



The Effect of Pesticides on Cholinesterase Activity by Using Fish as a Biomarker

Nursabrina Mohd Hayat¹, Mohd Khalizan Sabullah¹, Mohd Yunus Shukor¹, Mohd Arif Syed¹, Farrah Aini Dahalan², Khalilah Abdul Khalil³ and Siti Aqlima Ahmad^{1*}.

1)Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia. 2)The School of Environmental Engineering, Universiti Malaysia Perlis, Kompleks Pengajian Jejawi 3, 02600 Arau, Perlis, Malaysia. 3)Department of Biomolecular Sciences, Faculty of Applied Sciences, Universiti Teknologi MARA, Sec. 2, 40150 Shah Alam, Selangor, Malaysia.

*Corresponding author: Siti Aqlima Ahmad. Tel: +603-89468292, E-mail: aqlima@upm.edu.my

History

Received: 26.5.2014

Revised: 24.7.2014

Accepted: 28.7.2014

Online: 30.7.2014

Keyword

Pesticide

Biomarker

Cholinesterase

Fish

Review

Many water bodies have been polluted by direct or indirect release of natural or artificial molecules which aimed to kill or mitigate any harmful organism. Non target living organisms were also affected by these compounds known as pesticides. They can cause poisoning in the human body through the inhibition of cholinesterase enzyme (ChE) which plays the role in human detoxifying process. The inhibition reaction of cholinesterase is very important in human nervous system although they are far more lethal against insects and small animals. Water rehabilitation followed by monitoring is the best technique to ensure a safer environment. As the first method, preliminary screening of contamination can be carried out using enzymatic biosensor before a high performance technology is needed for analytical quantification. ChE-based biomarker was considered as an effective method for the monitoring of environmental contamination in compound especially pesticides in agriculture. Fish was documented to be very sensitive toward toxicants, thus fish is selected as the biomarker along with ChE to indicate the existence of toxicant. This review gives a highlight on the capability of ChE-based biosensor for detecting contamination caused by pesticides which can later become one beneficial method for bioremediation.

Pesticides can be termed as herbicide, insecticide, nematocide, termiticide, molluscicide, piscicide, avicide, rodenticide, predacide, bactericide, antimicrobial, fungicide, disinfectant, and sanitizer [1]. In pest management, pesticides was considered as an effective chemical application. There was no doubt that a non-target organism can also be affected by this compound. Organochlorines (OC), Organophosphates (OP), and Carbamates (CB) are three group of chemical families which was grouped under insecticides. Utilisation of OPs and CBs for pest management in modern agriculture was due to their low persistence and high insecticidal [2]. Both compound have greater acute toxicity, posing risk to people who may be exposed to a large quantities and they were also degraded by environmental factor rapidly compared to Ocs [3]. OPs, which shows a low environmental persistence have come into widespread use since 1930s. This compound poses a potential hazard to human health because of its high acute toxicity. CBs and OPs undergo serine carbamylation or phosphorylation at the active site of the enzyme [4] which disrupt the process and leads to the blocking of acetylcholine metabolism. Numerous

analysis methods have been developed for OPs and CBs quantification in contaminated samples that give a reliable, precise and high sensitivity results in gas chromatography [5], liquid chromatography [6], ultraviolet spectroscopy [7], gas-mass spectroscopy [8], fluorimetry [9] and surface plasmon resonance (SPR) [10]. Chlorpyrifos and fenitrothion are examples of OP that cause ChE inhibition after biotransformation into its metabolites [11]. Acetylcholinesterase (AChE) is a fast acting enzyme that involved in hydrolysis of neurotransmitter, acetylcholine (ACh) located at the synaptic cleft. Inhibition of AChE cause ACh accumulation which leads to hyperexcitation, convulsions, and death [12]. AChE is the target for many insecticides, such as OPs and CBs due to its critical function in the nervous system [13]. Butyrylcholinesterase (BChE), in the absence of AChE helps in regulating cholinergic transmission by the scavenging process of OP and CB inhibitors comes from esteratic activity of BChE. Excess substrate will inhibit AChE while BChE exhibits the substrate activation in excess substrate which distinguishes AChE from BChE [14]. Thus, this review focuses more on how

cholinesterase can become one of the potential biosensor that is sensitive to toxicants, discuss more on pesticides.

Acetylcholinesterase (AChE)

AChE (E.C. 3.1.1.7) is the key enzyme in the nervous system of animals. It can be found in various part of the body, notably in neuromuscular junction and brain tissue [15]. Majority of acetylcholinesterase can be found in the brain muscle [16]. Other than nerve tissue, AChE also present in liver, red blood cells and muscles with the same or different morphology. It exists in the form of disulfide-linked oligomers mixed with collagen or lipid-containing protein subunits [17]. AChE is a tetrameric molecule, globular, and it also exhibit identical solubility or amphiphilic molecular forms. The examples of AChE in molecular form are amphiphilic dimers, hypophobic-tailed tetramers and collagen-like tailed forms [18]. It is an ammonium containing compound and was the first neurotransmitter to be isolated in 1920 [19]. The three dimensional structure of AChE was first determined using the protein from *Torpedo californica* [20]. The main function of AChE is to terminate synaptic signal that was sent in the form of ACh. AChE active site is located at the bottom of a deep gorge which largely lined by aromatic residues. AChE active centre has two functional sub sites called esteratic and anionic site [21]. AChE in addition, consists of one or more binding site known as peripheral binding site for ACh and other ligand located near the entrance of active site gorge [21, 22] (Figure 1). The esteratic site is where the choline being displaced by esterification of carboxyl group of ACh and hydroxyl group of serine occurred. It consists of three amino acid residues which are serine, histidine and glutamic acid and they are located at the bottom of active site gorge [21]. On the wall of the active site gorge, aromatic amino acid residues embedded in anionic site. Dipole-dipole interaction between the electrons of aromatic rings and the quaternary nitrogen of ACh induced the substrate recognition. A rapid translation of substrate from the surface to the active site can be achieved by an 'aromatic guidance' provided by the aromatic acid residues [23]. Peripheral anionic site influence the rates of catalysis due to its conformational changes suggested by Bourne et al., [22]. The binding of any ligand at peripheral anionic site will blocked the entry of substrates and the exit of products from the active site which caused substrate inhibition [21]. ACh consists of two units, choline and acetic acid that are connected by ester linkage. It acts as a nerve impulse transmitter and was packaged in a vesicle called vesicular acetylcholine transporter (vAChT). Action potential will trigger the movement of vAChT and ACh will be released in the synaptic cleft. The binding of ACh to muscarinic receptors situated on the membrane of pre-synaptic and post synaptic neuron causes the opening of ion channel and trigger a respond as the

movement of ion into synaptic membrane is allowed, followed by depolarisation of the membrane [24]. The arrival of nerve impulses depolarises the presynaptic membrane at the synaptic knob, opening calcium channels and increases the permeability of the membrane to calcium (Ca^{2+}) ions [25]. Ca^{2+} rushes into the synaptic knob, causing the synaptic vesicles to fuse with the presynaptic membrane and release their content into the synaptic cleft (Figure 1). ACh diffuses across the synaptic cleft which later creates a delay about a fraction of milliseconds, and attach to a specific protein on the postsynaptic membrane that recognises the acetylcholine molecular structure. There will be a change in the shape of receptor site, which results in the opening of ion channels in the postsynaptic membrane due the arrival of the ACh [26]. AChE then hydrolyses ACh into acetic acid and choline and terminates the response because ACh does not bind with the muscarinic receptor anymore. The recycle of choline will occur for synthesising a new ACh in presynaptic neuron. AChE differs from BChE by means of inhibitor reactivity, substrate specificity, kinetic properties and distribution in tissue [27, 28]. AChE is enzymes which preferentially hydrolyse acetyl esters such as ACh or acetyl- β -methylcholine, while butyrylcholinesterases (BChE) are those which demonstrate a preference for other types of esters such as butyrylcholine or propionylcholine [29]. Due to its sensitivity towards the OP and CB groups, AChE has been manipulated to be used in biomonitoring and bioassay for those contaminants and has been recently used in various species of fish [30-32]. AChE activity in invertebrates such as shellfish [33, 34] and crustaceans [35] has been proved to be useful in biomonitoring programme. It has been discovered that AChE from *Pangasius pangasius* is useful on detecting heavy metals and its sensitivity towards heavy metals such as copper, silver and chromium [36].

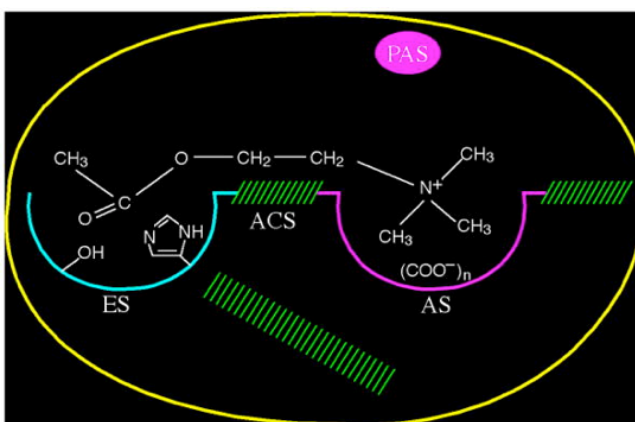


Figure 1: Schematic representation of the binding sites of AChE. ES, esteratic site; AS, anionic substrate binding site; ACS, aromatic cation binding site; PAS, peripheral anionic binding site. Putative hydrophobic binding regions are represented by the hatched areas. The esteratic and anionic sites of the catalytic centre were spanned by ACh. (Adapted from Dvir et al., 2010 [21])

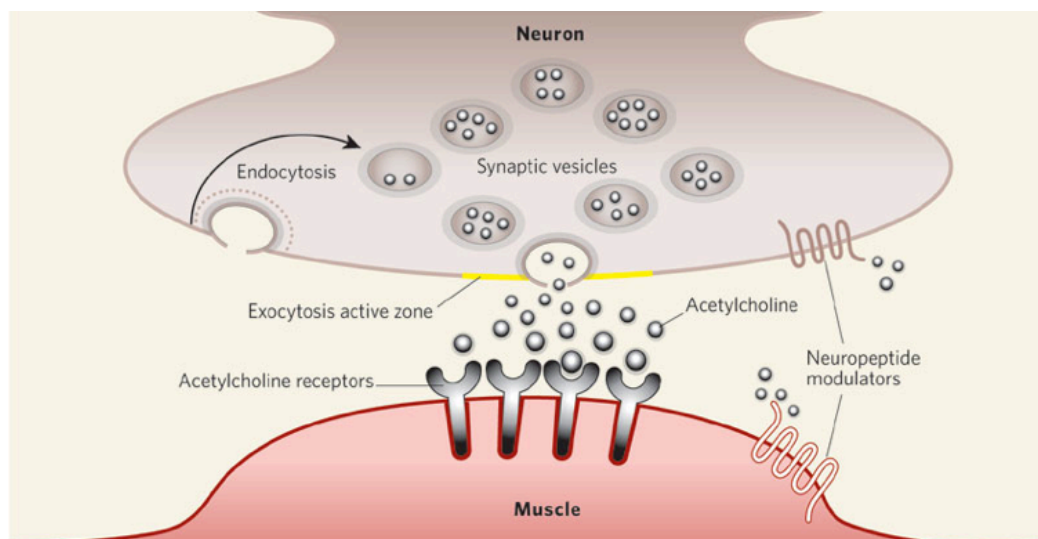


Figure 2: Signal transmission at the neuromuscular junction (Adapted from Bargmann [37]).

Butyrylcholinesterase (BChE)

Pseudocholinesterase or Butyrylcholinesterase (BChE; E.C. 3.1.1.8) is one of the serine hydrolase that primarily targets butyrylcholine (BCh) [28, 39]. The presence of BChE is important in the regulation of the cholinergic system [40–42]. BChE is a non-specific cholinesterase mainly found in blood plasma and is similar to AChE. BChE was known to be synthesised in liver and distributed in liver, intestine, heart, kidney and lung [42]. BChE contains peripheral anionic site, omega loop, choline binding site, oxyanion hole, catalytic triad of esteratic site, and acyl binding site (Figure 3). The monomer of BChE consists of approximately 574 amino acids and is 20Å deep [43]. Peripheral anionic site of BChE is made up of Asp70 and Tyr332 residues that involved in the binding of positively charged substrate molecules with the Tyr332 residue during the initial state. The Asp70 residue helps to induce the conformation alteration of the substrate's monomer which is being carried to the choline binding site through the alteration of the arms of omega loop. Acyl binding site helps to stabilise the substrate and facilitate the hydroxylation process through binding with the acyl group of the substrate. Inhibition of BChE by the phosphorylation of Ser198 was caused by OP or CB. BChE catalyses the hydrolysis of acetylcholine efficiently and it can compensate the lacking of AChE to allow the continuation regulation of cholinergic neurotransmission [44–46]. BChE detoxifies those inhibitors and act as endogenous scavengers before these chemicals reach acetylcholinesterase at physiologically relevant target sites [14]. Studies proved that BChE can act as detoxification enzyme against anticholinesterase agents, such as OP pesticides [14]. Rodriguez-Fuentes et al., [47] observed that BChE

contributed 37% of the total ChE activities in the muscle of flat fish, but no data was available when considering the BChE activity in invertebrates. In vivo and in vitro biomarker of pesticides can be made using BChE. The exposure of living specimens toward the analysed substance for a period of time has been indicated, as in vivo approach followed by tissue sample analysis through the dissection on specimen, while in vitro approach makes use of the extracted tissues from the specimen and directly exposes them with the analysed substance [48]. A decrease in BChE activity in aquatic organisms followed by the assay on the analysed tissues is capable of indicating the presence of contaminant in the aquatic environment. Thus, the pollution level of aquatic environment can be determined [48, 49]. The use of in vitro assay for the detection of insecticides was normally done using AChE but BChE have also been proved by in several studies as shown in Table 1.

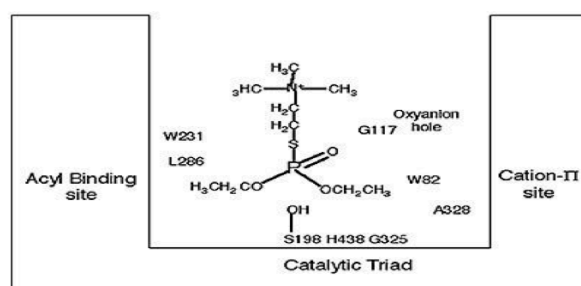


Figure 3: A schematic representation of the BChE active site. The figure shows the catalytic triad (S198, H438, and E325) and residues, which figured prominently in the mutants which retained OP hydrolase activity.

Table 1: Pesticide IC₅₀ for in vitro cholinesterase in previous studies (Assis et al., 2012 [48])

Species	IC ₅₀	Source	Reference
<i>Alburnus alburnus</i>	1.39	Serum	[49]
<i>Leuciscus idus</i>	0.35		
<i>Abramis ballerus</i>	0.18		
<i>Abramis brama</i>	0.22		
<i>Rutilus rutilus</i>	0.35		
<i>Blicca bjoerkna</i>	0.18	Muscle	[50]
<i>Limanda yokohamae</i>	88.23		
<i>Ictalurus furcatus</i>	10	Liver	[51]
	1.610	Muscle	
<i>Gasterosteus aculeatus</i>	0.1	Liver	[52]
	0.1	Muscle	
	0.1	Gills	

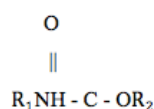
Organophosphates (OPs)

OPs are phosphoric or phosphonic acid derivatives. They are also irreversible inhibitors targeted on cholinesterase activities [54]. According to Gallo and Lawryk [55], not all OP have the anticholinesterase effect. It is the first potent synthetic AChE inhibitors that has introduced a new era in both chemical weapon and pesticide management and are widely used currently [56]. OPs are mostly polar, water-soluble chemicals and composed of a few lipophilic compounds. OPs usually being absorbed rapidly through the inhalation or ingestion but dermal absorption of OP are slower. Prolonged exposure to OP can cause severe poisoning because it can accumulate in fat, liver, kidneys and salivary glands. There are two groups of OPs, which are phosphorothioates (P=S), such as diazinone and parathion, and phosphates (P=O), such as dichlorvos. Phosphates are already biologically active, while phosphorothioates need to be activated to their oxon. Enzyme in the liver, known as cytochrome P450 monooxygenase activate the phosphorothioates naturally. Carboxylesterase and A-esterases such as paraoxonase can deactivate the activated OP that inhibits AChE [48]. Some OP such as dichlorvos may not be as persistent as the others depending on their lipophilicity. In fat, dichlorvos was stored briefly and can be easily eliminated in a few hours. However, because of their extensive storage in fat, the oxon of chlorpyrifos may stay in the body for days [57]. AChE reaction can be inhibited by OP through serine residue at the active site of the enzyme which can cause an irreversible inhibition [58]. OP inhibits cholinesterase by phosphorylating cholinesterase to form a highly stable phosphorylated enzyme. OPs first binding with the catalytic serine of AChE as a trigonal bipyramidal intermediate. The administration of oximes can reverse the initial structure

in some circumstances but it may persist for a period of days [59]. The active site of histidine being forced into a position unsuitable for catalysis. Steric exclusion then occurs and this explained the slow hydrolysis of the initial organophosphate complex to the aged form [60] (Figure 4). ACh accumulated in synapse which avoids the normal nerve impulse transmission because of the absence of functional AChE and leads to the loss of muscular coordination, seizure, and death. The discharge of OP pesticides into aquatic system may affects to the non-target organisms [61] and give threat to the entire ecosystem including food web due to bioconcentration to such non-target organisms [62, 63]. After the inhibition by OP, the reactivation of AChE become slow and was considered irreversible. Therefore new enzyme synthesis is crucial for recovery [35, 61]. Thus, ChE is an effective biochemical indicator of toxic stress and serves as sensitive parameter for testing water quality for the presence of various toxicants affecting its activity [64-66]. Environmental contamination can be monitored by OP poisoning that is utilised by the same principle of irreversible inhibition of AChE.

Carbamates (CBs)

CBs are carbamic acid derivatives that are widely used in household applications and agricultural field which capable to inhibit the cholinesterase activities and cause an excessive accumulation of ACh, leading to neurological dysfunction [68]. Neuromuscular paralysis was caused by the excessive accumulation of ACh in the synaptic cleft which was affected by the overstimulation of specific signalling process [69]. The general formula of the carbamates is:



where R_1 and R_2 are alkyl or aryl groups. Carbamates are said to be an effective insecticides because of their ability to inhibit AChE in the nervous system. They can also inhibit other esterases. They can inhibit serine-esterases

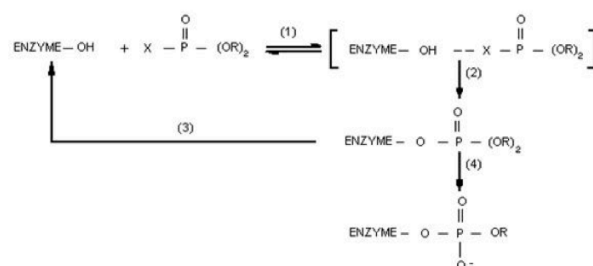


Figure 4: Schematic overview of cholinesterase inhibition by OPs. (1) Formation of Michaelis complex, (2) Phosphorylation of cholinesterase enzyme occurs, (3) Reactivation reaction followed. (4) "Aging" enzyme formed (Adapted from WHO, 1986 [67]).

or beta-esterases which have serine in their catalytic centre. The unstable carbamylation of the enzyme and the rapid regeneration of AChE compared with that from a phosphorylated enzyme make CB are less dangerous with regard to human exposure than OP. Carbamylation can cause the inhibition of CB which is similar to phosphorylation of OP [70]. The esteratic site of AChE will be esterified by CB and hindered by the binding of substrate to AChE which caused a reversible inhibition. AChE became unstable as the result of carbamylation process and generated into its active form rapidly [24]. CBs undergo serine carbamylation at the active site of the enzyme and block the metabolism of acetylcholine [71]. AChE inhibited after an enzyme-inhibitor complex was formed with subsequent carbamylation of the serine hydroxyl. It can exhibit its reversible effects as insecticide and cause intoxication by targeting cholinesterase [70]. The examples of CB are bendiocarb, carbaryl and carbofuran [72]. CBs are relatively toxic to mammals because they give adverse effect to AChE [73].

Biomarkers

In order to monitor the effects of contaminants, inhibitive ChE-based assay has been used for years on either human or wild life as a multiple detection marker of toxicant exposure. A vast number of deleterious xenobiotics including heavy metals, pesticides, industrial chemicals, and pathogens, that bioaccumulate in marine organisms, may cause toxicity to fish, handlers and eventually mankind as the ultimate consumer [74-76]. In order to monitor environmental contamination, stress indicators at cellular and tissue levels have been developed in fish and other aquatic organisms in the recent past [77-79]. The investigation of cholinesterase or AChE activity in fish tissues as early-warning biomarker was made recently for the assessment of pollution in ponds or lakes which receive sewage wastewater revealed site and tissue-specific variations in AChE responses. Biochemical changes occur more quickly than physiological responses and provide information on the sensitivity of organisms with regard to uptake, biotransformation, and detoxification patterns [80]. Toxicological effects of OP and CB can be measured by referring to the half maximal inhibitory which indicates the amount of concentration that can inhibit the biochemical or biological function by half after exposing the enzyme to certain series of inhibitors with different concentrations. This method is usually used for the detection at lower contamination levels for in vivo and in vitro effect of pesticides. Rehabilitation can be applied when there is a warning signal given by a biomarker test. Replicates of the sample that shows a high inhibition when tested using a biomarker will be chosen for validation and quantification by using a high performance technologies. However, sample which shows low or no inhibition will be tested again from the

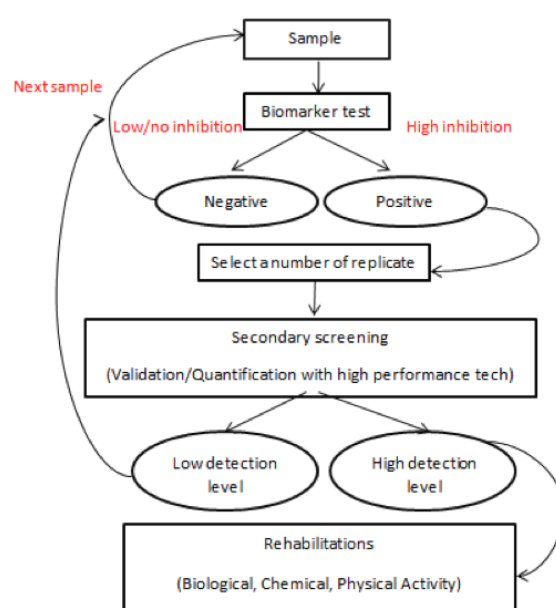


Figure 6. Schematic diagram of biomarker stages. Preliminary screening of the sample was done by exposing the sample to a high and low inhibition level.

start. This step will give an early warning signal when the result is positive. Following the secondary screening, with the use of advance or high performance technologies, rehabilitation can take place when the result is near or exceeds the maximum residue limit where the removal process of pollutions and contaminants from environmental media takes place such as environmental clean-up, groundwater remediation, land remediation, and brownfield site preparation (Figure 6). Moulton et al., [81] recommended a 30% decrease in AChE activity which is significant to indicate the over exposure of freshwater mussels to OP. Coppage et al., [82] documented that the inhibition of brain AChE in the range of 70-80% is critical to fishes. 20% or greater depression in AChE activity in birds, fish or invertebrates has been generally accepted to indicate exposure to organophosphate insecticides, according to Day and Scott [83]. In most species of fish, Fulton and Key

Table 2. Advantages and disadvantages of biomarkers (Adapted from NeuroRx Article [84])

Advantages	Disadvantages
Economical	Timing is critical
Precision of measurement	Expensive (cost of analyse)
Less biased than questionnaires	Storage (Longevity of sample)
Gives a rapid warning signal	Laboratory errors

Key [61] suggested that brain AChE inhibition of >70% levels are associated with mortality. However, selected species appeared to possess the capability to tolerate much higher levels (>90%) of brain inhibition. However, these effects were observed only when brain AChE inhibition is at near-lethal levels, as being suggested by most studies. Biomarkers offer distinct and obvious advantages. It can allow an integrated measurement of bioavailable contaminants causing biochemical responses. Thus, it provides early indicators of potential pollution. The advantages and disadvantages of biomarkers are being discussed as in Table 2.

Conclusion

The study on the exposure of pesticides and its effect on cholinesterase enzyme activity provides a biomarker that is sensitive for the detection of toxicants and could become one of the method to eliminate pesticides pollution which gave a great deal of threats to our water bodies. This method improves the understanding of the adverse effect caused by the toxicants in contaminated water bodies that are polluted by pesticides. Thus, this method can be used as a preliminary screening to remove any possible pollutants came from sewage, fertiliser, and industrial waste for biomonitoring programme that give promising results.

References

1. Randall C. National Pesticide Applicator Certification Core Manual (NASDARF). National Association of State Departments of Agriculture Research Foundation, Washington, DC, Ch.1. 2013.
2. Singh M, Kaur M, Kukreja H, Chugh R, Silakari O and Singh D. Acetylcholinesterase inhibitors as Alzheimer therapy: from nerve toxins to neuroprotection. *Eur. J. Med. Chem.* 2013; 70: 165–88.
3. Jeyaratnam J. Health problems of pesticide usage in the third world. *Br. Med. J. Ind. Med.* 1985; 42: 505–506.
4. Jokanovic M, Antonijevic B. and Vusinic S. Epidemiological studies of anticholinesterase pesticide poisoning in Serbia. In: *Anticholinesterase Pesticides: Metabolism, Neurotoxicity and Epidemiology*, (Satoh T and Gupta RC, editors), Chapter 35, John Wiley and Sons. 2010; 481–494.
5. Asensio-Ramos, Hernandez-Borges MJ, Ravelo-Perez LM and Rodriguez-Delgado MA. Evaluation of a modified QuEChERS method for the extraction of pesticides from agricultural, ornamental and forestal soils. *Anal. Bioanal. Chem.* 2010; 396: 2307–2319.
6. Cappiello A, Famiglini G, Mangani F, Palma P and Siviero A. Nano high-performance liquid chromatography electron ionization mass spectrometry approach for environmental analysis. *Anal. Chim. Acta.* 2003; 493: 125–136.
7. Ganzera M, Schneider P. and Stuppner H. Inhibitory effects of the essential oil of chamomile (*Matricaria recutita* L.) and its major constituents on human cytochrome P450 enzymes. *Life Sci.* 2006; 78: 856–861.
8. Wong JL and Wessel GM. FRAP analysis of secretory granule lipids and proteins in sea urchin egg. In *Exocytosis and Endocytosis*. New Jersey. Human Press. 2007; 61–74.
9. Van Dam EW, Roelfsema F, Veldhuis JD, Helmerhorst FM, Frolich M, Meinders AE, Krans HM and Pijl H. Increase in daily LH secretion in response to short-term calorie restriction in obese women with PCOS. *Am. J. Physiol. Endocrinol. Metab.* 2002; 865–872.
10. Chand RS and Gupta BS. Surface plasmon resonance based fiber-optic sensor for the detection of pesticide, *Sens. Actuators. B.* 2007; 661–666.
11. Tahara H, Kusuhara H, Endou H, Koepsell H, Imaoka T, Fuse E, and Sugiyama Y. A species difference in the transport activities of H₂ receptor antagonists by rat and human renal organic anion and cation transporters. *J. Pharmacol. Exp. Ther.* 2005; 315: 337–345.
12. Tong F, Islam RM, Carlier PR, Ma M, Ekström F and Bloomquist JR. Effects of anticholinesterases on catalysis and induced conformational change of the peripheral anionic site of murine acetylcholinesterase. *Pest. Biochem. Physiol.* 2013; 106: 79–84.
13. Shelton, JF, Hertz-Picciotto, I and Pessah, IN. Tipping the balance of autism risk: potential mechanisms linking pesticides and autism. *Environ. Health Perspect.* 2012; 120: 944–951.
14. Cokugras AN. Butyrylcholinesterase: structure and physiological importance. *Turk. J. Biochem.* 2003; 28: 54–61.
15. Radic Z. and Taylor P. Structure and function of cholinesterases. In: *Toxicology of Organophosphates and Carbamate Compounds*. Elsevier, Amsterdam. 2006; 161–186.
16. Massoulié J. The origin of the molecular diversity and functional anchoring of cholinesterases. *Neurosignals.* 2002; 11: 130–143.
17. Chen VP, Xie HQ, Chan WK, Leung KW, Chan GK, Choi RC, Bon S, Massoulié J and Tsim KW. The PRiMA-linked cholinesterase tetramers are assembled from homodimers: hybrid molecules composed of acetylcholinesterase and butyrylcholinesterase dimers are up-regulated during development of chicken brain. *J. Biol. Chem.* 2010; 285: 27265–27278.
18. Bon S, Dufourcq J, Leroy J, Cornut I and Massoulié J. The C-terminal 10 peptide of acetylcholinesterase forms an alpha helix that supports homomeric and heteromeric interactions. *Eur. J. Biochem.* 2004; 271: 33–47.

19. Mushigeri SB and David M. Fenvalerate induced changes in ACh and associated AChE activity in different tissues of fish *Cirrhinus mrigala* (Hamilton) under lethal and sub-lethal exposure period. *Environ. Toxicol. Pharmacol.* 2005; 20: 65–72.
20. Sussman JL, Harel M, Frolov F, Oefner C, Goldman A, Tokar L. and Silman I. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine binding protein. *Science*.1991; 253: 872–879.
21. Dvir H, Silman I, Harel M, Rosenberry TL and Sussman JL. Acetylcholinesterase: From 3D structure to function. *Chem.-Biol. Interact.* 2010; 187: 10–22.
22. Bourne Y, Radic Z, Kolb HC, Sharpless KB, Taylor P. and Marchot P. Structural insights into conformational flexibility at the peripheral site and within the active center gorge of AChE. *Chem.-Biol. Interact.* 2005; 157–165.
23. Sussman JL and Silman I. Acetylcholinesterase: structure and use as a model for specific cation – protein interactions. *Curr. Opin. Struct. Biol.* 1992; 721–729.
24. Seidman S and Soreq H. Acetylcholinesterase – new roles for an old factor. *Nat. Rev. Neurosci.* 2. 2001; 294–302.
25. Poli A, Di Iorio P, Beraudi A, Notari S, Zaccanti F, Villani L and Traversa U. The calcium dependent (3H) acetylcholine release from synaptosomes of brown trout (*Salmo trutta*) optic tectum is inhibited by adenosine A1 receptors: effects of enucleation on A1 receptor density and cholinergic markers. *Brain. Res.* 2001; 892: 78–85.
26. Lehninger AL. *Biochemistry*. New Delhi: Kalyani Publications. 1993.
27. Mehrani H. Simplified procedures for purification and stabilization of human plasma butyrylcholinesterase. *Proc. Biochem.* 2004; 39:877–882.
28. Mesulam MM and Geula C. Acetylcholinesterase-rich neurons of the human cerebral cortex: cytoarchitectonic and ontogenetic patterns of distribution. *J. Comp. Neurol.* 1991; 306:193–220.
29. Massoulié J, Perrier N, Noureddinne H, Liang D. and Bon S. Old and new questions about cholinesterases. *Chem.-Biol. Interact.* 2008; 175: 30–44.
30. Dembélé K, Haubruge E. and Gaspar C. Concentration effects of selected insecticides on brain acetylcholinesterase in the common carp (*Cyprinus carpio* L.). *Ecotoxicol. Environ. Saf.* 2000; 45: 49–54.
31. Matozzo V, Tomei A. and Marin MG. Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarum* from the Lagoon of Venice. *Mar. Biol.* 2005; 50: 1686–1693.
32. Narbonne JF, Aarab N, Clearandeanu C, Daubeze M, Narbonne J, Champeau O and Garrigues P. Scale of classification based on biochemical makers in mussels : application of pollution in Mediterranean coast and temporal trends. *Int. J. Biol. Markers.* 2005; 10: 58–71.
33. Brown P, Long S, Spurgeon D, Svendsen C and Hankard P. Toxicological and biochemical responses of the earthworm to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. *Chemosphere.* 2004; 57: 1675–1681.
34. Lehtonen M, Vorobyev VA, Hugdahl K, Tuokkola T and Laine M. Neural correlates of morphological decomposition in a morphologically rich language: an fMRI study. *J. Brain Lang.* 2006; 98: 182–193.
35. Bocquene, G., Roig, A. and Fournier, D. Cholinesterase from, the common oyster (*Crassostrea gigas*). Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. *FEBS Lett.*1997; 407: 261–266.
36. Tham LG, Perumal N, Syed MA, Shamaan NA and Shukor MY. Assessment of *Clarias batrachus* as a source of acetylcholinesterase (AChE) for the detection of insecticides. *J. Environ. Biol.* 2009; 30: 135–138.
37. Bargmann C. Neuroscience: Genomics reaches the synapse. *Nature.* 2005; 436: 510–517.
38. Abou-Donia MB, Abdel-Rahman AA and Goldstein LB. Sensorimotor deficits and increased brain nicotinic acetylcholine receptors following exposure to chlorpyrifos and/or nicotine in rats. *Arch. Toxicol.* 2003; 77: 452–458.
39. Barbosa M, Rios O, Vela'squez M, Villalobos J and Ehrmanns J. Acetylcholinesterase and butyrylcholinesterase histochemical activities and tumor cell growth in several brain tumors. *Surg. Neurol.* 2001; 55: 106–112.
40. Darvesh S, Hopkins DA. and Geula C. Neurobiology of butyrylcholinesterase. *Nat. Rev. Neurosci.* 2003; 4: 131–138.
41. Giacobini E. Butyrylcholinesterase: its role in brain function. In: Giacobini E, editor. *Butyrylcholinesterase: Its Function and Inhibitors*. London, UK: Martin Dunitz. 2003; 1–20.
42. Duysen EG, Li B, Xie W, Schopfer LM, Anderson R.S, Broomfield CA and Lockridge O. Evidence for nonacetylcholinesterase targets of organophosphorus nerve agent: supersensitivity of acetylcholinesterase knockout mouse to VX lethality. *J. Pharmacol. Exp. Ther.* 2001; 299: 528–535.
43. Montenegro G, Díaz-forestier J, Fredes C and Rodríguez S. Phenolic profiles of nectar and honey of *Quillaja saponaria* (Quillajaceae) as potential chemical markers. *J. Biol. Res.* 2013; 562: 177–182.
44. Li JG, Benovic JL and Liu-Chen LY. Mechanisms of agonist-induced down-regulation of the human kappa-opioid receptor: internalization is required for down-regulation. *Mol. Pharmacol.* 2000; 58: 795–801.

45. Xie WH, Altamirano CV, Bartels CF, Speirs RJ, Cashman JR and Lockridge O. An improved cocaine hydrolase: the A328Y mutant of human butyrylcholinesterase is 4-fold more efficient. *Mol. Pharmacol.* 1999; 55:83–91.
46. Greig NH, Lahiri DK and Sambamurti K. Butyrylcholinesterase: An important new target in Alzheimer's disease therapy. *Int. Psychog.* 2002; 1: 77–91.
47. Rodríguez-Fuentes G, Armstrong J, Schlenk D. Characterization of muscle cholinesterases from two demersal flatfish collected near a municipal wastewater outfall in Southern California. *Environ. Toxicol. Environ. Saf.* 2008; 69: 466–471.
48. Assis CRD, Linhares AG, Oliveira VM, França RCP, Carvalho EVMM, Bezerra RS and de Carvalho LB. Comparative effect of pesticides on brain acetylcholinesterase in tropical fish. *Sci. Total Environ.* 2012; 441: 141–150.
49. Chuiko GM, Podgornaya VA and Zhelnin YY. Acetylcholinesterase and butyrylcholinesterase activities in brain and plasma of freshwater teleosts: cross-species and cross-family differences. *Comp. Biochem. Physiol. B.* 2003; 135: 55–61.
50. Schopfer LM, Boeck AT, Broomfield CA and Lockridge O. Mutants of human butyrylcholinesterase with organophosphate hydrolase activity; evidence that His117 is a general base catalyst for hydrolysis of echothiophate. *J Med. Chem. Def.* 2004; 2: 20.
51. Jung JH, Addison RF and Shim WJ. Characterization of cholinesterases in marbled sole, *Limanda yokohamae*, and their inhibition in vitro by the fungicide iprobenfos. *Mar. Environ. Res.* 2007; 63: 471–478.
52. Aker WG, Hu X, Wang P and Hwang HM. Comparing the Relative Toxicity of Malathion and Malaoxon in Blue Catfish (*Ictalurus furcatus*). *Environ. Toxicol.* 2008; 23: 548–554.
53. Wogram J, Sturm A, Segner H and Liess M. Effects of parathion on acetylcholinesterase, butyrylcholinesterase, and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatus*) following short-term exposure. *Environ. Toxicol. Chem.* 2001; 20: 1528–1531.
54. Sidell FR, Borak J. Chemical warfare agents: II. Nerve agents. *Ann. Emerg. Med.* 1992; 21: 865–871.
55. Gallo MA and Lawryk NJ. Chapter 16. Organic Phosphorus Pesticides In H.W. (eds), *Pesticides studied in man*. Baltimore: Williams and Wilkins. 1991; 917–1123.
56. Jordaan MS, Reinecke SA and Reinecke AJ. Biomarker responses and morphological effects in juvenile tilapia *Oreochromis mossambicus* following sequential exposure to the organophosphate azinphos-methyl. *Aquat. Toxicol.* 2013; 144–145.
57. Marrs TC, Rice, P and Vale JA. The role of oximes in the treatment of nerve agent poisoning in civilian casualties. *Toxicol. Rev.* 2006; 25: 297–323.
58. Silman I and Sussman JL. Acetylcholinesterase: “classical” and “non-classical” functions and pharmacology. *Curr. Opin. Pharmacol.* 2005; 3: 293–302.
59. Eddleston M, Karalliedde L, Buckley N, Fernando R, Hutchinson G, Isbister G, Konradsen F, Murray D, Piola JC, Senanayake N, Sheriff R, Singh S, Siwach SB and Smit L. Pesticide poisoning in the developing world—a minimum pesticide list. *The Lancet.* 2002; 360: 1163–1167.
60. Millard CB., Lockridge O and Broomfield CA. Organophosphorus acid anhydride hydrolase activity in human butyryl-cholinesterase: synergy results in a somanase. *J. Biochem.* 1999; 37: 237–247.
61. Fulton MH and Key PB. Lethal and sublethal effects of chlor-pyrifos exposure on adult and larval stages of the grass shrimp, *Palaemonetes pugio*. *J. Environ. Sci. Health B.* 1993; 28: 621–640.
62. Odenkirchen EW and Eisler R. Chlorpyrifos hazards to fish, wildlife, and invertebrates: a synoptic review. Report No. 85, U.S. Fish and Wildlife Services Biol. Rep. 1998; 17–23.
63. Bretau S, Toutant JP and Saglio P. Effects of carbofuran, diuron, and nicosulfuron on acetylcholinesterase activity in goldfish (*Carassius auratus*). *Ecotoxicol. Environ. Saf.* 2000; 47: 117–124.
64. Bocquene G and Cadiou Y. Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. *Mar. Ecol. Prog. Ser.* 1992; 91: 77–82.
65. Narbonne JFP, Garrigues PD, Ribera C, Raoux A, Mathieu P, Lamaine JP and LauFaurie M. Mixed-function oxygenase enzymes as tools for pollution monitoring. Field studies on the French coast of the Mediterranean Sea. *Comp. Biochem. Physiol. Part C: Comp. Pharmacol.* 1991; 100: 37–42.
66. Kirby MF, Morris S, Hurst M, Kirby SJ, Neall P, Taylor T and Fagg A. The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotoxic contamination in U.K estuaries. *Mar. Pollut. Bull.* 2000; 40, 780–791.
67. WHO. Environmental Health Criteria, 63: Organophosphorus insecticides: a general introduction. Geneva, World Health Organization. 1986: pp. 181.
68. Chang SK, Arendt BK, Darce JR, Wu X and Jelinek DF. A role for B₂L₂S in the activation of innate immune cells. *Blood.* 2006; 108: 2687–2694.
69. Beauvais SL, Jones, SB, Brewer SK and Little EE. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioural measures. *Environ. Toxicol. Chem.* 2000; 19: 1875–1880.
70. Colović MB, Krstić DZ, Lazarević-Pašti TD, Bondžić AM and Vasić, VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. *Curr. Neuropharmacol.* 2013; 11: 315–335.

71. Shi MA, Yuan JZ, Wu J, Zhuang PJ, Wu XF, Tang ZH. Kinetic analysis of acetylcholinesterase in a propoxur-resistant strain of housefly (*Musca domestica*) from Shanghai, China. *Pest. Biochem. Physiol.* 2002; 72: 72–82.
72. Msagati TAM and Mamba BB. Monitoring of N-methyl carbamate pesticide residues in water using hollow fibre supported liquid membrane and solid phase extraction. *Phys. Chem. Earth.* 2012; 50-52: 149–156.
73. McKenzie KM, Mee JM, Rogers CJ, Hixon MS, Kaufmann GF, Janda KD. Identification and Characterization of Single Chain Anti-cocaine Catalytic Antibodies. *J. Mol. Bio.* 2007; 365: 722–731.
74. Hejkal TW, Gerba CH, Henderson J and Freeze N. Bacteriological, virological and chemical evaluation of a wastewater aquaculture system. *Water Resour. Res.* 1983; 17: 1749–1755.
75. WHO\ FAO. National Research Council Recommended Dietary Allowances 10th Edn. National Academy Press. 1989. Washington, DC. USA.
76. Almroth C, Sturve B, Stephensen J, Fredrik Holth E and Förlin L. Protein carbonyls and antioxidative defenses in corks wing wrasse (*Symphodus melops*) from a heavy metal-polluted and PAH-polluted site. *Mar. Environ. Res.* 2008; 66: 271–277.
77. Al-Ghais SM. Acetylcholinesterase, glutathione and hepatosomatic index as potential biomarkers of sewage pollution and depuration in fish. *Mar. Pollut. Bull.* 2013; 74: 183–186.
78. Lam PKS and Gray JS. The use of biomarkers in environmental monitoring programmes. *Mar. Pollut. Bull.* 2003; 46: 182–186.
79. Facey K, Bradbury I, Laking G and Payne E. Overview of the clinical effectiveness of positron emission tomography in selected cancers. United Kingdom. Gray Publishing. *Health Technol. Assess.* 2007; 11: 3–4.
80. Galloway JN and Cowling EB. Reactive nitrogen and the world: Two hundred years of change. *Ambio.* 2002; 31: 64–71.
81. Moulton CA, Fleming WJ and Purnell CE. Effects of two cholinesterase-inhibiting pesticides on freshwater mussels. *Environ. Toxicol. Chem.* 1996; 15: 131–137.
82. Coppage DL, Mathews E, Cook GH and Knight J. Brain acetylcholinesterase inhibition in fish as a diagnosis of environmental poisoning by malathion, O-o-dimethyl S-(1, 2 Dicarbethoxylthyl) Phosphorodithioate. *Pestic. Biochem. Physiol.* 1975; 5: 536–542.
83. Day KE and Scott IM. Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. *Aquat. Toxicol.* 1990; 18: 101–113.