INHIBITION AND RECOVERY OF ACETYLCHOLINESTERASE ACTIVITY IN THE GILLS OF THE CARP, CIRRHINUS MRIGALA EXPOSED TO ‘NUVAN®’

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ABSTRACT

The polymorphic nature of cholinesterases (ChEs) present in the gills of an Indian Major Carp, Cirrhinus mrigala, has been characterized and acetylcholinesterase (AChE) was found to be in greater proportion. A significant decline in the gill AChE activity of the fish was observed on exposure to different sublethal concentrations of ‘Nuvan®’. At 4h of exposure, the AChE activity decreased up to 68.81% at 5mg/l and 77.88% at 15 mg/l. At 4d, the inhibition was 87.1% at 5mg/l and 90.38% at 15mg/l. The inhibition of AChE activity is associated with influence on transmission of nerve impulses, resulting in improper functioning of gills. A gradual increase in the activity of gill AChE at different durations of recovery i.e. 2dr to 16dr, compared to that at 4d is observed. During recovery, the activity of the enzyme in fish exposed to 5mg/l and 15mg/l of ‘Nuvan®’, compared to those in control fish, are 68.54% and 48.33% respectively.

The study shows that the activities of the enzyme in the gills remain significantly lower even after long periods of recovery than those at controls. This indicates that the gills are unable to attain their normal metabolism even after long recovery periods. Therefore, analysis of sensitive indicators of insecticide exposure could provide useful guidelines which may help in minimizing the level of environmental pollution protecting the viability of fish populations.

KEYWORDS: Gills, Cirrhinus mrigala, Acetylcholinesterase, ‘Nuvan®’, Organophosphosphate

INTRODUCTION

Fish being highly sensitive to their environment are considered one of the most useful indicators of pollution in water bodies. A polluted ecosystem may threaten fish population and thus could have direct effect on human nutrition and health. ‘Nuvan®’, a commercial formulation (Syngenta India Ltd.), containing active ingredient dichlorvos [(O, O-Dimethyl O-2, 2-Dichlorovinyl phosphate (DDVP)), an organophosphorus insecticide, may be released in water bodies through uncontrolled disposal of wastewaters generated during its production by manufacturers and also as the result of spills or other accidents. It is frequently used for many years in agricultural and veterinary applications and often end up in aquatic habitats carried by wind, runoff, or through uncontrolled waste disposal. ‘Nuvan®’ is released on surface water as it is commonly used in fish farming to eradicate crustacean ectoparasites (Grave et al., 1991a, b; Jackson & Costello, 1992; Roth et al., 1993; Mladineo et al., 2006); and to treat culture ponds for the eradication of freshwater fish predators prior to the stocking of spawn - fry, fingerlings or juveniles of carps (Konar, 1964; Srivastava & Konar, 1966).

The mode of action of organophosphorus insecticides is based on the inhibition of the enzyme acetylcholinesterase (AChE) (Sismeiro-Vivas et al., 2007). Acetylcholinesterase (AChE; Enzyme Commission E.C.3.1.1.7) is a key enzyme that is responsible for catalyzing the hydrolysis of the neurotransmitter acetylcholine at the nerve synapse.
Inhibition of AChE creates a surplus of neurotransmitter, acetylcholine, causing prolonged excitatory postsynaptic potential. This results in repeated uncontrolled firing of neurons, leading to hyper-stimulation of the nerve/muscle fibres, which leads to paralysis and eventually death (Chebbi & David, 2009). In the literature, reports are available showing the inhibition of AChE activity in the gills of fish species exposed to a variety of organophosphorus insecticides. These include studies on the effect of sublethal concentrations of the insecticides on the gills of fish species - *Tilapia mossambica* exposed to malathion (Sahib et al., 1980); *Ictalurus punctatus* exposed to chlorpyrifos, parathion and S,S,S-tributyl phosphorotrithioate (Straus & Chambers, 1995); *Gasterosteus aculeatus* exposed to parathion (Wogram et al., 2001); *Oreochromis mossambicus* exposed to profenofos (Rao et al., 2003a); *Oreochromis mossambicus* exposed to chlorpyrifos (Rao et al., 2003b); *Catla catla, Labeo rohita and Cirrhinus mrigala* exposed to chlorpyrifos (Tilak et al., 2005); *Oreochromis mossambicus* exposed to 2-butenoic acid-3-(diethoxyphosphinothionyl) methyl ester (Rao, 2004); *Catla catla, Labeo rohita and Cirrhinus mrigala* exposed to chlorpyrifos (Tilak et al., 2005).

Most studies performed previously have focussed on the potential deleterious effects of the active ingredients. In addition, inert ingredients, may also contribute significantly to the overall toxicity of the marketed commercial formulations of the insecticides. Hence, toxicity testing of commercial formulation containing both active and inert ingredients is likely to provide more realistic report on the overall ecotoxicological impact of insecticides on sensitive non-target organisms such as fish (Peraira et al., 2009). Therefore, in the present investigation, efforts have been made to study inhibitory pattern of AChE activity in the gills of *Cirrhinus mrigala* exposed to sublethal concentrations of the commercial formulation, ‘Nuvan®’.

This study examined the presence of ChEs, its polymorphic forms and the effect of ‘Nuvan®’ on AChE activity in the gills of an Indian major carp, *Cirrhinus mrigala* (Family, Cyprinidae; Order, Cypriniformes; Taxonomic Serial Number 163679) (Integrated Taxonomy Information System, 2008). *Cirrhinus mrigala* is an economically important valuable food fish that thrives well in ponds preferably clear waters and is cultured on large scale in India. Evaluation of the effect of ‘Nuvan®’ on the AChE activity in the gills of the fish could serve as a useful biomarker and help the aquaculturists to assess the level of insecticide contamination in the aquatic environment.

**MATERIALS AND METHODS**

Live specimens of the fish *Cirrhinus mrigala* (mean ± S.D. standard length, L₅ 125.59 ± 6.3mm, weight 22.61 ± 3.86 gm; n = 378) were collected from local ponds at Varanasi. The fishes were kept at controlled room temperature (25 ± 1°C) and photoperiod on 12L:12D cycle, in glass aquaria containing water, aerated continuously; and were acclimatized with the laboratory conditions for 15 days prior to the commencement of the experiments. The fishes were fed *ad libitum* with commercial food pellets (Tokyu®). Water quality characteristics were determined following APHA, AWWA, WPCF (1985). The quality parameters (mean ± SD) of water used for acclimation, in controls and for the preparation of test solution were: temperature, 25 ± 1°C; dissolved oxygen, 7.43 ± 0.06 mg/l; pH, 7.05 ± 0.006; alkalinity, 288 ± 11.5 ppm and hardness as CaCO₃, 170.67 ± 1.16 mg/l.

**Test Chemical**

‘Nuvan®’, a commercial formulation (Syngenta India Ltd.), containing 76% w/w dichlorvos, an
organophosphorus insecticide, as an active ingredient was used in the study. The stock solution was prepared by dissolving ‘Nuvan®’, (100mg/l) in water. The test concentrations of ‘Nuvan®’ were selected in order to understand and to compare the short-term toxic effect of the insecticide at a lower concentration 5mg/l (i.e. 25% 96h LC50 value) and at higher concentration 15mg/l (i.e. 75% 96h LC50 value). The doses were selected randomly based on the survivability response of the fish (Srivastava et al., 2012).

**Experimental Design**

The fishes were exposed to the sublethal test concentrations (5mg/l and 15mg/l) of ‘Nuvan®’, using 24-h renewal bioassay method (APHA, AWWA, and WPCF, 1985). Fish exposed to the insecticide (experimental fish) as well as those without being exposed (control fish) for different intervals — 4h, 8h, 12h, 1 day (d), 2d, 3d, and 4d were cold anesthetized following Mittal and Whitear (1978). Another group of the fish, exposed to the test concentrations of the insecticide for 4d, was transferred to fresh water to recover for different intervals — 2 days of recovery (2dr), 4dr, 6dr, 8dr, 12dr and 16dr and was cold anesthetized

**Preparation of Tissue Extracts**

From each set, 3 fishes were selected randomly at each interval and were cold anaesthetized following Mittal and Whitear (1978). The fishes were kept in water at room temperature (25 ± 2°C); crushed ice was added to the water gradually, at such a rate that the temperature fell a few degrees every few minutes. At 10 ± 1°C the fishes became immobile and unresponsive to touching or pricking and the fish were taken out of water without struggle to excise the gills. The fish were euthanized after sampling. Gills excised from each fish were processed separately. Excised pieces of gills were, rinsed in 0.1 M phosphate buffer (at 4°C), blotted dry using Whatmann filter paper (Grade1) and weighed. The tissues were then homogenized (10%, w/v) under ice cold condition in 0.1 M phosphate buffer (pH 7.1), using a glass homogenizer kept in a beaker filled with ice flakes. The homogenates were centrifuged at 4°C for 15 min at 8,000 x g. The corresponding supernatants were used fresh, to determine protein contents and for enzyme assay.

**Determination of Protein Contents**

Protein contents in supernatants of fish tissue homogenates were determined according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

**Characterization and Assay of Cholinesterases in Fish Gills**

Characterization of gill cholinesterases was carried out following the method of Ellman et al. (1961) using acetylthiocholine iodide (ASChI) as substrate. Briefly, 400µL of the supernatant were pre-incubated for 5 min with 100µL of 0.01 M of 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) in 2.60mL of 0.1 M phosphate buffer, pH 7.1. Absorbance was measured at 412 nm on a UV spectrophotometer (Genesys 10 UV spectrophotometer, Thermo Fisher Scientific, U.S.A.). When absorbance reaches a stable value, it is recorded as the basal reading. AChE activity was then measured with 20µL of substrate, acetyltihiocoline iodide (ASChI) and change in absorbance was recorded for a period of 10 min at an interval of 2 minute each.

Kinetic parameters of gill ChE was investigated by increasing the substrate concentration (from 0.02mM to 20.48mM). Eserine salicylate, Iso-OMPA (tetrakisopropyl pyrophosphoramide) and BW284C51 {1, 5 bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide} were used as specific inhibitors of total cholinesterase (ChE), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE), respectively. Stock solutions of eserine salicylate and iso-OMPA were prepared in ethanol, and BW284C51 was dissolved in double distilled water. Working concentration range of
the inhibitors for ChE characterization was 0.05 µM to 128 µM.

Acetylcholinesterase (AChE) activities in the control as well as exposed gill tissues were assayed following the similar method in the presence of 125 µM of iso-OMPA to inhibit BChE in reaction mixture. All the experiments were repeated three times to determine its reproducibility.

**Statistical Analysis**

In each set of experiment, data obtained from the fishes kept (a) under control conditions (b) under experimental conditions i.e. fishes exposed to ‘Nuvan®’ at the concentrations 5mg/l or 15mg/l; and (c) during recovery periods, were pooled separately. Further, estimations were based on the data obtained from three fishes. Each set of the experiment was repeated three times to determine the reproducibility.

Average total protein content and AChE activity with standard deviations (S.D.) was estimated using Microsoft Excel 2007 on an Intel® Core™ 2Duo computer. To determine the kinetic parameters of ChE activity, i.e., the apparent Michaelis-Menten constant (Km) and the maximum substrate hydrolysis velocity (Vmax), substrate concentration versus reaction velocity curves were analysed using the Solver feature of Microsoft Excel (ver. 2007) by fitting experimental data to the Michaelis-Menten equation (http://www-biol.paisley.ac.uk/kinetics) (Tu et al., 2009).

In addition, IC50 (concentration of inhibitors at which 50% inhibition of the enzymatic activity occurs) values were calculated using log-probit analysis. The results were expressed throughout as mean ± S.D. Statistical comparisons were made between data obtained from control and experimental conditions using one-way ANOVA followed by Dunnett post hoc test, through Statistical Package for the Social Sciences (SPSS) for Windows (standard version 11.5) software. P<0.05 was accepted as the level of statistical significance.

**RESULTS**

**Protein Contents**

In control fish, the total protein content in the gills is found to be 21.65 ± 3.7 mg/g. In the fishes exposed to ‘Nuvan®’ at 5mg/l or 15mg/l, protein contents in the gills at different intervals, in general, increases. Further, the increase in the total protein contents fluctuates within narrow limits at different intervals.

In fishes exposed to 5mg/l and 15mg/l, the increase in total protein contents at different intervals compared to that of the controls is statistically significant (Figure 1). In the fishes exposed to 5mg/l or 15mg/l of ‘Nuvan®’ for 4d and then on their transfer to freshwater, the total protein contents in the gills even up to 16dr, in general, remain significantly high, similar to that at 4d (Figure 1)

**Gill Cholinesterase Characterization**

The estimated gill ChE kinetic parameters for the substrate acetylthiocholine iodide (ASChI) are shown in Table 1. Maximum rate of ASChI hydrolysis (Vmax) was observed to be 35.02 ± 0.90 nanomoles/min/mg of protein. In addition, an effect of substrate concentration on the ChE activity was observed and shown as Michaelis-Menten curve (Figure 2). Reaction velocity increased with increase in substrate concentration up to 2.56 mM and substrate inhibition was found at concentration equal or higher than 5.12 mM. Km value (Michaelis-Menten constant), which represents the substrate concentration required to attain half of the maximum reaction velocity (Vmax) was observed to be 0.0808 mM for gill ChE using ASChI.

In order to confirm the identity of the ChE present in the gills of *C. mrigala*, specific inhibitors i.e., eserine salicylate (total ChE inhibitor), BW284c51 (AChE inhibitor) and iso-OMPA (BChE inhibitor) were used in the enzymatic
reaction mixtures employing ASChI as substrate. The effects of these inhibitors on the gill ChE activity are shown in Figure 3. In addition, IC\textsubscript{50}-30 min values of each inhibitor for gill ChE were also summarized in Table 1. The results from these inhibition assays showed an almost total inhibition (\~96% at 128 \mu M conc.) of gill ChE activity by eserine salicylate. This inhibition was statistically highly significant relative to controls ($F = 2901.86; df = 11,35; p \leq 0.05$) and confirms that most of the ASChI hydrolysing activity in the gill of \textit{C. mrigala} is due to ChE. AChE specific inhibitor, BW284c51 also significantly inhibited the gill ChE activity ($F = 9201.5; df = 11,35; p \leq 0.05$).

However, maximum inhibition by this inhibitor was found to be approx. 75% at even at 128 \mu M concentration. Inhibition by iso-OMPA was observed to be significant ($F = 38.813; df = 11,35; p \leq 0.05$), although the inhibition value was smaller (approx. 37% at 128 \mu M conc.) than that for eserine and BW284c51. In all the experiments with selective inhibitors, no significant differences between water and solvent controls were found.

**Acetylcholinesterase Activity**

In control fish, the specific activity of Acetylcholinesterase (AChE) in the gills is found to be 14.30 ± 1.48 nmoles min\textsuperscript{-1} mg\textsuperscript{-1} protein. This value in control fish is considered as 100% activity of AChE in the gills for comparison with those in the fish exposed to ‘Nuvan\textsuperscript{®}’ for different durations.

In the fishes exposed to ‘Nuvan\textsuperscript{®}’, the AChE activity in the gills, at 4h compared to that of control, shows a sharp statistically significant decline by 68.81% at 5mg/l and 77.88% at 15mg/l. At subsequent intervals, the activity of the enzyme further declines and reaches 87.1% and 90.38% at 5mg/l and 15mg/l respectively at 4d of exposure.

The fishes exposed to 5mg/l or 15mg/l of ‘Nuvan\textsuperscript{®}’ for 4d and then on their transfer to fresh water, show a gradual increase in the residual activity of AChE in the gills at different durations of recovery i.e. 2dr to 16dr, compared to that at 4d. Nevertheless, the increase in the activity of the enzyme at each of these durations is significantly lower than that in control fish. At 2dr the activity of the enzyme, compared to that at 4d, increases and is found to be 29.76% and 21.11% at 5mg/l and 15mg/l respectively. At subsequent intervals, the activity of the enzyme continues to increase gradually and at 16dr it is found to be 68.54% and 48.33% at 5mg/l and 15mg/l respectively (Figure 4).

**DISCUSSIONS**

In \textit{C. mrigala}, exposed to ‘Nuvan\textsuperscript{®}’ at 5mg/l as well as 15mg/l, the total protein content in the gills even up to 16dr appear significantly increased compared to those in the controls. This reflects that the gills are not able to attain their normal metabolism even up to long recovery period in freshwater. Susan et al. (2010) reported that the insecticides probably act continuously on the animal system for longer periods, thereby rendering detoxification mechanism less efficient thus making recovery slow at sublethal concentrations.

Substrate and inhibitor specific characterizations revealed that the measured esterase activity in the gills of the carp, \textit{C. mrigala} is mainly due to cholinesterases as it was almost completely inhibited (\~96% inhibition) by eserine salicylate at \mu M concentration range. The residual activity, which is a very small proportion, may be due to other classes of the esterase enzyme family, such as aryl-esterases or carboxyl-esterases. The enzyme inhibition at a higher substrate concentration is typical of AChE, thus suggesting that the detected esterase was indeed AChE (Habig and Di Giulio, 1988; Galloway et al., 2002). The inhibition assays with BW284c51 and iso-OMPA suggest that greater levels of enzymatic activity are due to the presence of acetylcholinesterase (AChE), and it also confirms the presence of another type of ChE, i.e., BChE in the gills of \textit{C. mrigala} in smaller proportion. Extensive nerve innervations and profuse blood supply are the major sources of AChE activity in the gills of the fishes. On the other hand, blood serum may be responsible for its
significant BChE activity. This type of polymorphic nature of ChE in the gills of fishes has not been described previously. However, a greater proportion of AChE type than the BChE in brain, liver and muscle of Haemulon plumieri was reported by Leticia and Gerardo (2008). Nigam et al. (2012) reported the polymorphic nature of ChE in the skin mucus of Cirrhinus mrigala, Labeo rohita and Catla catla. Similar results have also been reported on polymorphic forms of ChEs in various fish species (Sturm et al., 1999; Dembélé, 1999; Varo et al., 2000, 2003, 2007; Nunes et al., 2005).

The fish gill is a sophisticated organ that performs a vast array of processes including respiration and receives extensive innervations from the nervous system in the form of afferent (sensory) and efferent (motor) pathways (Jonz and Zaccone, 2009). According to the present investigation, a significant inhibition of the AChE activity, 87.1% - 90.38 %, in the gills of the fish Cirrhinus mrigala exposed to ‘Nuvan®’ at the sublethal concentrations (5mg/l and 15mg/l) for 4d is observed. This inhibition could be considered to have a significant influence on the transmission of nerve impulses, which may consequently, result in the improper functioning of the gills and influence the efficiency of gas exchange across the gills.

Present investigation reveals that in C. mrigala exposed to ‘Nuvan®’ at 5mg/l as well as at 15mg/l for 4d and then transferred to freshwater for recovery, the AChE activity in the gills, compared to those in the controls, show a gradual and significant recovery. Nevertheless, even after prolonged period of recovery the activity of the enzyme remains significantly lower than that of controls. This, as suggested by Boone & Chambers (1996) could be due to much of the AChE, which has become irreversibly inhibited (“aged”); aging is a dealkylation of the phosphorylated AChE which renders it resistant to hydrolysis. Further, the slower rate of recovery may be due to lower amounts of reserve AChE and/or a slower synthesis of new AChE. Chebbi & David (2009) also reported that AChE activity in the gill, brain, muscle and liver of Cyprinus carpio after exposure to one fifth and one tenth of lethal concentration of Quinolphos still remains significantly lower than that of the controls even after seven days in freshwater.

Significantly inhibited AChE activity even after prolonged recovery periods has also been recorded in different tissues of several fish species exposed to a variety of organophosphates e.g. in the brain, gill, liver muscle and plasma of Ictalurus punctatus - chlorpyrifos, parathion and DEF® (S,S,S-tributyl phosphorotrithioate) (Straus & Chambers, 1995); and in the gill and muscles of Oreochromis mossambicus - RPR-II (Rao, 2006); Rao et al. (2003a, b) reported that in Oreochromis mossambicus, exposed to 96h LC50 value of profenofos, the AChE activity recovers completely in 20.7d in the brain and in 23.3d in the gill. They reported that in the fish exposed to chlorpyrifos recovery in these tissues is in 19.4d and 29.2d respectively.

In conclusion, the major form of ChE in the gills of C. mrigala is AChE, which was significantly inhibited by sublethal concentrations of ‘Nuvan®’. The fish were able to survive on the inhibition of AChE activity, even up to 87.1% and 90.38%, in the gills. This suggests that acetylcholinesterase is definitely a target of organophosphate insecticides but it is not the only physiologically important target. Furthermore, gill AChE of the fishes may be used a biomarker for the assessment of organophosphate exposure in fishes.

ACKNOWLEDGEMENTS

This work was supported by a grant to Dr. Swati Mittal (Principal Investigator) under the Major Research Project sponsored by the University Grants Commission, Government of India. Ms. Nidhi Srivastava was supported as Project Fellow under the project. Mr. Ashwini Kumar Nigam was supported as Senior Research Fellow under the C.S.I.R.-NET Fellowship Scheme, Council of Scientific and Industrial Research, Government of India. Dr. Usha Kumari is supported as Senior Research Associate sponsored by the Council of Scientific and Industrial Research, Government of India.
REFERENCES


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APPENDICES

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**Figure 1:** Total Protein Content (mg/g of Tissue; Mean ± S.D.) in the Gills of *C. mrigala* at Different Intervals in Controls, Exposure to 5mg/l and 15mg/l of ‘Nuvan®’ and Recovery. Symbols: 0h, Prior to Exposure to ‘Nuvan®’; h, Hour; d, Day; dr, Day of Recovery; mg/l, Milligram/Liter; Asterisk ‘*’ on Bars Indicates Significant Difference from Control (P < 0.05). n=378

**Figure 2:** Michaelis–Menten Plot Showing Cholinesterase Activity in Gills of *C. mrigala* at Different Substrate (Acetylthiocholine Iodide) Concentrations
Figure 3: Effects of Inhibitors—Eserine Salicylate, Iso-OMPA, and BW284C51 at Different Concentrations on Cholinesterase Activity in Gills of *C. mrigala*. Values are Mean±SD. Asterisk (*) on Bars Indicates Significant Difference from Control (P < 0.05). Symbols: 0, Control; 0’, Ethanol Solvent Controls for Eserine Salicylate and Iso-OMPA.

Figure 4: Percent Residual Activity of Acetylcholinesterase (Mean ± S.D.) in the Gills of *C. mrigala* at Different Intervals in Controls, Exposure to 5mg/l and 15mg/l of ‘Nuvan®’ and Recovery. Symbols: 0h, Prior to Exposure to ‘Nuvan®’; h, Hour; d, Day; dr, Day of Recovery; mg/l, Milligram/Liter; Asterisk ‘*’ on Bars Indicates Significant Difference from Control (P < 0.05). n=378

Table 1: Estimated Km (mM), Vmax (nmoles/min/mg of Protein) and IC\textsubscript{50}-30min Values (µM) Using the Substrate Acetylthiocholine Iodide (ASChI) for the Gill Cholinesterase of *Cirrhinus mrigala*

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