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Comparison of Acetylcholinesterase and Glutathione S-Transferase Activity in *Chironomus riparius* Meigen Exposed to Chemical-Spiked Sediments

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Biochemical biomarkers may identify effects at a subcellular level before they are apparent at higher levels of biological organization (McCarthy and Shugart, 1990). Two such biomarkers are the enzymes acetylcholinesterase (AChE) and glutathione S-transferase (GST). The inhibition of AChE by neurotoxic compounds has been widely used as a sensitive biomarker in wildlife and humans (Crane et al., 1995). AChE is inhibited by phosphate and carbamate esters that are commonly used as insecticides because they bind to the enzyme. This leads to the accumulation of acetylcholine in the cholinergic synapse and disruption of normal nervous system function (Habig and Di Giulio, 1991). GSTs are a family of detoxicating enzymes that catalyse the conjugation of reduced glutathione (GSH) with a group of compounds having electrophilic centres. These can include nitrocompounds (Usui et al., 1977), organophosphates (Motoyama and Dauterman, 1977; Usui et al., 1977) and organochlorines (Clark et al., 1986). The GSH conjugation products become less toxic and more water-soluble so that they can be easily excreted from cells after further metabolism.

Chironomus riparius Meigen larvae are widely used in sediment toxicity tests because they are easy to culture, sensitive to many pollutants and have a short life cycle (Ingersoll and Nelson, 1990). Their ability to burrow into sediments makes them good biological indicators for toxicants that may be adsorbed to sediments, and changes in cholinesterase activity after exposure to toxicants have been reported by several workers (Day and Scott, 1990; Detra and Collins, 1991; Ibrahim et al., 1998).

In a previous study (Ibrahim et al., 1998) we tested the response of *C. riparius* AChE activity to a range of different chemicals and found that this biomarker was inhibited when exposed to organophosphates and carbamate pesticides, as would be expected from its mode of action. However, there was some evidence that permethrin, a synthetic pyrethroid, also inhibited AChE, albeit to a lesser extent. The exposures in the earlier study were via water only. In the study reported here we again tested compounds that are widely used in agriculture or industry. However, this time the chemicals were spiked onto sediment to provide a more a more realistic route of exposure for benthic species such as as *C. riparius*. GST as well as AChE was measured, to assess its usefulness as a biomarker in *C. riparius*

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Four chemicals were used: lindane, pirimiphos methyl, permethrin and zinc. Lindane (hexachlorocyclohexane) is an organochlorine insecticide with a very wide biological spectrum of activity (Hassall, 1990). Organochlorines are persistent pesticides and lindane is one of sixteen compounds classified as a Persistent Organic Pollutant by the United States Environment Protection Agency (USEPA, 1998). Pirimiphos methyl (*o*-2-diethylamino-6-methylpyrimidin-4-yl *o,o*-dimethyl phosphorothioate) is a broad-spectrum organophosphorus insecticide with moderate toxicity and rapid degradation in the environment (Hassall, 1990). Permethrin, a synthetic pyrethroid, is effective against a wide range of phytophagous insects and is especially useful in domestic hygiene as a pest control agent for mosquitoes (Hassall, 1990). Zinc, a heavy metal, is widely distributed in the environment and a common aquatic pollutant (Crane and Maltby, 1991).

The objective of this study was to determine whether the inhibition of AChE or the induction of GST in *C. riparius* could be used as sensitive, rapid and specific biomarkers when animals were exposed to sediment spiked with lindane, pirimiphos methyl, permethrin and zinc.

MATERIALS AND METHODS

Sediment was collected from the River Colne, Staines, Middlesex, using a stainless steel shovel to scoop samples into high-density polyethylene containers. Sediments were then stored at 4^0C for less than 24 hrs before sieving. Large particles in the sediment were removed by hand and the sediment was sieved through a 250 μm. Sediment dry weight was obtained by drying a 100 g sample at 300^0C. The amount of organic matter present in the sediment was 5.2%, determined by loss on ignition (Allen et al., 1974).

Sediment samples were spiked with lindane, pirimiphos methyl and permethrin (Greyhound Chemicals) to produce a control plus 0.1, 0.5, 1 and 5 μg/g sediment dry weight treatments. This was done by adding a measured amount of insecticide dissolved in acetone to a known weight of sediment, and mixing with a food mixer for 45 min. Zinc chloride (Sigma, Analar) dissolved in distilled water was prepared in the same way at concentrations of 0, 0.1, 0.5, 1, 5 mg Zn/g sediment dry weight.

Twenty grams of each spiked sediment was then weighed out into replicate 150 ml beakers, and 60 ml of local stream water was poured gently over the sediment in each beaker. Two replicates were prepared for each concentration and chemical.

Beakers were arranged at random on a bench, left for 2-4 h to allow the sediment to settle, covered with polyethylene film (Sainsbury food wrap, London) to reduce evaporation, and aerated overnight. *C. riparius,* initially obtained from WRc plc (Medmenham, UK), were cultured according to standard methods (ASTM, 1999). Eight fourth instar larvae were added at random to each beaker. The experiment was conducted at 20°C with a 16L:8D photoperiod and an illuminance of 500-1000 lux.

After 24 and 48 hrs, one of the duplicate vessels for each chemical and concentration was tipped into a tray. Chironomids were removed from the sediment using a soft paintbrush rinsed with distilled water between each test vessel. Animals were dried using unbleached tissue paper. They were then placed individually in Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80^{0}C before enzyme activity measurement. The sorting tray was wiped and rinsed with distilled water between test vessels.

Each snap-frozen larva was homogenised in 0.02M ice-cold phosphate buffer, pH 8 (PB) containing 1% triton-X-100. Tubes were centrifuged (Biofuge, Heraeus instruments) at 13,000g and 4 ^{0}C for 4 min. The AChE assay was based on the method of Ellman et al. (1961) modified for use in a microtitre plate (Fisher et al., 2000). The 96-well microtitre plate (MPO1, Life Science (UK) Ltd, Greiner) was loaded with 100 µl of 8 mM di-thio-nitrobenzene (DTNB, Sigma D-8130) in PB and 0.75 mg/ml $NaHCO_3$. Three replicates of 50 µl of assay blank (PB pH 8 containing 0.1% triton-x-100) or quality control enzyme (eel cholinesterase, Sigma C-3389 made up on ice in a cold room as a nominal 0.5 unit/ml in ice-cold PB), or homogenate supernatant were added. This was followed by the addition of 50 µl of 16 mM acetylthiocholine iodide (ATCI, Sigma A-5751) in PB. The microtitre plate was inserted into the plate reader (Labsystems iEMS Reader MF) and incubated for 5 min at 30^{0}C. There was a pre-measurement mix for 2.5 min at 1150 rpm. The activity rate was measured as change in OD/min at 412 nm. The molar extinction coefficient of 8160 for a light path of 0.6 cm was used for activity calculation.

GST analysis was based on the method of Habig et al. (1974), modified for use in microtitre plates. The microtitre plate was loaded on ice with three replicates of 50 µl of assay blank (PB containing 0.1% triton-X-100 and 0.1% (V/V) phenylmethylsulphonylfloride (PMSF, Sigma P-7626)), standard enzyme (1 unit/ml of Equine GST (Sigma G-6511) in ice cold phosphate buffer pH 6.5 containing 1 mg/ml bovine serum albumin (BSA, fraction V, 96-99% Sigma A-2153), and samples (diluted with two volumes of PB adjusted to produce a final pH of 6.5, containing 0.15% (v/v) PMSF)). The plate was then incubated for 5 min at 30^{0}C in the plate reader. Meanwhile the substrate mixture was made by adding 7 ml of 20 mM reduced glutathione (rGSH, Sigma G-4251) in 0.1 M pH 6.5 potassium dihydrogen phosphate KH_2PO_4 (anhydrous, 99% purity, Sigma P5379), 1 mM ethylenediamine tetra acetic acid (EDTA, Sigma ED4SS) to 12.5 ml 0.02M sodium hydrogen phosphate buffer, pH 6.5. After 5 min incubation of the solution at 30^{0}C in a thermostatic bath (Grant Instruments), 1.4 ml of 40 mM 1-chloro-2,4-dinitrobenzene (CDNB, Sigma C-6396) in 95% ethanol was added and mixed thoroughly. 150 µl of the substrate mixture was then added to the microtitre plate and the plate was incubated for a further 2 min at 30^{0}C accompanied by intermittent shaking at 1150 rpm. The measurement period was 10 min at 30^{0}C. The rate was measured as a single wavelength kinetic change in OD/min at 340 nm. The molar extinction coefficient of 5760 for a light path of 0.6 cm was used for the activity calculation.

Both the activities of GST and AChE were expressed as activity per unit protein. The protein assay was a microtitre plate version of the bicinchoninic acid (BCA) assay (Pierce). The BCA working reagent mixture was incubated in a water bath at 30^0C for 10 min prior to the addition of 200 µl to 20 µl of supernatant homogenate or standard protein solutions (0, 100, 200, 400, 600, 800, 1100, 1500 µg/l of BSA in PB pH 8 containing 0.1% triton-x-100). The plate was then inserted into the preheated plate reader and the reaction run for 10 min at 30 ^{0}C. The rate was measured as change in OD/min at 550 nm. Protein concentration was calculated in relation to a regression line drawn through points obtained from the standard curve.

Analysis of variance (ANOVA) was used to analyse differences between treatments. Biomarker data were $\log_{10} + 1$ transformed before analysis. Multiple comparisons using the Tukey Honestly Significant Difference test were used to detect significantly different treatment pairs (Zar, 1996).

RESULTS AND DISCUSSION

Exposure to permethrin and zinc had no effect on fourth instar *C. riparius* larvae. Exposure to pirimiphos methyl resulted in AChE inhibition after both 24 and 48 hr (Figure 1a and 1b). AChE activity decreased with increasing concentration of pirimiphos methyl after 24 hr ($F_{4,50} = 8.921$, $P < 0.01$); a significant difference was evident between the 5 µg/g treatment and the control, 0.1 µg/g and 0.5 µg/g treatments ($q=10.729$, 10.802 and 11.839, $p<0.05$), and between the 1 µg/g and the control, 0.1 µg/g and 0.5 µg/g treatments ($q= 12.6$, 12.511 and 14.361, $p<0.05$). After 48 hr exposure there remained a highly significant difference between treatments ($F_{4,46} =26.537$, $P<0.01$), because of differences between the 1 µg/g treatment and the control, 0.1 µg/g and 0.5 µg/g treatments ($q = 9.623$, 9.352 and 9.233, $p<0.05$), and between the 5 µg/g and the control, 0.5 µg/g and 1 µg/g treatments ($q = 4.909$, 4.603 and 4.628, $p<0.05$). It is well established that organophosphates are inhibitors of AChE, and can inhibit cholinesterase activity of *C riparius* (Callaghan et al., in press; Detra and Collins, 1991; Ibrahim et al., 1998).

There was also a significant difference in AChE activity for larvae that were exposed to lindane for 24 hr ($F_{4,50} = 3.12$, $p=0.0228$). This was because there was a difference between the 1µg/g treatment and the control ($q = 4.657$, $p<0.05$). Lindane is not a specific cholinesterase inhibitor, and the inhibition in this study was not monotonic: there was no significant inhibition at the highest concentration (5µg/g). To our knowledge no other workers have found that lindane or other organochlorines inhibit AChE, so this result may be a type I statistical error. Such results demonstrate that caution needs to be exercised if biomarker responses are used as diagnostic tools in field situations. Indeed, Olsen et al. (in press) found that AChE and GST activities could differ by almost 2 fold in *C. riparius* larvae deployed at apparently uncontaminated field sites.

No effects on GST activity were found in the present study when *C. riparius* were

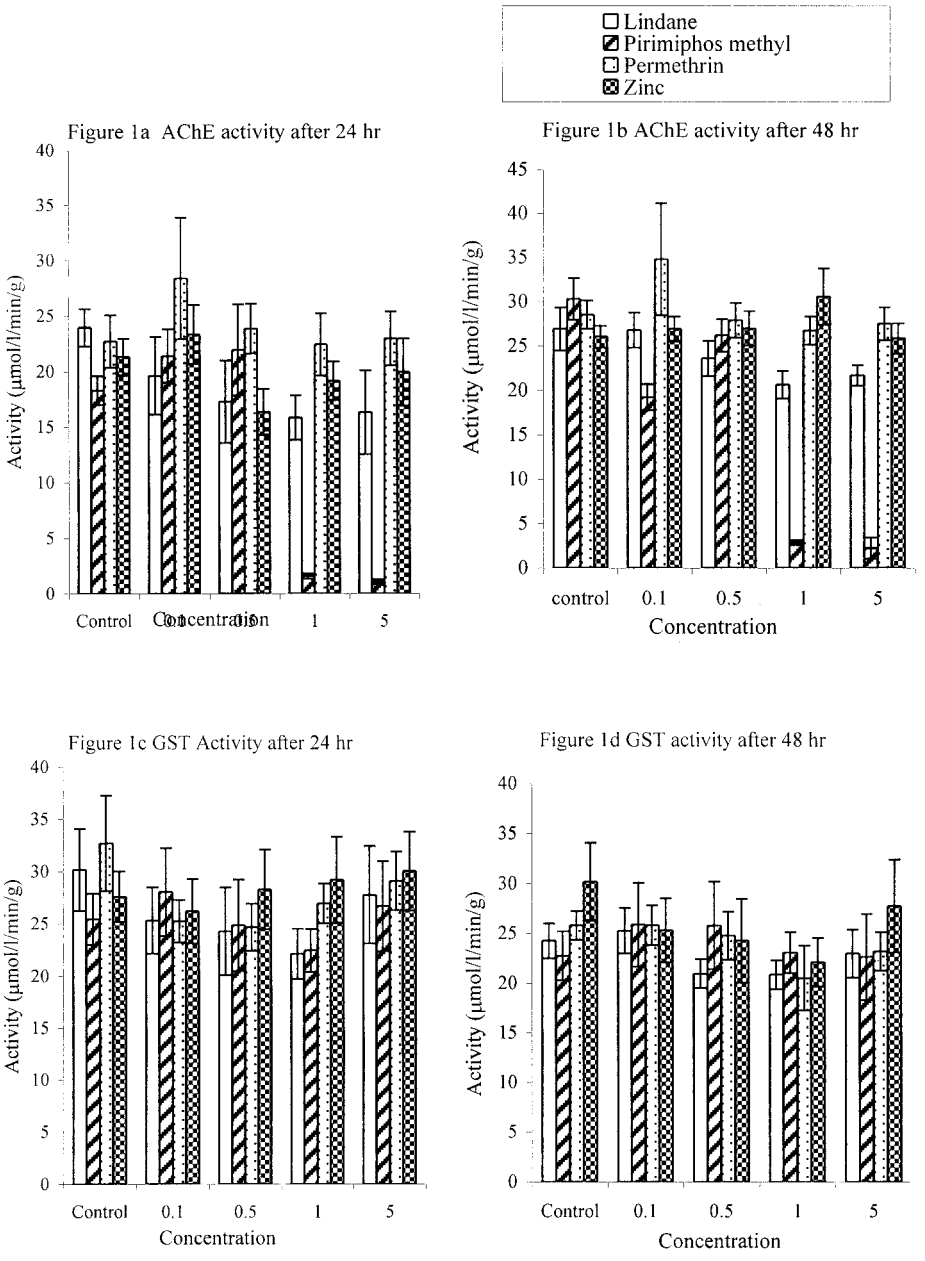

Figure1. AChE and GST activities in *C. riparius* larvae exposed to zinc, permethrin pirimiphos methyl or zinc for either 24 or 48 hours. Error bars are standard errors Concentrations are mg/g except for zinc (mg/g).

exposed to the four chemicals (Figure 1c and 1d). This is in contrast to work with other invertebrates. Housefly GST was induced by organophosphate and organochlorine insecticides (Clark et al., 1986; Hayaoka and Dauterman, 1982) and by phenobarbital (Ottea and Plapp, 1981). GST induction has also been documented in the American cockroach and the bulb mite after insecticide exposure (Capua et al., 1991; Usui et al., 1977). Our results with GST could be attributed to the molecular and physical properties of the enzyme. Glutathione s-transferase appears to have more than one isoenzyme (Motoyama and Dauterman, 1977). Clark et al. (1986) found that GST purified from two strains of housefly had two forms each, with each of these forms specific towards certain pesticides. This suggests that all GSTs are not inducible by all xenobiotics and chironomids may lack enzymes specific to the three insecticides used in the study. A second reason could be that the substrate used in this study, CDNB, is not specific for the GST induced by these insecticides. It seems from this and other studies performed in our laboratories (Hirthe et al., in press) that *C. riparius* GSTs measured in this assay are not sensitive to several different insecticides. Stegeman et al. (1992) also pointed to conflicting results in studies on induction of GST in various aquatic invertebrates.

The conclusions from this study are that the measurement of AChE in individual *C. riparius* larvae exposed to contaminated sediment can provide a rapid measure of exposure to organophosphorus pesticides. However, researchers should be aware of the danger of false positive results, particularly in field situations. The use of GST as a biomarker in *C. riparius* cannot be recommended since, in this and other studies, it does not respond even to high concentrations of insecticides.

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