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In vitro and *in vivo* inhibition of *Gammarus pulex* acetylcholinesterase by diazinon

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Abstract

This study was designed to investigate the effect of the *in vivo* and *in vitro* exposure to the organophosphorothioate pesticide diazinon, its metabolite diazinon oxon and diazinon in a commercial formulation on the AChE activity of the freshwater amphipod *Gammarus pulex*. Only diazinon oxon at concentrations of $\geq 50x10^3$ nM oxon caused a significant inhibition of acetylcholinesterase (AChE) activity *in vitro*. However, diazinon oxon had no significant impact on the enzyme activity following *in vivo* exposure. The latter mode of exposure to either $\geq$ 10 nM of diazinon or $\geq$ 5 nM of the diazinon in the commercial formulation caused a significant reduction in AChE activity of treated organisms. This study supports the finding of previous studies that suggested AChE activity in aquatic animals as a valuable biomarker of diazinon.

1- Introduction

Organothiophosphate pesticides (OPs) have been widely employed in modern agriculture to protect crops and seeds from the unwanted presence of insect pests (Pimentel, 2009). A faction of these chemicals can reach other environmental compartments such as water bodies and thus impact non-target organisms (Adedeji *et al.*, 2009). The toxicity of OPs is mainly caused by the inhibition of Acetylcholinesterase (AChE), which is an important enzyme in the nervous system of animals. This enzyme is responsible for the hydrolytic degradation of the neurotransmitter acetylcholine (ACh). As a result of AChE inhibition, ACh can accumulate in the synaptic cleft and cause abnormal activity in both neuronal and muscular tissue (Roex *et al.*, 2003). This can

consequently lead to dysfunction in the impacted organisms, such as behaviour changes, paralysis and death (Fulton and Key, 2001; Xuereb *et al.*, 2007). Therefore, determination of AChE activity is employed in clinical practice and environmental biomonitoring investigations to examine the effect of OPs exposure on biota under both laboratory and field conditions (Xuereb *et al*., 2007; Falfushynska and Stolyar, 2009; Mdegela *et al*., 2010). Studying alterations in AChE activity enables detecting of sublethal responses to OPs exposure at biochemical level before more crucial physiological and population impacts become obvious. In addition, the measurement of AChE activity provides a sensitive parameter to test the presence of OPs (Kuhn and Streit, 1994).

Monitoring of AChE in aquatic fauna might be more useful than the use of analytical chemistry alone. The latter approach can only provide data regarding the concentrations of contaminants in water ecosystems but do not offer information on the toxicity and the potential impact of pollutants on the ecosystems. In addition, the majorities OPs have relatively short half-lives in the aquatic environment and are not water soluble. Thus, the environmental level of these chemicals might reduce below detectable concentrations in a short period (Post and Leasure, 1974; Fulton and Key, 2001).

In invertebrates, the inhibition of AChE by OPs exposure was observed in various extracts such as whole body tissues, muscle tissue, nerve ganglia, and hemolymph (Dembélé *et al*., 2000; Fulton and Key, 2001; García-de La Parra *et al*., 2006; Xuereb *et al*., 2009; Tu *et al*., 2010). Among aquatic invertebrates, *Gammarus* is one of the most sensitive genera of organisms to anti-cholinesterase compounds (Kuhn and Streit, 1994; Xuereb *et al.*, 2007). Alterations in the ACh neuronal of pathways can disturb higher integrated processes. Xuereb *et al*. (2009) observed that reduction in feeding rate and movement activity of *Gammarus fossarum* were directly related to levels of AChE inhibition for the OP pesticide chlorpyrifos and the carbamate pesticide methomyl (Xuereb *et al*., 2009). Several studies have examined the effect of the organothiophosphate pesticide, diazinon, on AChE activity in aquatic animals. For instance, Sharbidre *et al*. (2011) reported that exposure to 0.5 µM of diazinon caused a significant reduction brain in AChE activity

of the freshwater guppy fish *Poecilia reticulate* but did not affect the muscle and gill AChE activity of exposed animals. In another study, AChE activity in the plasma of the rainbow trout *Oncorhynchus mykiss* was significantly decreased following 7 days of exposure to 0.33 µM of diazinon (Banaee *et al*., 2011). Pan and Dutta (1998) observed that AChE activity in brine of juvenile largemouth bass *Micropterus salmoides* was 10-times more sensitive to diazinon than median lethal test.

Alteration in AChE activity in aquatic biota by insecticides was observed following both *in vivo* and *in vitro* exposure (Straus and Chambers, 1995; Kuhn and Streit, 1994; García-de La Parra *et al*., 2006; Gagnaire *et al*., 2008; Xuereb *et al*., 2009). *In vivo* assays are more environmentally relevant approach to test the influence of pollutants on biochemical biomarkers. Although this approach is more time consuming than *in vitro* assays, important factors that may affect the uptake of pollutants and their availability to an organism are taken into account (Ibrahim *et al*., 1998). Therefore, *In vivo* technique may provide a better understanding of biomarker responses to contaminant exposure under natural conditions.

Gammarids are important freshwater detritivores, involved in the decomposition process of leaves and other decaying materials, and serve as an important food source for fish Kuhn and Streit, 1994). The aim of this study was to investigate the effects of the OP pesticide diazinon, its metabolite diazinon oxon and diazinon in a commercial formulation on AChE activity of *G. pul*ex.

2- Material and methods

2.1- Animals collection and maintenance

Samples were collected in October, 2009. A hand net was used to obtain *G. pulex* from a single population in a freshwater slow-running unpolluted stream located at the Creswell Crags nature reserve, Derbyshire. Immediately after sampling, specimens were stored in 25 liters plastic pins containing stream water and quickly transferred to the laboratory. Specimens were kept at least 7 days for acclimatisation in oxygenated aquaria containing de-chlorinated tap water. Animals' acclimation and all subsequent experiments were performed at 15 °C under a 12 h light: dark cycle. Animals were fed

with conditioned alder leaves (*Alnus glutinosa*) during acclimation up to 24 h prior to experiment. During acclimation, de-chlorinated tap water was renewed every three days.

2.2- Acetylcholinesterase activity

The activity of AChE in *G. pulex* was measured employing the colourimetric method based on Ellman *et al*. (1961), modified for microplates. All preparation procedures were performed at 4 °C. Treatments were performed using three replicates, each containing 3 randomly chosen adult intermoult *G. pulex* (both sexes; approximately 30±5 mg wet weight). Examination of the sex of *G. pulex* may induce stress and/or damage to organisms. Tested organisms were homogenized in 4 mL of ice-cold phosphate buffer (0.1 M; pH 7.8) containing 0.1% v/v Triton X-100, with an Ultra-Turrax® T25 Basic blender at 24,000 rpm for 10 sec. To obtain a clear supernatant, reducing the effects of light scattering, the homogenate was centrifuged at 9000 g for 15 min at 4°C. The clear supernatant was carefully collected, placed into clean 1.5 mL microcentrifuge tubes and kept at 4°C until used in the assay. For sample preparation, all substances were kept on ice to maintain their stability. Either 16 µL of the supernatant or homogenisation buffer were placed in the wells of a 96-well microplate. Then 285 µL of the reagent mix (0.1 M PBS pH 7.8, containing 0.2 mM DTNB) as chromogenic reagent, and 2 mM of acetylthiocholine iodide (ATCh) as a substrate were added. Two blanks, one without substrate and one without sample, were used to measure the reaction of thiol groups, and spontaneous substrate hydrolysis. The measurement of enzyme activity was carried out using a rate assay by following the rate of change of absorbance at 415 nm for 20 min (measurement once every 30 sec) at 25°C.

In order to assess the *in vitro* inhibition of AChE activity, non-pesticides treated *G. pulex* were homogenised as previously described and the 9000 g supernatant kept for measurement of AChE activity. Homogenates were *in vitro* exposed to 0, 10, 100, 1000, 10000or 100000 nm of diazinon, diazinon oxon or the commercial diazinon formulation. They were immediately vortex mixed and placed on ice for 15 min to allow interaction with the gammarid AChE. The enzyme activity was then determined as described above. For *in*

vivo exposure gammarids were exposed to diazinon (0, 10, 25 or 50 nM), diazinon oxon (0, 10, 25 or 50 nM) or the commercial diazinon formulation (0, 5, 10, 25 or 50 nM) for up to 24 h. These concentrations were selected based on the results of the 96 h LC_{50} (median lethal concentration) test (data is not shown). For the time course experiment, AChE activity was measured in organisms exposed to 0, 10 or 25 nM of diazinon, diazinon oxon or the commercial diazinon formulation following 2, 4, 6, 12, 16 and 24 h of exposure.

The enzyme activity was adjusted according the protein content of the sample, which was measured using the bicinchoninic acid (BCA) protein assay.

2.3- Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM) calculated from independent replicates. Wilk test and Hartley–Cochran–Bartlett tests were employed to test the normal distribution of the variables and the variance homogeneity, respectively. Differences between data groups were evaluated by analysis of variance (ANOVA), after which results were analysed using a post hoc Tukey test with 95% confidence limits to determine significance differences between the control and exposure groups.

3- Results

3.1- *In vitro* exposure

Exposure to up to $10x10^4$ nM of diazinon or diazinon in the commercial formulation had no significant effects on AChE activity in *G. pulex* extracts (Figure 1). In contrast, the enzyme activity in *G. pulex* extracts treated with $50x10^3$ nM or higher concentrations of diazinon oxon was significantly lower than that of untreated organism ($p<0.001$), with an IC_{50} value of 3 µM.

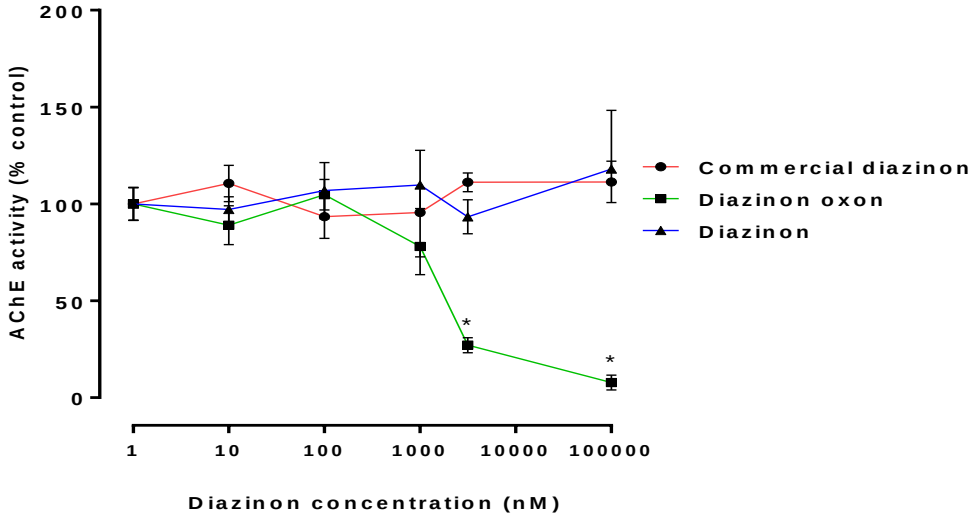

Figure 1 Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the *in vitro* acetylcholinesterase activity of *G. pulex*. Means ± SEM., n = 3,* significantly different from control ($p<0.001$).

3.2- *in vivo* exposure

3.2.1- Twenty four hour exposure

As shown in Figure 2, the AChE activity of *G. pulex* was not significantly reduced following exposure to up to 50 nM of diazinon oxon. In contrast, exposure to 10 nM or higher concentrations of diazinon or 5 nm or higher concentrations of the diazinon in the commercial formulation caused a significant reduction in the enzyme activity (Figure 2). The IC_{50} values were 10 and 17 nM for diazinon in the commercial formulation and diazinon, respectively.

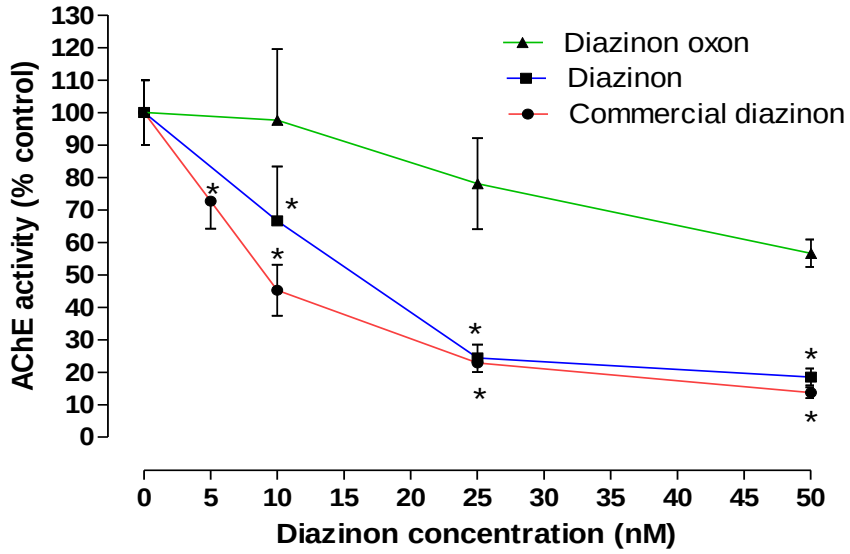

Figure 2 Effect of diazinon, diazinon oxon and diazinon in the commercial formulation on the acetylcholinesterase activity of *G. pulex* after 24 h exposure. Means ± SEM, n = 3; * significantly different from control ($p<0.001$).

3.2.2- Time course experiment

Under the current study conditions, AChE activity in *G. pulex* was not significantly affected following exposure to 10 nM diazinon (Figure 3). However, a significant reduction in the enzyme activity was observed following exposure to 25 nM diazinon for 12 h or longer period ($P< 0.001$). In contrast, up to 24 h of exposure to diazinon oxon had no significant effect on the AChE activity of treated animals (Figure 4).

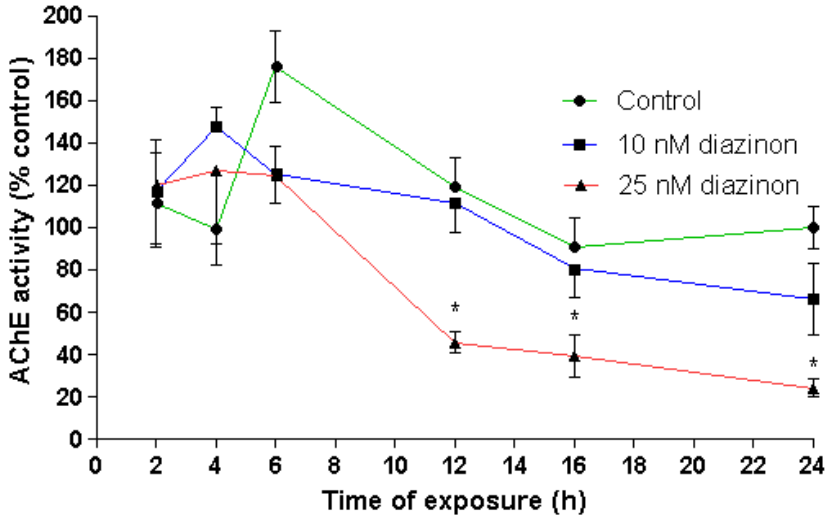

Figure 3 Time course for the effect of diazinon on the acetylcholinesterase activity of *G. pulex*. Means ± SEM, n = 3; * significantly different from control p<0.001).

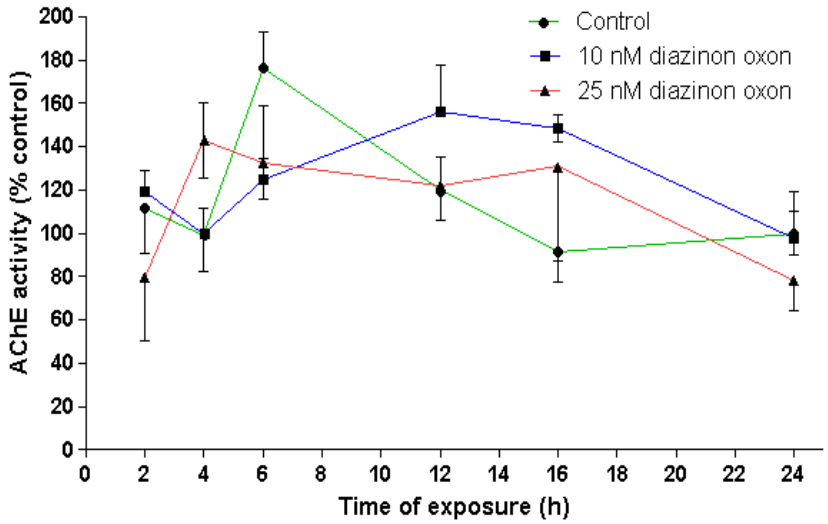

Figure 4 Time course for the effect of diazinon oxon on the acetylcholinesterase activity of *G. pulex*. N = 3.

Exposure to 25 nM of diazinon in the commercial formulation caused significant inhibition in AChE activity in test animals following 16 h or longer period. However, lower tested diazinon in the commercial formulation (10 nM) took 24 h to induce significant reduction in the enzyme activity of exposed organisms (Figure 5).

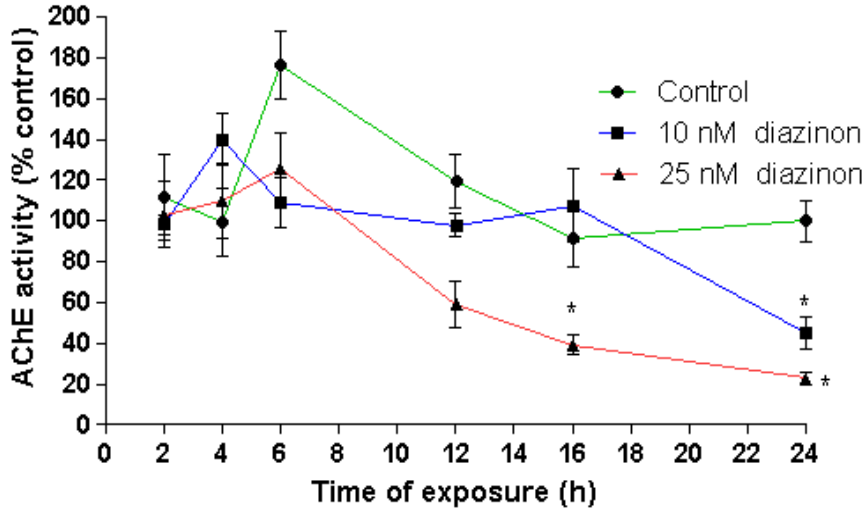

Figure 5 Time course for the effect of diazinon in the commercial formulation on the acetylcholinesterase activity of *G. pulex*. (Means ± SEM, n = 3, * significantly different from control ($p<0.001$).

4- Dissection

In the current study, initial investigations indicated that the 9000 g supernatant of whole body extracts of *G. pulex* can provide a good model to examine the impact diazinon on AChE activity. AChE activity of *G. pulex* of tested organisms was inhibited following an *in vivo* exposure to diazinon and diazinon in the commercial formulation but not diazinon oxon. This suggests that, under *in vivo* exposure, the sensitivity of this enzyme in *G. pulex* is higher to diazinon and diazinon in the commercial formulation than that to diazinon oxon. The inhibition of AChE activity, observed in the current study, might be caused by direct action of diazinon and diazinon in the commercial formulation exposure on the active site of this enzyme. A significant inhibition in AChE activity not only indicates that the organism has been exposed, but also that a high enough

dose of the toxin has reached the target site to cause a physiological impact (Edwards and Fisher, 1991; Fulton and Key, 2001). Inhibition of AChE reduces the hydrolytic degradation of Ach, which can accumulate in the synaptic cleft leading to abnormal activity in muscular tissue. This might include spasms and paralysis which lead to muscular convulsions (Fulton and Key, 2001; Üner *et al*., 2006).

Under in vitro exposure conditions, the enzyme activity was not significantly affected by diazinon or diazinon in the commercial formulation. In contrast, in vitro exposure to 10 µM diazinon oxon caused almost fill inhibition (92%) in AChE activity. The absent of significant effect on AChE by diazinon oxon following *in vivo* exposure may be explained by reduction in uptake rate and/or increase in degradation of diazinon oxon *in vivo*. In an earlier study, toxicity of metabolite diazinon oxon to in *Daphnia magna* was hardly detected during exposure to diazinon for 21 h (Kretschmann *et al*., 2011). The authors suggested that, diazinon oxon is accumulated lower than diazinon. In another study, 50% reduction in AChE activity caused significant decrease in the survival of *G. fossarum* exposed to chlorpyrifos (Xuereb *et al*., 2009). However, the same study, did not observe decrease in the survival of test organisms following exposure to the carbonate pesticide methomyl, which reduced AChE activity by 66%. This suggests that acute toxicity caused by chlorpyrifos might not be based on AChE but on other toxicity pathways (Xuereb *et al*., 2009).

In line with the findings of this study, earlier studies presented similar results, in which AChE of aquatic organisms was reduced following exposure to diazinon. For instance, 24 h exposure to 0.6 µM diazinon led to significant inhibition in AChE (63%) of largemouth bass *Micropterus salmoides* (Üner *et al*., 2006). The same study observed that the enzyme activity in brain of the Nile tilapia *Oreochromis niloticus* was significantly reduced (93%) following a week of exposure to 3.3 µM diazinon. Likewise, exposure to 0.0014 µM chlorpyrifos caused a significant decrease in AChE activity in *G. fossarum* (Xuereb *et al*., 2009). Kuhn and Streit (1994) observed significant reduction in AChE activity in *G. pulex* following 24 h exposure to 0.004 and 0.003 µM of fenitrothion and parathion–methyl, respectively. Similarly, a significant inhibition in the enzyme activity in *G. pulex* was observed following 8 day exposure to 458.5 µM of parathion–methyl.

In vivo exposure to chlorpyrifos caused significant decline in AChE of *G. pulex* activity with a 96-h IC_{50} of 0.99 nM. However, under *in vitro* exposure, a much higher concentration (100 µM) of chlorpyrifos was needed to induce significant inhibition (95%) in the enzyme activity of exposed organisms (Xuereb *et al*., 2007). This suggests the involvement of bioaccumulation and biotransformation mechanisms. After accumulation in *G. pulex*, chlorpyifos is rapidly eliminated (Ashauer *et al*., 2006). The authors reported that approximately 50% of this pesticide was eliminated in test organisms within the first 3 days of the depuration phase. In another study, Berger and Sultatos (1997) reported that, exposure to insecticides can inhibit the P450-dependent metabolism of certain endogenous substrates in mammalian.

In contrast to these findings, AChE activity in D. magna was not significantly altered by 2 days exposure to 0.023 µM diazinon (Jemec *et al*., 2007). The authors suggested that diazinon might be metabolised in exposed organisms. In addition, diazinon toxicity is species-dependent. Keizer *et al*. (1995) reported that diazinon toxicity is affected by the level of bioactivation of this pesticide by conversion to the potent diazinon oxon, detoxification in the species, and the affinity of AChE for diazinon within *D. magna*.

Different sensitivity of aquatic organisms to diazinon may be attributed to differences in absorption rate of this pesticide. For instance, while the LC_{50} concentration of diazinon to killifish was 14-times lower than that to loach *Misgurnus anguillicaudatus*, inhibition of AChE by diazinon oxon in killifis was 22-times lower than that in loach (Oh *et al*., 1991).

The present investigation indicated that the pesticide diazinon can inhibit the activity of AChE in *G. pulex*. Inhibition of AChE activity can interrupt the central and peripheral nervous system, causing several behavioural impacts, such as hyperactivity, asphyxia, and eventually mortality (Roex *et al*., 2003; Xuereb *et al*., 2009). Therefore, it is suggested that AChE in *Gammarus spp* could be effectively employed as a biomarker of pesticide toxicity.

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