universidade Federal do Paraná, Caixa Postal 19020, Curitiba, 81531-980-PR. Brasil. deborarebechi@hotmail.com; rrichardivs@gmail.com; maiaravicentini@gmail.com

²Laboratório de Toxicologia Ambiental, Departamento de Farmacologia, Universidade Federal do Paraná, Curitiba, Paraná, Brasil. guiloski@ig.com.br; helassis@ufpr.br

³Corresponding author: mnnavarro@ufpr.br

**ABSTRACT.** Low malathion concentrations influence metabolism in *Chironomus sancticaroli* (Diptera, Chironomidae) in acute and chronic toxicity tests. Organophosphate compounds are used in agro-systems, and in programs to control pathogen vectors. Because they are continuously applied, organophosphates often reach water sources and may have an impact on aquatic life. The effects of acute and chronic exposure to the organophosphate insecticide malathion on the midge *Chironomus sancticaroli* are evaluated. To that end, three biochemical biomarkers, acetylcholinesterase (AChE), alpha (EST-α) and beta (EST-β) esterase were used. Acute bioassays with five concentrations of malathion, and chronic bioassays with two concentrations of malathion were carried out. In the acute exposure test, AChE, EST-α and EST-β activities declined by 66, 40 and 37%, respectively, at 0.251 µg L⁻¹ and more than 80% at 1.37, 1.96 and 2.51 µg L⁻¹. In chronic exposure tests, AChE and EST-α activities declined by 28 and 15% at 0.251 µg L⁻¹. Results of the present study show that low concentrations of malathion can influence larval metabolism, indicating high toxicity for *Chironomus sancticaroli* and environmental risk associated with the use of organophosphates.

**KEY WORDS.** Acetylcholinesterase; bioindicator; esterases; organophosphate; toxicity.

Organophosphates are used worldwide to eliminate insect vectors of parasites and pathogens to humans and domestic animals, as well as to control agricultural pests. These compounds affect the central nervous system, causing spasms and death (Bartling et al. 2007). Malathion, an organophosphate insecticide, is highly toxic and has low specificity, which means that it affects a wide variety of organisms. From an ecological perspective, there is a concern about malathion’s potential adversely affect non-target organisms, especially aquatic invertebrates (Xuereb et al. 2009).

Immature Chironomidae (Diptera) inhabit the benthic compartment of aquatic ecosystems (Di Veroli et al. 2012a; Lagauzère et al. 2009), and are important in the food chain dynamics as intermediates between producers and secondary consumers (Porinchu & MacDonald 2003). Because they are sensitive to various pollutants (Preston 2002), reproduce easily and have short life cycles (Fonseca & Rocha, 2004), chironomids are often used as indicators of acute and chronic toxicity in sediments and water contaminated with a variety of pollutants (eg. Al-Shami et al. 2010; Choung et al. 2013; Di Veroli et al. 2012b; Ebau et al. 2012; Jun et al. 2012; Roulier et al. 2008).

In aquatic ecosystems, the concentration of pollutants may not be high enough to cause mortality in animal communities. However, even in low concentration pollutants can cause damage to the entire ecosystem and its food chain. Consequently, sensitive markers should be used to assess the environmental health with respect to pollutants (Domingues et al. 2010). For this reason, the biomarker approach has become increasingly more popular. Its use is based on the assumption that low concentrations of toxic substances cause cellular responses in organisms before harmful effects can be observed in larger scales (Azevedo-Pereira et al. 2011; Domingues et al. 2010; Park et al. 2012).

*Chironomus sancticaroli* Strixino & Strixino, 1981 is an endemic species from Brazil (long considered synonymous with *Chironomus xanthus* Rempel, 1939) (Trivinho-Strixino 2011) and has been used as a model in several studies (Moreira-Santos et al. 2005; Printes et al. 2011). The activities of enzymes that indicate neurotoxicity and detoxification are used in the midge, such as AChE (acetylcholinesterase) and GST (glutathione S-transferase), respectively.

The enzyme AChE is well-known and is used as a marker for exposure to organophosphates and carbamates (both inhibit this enzyme, resulting in a general nervous system failure (Fulton & Key 2001)). GST, on the other hand, is a family of detoxifying enzymes that catalyze the combination of reduced glutathione (GSH) with a group of compounds having electrophilic centers (such as organophosphates and organochlorides). The products of this combination are usually less toxic and more soluble in water, hence are more easily excreted from cells (Crane et al. 2002). Increased activity of this enzyme is used as a marker of organic contamination (Domingues et al. 2010), even though its effects were not documented in some studies using Chironomidae (Callaghan et al. 2001; Crane et al. 2002; Rakotondravelo et al. 2006; Printes et al. 2011).
Other biochemical markers can be used in studies on the effects of pesticides, for instance the metabolic enzymes alpha esterase and beta esterase, which bind to organophosphates and carbamates and slowly transform them (Hemingway & Ranson 2000). The major objectives of the current study were to evaluate and compare the acute and chronic effects of malathion on immature C. sancticaroli by measuring the activity of the enzymes acetylcholinesterase, alpha and beta esterase that might indicate short- or long-term exposure.

**MATERIAL AND METHODS**

Midge larvae were obtained from a colony of the Laboratory of Medical and Veterinary Entomology, Federal University of Paraná (UFPR). This colony is maintained following the protocol of Maier et al. (1990), with modifications in the temperature (25°C ± 2) and photoperiod (12 h light: 12 h dark). Voucher specimens of this colony are deposited in the Pe. Jesus Santiago Moure Entomological Collection of the Department of Zoology, UFPR (DZUP) with accession numbers from 249269 to 249276.

To estimate lethal malathion concentrations for Chironomus sancticaroli, bioassays were carried out in 300 ml glass flasks. Each flask was provided with the following: 200 ml test solution, 24 g sediment (sand composed of quartz and feldspar, granulometry 0.063 – 2.36 mm, > 50% of the sand grains were ~1.18 mm) and ten, 4th instar larvae. Four replicas of each nominal concentration of malathion (0.3, 0.9, 1.5, 2.1, 2.7, 3.3, 3.9, 4.5 µg L⁻¹) and one control with 100% ethanol were used. Three bioassays were performed, each including 960 larvae (120 larvae per concentration). Bioassays lasted 96 hours and were kept in a BOD chamber at constant temperature (25 ± 2°C) and photoperiod (12 h light/12 h dark). The lethal concentrations of the insecticide were estimated with 50 larvae per concentration, with a total of 900 larvae acutely exposed and 450 larvae chronically exposed.

Larvae of acute and chronic toxicity bioassays were stored in a freezer at -80°C and were subsequently homogenized in 300 µl 0.1 M pH 7.5 potassium phosphate buffer (for the enzyme AChE) and in 150 µl 0.2 M pH 7.2 potassium phosphate buffer (for the enzymes EST-α and EST-β), followed by centrifugation at 12,000 x g for 1 minute at 4°C.

For the enzyme AChE we followed Ellman et al. (1961) modified for microplates following Silva de Assis (1998). The EST-α and EST-β activities followed the methodology by Valle et al. (2006). Total protein per larva was measured following Bradford’s (1976) protocol. The biochemical analyses were carried out on a BioTek microplate reader.

Statistical analyses were carried out in the program R (R Development Core Team 2011). A generalized linear model (GLM) was adjusted through a Gamma error distribution using MASS libraries (Venables and Ripley 2002) and effects (Fox 2003). Given that the data met the assumptions of independence, normality, and homoscedasticity, One-way ANOVA and Tukey contrasts (p ≤ 0.05) were used for a posteriori comparisons using a multcomp library (Hothorn et al. 2008).

**RESULTS**

Malathion concentrations ranging from 0.95 to 7.51 µg L⁻¹ were lethal to Chironomus sancticaroli. Based on this lethal range, we selected concentrations to expose the organisms for biomarker evaluation. The lethal concentration 50 (LC₅₀) was 2.51 µg L⁻¹, from which we obtained the value of the no observed effect concentration (NOEC) of 0.25 µg L⁻¹ (Table 1).

Table I. Lethal concentrations of the malathion compound for Chironomus sancticaroli expressed in µg L⁻¹ and confidence interval (CI).

<table>
<thead>
<tr>
<th>Lethal concentrations</th>
<th>Concentrations (µg L⁻¹)</th>
<th>CI (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC2</td>
<td>0.95</td>
<td>0.81 – 1.08</td>
</tr>
<tr>
<td>LC10</td>
<td>1.37</td>
<td>1.22 – 1.51</td>
</tr>
<tr>
<td>LC30</td>
<td>1.96</td>
<td>1.81 – 2.09</td>
</tr>
<tr>
<td>LC50</td>
<td>2.51</td>
<td>2.36 – 2.65</td>
</tr>
<tr>
<td>LC70</td>
<td>3.21</td>
<td>3.03 – 3.42</td>
</tr>
<tr>
<td>LC90</td>
<td>4.59</td>
<td>4.24 – 5.05</td>
</tr>
<tr>
<td>LC99</td>
<td>7.51</td>
<td>6.62 – 8.82</td>
</tr>
</tbody>
</table>

In order to understand the effects caused by short- and long-term exposure to malathion we exposed C. sancticaroli to two nominal concentrations (0.1 and 0.25 µg L⁻¹), both acutely and chronically. At the concentration 0.1 µg L⁻¹ (the maxi-
mum allowed by Brazilian legislation), no mortality occurred among 4th instar larvae exposed for a brief interval (96h). Nonetheless, this exposure still led to a significant decrease in the activity of the enzymes AChE and EST-β (46 and 25%, respectively, Fig. 1A). When exposure was continuous, beginning with the egg stage, this insecticide concentration caused a reduction in enzymatic activity (AChE, EST-α and EST-β decreased 15, 8 and 5%, respectively) which was not significantly different from the control (Fig. 1B).

Mortality did not occur when the organisms were exposed to acute toxicity bioassays at 0.25 g L⁻¹ (NOEC), but enzyme activities significantly decreased (66, 40 and 37% for AChE, EST-α and EST-β, respectively, Fig. 1A). When organisms were chronically exposed to the same concentration, enzymatic activity declined again for AChE and EST-α by 28 and 15%, respectively (Fig. 1B).

A concentration-dependent relationship was found in the present study for the three enzymes (Fig. 1A and Table II). After the sub-lethal concentration LC₁₀ (1.37 g L⁻¹), some mortality occurred (Table II) and enzyme activity of the three enzymes decreased more than 78% in survivors.

### Table II. Percent inhibition of the enzymes AChE, EST-α and EST-β and the mortality observed in Chironomus sancticaroli exposed to five concentrations of malathion. There was no mortality in the control.

<table>
<thead>
<tr>
<th>Concentration (µg L⁻¹)</th>
<th>Inhibition (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Chronic</td>
</tr>
<tr>
<td></td>
<td>AChE EST-α EST-β</td>
<td>AChE EST-α EST-β</td>
</tr>
<tr>
<td>0.10</td>
<td>47</td>
<td>17</td>
</tr>
<tr>
<td>0.25</td>
<td>67</td>
<td>41</td>
</tr>
<tr>
<td>1.37</td>
<td>97</td>
<td>86</td>
</tr>
<tr>
<td>1.96</td>
<td>98</td>
<td>89</td>
</tr>
<tr>
<td>2.51</td>
<td>98</td>
<td>91</td>
</tr>
</tbody>
</table>

*Indicates *p* <0.05 (using one-way ANOVA and Tukey).

**DISCUSSION**

Neurotoxicity and metabolic detoxification caused by pesticides are often detected with the help of marker enzymes. In the present study, the activity of three marker enzymes was impacted by malathion, even in the concentrations permitted by the Brazilian law.

Inhibition of the activity of the three marker enzymes used in this study, even when the concentrations of malathion
are very low, may affect the metabolism and thus the fitness of organisms. Rakondravelo et al. (2006), studying the effects of exposing *Chironomus tentans* Fabricius, 1805 for 20 days to concentrations of 0.1 µg L⁻¹ of the organophosphate insecticide chlorpyrifos, reported that the activity of the following enzymes (percentage of inhibition in parenthesis) was inhibited: AChE (60%), EST-α (31%), and EST-β (49%). In our work we also detected enzymatic inhibition, though in a smaller scale, which is consistent with previous tests that found chlorpyrifos to be more harmful to Chironomidae than malathion (Stevens 1992).

As expected, the no observed effect concentration (NOEC) did not result in mortality. However, enzymatic alterations were detected. A similar outcome was achieved by Beyers & Sikoski (1994), who obtained a 24% inhibition of the AChE activity after exposing the fish *Pychochilus lucius* Girard, 1856 to the NOEC calculated for the species. Jin-Clark et al. (2008) exposed *Chironomus tentans* to a concentration of 0.25 µg L⁻¹ of the organophosphate insecticide chlorpyrifos, obtaining 70% inhibition of the AChE. According to Day & Scott (1990) a 50% inhibition of the AChE generally affects the survival of organisms. Even though vulnerability differs among individuals, our results indicate that our *C. sancticaroli* study population may have been affected.

According to Buchwalter et al. (2004), different larval instars of the same species differ in their vulnerability to xenobiotics. Usually, younger larvae are more vulnerable than older ones. Therefore, it is expected that chronic exposure to low concentrations of xenobiotics would lead to stronger enzymatic inhibition when compared to acute exposure to the same substance in the same concentration (Rakotondravelo et al. 2006). However, our results do not indicate that. Greater enzymatic inhibition resulted after acute exposure to malathion in the concentrations of 0.1 and 0.25 µg L⁻¹, when compared to chronic exposure. This may be due to the biochemical detoxification mechanism of our subjects, which partially recover after the initial exposure phase. This detoxification mechanism ensures survival at least when concentrations of the insecticide are low (Hemingway and Karunaratne 1998).

Several studies have confirmed a direct relationship between xenobiotic concentrations and enzyme inhibition (Callaghan et al. 2001; Forcella et al. 2007; Mehler et al. 2008; Printes et al. 2007). Such concentration-dependent relationship was found in this study for the three enzymes analyzed. Esterases, which eliminate organophosphates from the body using hydrolysis or sequestration of compounds, are considered the main enzymes in the detoxification of these compounds. Therefore, inhibition of these enzymes can minimize the effects of the insecticide on the AChE, providing greater chances of survival for individuals (Rakotondravelo et al. 2006).

The significant reduction in AChE activity (Table II) reveals that larvae are able to survive inhibition of this enzyme, at least for short periods. Similar results were found by Domingues et al. (2007), who reported that larvae of *Chironomus riparius* Meigen, 1804 exposed to the organophosphate dimethoate remained alive after 66% inhibition of the AChE. Berra et al. (2006) observed that *Hydropsyche pellucidula* Curtis, 1834 (Trichoptera, Hydropsychidae) is able to survive the fenitrothion even after 97% enzymatic activity inhibition.

Although larvae do survive high levels of enzyme inhibition, a behavioral effect observed in organisms exposed to organophosphate compounds is limited mobility or paralysis, which is attributed to inhibition of the AChE. The latter is responsible for proper nervous system functioning (Azevedo-Pereira et al. 2011). Paralysis, which was mainly observed in organisms exposed to higher concentration of malathion, can have serious ecological consequences. Paralyzed larvae have difficulties feeding and are unable to protect themselves in the substrate using their silk pipe constructions, becoming more susceptible to predation and to being carried away by the water currents, affecting population density (Callaghan et al. 2001).

In micro-quantities, malathion can be considered toxic to *C. sancticaroli* and impact populations of this species, also disrupting the food chain. Furthermore, malathion residues found in aquatic environments affect the entire aquatic community and, consequently, humans. Studies have reported that malathion is associated with Parkinson’s disease, neurocognitive disorders in children, and is capable of triggering a metabolic predisposition to weight gain (Holtcamp 2012; Moretto & Colosio 2013).

In conclusion, our study shows that the insecticide malathion in very toxic to the midge *C. sancticaroli*, after short- and long-term exposure. A continual release of this compound, even at the sub-lethal concentrations accepted by the Brazilian legislation, can influence the metabolism of the midge, with consequences to individual behavior, growth and survival. The entire food web may be also influenced by the effects of malathion on larvae of this midge.

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