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Acetylcholinesterase in Invertebrate Fauna as a Biomarker for Pesticide Pollution

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***Abstract*: Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine. AChE is the target site of inhibition by organophosphorus and carbamate pesticides. In particular, organophosphorous pesticides inhibit the enzyme activity by covalently phosphorylating the serine residue within the active site group. They irreversibly inhibit AChE, resulting in excessive accumulation of acetylcholine, leading to the hyperactivities and consequently paralysis of the neural and muscle system. Therefore, monitoring of AChE inhibition is widely used as a biomarker of organophosphorous and carbamate exposure either in aquatic or terrestrial environments. Recently, new insights are emerging in the use of AChE as biomarker in environmental biomonitoring. A number of important contaminants other than carbamate and organophosphorus pesticides have recently been shown to have anticholinesterase properties, including heavy metals, detergents, hydrocarbons, and herbicides. It is also worth noting that not only different compounds may reach levels of significance in terms of anticholinesterase effect, but, moreover, combinations of different chemical classes were shown to be highly synergistic in their ability to inhibit AChE activity.**

***Keywords*: Acetylcholinesterase, Invertebrate, Fauna, Pesticide, Biomarker.**

1. Introduction

The measurement of cellular and sub cellular responses to chemical contaminants (referred to as biomarkers) in living organisms represents a recent tool in environmental monitoring responding to the need to detect and assess the effects of chemical contaminants on the biota (Peakall, 1992; Legadic et al., 2000). Typically, biomarkers are defined as quantitative measures of changes in biological systems that respond to either (or both) the exposure to xenobiotic substances that lead to biological effects (Lam and Gray, 2003). The use of the term biomarker is generally restricted to cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect. One of the most important features of molecular/cellular biomarkers is that they have the potential to anticipate changes at higher levels of biological organisation, i.e., population, community or ecosystem. Thus these early warning responses can be used in a predictive way, allowing the initiation of bioremediation strategies before irreversible environmental damage of ecological consequences occurs (Moore et al., 2004). Biomarkers are then defined as short-term indicators of long-term biological effects.

In the last two decades, the biomarker approach has been incorporated worldwide into several pollution monitoring programmes for the ecotoxicological assessment of marine, terrestrial and fresh water ecosystems (McCarthy and Shugart, 1990; Peakall, 1992; Peakall and Shugart, 1993; Walker, 1998a).

The interest in the use of biomarkers in environmental risk assessment (ERA) has been increasing steadily (Adams, 2002). The process of ERA is continually developing in eco-toxicological studies in order to address changing needs and diverse toxicological issues. Allan et al. (2006) recently proposed the use of biomarkers on sub-individual level in water quality monitoring regulated by the European Union's Water Framework Directive (Directive 2000/60/EC, 2000) in order to ―provide more realistic assessment of impacts and exposure of aquatic organisms to specific contaminants present in water‖. Molecular and cellular biomarkers share important characteristics useful for the use in ERA, such as sensitivity, specificity, simplicity of measurement and a potential linkage to higher level effects, although in some cases this last aspect needs more investigation (Peakall and Walker, 1994; Peakall, 1999; Walker, 1999).

Among the various types of biomarkers studied, the inhibition of acetylcholinesterase (AChE) activity receives special attention in eco-toxicological studies. Moreover, recent increasing knowledge about this enzyme, its functions, its sensitivity to several classes of pollutants and its molecular expression have provided new insights into the application of this biomarker in environmental monitoring and assessment. In the present study one such attempt has been made to estimate the acetyl cholinesterase enzyme activity in crabs and snails, which serve as a bio-indicator to assess the pollution in particular.

2. Materials and methods

A. *Assay of Cholinesterase*

The acetylcholine remaining after hydrolysis in converted to hydroxamic acid by treatment with alkaline hydroxylamine. Hydroxamic acid with ferric chloride in acid solution gives a

red purple complex. The intensity of colour is proportional to the concentration of acetylcholine present in it. The following reagent are required to prepare the buffer is dissolve 16.72 clear crystal of Na_2HPo_4(Disodium hydrogen phosphate) and 2.72 g of KH_2PO_4 (Potassium dihydrogen phosphate0 in one litre of distilled water and adjust the pH to 7.2. To prepare Acetylcholine standard solution, dissolve 0.111g AChCl (Acetylcholin chloride) in 100 ml if buffer solution. Prepare before use and a drop of toluene. To prepare Hydroxylamine hydrochloride solution, dissolve 1.398g of NH_2OH HCL in 100 ml of distilled water and keep it in refrigerator. To prepare Sodium hydroxide solution 3.5M, dissolve 14g NaOH in 100 ml of distilled water. To prepare alkaline hydroxylamine solution, reagents are mixed in 1:1 ratio before use. To prepare Hydrochloric acid dilute HCL (Sp.gr.1.18) with 2 volume of distilled water. To prepare Ferric chroride solution 0.37M, to dissolve 10g $Fecl_36H_2O$ in 100 ml of 0.1N HCL.

B. Procedure

1) Preparation of Achl standard curve

- Pipette out 0 (control A) 0.2,0.4, 0.6, 0.8 and 1.0 ml aliquots of AChCl (b) solution into a series of test tubes.
- Add phosphate buffer(a) to these test tubes to make the volume in each tube to 1 ml and incubate it for 30 min at 37^0c.
- Add 2 ml of alkaline hydroxylamine solution (a) to each tube and mix it by shaking thoroughly.
- After one minute add 1.0 ml solution of HCL and 1.0 ml of $FeCl_3$ solution and shake it vigorously.
- Measure the absorbance of 540nm using control (A) to adjust the instrument to Zero.

2) Determination of acetylcholine

- The test samples used in the present study were discussed and the tissues of the respective species were taken and homogenated in phosphate buffer of pH 7.2
- The tissue homogenate was spun down in a refrigerated centrifuge at 22000 rpm for one hour. The supernatant can be used without further treatment and can be kept frozen for some months without loss of activity.
- Steps as in the preparation of Ach standard curve were followed for the rest of the analysis

3) Determination of cholinesterase activity

- Dilute O (Controls B) 0.2, 0.4, 0.6, 0.8, 1.0 of enzyme homogenate to 25 ml with buffer solution (a)
- Pipette out 1.0 ml of each of these dilution into test tube and add 1.0 ml of distilled water to each tube and keep the tubes at 37^0c for 30 minutes
- Add 1.0 ml of AChl (b) to each tubes at a time interval of 1.0 minute and incubate for 30 minutes and shake the tubes vigorously and frequently while in incubation
- Add 2 ml of alkaline hydroxylamine solution at intervals of one minute and shake well
- After one minute add 1.0 ml of HCL solution and then 1.0 ml of $FeCl_3$ solution and shake vigorously

3. Analysis and interpretation

A. Bioassay of acetyl cholinesterase in control specimens studied

It is known that AChE enzyme is a neurotransmitter. It has been quantitatively and qualitatively estimated in several invertebrates and vertebrates. This enzyme helps the organism for neural transmission. An attempt has been made in this present investigation on bioassay of two invertebrate species namely the snail and crab. The specimen were collected from water bodies where there is no contamination of agriculture irrigated water. The methodology for the bioassay of AChE is already detailed in the section of materials and methods. The bioassay values of these specimens are served as control values.

Table-1 gives the data on the AChE activity of the snail under investigation from the data it is inferred that the mean value of the AChE activity of the control specimen is 0.79±0.062 µmol /min/µl.

Table 1
Estimation of AChE in Snail as Sample (control Batch)

Experiment	Enzyme activity µ mol/min/µl.
1	0.753
2	0.774
3	0.719
4	0.831
5	0.920
6	0.790
mean	0.79±0.062

Table 2
Estimation of AChE in Crab as Sample (control Batch)

Experiment	Enzyme activity µ mol/min/µl.
1	0.772
2	0.824
3	0.800
4	0.683
5	0.732
6	0.758
mean	0.76±0.032

B. Bioassay of AChE in experimental invertebrate specimens collected from ponds associated with agriculture land

The sample specimen of snail and crab were collected from the ponds. These ponds are situated near agriculture fields where the discharge of irrigated watering AChE these ponds

In the 1st phase of experiment specimen were collected before sowing of the agriculture crops. The specimens were subjected to the very same bio assay observation as detailed in

the previous chapter. Six connective records were made to arrive a concordant mean value. The very same procedure was adopted for both the cases.

The results of above studies are presented in Table-3 and Table-4. From the data presented in these table, it is evident that the mean AChE enzyme activity for snail was 0.76±0.032 μ mol/min/μl and that of crab was 0.73±0.095 μ mol/min/μl. The comparative analysis of these data among with data obtained for control indicates that these organisms were having reduced level of AChE enzyme activity than their control values. This would suggest that in the pond where the specimen was collected are already polluted with pesticides. The reduced level of AChE may be presumed due to the contamination of pesticide chemical.

Table 3
SNAIL BATCH-I sample period before sowing

Experiment	Enzyme activity μ mol/min/μl.
1	0.727
2	0.746
3	0.692
4	0.785
5	0.884
6	0.764
Mean	0.76±0.032

Table 4
CRAB BATCH-I samples period before sowing

Experiment	Enzyme activity μ mol/min/μl.
1	0.754
2	0.708
3	0.774
4	0.657
5	0.798
6	0.705
Mean	0.73±0.095

C. Bioassay of AChE in experimental invertebrate specimen collected from ponds associated with agricultural land which were analyzed during the period of harvesting

In order to find out the pesticide toxicity inhibition on AChE on these tested organism, the same bioassay of AChE were also been tried on snails and crabs collected with an interval of six months period. At the time of collection, harvest was going on in the agriculture fields. There was a discharge of irrigated water in these ponds under study. The same enzyme values in these specimens were 0.22±0.105 μ mol/min/μl and 0.15±0.089 μ mol/min/μl during the harvest period. Forgoing observation reasonably to suggest the pesticidal contamination due to the discharge of irrigated run-off water, which may cause the significant reduction in AChE activity.

Monitoring of pesticide contamination through AChE enzyme activity. In order to observe a series of bioassays on AChE activities on the specimen under investigations were also been extended. The study was carried out for six-month duration. Bioassays were made in periodic intervals. The same analytical procedure explained in earlier chapters was adopted Subsequent series of observation during the period of study were also made and recorded. The data was presented in the form of Table and graph. It is evident that the reduction of AChE activity is proportional to the period of exposure in the case of experimental specimens.

Table 5
SNAIL- BATCH II Samples period at the time of harvest

Experiment	Enzyme activity μ mol/min/μl.
1	0.303
2	0.118
3	0.214
4	0.086
5	0.398
6	0.244
Mean	0.22±0.105

Table 6
CRAB- BATCH-II Sample period at the time of Harvest

Experiment	Enzyme activity μ mol/min/μl.
1	0.053
2	0.166
3	0.128
4	0.081
5	0.160
6	0.333
Mean	0.15±0.089

In the case of crab also the very same trend was observed. The graph and the data exhibit the experimental data for all activities. By keeping hold all the above data it would be presumed that agricultural professional activities may have brought a continuous discharge of pesticide compound to the freshwater ponds under investigation. Table 7 & 8 shows the comparative data on the AChE activity of the snail and crab collected in the present study.

Table 7
Comparative data on the ache activity of the snail collected in the present study

Control	0.79±0.063
Batch I	0.76±0.032
Batch II	0.22±0.105

Table 8
Comparative data on the ache activity of the crab collected in the present study

Control	0.79±0.032
Batch I	0.73±0.095
Batch II	0.15±0.089

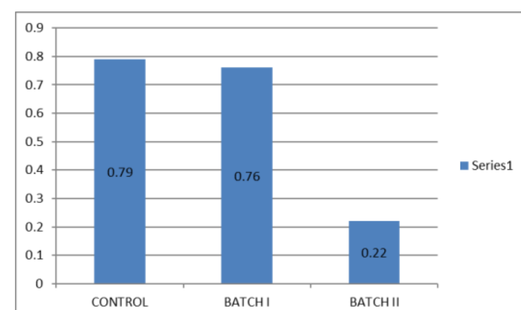

Fig. 1. Graph-1: Comparative data on the ache activity of the snail collected in the present study

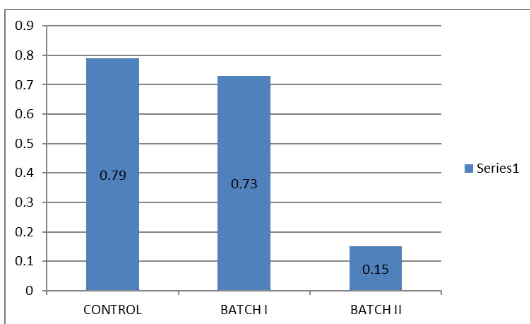

Fig. 2. Graph-2: Comparative data on the ache activity of the crab collected in the present study

4. Discussion and Conclusion

The bioassay studies done on AChE enzyme inhibition in Snail and Crab in the present study shows a well pronounced and a gradual decrease of the enzymatic activity in the experimental samples analysed during the study period.

The enzyme activity is control samples of snail was noted as 0.79±0.63 where as in Batch-I samples the activity was 0.76±0.032 μ mol/min/μl respectively. The same trend was observed in case of control and batch-I samples of crabs as 0.76±0.032 μ mol/min/μl and 0.73±0.095 μ mol/min/μl respectively.

The slight change in variation in enzyme activity of the two species i.e. snails and crabs can be distributed that the agricultural field was less contaminated with pesticide to a range that could not affect the invertebrate species of the study.

In contrast it was interesting to know that there was a pronounced decrease in enzyme activity in the experimental samples of snail and crabs are analyzed and the values are 0.22±0.105 μ mol/min/μl and 0.15±0.089 μ mol/min/μl respectively.

It may be suggested that during this period the fields received the agricultural run -off water heavily contaminated with pesticide and this attribute to the decrease in AChE enzyme activity in the experimental samples investigated. By the above results it can be specified that this type of observations would give a circumstantial evidence of the pesticide pollution and the bio augmentation.

- The present investigation may have the following implications;
- Retrospective bio monitoring of the pesticide pollution.
- The application of environmental biotechnological methods to estimate the effect of organophosphorus compounds.
- Environmental implications of the pesticide toxicity.
- Decision on the utilization of pesticide for the policy makers.
- Dissemination for the application of bio-pesticides.
- Soil micro and macro fauna as bio indicator of the pollution load.

Although AChE inhibition has been measured in the tissues of a variety of invertebrate species following organophosphate exposure, the relation between AChE inhibition and lethality is less distinct.

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