Acetylcholinesterase activity in the freshwater shrimp *Caridina nilotica* as a biomarker of Roundup® herbicide pollution of freshwater systems in South Africa

P. K. Mensah, W. J. Muller and C. G. Palmer

**ABSTRACT**

The use of *Caridina nilotica* whole-body acetylcholinesterase (AChE) activity as a potential biomarker of Roundup® pollution of aquatic ecosystems was investigated. Forty days post hatch (dph) shrimps were exposed to different concentrations of 0.0, 4.3, 6.7, 10.5, 16.4, 25.6 and 40.0 mg/L in a 96 h acute toxicity test; and 0.0, 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L in a 21 d chronic toxicity test. Whole-body AChE activities were determined at the end of the exposure periods by spectrophotometric assay of sample extract; activities were then normalized against protein contents in the samples and expressed in nanomoles of substrate hydrolyzed. Results of both tests showed that AChE activity was concentration-dependent. Mean AChE activities and standard deviations (±SD) for 96 h acute toxicity were 3.6239 (±0.4185), 3.4157 (±1.1842), 2.537 (±1.3989), 2.4253 (±1.4202), 2.4127 (±1.9097), 2.0017 (±1.1080) and 2.316 (±0.4001) nmol/min/mg protein; while activity levels for 21 d test were 3.6907 (±0.3401), 2.8473 (±0.713), 2.9134 (±0.9879), 2.6738 (±0.7117), 2.3019 (±0.4464) and 2.1478 (±0.864) nmol/min/mg protein. Reference basal AChE activity for 40 dph *C. nilotica* based on the two control groups was estimated as 3.6907 (±0.3401) nmol/min/mg proteins. The present work provides ecotoxicological basis for the possible use of AChE activity in *C. nilotica* as a biomarker for monitoring Roundup® pollution in freshwater systems.

**Key words** | AChE activity, biomarker, *Caridina nilotica*, Roundup®, South Africa

**INTRODUCTION**

In recent years, there has been global concern on the effect of toxic chemicals on aquatic biota due to the upsurge in contamination of aquatic ecosystems by heavy metals and organic compounds such as hydrocarbons and pesticides (Romero *et al*. 2011). Potential problems associated with the use of herbicides include injury to non-target vegetation, injury to crops, residue in soil or water, toxicity to non-target animals and concerns for human health and safety (Radojevic *et al*. 2007). The crustacean *Caridina nilotica* (Decapoda: Atyidae) is a widely distributed freshwater shrimp found in South African inland waters (Day 2001; Hart *et al*. 2001). It thrives in temperatures between 10 and 30°C and is considered an important role player in the freshwater ecosystems as it forms part of most food webs. Its omnivorous mode of feeding is useful in enhancing aquatic macrophyte photosynthesis and recycling of organic matter (Hart *et al*. 2001). It has been identified as a potential standard toxicity test organism for producing ecologically relevant toxicity test data (Gola & Muller 2008). *C. nilotica* acute toxicity test methods have been developed for neonate, juvenile and adult stages, while chronic toxicity test methods for embryo, partial life-cycle and full life-cycle are currently under development by the Unilever Center for Environmental Water Quality (UCEWQ), Institute for Water Research (IWR), Rhodes University, Grahamstown, South Africa (Mensah *et al*. 2011). However, there is no toxicity test method that uses biochemical endpoint of *C. nilotica* to assess environmental water quality in South Africa. Hence, the aim of this study was to investigate acetylcholinesterase (AChE) activity as a potential biomarker to detect pollutant-induced stress in the freshwater shrimp *C. nilotica*. The main physiological function of the enzyme AChE is to hydrolyze acetylcholine (ACh), a neurotransmitter of cholinergic synapses, during transduction of nerve
impulses. Inhibition of AChE prevents the hydrolysis of ACh in nerve synapses and neuromuscular junctions, causing accumulation of excess ACh at these sites. This results in overexcitation of the synaptic and muscular tissues, which may lead to behaviors such as hyperactivity, asphyxia and finally death. The use of AChE activity is therefore regarded as a good biomarker for the detection of a range of toxic compounds including insecticides, herbicides, surfactants and metals in aquatic animals (Richardson et al. 2010).

Glyphosate formulated herbicides are among the leading chemicals used all over the world to control aquatic weed (Romero et al. 2011). Roundup® is the most popular and widely used glyphosate-based herbicide in South Africa and most parts of the world (Bold 2007; Romero et al. 2011). It is composed of isopropylamine (IPA) salt of glyphosate as the active ingredient; the surfactant polyoxyethylene amine (POEA); and water.

Glyphosate inhibits the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) found in the shikimic acid (shikimate) pathway of plants, thereby retarding the production of aromatic amino acids such as phenylalanine, tryptophan and tyrosine (Stenersen 2004). These essential substances are necessary for the organism’s survival and propagation. The shikimate pathway is absent in animals, which may account for animals’ low susceptibility to glyphosate toxicity. However, high glyphosate doses have been thought to alter mitochondrial activity of animals, possibly by uncoupling of oxidative phosphorylation during cellular respiration (WHO 1994). Glyphosate may also be an endocrine disrupting chemical (EDC) as it has been reported to interfere with steroidogenic acute regulatory (StAR) protein expression and serum growth hormone, thereby inhibiting steroidogenesis and growth respectively in animals (Walsh et al. 2000; El-Shebly & El-kady 2008).

The toxicity of glyphosate-based herbicides to non-target aquatic organisms is of great concern to ecotoxicologists. This is because these bioactive chemicals have high water solubility and are used extensively in the environment, especially in shallow water systems (Tsui & Chu 2003). Glyphosate herbicides are thought to reach concentrations of 2.8 mg/L acid equivalence (a.e.) in aquatic habitats due to accidental overspray but a concentration of 1.7 mg/L a.e. has been reported in the USA (Giesy et al. 2000). Although glyphosate has not been cited often in South African literature, it has long been found since the 1990s in the Hex River Valley of the Western Cape (Maharaj 2005). In recent years, the use of glyphosate herbicides has increased tremendously in South Africa, as farmers and Working for Water (WfW) programme of the South African National Department of Water and Environmental Affairs (DWEA) use glyphosate formulated herbicides such as Mamba, Tumbleweed, Ecoplug, Touchdown and Roundup® to control aquatic invasive plant species (Bold 2007). Roundup® was chosen as the test chemical for this study because it is the most popular and widely used herbicide in South Africa and most parts of the world (Bold 2007; Romero et al. 2011); glyphosate has been detected in surface waters long after being used to kill aquatic weeds, though it is thought to rapidly dissipate from surface waters and adsorbs strongly to soils and sediments (Glusczak et al. 2007); aquatic ecotoxicologists are concerned about its potential impact on the environment due to increased cultivation of genetically modified glyphosate-resistant crops in recent years (Kolpin et al. 2006); it’s mode of action was designed to affect only plants (Stenersen 2004), but various studies in recent years have reported adverse impact on non-target animals (Giesy et al. 2000; Tsui & Chu 2003; El-Shebly & El-kady 2008).

**MATERIALS AND METHODS**

**Test organism**

Forty days post hatching (dph) shrimps of mean length 10.123 (± 0.227) mm were obtained from a culture maintained at the UCEWQ. Organisms were acclimatized for 24 h in a controlled environment of temperature 24 ± 1 C and 14:12 h light:dark regime before being transferred individually into experimental vessels with the aid of a glass pipette (volume capacity = 10 mL; internal diameter = 2.0 mm). The shrimps had very tiny body width (<1.0 mm), which made sucking them up into the pipette very easy.

**Test chemical**

Liquid Roundup®, active ingredient: 360 g glyphosate (glyphcine) a.e./L (contains 480 g isopropylamine salt of glyphosate/L, registered and distributed by Monsanto South Africa (Pty Ltd.), was purchased from a local chemical shop in Grahamstown, South Africa. As the manufacturers recommend 2–4% application rate, a 2% stock solution was prepared by dissolving 20 mL Roundup® (in a 1,000 mL Schott Duran bottle) with distilled water to the 1,000 mL mark to obtain a concentration of 7,200 mg/L a.e. Appropriate dilutions of the stock solution were made with dechlorinated tap water to obtain the desired nominal
exposure concentrations just before start of the experiments. Thus, all units for Roundup® concentrations reported in this study were in mg/L a.e., just as the unit of the stock solution.

**Acute toxicity and chronic toxicity tests**

*C. nilotica* 96 h acute and 21 d sublethal toxicity tests were conducted with Roundup® at concentrations 0.0 (control), 4.3, 6.7, 10.5, 16.4, 25.6 and 40 mg/L for 96 h test; and 0.0, 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L for 21 d sublethal test, in 600 mL glass beakers. Tests were conducted according to protocol developed by the UCEWQ (Gola & Muller 2008). Each concentration contained 10 shrimps and was replicated three times. Dead shrimps were recorded twice daily and removed from experimental vessels. Shrimps were not fed during the experimental period. Each test was repeated three times and results pooled for analysis. At the end of each test period, all surviving shrimps were quickly transferred individually into Eppendorf tubes, immediately frozen in liquid nitrogen and stored at −80 °C until the AChE activity was measured. Water quality parameters in both experiments were measured: pH ranged from 8.34 to 8.55, DO (dissolved oxygen) from 5.89 to 5.99 mg/L, EC (electrical conductivity) from 0.90 to 0.97 mS/cm and temperature from 24.00 to 24.50 °C. These ranges of values were all within the acclimated conditions of the culture maintained in the laboratory.

**Measurement of acetylcholinesterase (AChE) activity**

Measurement of shrimp whole-body AChE activity was based on the method described by McLoughlin et al. (2000). Whole shrimp body lengths were measured, homogenized with 30 μL 1% Triton-X-100 in 0.02 M phosphate buffer solution (PBS) (pH 8), and diluted with 270 μL 0.02 M PBS (pH 8). The homogenate was centrifuged at 14,000 g at 4 °C for 15 min. Clear supernatant (50 μL) and 100 μL of the chromogenic Ellman’s reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (0.008 M, pH 7) were added to a 96-well microtiter plate. Measurement of enzyme activity was initiated by adding 50 μL of acetylthiocholine iodide solution (0.016 M). The final volume was 200 μL. Spontaneous substrate hydrolysis was assessed using two controls, a blank without acetylthiocholine and a blank without the sample. Absorption of the 2-nitro-5-thiobenzoate anion, formed from the reaction, was recorded at 405 nm every 30 s for 10 min (at 30 °C) using a microtiter plate reader (PowerWave, Bio-Tek Instruments Inc, USA). Enzyme activity was expressed as micromoles of acetylthiocholine hydrolyzed per minute per milligram of protein. Protein levels for AChE were estimated by the Bradford method (1976), using bovine serum albumin (BSA) as standard. Absorption kinetics were calculated in a linear range, and then converted to nanomoles per minute using the equation: $\frac{\text{AChE activity rate (nmol/min)}}{\text{mg protein}} = \frac{\Delta \text{abs} / \text{min}}{\text{volume (L)}} = \varepsilon \times L$, where $\varepsilon = 1.36 \times 10^4$ L/mol/cm is the molar extinction coefficient of DTNB, $L$ is the path length in cm, and $\Delta \text{abs}$ is the change in absorbance per minute (Ellman et al. 1961). Each sample and standard was analyzed in quadruplicate.

**Statistical analysis**

Data from both 96 h acute and 21 d chronic toxicity tests were analyzed for normality using the Kolmogorov–Smirnov tests ($p < 0.05$). One-way analysis of variance (ANOVA) was used to test the hypothesis that the mean AChE activities across the different tested concentrations were equal. In the presence of statistical significance difference, Newman–Keuls multiple comparison post-hoc tests were used to compare means of any two groups. Student’s $t$-test was used to compare mean control groups AChE activity values between 96 h and 21 d toxicity tests, and Pearson’s correlation was used to compare the relationship between protein content and AChE activity. A Pearson’s correlation value ($R^2$) of greater than 0.5 implies a strong and positive relationship. Statistics were performed using Statistica Version 9 and all statistical decisions were made at alpha $= 0.05$ a priori.

**RESULTS**

In order to rectify potential enzyme concentration variations that might result from output differences during the extraction process, AChE activities are subjected to normalization by dividing with the protein content and are expressed as nmol/min/mg protein (Xuereb et al. 2009a). Normalization is based on the assumption that changes in AChE activity and total protein contents are proportionate as the former is composed of protein. Thus, a positive relationship should exist between AChE activity expressed in nmol/min and total protein content expressed in mg/mL (Xuereb et al. 2009a). The non-normalized and normalized AChE activity levels for 96 h acute and 21 d chronic toxicity tests are presented in Table 1. Pearson’s correlation analysis of the relationship between protein contents and non-normalized AChE activities of whole-body shrimp in the 96 h acute toxicity showed no significant linear
relationships between AChE activities and protein contents ($p > 0.05$, $R^2 = 0.1128$). However, Pearson’s correlation coefficient indicated a statistically significant linear relationship between protein contents and normalized AChE activities ($p < 0.05$, $R^2 = 0.6047$). For 21 d chronic toxicity test, Pearson’s correlation indicated no significant linear relationship between protein contents and non-normalized AChE activities ($p > 0.05$, $R^2 = 0.0880$), but a statistically significant linear relationship between protein contents and normalized AChE activities ($p < 0.05$, $R^2 = 0.5274$) was found.

The normalized AChE activities were tested against the hypothesis that mean values across different concentrations were equal. The null hypothesis was accepted when one-way ANOVA revealed that the average AChE activity across different Roundup® concentrations was equal for both 96 h acute toxicity and 21 d chronic toxicity tests ($p > 0.05$). The mean AChE activity and standard deviations ($\pm$ SD) for control, 4.3, 6.7, 10.5, 16.4, 25.6, 40.0 mg/L respectively (Figure 1). For the 21 d chronic toxicity test, the mean AChE activity and standard deviations ($\pm$ SD) were found to be 3.6907 ($\pm$ 0.3401), 2.8473 ($\pm$ 0.713), 2.6738 ($\pm$ 0.7117), 2.3019 ($\pm$ 0.4464) nmol/min/mg protein for concentrations 0.0 (control), 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L respectively (Figure 2). Basal AChE activity was evaluated by Student’s $t$-test analysis of the mean activity between 96 h and 21 d toxicity tests control groups. The means of the two control groups were not significantly different ($t(4) = 0.44$, $p > 0.05$), and the basal AChE activity was estimated as 3.6907 ($\pm$ 0.3401) nmol/min/mg protein.

### DISCUSSION

The use of biomarkers in aquatic invertebrates as water pollution indicators has received widespread recognition in

<table>
<thead>
<tr>
<th>Period</th>
<th>Concentration (mg/L)</th>
<th>Non-normalized AChE ($\pm$ SD) (nmol/min)</th>
<th>Normalized AChE ($\pm$ SD) (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 h</td>
<td>0.0</td>
<td>1.1697 ($\pm$ 0.4040)</td>
<td>3.6239 ($\pm$ 0.4185)</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>0.919 ($\pm$ 0.2549)</td>
<td>3.4157 ($\pm$ 1.1842)</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>0.7003 ($\pm$ 0.1493)</td>
<td>2.537 ($\pm$ 1.3989)</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>0.88 ($\pm$ 0.5169)</td>
<td>2.4253 ($\pm$ 1.4202)</td>
</tr>
<tr>
<td></td>
<td>16.4</td>
<td>0.9587 ($\pm$ 0.3555)</td>
<td>2.4127 ($\pm$ 1.9097)</td>
</tr>
<tr>
<td></td>
<td>25.6</td>
<td>1.0553 ($\pm$ 0.4876)</td>
<td>2.0017 ($\pm$ 1.1080)</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>1.214 ($\pm$ 0.3329)</td>
<td>2.316 ($\pm$ 0.4001)</td>
</tr>
<tr>
<td>21 d</td>
<td>0.0</td>
<td>1.4793 ($\pm$ 0.4134)</td>
<td>3.6907 ($\pm$ 0.3401)</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>1.2305 ($\pm$ 0.3503)</td>
<td>2.8473 ($\pm$ 0.713)</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>1.1204 ($\pm$ 0.2196)</td>
<td>2.9134 ($\pm$ 0.9879)</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>1.1062 ($\pm$ 0.2957)</td>
<td>2.6738 ($\pm$ 0.7117)</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>1.6636 ($\pm$ 0.1767)</td>
<td>2.3019 ($\pm$ 0.4464)</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>2.1698 ($\pm$ 0.2726)</td>
<td>2.1478 ($\pm$ 0.864)</td>
</tr>
</tbody>
</table>
Biomarkers are valuable tools for environmental assessment because they make it possible for chemical stress to be expressed in biological terms (Xuereb et al. 2009a,b; Richardson et al. 2010). AChE activity levels in organisms exposed to toxic chemicals may be concentration-dependent and/or time-dependent since exposure time and concentration used are important determinants of a chemical’s toxicity. In this study, AChE activity levels decreased with increasing concentrations in both 96 h acute toxicity and 21 d chronic toxicity tests (Figures 1 and 2), which seems to suggest that AChE activity levels in C. nilotica is concentration-dependence. In a separate study, Beltran & Pocsidio (2010) reported concentration-dependence of AChE when they evaluated effect of the pesticide malathion on the freshwater bivalve mollusc Corbicula fluminea. Glusczak et al. (2007) reported reduction in AChE activity levels of Silver catfish (Rhamdia quelen) brain after exposure to commercial formulation Roundup® of concentrations 0.0 (control), 0.2 or 0.4 mg/L for 96 h. Their results concur with the current study that the herbicide Roundup® causes reduction in AChE activity levels of aquatic animals in a concentration-dependent manner. In another study, Xuereb et al. (2009b) exposed Gammarus fossarum to the organophosphorous pesticide chlorpyrifos (CPE) and the carbamate pesticide methomyl (MT) for 96 h. They used concentrations of 0.125 – 1 μg/L of CPE and 10 – 160 μg/L of MT. They reported decrease in AChE activity levels in G. fossarum as concentrations were increased for both pesticides.

Results of the current study showed no significant difference between mean AChE activity levels between 96 h acute toxicity and 21 d chronic toxicity tests, which were found to range between 2.6761 (± 1.1772) and 2.7736 (± 0.8017) respectively. This suggests that AChE activity is not time dependent, and therefore Roundup® effect on AChE levels in C. nilotica does not depend on acute or chronic exposure. This seems to be supported by previous studies. In the study of Xuereb et al. (2009b), they reported that CPE caused decreased AChE levels over the 96 h period, which was contrary to the current study where time did not affect the enzyme’s activity in both 96 h and 21 d toxicity tests. However, their study with MT revealed that AChE activity levels remained constant over the exposure period, which is in agreement with this study. Such observed phenomena may be attributed to the length of exposure period, toxicity of the chemical and intrinsic biotic factors of the organism involved.

In order to use appropriately an enzyme activity as a biomarker of contamination, good knowledge of its variability in terms of intrinsic biotic and environmental factors is required. Thus, it is necessary to establish reference basal enzyme activity levels in the test organism before an enzyme activity is used as a biomarker of pollution. Basal enzyme activity is often derived from the control groups in exposure studies (Xuereb et al. 2009a,b). In the current study, the reference basal AChE activity range was established from the control groups mean enzyme activities, which was found to be 3.6907 (± 0.3401) nmol/min/mg protein. Therefore, this was used as the reference point at which AChE activity levels of the current study were evaluated. Based on this, AChE activity levels in nmol/min/mg protein in 96 h acute toxicity test were transformed to percent activity levels to obtain 100, 94, 70, 67, 55 and 64% for control, 4.3, 6.7, 10.5, 16.4, 25.6 and 40.0 mg/L respectively. This implies percent activity levels reduced by 0, 24, 22, 29, 39 and 43% in the respective Roundup® concentrations. Percent AChE activity levels for shrimps in the 21 d chronic toxicity were 100, 76, 78, 71, 61 and 57% for control, 2.2, 2.8, 3.4, 4.3 and 5.4 mg/L respectively, which means AChE activity levels reduced by 0, 24, 22, 29, 39 and 43% in the respective Roundup® concentrations. Many studies have reported AChE activity levels in percentages and have demonstrated that reduction in AChE activity levels is proportional to increased toxicant exposure concentrations. Printes & Callaghan (2004) demonstrated that the organophosphates chlorpyrifos, malathion and parathion caused reduction in Daphnia magna AChE activity levels by up to 50% with accompanying adverse effects on mobility, while acephate caused 70% reduction in D. magna AChE activity levels with no adverse effects. Xuereb et al. (2009b) reported that chlorpyrifos caused more than 50% AChE activity inhibition in G. fossarum, while the median lethal concentration (LC50) produced 70% AChE activity inhibition. They also reported that 160 μg/L of MT caused 66% AChE inhibition in G. fossarum.

As stated earlier, AChE activities are generally normalized against the protein content in sample extracts and expressed in nanomoles of substrate hydrolyzed (nmol/min/mg protein) (Xuereb et al. 2009a,b; Richardson et al. 2010). Conversely, the natural variation of structural protein contents that is related to physiological changes such as reproductive status constitutes a source of variability, which may cause imprecision in estimating the basal level of AChE activity (Xuereb et al. 2009a). The robustness of the basal activity estimated for this study lies in the fact that the shrimps used were lab-cultured and may not be affected much by environmental factors as the experiments.
were conducted under controlled conditions. Moreover extracting AChE from the whole body means getting access to the total protein content present in the organism, as against extraction from specific tissues from large animals (Printes & Callaghan 2003; Richardson et al. 2010). Thus, the use of total protein content to normalized AChE activity levels ensured that the natural variation of structural protein contents would have little or no impact on the reported levels of activity.

**CONCLUSION**

*Caridina nilotica* is a good sentinel toxicity test organism for aquatic ecotoxicological studies. The present study provides some necessary ecotoxicological data that could be the basis for the possible use of *C. nilotica* AChE activity levels for monitoring by pollution of aquatic ecosystems Roundup® and other glyphosate formulated herbicides. The establishment of the basal AChE activity level for 40 dph *C. nilotica* has provided a reference value with which relative AChE activity levels can be estimated in a polluted environment. If minimum and maximum reference thresholds are estimated below and above the basal reference value respectively, then any decrease or increase of AChE activity levels may be deemed a consequence of present or past pollution.

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