JEB
Journal of Environmental Biology

Journal Website : www.jeb.co.in
E-mail : editor@jeb.co.in
ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Potential use of acetylcholinesterase, glutathione-*S*-transferase and metallothionein for assessment of contaminated sediment in tropical chironomid, *Chironomus javanus*

A. Somparn[1], C.B. Iwai[1,2*] and B. Noller[3]

[1]Department of Plant Sciences and Agricultural Resources, Land Resources and Environment Division, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand,40002.

[2]Integrated Water Resource Management Research and Development Center in Northeast Thailand, Khon Kaen, Thailand,40002.

[3]The University of Queensland, Centre for Mined Land Rehabilitation (CMLR), Brisbane, 4072 Australia.

*Corresponding Author's E-mail : chulee_b@kku.ac.th

Publication Info

Paper received:
11 May 2014

Revised received:
14 February 2015

Accepted:
20 March 2015

Abstract

Heavy metals and organophosphorus insecticide is known to act as disruptors for the enzyme system, leading to physiologic disorders. The present study was conducted to investigate the potential use of these enzymes as biomarkers in assessment of contaminated sediments on tropical chironomid species. Acetylcholinesterase (AChE), glutathione-S-transferase (GST) and metallothionein (MT) activity was measured in the fourth-instar chironomid larvae, *Chironomus javanus*, Kieffer, after either 48-hr or 96-hr exposure to organophosphorus insecticide, chlorpyrifos (0.01- 0.25 mg kg^{-1}) or heavy metal cadmium (0.1-25 mg kg^{-1}). Exposure to chlorpyrifos (0.01 mg kg^{-1}) at 48 and 96 hr significantly of AChE activity (64.2 % - 85.9%) and induced GST activity (33.9 - 63.8 %) when compared with control ($P<0.05$). Moreover, exposure to cadmium (0.1mg kg^{-1}) at 48 and 96 hr also showed significant increas GST activity (11.7 - 40 %) and MT level (9.0 % - 70.5 %) when compared with control ($P<0.05$). The results indicated the impact of enzyme activity on chlorpyrifos and cadmium contamination. Activity of AChE, GST and MT could serve as potential biomarkers for assessment and biomonitoring the effects of insecticide and heavy metal contamination in tropical aquatic ecosystems.

Key words

Acetylcholinesterase, Cadmium, Chlorpyrifos, Glutathione *S*-transferase, Metallothionein.

Introduction

Measurement of toxicant concentration is the most common and simplest way to assess environment quality and occurrence of biological effects from exposure to pollution (Van der Oost *et al*., 2003). However, use of biochemical markers such as enzyme activity, which is closely related to molecular biology, immunological or physiological activity of an organism, has been proposed as sensitive 'early warning' tool for assessing biological effects (Callaghan *et al*., 2000; Van der Oost *et al*., 2003). Acetylcholinesterase (AChE), a carboxylesterase enzyme, has a key role in maintaining normal nerve function in the invertebrate organisms (Callaghan, 2001) and is inhibited by neurotoxic compounds such as phosphate and carbamate esters (commonly used as insecticides) that bind to the enzyme and lead to accumulation of acetylcholine in the synapse resulting in disruption of normal functioning of nervous system (Olsen *et al*., 2001). AChE inhibition has been used as a specific biomarker for organophosphate and carbamate pesticides (Domingues, 2008). Glutathione-*S*-transferases (GST) represents a family of enzymes that catalyzes the conjugation of various compounds having electrophilic centers (nitro compounds, organophosphates and organochlorines) with tripeptide glutathione. Glutathione conjugation products are rendered less toxic and become more soluble in water, facilitating their excretion (Olsen et al.,2001; Hyne and Maher, 2003). Increase in GST

activity has been used as a biomarker of organic contamination (Domingues, 2008). Metallothioneins (MTs) are small (6–7 kDa) cytosolic proteins involved in metal homeostasis and detoxification processes that contain about 60 amino acids (none of which is aromatic) and high content of cysteine residues (Coyle *et al*., 2002; Costa *et al*., 2008). The thiol groups (-SH) of cysteine residues enable MTs to bind particular heavy metal for their storage, transport or detoxification (Amiard *et al*., 2006). In organisms, it has been reported that MT level increase every 48 hr after being exposured to heavy metals ions. Thus, this response can be used for the monitoring the effects of heavy metal contamination in the environment (Rome´o *et al*., 2000; Strouhal *et al*., 2003; Kraemer *et al*., 2005; Prusa *et al*.,2006).

Application of biomarker data is relatively new in ecotoxicological study but has limitations in tropical countries such as Thailand due to availability of ecotoxicological data on aquatic organisms. Similar to the situation with many developing countries, there has been a revision of overseas ecotoxicological data, especially from temperate locations, regarding the significance of contaminants affecting organism in the environment (Dominues *et al*., 2007). Direct comparison of temperate-zone climate with tropical ecotoxicological data may not be suitable, because organisms from tropical locations may be more or less sensitive to contaminants than surrogate species. The characteristics of sediment and water in river systems may differ from other temperate locations that might influence toxicity and sensitivity of organisms to contaminants (Somparn *et al*., 2010). The assessment of toxic effects of pesticides and heavy metals in tropical climate ecosystems should be performed with local species, since their sensitivity to toxicants may differ considerably from those of laboratory standardized organisms from temperate-zone studies.

Chironomid species have been adopted as test species for ecotoxicological assessment in both water and sediment because they are an important component of freshwater ecosystems, which represents one of the most widely distributed groups of benthic organisms adapted to almost every type of aquatic ecosystem (Vos, 2000). These species are important prey for juvenile and adult fish and aquatic birds and can easily be cultured under laboratory conditions with relatively short life cycle (Taenzler, 2007). Two species of chironomid, typically used in ecotoxicity testing, are *C. tentans* and *C. riparius* (Watts and Pascoe, 2000). Since these species are not commonly found in Thailand freshwater ecosystem, local chironomid species was used in study to represent an accurate adverse effect of pollution on the tropical invertebrate species in Thailand. Therefore, the aim of the present study was to investigate the effects of chlorpyrifos and cadmium on enzyme activity (AChE, GST and MT) in tropical chironomid, *Chironomus javanus*.

Materials and Methods

Test organisms: Chironomid larvae *(Chironomus javanus*) were collected in the Ecotoxicology and Environmental Sciences Laboratory, Khon Kaen University, Thailand were since 2009.The culture was modified from OECD (2004). In brief, test species where maintained at 25±1.0°C and kept under static conditions at 6:18 hr (light- dark) photo-period. The culture water had a dissolved oxygen (DO) concentration at 5 -7 mgl^{-1} and pH of 7-8. Test species was fed with 0.5 mg of ground commercial fish food (TetraMin®, Melle,Germany). Continuous gentle aeration was provided, using an air pump to maintain optimum oxygen level, and the culture was maintained till further use.

Study design: AChE, GST and MT assays were performed to determine the activity of enzymes in chironomid larvae after exposure to chlorpyrifos and cadmium for 48 and 96hr at 25±1.0°C. Artificial sediment was spiked with chlorpyrifos to give test concentration of 0.01, 0.05, 0.1 and 0.25 mg kg^{-1} and cadmium 0.1, 1, 10 and 25 mg kg^{-1}as described by OECD (2004) test procedure. Four replication of five 4^{th} instar larvae per treatment and control were used.

Measurement of AChE and GST activity: All the steps were carried out at 4°C. Whole frozen chironomid was homogenized in ice-cooled buffer (0.02 M phosphate buffer pH 8.0, containing 1% Triton X-100) using a tissue homogenizer. Homogenate was centrifuged at 14,000 ×*g* at 4°C for 15 min. And supernatant of each sample was stored at –20°C for not more than a week, until enzymatic activities were complete. Total protein content in homogenate was measured following the method of Bradford (1976), at 595 nm, using bovine serum albumin as standard protein.

AChE activity assay was based on the method described by Ellaman *et al*. (1961), with some modification. In brief, the sample was mixed with 8 mM 5,5-di-thio-nitrobenzene acid (DTNB) in phosphate buffer containing 0.75 mgl^{-1} $NaHCO_3$ and 50 µl of 16 mM acetylthiocholine iodide. After incubation at 30°C for 5 min, AChE activity was determined by kinetic measurement at 412 nm. Results were expressed as nmol mg^{-1} protein min^{-1}.

GST activity assay was based on the method of Habig et al. (1974), with some modification adapted to microplate. In brief, the sample was mixed with assay buffer containing 1-chloro-2,4-dinitrobenzene as substrate and glutathione in sodium phosphate buffer at pH 7.5. All GST activity assays were realized in conditions of linearity with respect to incubation time. The rate was measured as ΔOD/min at 340 nm. Extinction coefficient of 9.6 was used with a light path of 0.600 cm for activity calculations. The results were expressed as nmol mg^{-1} protein min^{-1}.

MT levels was estimated by the method of Viarengo *et al*. (1997). Chironomid larvae was homogenized in three volumes

of 0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, with added 0.006 mM leupeptine,0.5 mM phenylmethyl sulphonylfluoride (PMSF) and 0.01% B-mercaptoethanol. The homogenate was then centrifuged at 30,000g for 30 min at 4^{o}C to obtain a supernatant. The supernatant was then treated with 1.05 ml of cold absolute ethanol and 80 µl of chloroform of 1 ml of supernatant. The samples were and then at 6000 g for 10 min. This supernatant sample was then centrifuged at 3000 g for 5 min at 4 °C; the supernatant absorbance was evaluated at 412 nm following the method of Ellaman *et al*. (1961), and MT level was estimated by utilizing reduced glutathione (GSH) as standard. The data expressed as ng mg^{-1} protein

Statistical analysis: The data obtained from different experiments were analyzed using ANOVA by Statistical 8 software (Version 8,USA) to detect significant difference in variance (P<0.05) between treatment group of concentrations and control.

Results and Discussion

The result of the effect of chlorpyrifos on AChE and GST activity is tabulated of in Table1. Chlorpyrifos inhibited AChE activity in a concentration duration manner. After exposure to highest concentration (0.25 mg kg^{-1}) chlorpyrifos for 48 hr, the AChE activity in chironomid was inhibited up to 76.5 % and when exposed for 96 hr inhibition reached 91.1 % (Table 1). This inhibition effect might be due to binding of chlorpyrifos-inhibited AChE activity to active site serine, resulting in irreversible inhibition of enzymes and thereby increasing both the level and duration of action of the neurotransmitter acetylcholine. Accumulation of acetylcholine will results in prolonged muscle contraction and prolonged electrical activity at nerve endings causing uncontrolled movement (Olsen *et al*., 200; Handy and Galloway, 2003). Previous studies have reported chlorpyrifos the ability of inhibit AChE in *C. tentans* and *C. riparius* in a dose and time-dependent manner (Dominingues *et al*., 2008; Callaghan *et al*., 2002). These studies also indicate that chironomid was sensitive to low concentrations of chlorpyrifos (0.01 mg/ kg^{-1}) and inhibited AChE activity to over 50 % after 24 hrs exposure, and that AChE activity can be used as a biomarker for assessing chlorpyrifos contamination that has low persistence in the environment (PMRA, 2000).

At highest concentration GST activity increased by 272 % and 192% at 48 and 96 hr, respectively (Table 1). It is well established that chlorpyrifos is effective in the inducting GST activity in chironomid by catalyzing the conjugation reaction of molecules with electrophilic sites with reduced glutathione and with a group of compounds like organophosphates and organochlorines (Olsen *et al*, 2001; Kheir, 2001; Crane, 2002). GST can alter the structure of pesticide to less toxic form and enhance elimination of toxicity from the organism. (Olsen *et al*.,2001; Hyne and Maher,. 2003). It is also known that GST is important in detoxification of organophosphates and organochlorines, and is induced by these pesticides in many aquatic organisms (Crane *et al*., 2002). Printes *et al*. (2007) reported that *C. xanthus* increased GST activity after exposure to chlorpyrifos for 96 hr. Moreover, there were significant differences in GST activity over time with higher activity at 48 hr, compared to 96 hr (Table1) because the chironomid absorbs chlorpyrifos in its body which causes the chironomid to create GST and remove chlorpyrifos from the body, resulting in less toxicity of chlorpyrifos.

Table 2 shows MT level and GST activity in chironomid after exposure to cadmium at 48 and 96 hr. The level of MT in

Table 1.: Effects of chlorpyrifos concentration on AChE and GST activity of *C. javanus* at 48 and 96 hr.

Chlorpyrifos (mg kg-1)	AChE activity (nmol mg l^{-1} protein)		GST activity (nmol mg l^{-1} protein)	
	48hr	96hr	48hr	48hr
0	33.7±1.0a (0)	39.7±1.6b (0)	50.6±4.7 (100)	51.1±2.7 (100)
0.01	12.1±0.9*a (64.29)	5.6±0.9*b (85.9)	67.8±2.5*d (134)	83.7±3.6*c (164)
0.05	9.89±1.2*a (70.9)	5.6±1.2*b (85.9)	99.1±2.1*c (196)	69.6±2.4*d (136)
0.10	9.5±1.8*a (71.9)	5.1±1.0*b (87.1)	127±1.1*b (250)	90.9±1.5*c (178)
0.25	7.9±1.4*a (76.6)	3.5±1.3*b (91.1)	138±2.93*a (272)	98.1±2.2*b (192)

Note: Values are mean of four replications ±SD; * indicate significant difference from control (LSD, P < 0.05); different letters in superscript indicate significant difference among different values as each exposure time; Value in parentheses indicate values of % AchE inhibition and % GST induction relative to the control.

Table 2: Effect of cadmium on MT level and GST activity of *C. javanus* at 48 and 96 hr.

Cadmium (mg kg^{-1})	MT level (ng g^{-1}tissue)		GST activity (nmol mg l^{-1} protein)	
	48hr	96hr	48hr	48hr
0	110±1.2^{a} (100)	69.5±1.9^{b} (100)	19.6±1.7 (100)	20.1±1.2 (100)
0.1	120±1.6^{*d} (109)	118±1.1^{*e} (171)	21.9±1.9^{b} (112)	28.2±1.2^{*c} (141)
1	136±1.1^{*c} (123)	140±1.9^{*d} (201)	28.9±4.7^{*b} (147)	32.95±2.20^{*c} (164)
10	172±0.7^{*b} (155)	159±1.8^{*c} (229)	26.3±3.3^{*b} (134)	33.5±1.1^{*c} (205)
25	233±0.7^{*a} (211)	167±1.1^{*b} (240)	76.2±2.0^{*a} (388)	41.2±1.1^{*b} (205)

Note: Values are mean of four replications ±SD; * indicate significant difference from control (LSD, P < 0.05); different letters in superscript indicate significant difference among different values as each exposure time; Value in parentheses indicate values of % MT and % GST induction relative to the control.

control tissues was 110.39 and 69.47 ng g^{-1} after 48 hr and 96 hr, respectively. There was significant increase in MT level in the chironomid at of 0.1 mg kg^{-1} concentration at 48 and 96 hr as compared to control (Table 2). In the present study, cadmium administration to the chironomid resulted in rapid increase of MT level up to maximum cadmium dose of 25 mg kg^{-1}. MT is a protein that can eliminate heavy-metal toxicants from organisms (Nordberg and Nordberg, 2000; Amiard *et al*., 2006) and its.

Induction represents an important defense mechanism against the toxic effects of cadmium to the chironomid. Similarly, Fabrik *et al*. (2008) observed that less toxicity of cadmium on the *C. riparius* was correlated to decrease MT in concentration, indicating the role of MT on heavy metal detoxification. The effect of cadmium was evident when chironomid was exposed to lowest concentration (0.1 mg kg^{-1}) as there was significant effect of cadmium on chironomid. The results presented for exposure times 48 and 96 hr, showed that the MT level in chironomid at of 96 hr decreased when compared to 48 hr (Table 2), which indicated that MTs needed some time to be induced. The LOEC value for cadmium exposed to *C. javanus* was 0.1 mg kg^{-1} indicating sensitivity of biomarker to cadmium exposure.

Regarding GST, the mean value for GST activity in chironomid for control obtained from the experiment with cadmium at 48 and 96 hr is presented in Table 2. After 48 hrs of exposure to 25 mg kg^{-1} cd, there was a significant increase in GST activity in chironomid (Table 2). After 96 hr, a significant change was observed in GST activity in chironomid for all the tested concentrations of cadmium (Table 2). Previous data have reported different effects of cadmium on GST activity in aquatic organism. An increase in GST activity in chironomid, *Propsilocerus akamusi* exposed to 50 mmol l of cadmium was observed at 96 hr (Zheng *et al*., 2011), and induction of GST activity has also been observed in crayfish *Procambarus clarkii* that exposed to 100 $\mu g l^{-1}$ cadmium for 96 hrs. This study indicated that GST activity also responded to cadmium. For comparison of GST activity in chironomid between exposure times of 48 and 96 hr, the results showed that no effect of cadmium on GST activity in chironomid for an exposure time of 48 hr (Table 2). Jamee *et al*. (2007) described that GST activity was possible, since it depended on the type of chemical, time and dose exposure and showed that the values of both MT level and GST activity in *C. javanus* could serve as one of the several biomarkers of cadmium contamination. However, changing MT level in chironomid at 48 hr (cadmium 0.1 mg kg^{-1}) showed higher potential as a biomarker of cadmium exposure than GST activity (Table 2).

Taken together, AChE and GST activity, as well as MT levels, show an impact on chironomid and thus represent an early toxicity response to chlorpyrifos and cadmium contamination. Therefore, assessment of AChE and GST, activity as well as MT levels in chironomid could be promising biomarkers of chlorpyrifos and cadmium contamination in sediment. The assay will be an environmental risk-assessment tool for pesticide and heavy-metal contamination in the tropical ecosystem.

Acknowledgments

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Graduate School, Integrated Water Resource Management Research and Development Center in Northeast Thailand, Research center for environmental and hazardous substance management and Center of Excellence on Hazardous Substance Management, Khon Kaen University, Thailand.

References

Amiard, J.C., C. Amiard-Triquet, S. Barka , J. Pellerin and P.S. Rainbow: Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers *Aquat Toxicol*., **76**, 160-202 (2006).

Beauvais, S.L., S.B. Jones, J.T. Parris, S.K. Brewer and E.E. Little: Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorynchusmykiss*). *Eco. Environ. Saf.,* **49**, 84-90 (2001).

Bradford, M.A.: Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.,* **72**, 248-254 (1997).

Callaghan, A.,C. Fisher, A. Grosso, J. Holloway and M. Crane: Effect of temperature and pirimiphos methyl on biochemical biomarkers in *Chironomus riparius*, Meigen. *Eco. Environ. Saf*., **52**, 128-133 (2002).

Costa, P.M., T. Repolho, S. Caeiro, M.E. Diniz, I. Moura and M.H. Costa: Modeling metallothionein induction in the liver of *Sparus aurata* exposed to metal-contaminated sediments. *Ecotox Environ Safe*., **71**, 117-124 (2008).

Coyle, P.J., C. Philcox, L.C. Carey and A. M. Rofe: Metallothionein: The multipurpose protein., *Cell. Mol. Life Sci*., **59**, 627-647 (2002).

Crane, M., W. Sildanchandra, R. Kheir and C. Callaghan: Relationship between biomarker activity and developmental endpoints in *Chironomus riparius* Meigen exposed to an organophosphate insecticide. *Ecotox. Environ. Saf*., **53**, 361-369 (2002).

Domingues, I., L.Guilhermino, A.M.V.M. Soares and A.J.A. Nogueira: Assessing dimethoate contamination in temperate and tropical climates: potential use of biomarkers in bioassays with two chironomid species. *Chemosphere*., **69**, 145-154 (2007).

Domingues, I., S.Kriengkrai, Y. Amararatne, M.V.M. Soares and A.J.A Nogueira: In situ assay with the midge *Kiefferulus calligaster* for contamination evaluation in aquatic agro-systems in central Thailand. *Chemosphere*.,**71,** 1877-1887 (2008).

Ellman, G., K.Courtney, J.V. Andres and R. Featherstone: A new and rapid colorimetric determination of acetylcholi-nesterase activity. *Biochem. Pharmaco*.,**7**, 88-95 (1961).

Fabrik, I., Ruferova, Z., Hilscherova, K., Adam, V., Trnkova, L. and Kizek, R.: Determination of metallothionein in larvae of freshwater a midges (*Chironomus riparius*) using Burdick Reaction. *Sensors*., **8**, 4081-4094 (2008).

Handy, R.D., T.S. Galloway and M.H. Depledge: A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotox.,* **12**, 331-343 (2003).

Hyne, R.V. and W.A. Maher: Invertebrate biomarkers: Links to toxic is that predict population decline. *Ecotoxicol Environ Saf.,* **54**, 366-

374 (2003).

Jemec, A., D. Damjana,T. Tišler and S.Kristina: Biochemical biomarkers in environmental studies-lessons learnt from enzymes catalase, glutathione *S*-transferase and cholinesterase in two crustacean species. *Environ Sci Pollut Res.,* **17**, 71-81 (2009).

Kraemer, L.D., P.G.Campbell and L.Hare: Dynamics of cd and zn accumulation in organs and sub-cellular fractions in field transplantated juvenile yellow perch (*Percaflavescens*). *Environ. Pollut.,* **138**, 324-337 (2005).

Kheir, R., H. Ibrahim, J. Lewis, A. Callaghan and M. Crane: Comparison of acetylcholinesterase and glutathione-*S*-transferase activity in *Chironomus riparius* Meigen exposed to chemical-spiked sediments. *Bull Environ Contam Toxicol*., **66**,603-610 (2001).

Kovarova, J, R. Kizek , A. Vojtech, D. Harustiakova, O. Celechovska, and Z. Svobodova:Effect of Cadmium Chloride on Metallothionein levels in carp. *Sensors.,* **9**, 4789-4803 (2009).

Nordberg, M and G.F. Nordberg: Toxicological aspects of metallothionein. *Cell Mol Biol.,* **46**, 451-463 (2000).

OECD: Test guideline 218 sediment-water chironomid toxicity test using spiked sediment (2004) .

Olsen, T., L. Ellerbeck, T. Fisher, A. Callaghan and M.Crane: Variability in acetylcholinesterase and glutathione *S*-transferaseactivities in *Chironomus riparius* Meigen deployed in situ at uncontaminated field sites. *Environ Toxicol Chem.,* **20**, 1725-1732 (2001).

PMRA (Pest Management Regulatory Agency): Environmental assessment of chlorpyrifos. environmental assessment. Division, Pest Management Regulatory Agency, Ottawa, Ontario (2000).

Prusa, R., M.Svoboda, O.Blastik, V.Adam, O.Zitka, M.Beklova, T.Eckschlager and R.Kizek: Increase in content of metallothionein as marker of resistance to cisplatinin a human germ cell tumor cell line. *Clin. Chem.,* ***52***, 174-175 (2006).

Pery, A. R., R. V.Ducrot, R. Mons, C. Miege, J. Gahou, D. Gorini and J. Garric: Survival test with *Chrironomus riparius* exposured to spiked sediment can profit DEBtox model. *Water Res*., **37**, 2691-2699 (2003).

Printes, L. B. E. L. G. Espindola and M. N. Fernandes: Biochemical biomarkers in individu all larvae of *Chironomus xanthus* (Rempel, 1939) (Diptera, Chironomidae). *J. Braz. Soc. Ecotoxicol.,* **2**, 53-60 (2007).

Rome´o, M., M. Bennani,M.Gnassia-Barelli, M. Lafaurie and J.P. Girard: Cadmium and copper display different responses to oxidative stress in the kidney of the sea bass *Dicentrarchuslabrax*. *AquaToxico*., **48**, 185 -194 (2000).

Roesijadi, G.: Mercury-binding proteins from the marine mussel, *Mytilus edulis*. *Environ Health Perspect*., **65**, 45-48 (1986).

Somparn, A., C.B Iwai, and B. Noller: Development of the indigenous chironomid species ecotoxicology test : Tool for water quality management in Thailand. *IJERD*., **1**, 32-36 (2010).

Strouhal, M., R.Kizek, J.Vecek, L.Trnkova and M. Nemec: Electrochemical study of heavymetals and metallothionein in yeast *Yarrowialipolytica*. *Bioelectrochem.,* **60**, 29-36 (2003).

Taenzler,V., B. Eric ,D. Michael, P.Verena and W. Lennart: Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. *Ecotox*., **16**, 221-223 (2007).

Van der Oost, R., J.Beyer and N.P.E. Vermeulen: Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol*., **13**, 57-149 (2003).

Vos, J.H., M.A.G.Ooijevaar, J.E.Postma and W.Admiraal: Interaction between food availability and food quality during growth of early instar chironomid larvae. *J North Am Benthol Soc*., **19**, 158-168 (2000).

Watts, M.M.P. and D.Pascoe: A comparative study of *Chironomus riparius* Meigen and *Chironomus tentans,* Fabricius (Diptera: Chironomidae) in aquatic toxicity tests. *Arch Environ. Contam. Toxicol*., **33**, 299-306 (2000).

Wheelock, C.E., K.J. Eder, I.Werner, H. Huang, P.D. Jones, B.F. Brammell, A.A. Elskus and B.D. Hammock: Individual variability in esterase activity and CYP1Alevels in Chinook salmon (*Oncorhynchuststshawytscha*) exposed to esfenvalerate and chlorpyrifos. *Aqua Toxico.,* **74 ,** 172-192 (2005).

Zheng X, W.Long, Y. Guo and M. Enbo: Effects of cadmium exposure on lipid peroxidation and the antioxidant system in fourth-instar larvae of *Propsilocerus akamusi* (diptera: Chironomidae) under laboratory conditions. *J .Econ Entomo.,* **104**, 827-832 (2011).