①

Response of GSTase and Liver Esterase in Goldfish (Carrasius auratus) and Topmouth Gudegon (Pseudorasbora parva) after Sublethal Exposure to Cyhalofop-butyl and Profurite-aminium

ZHU Guo-nian[1*], WU Hui-ming[1],
Jilisa K. Mwalilino[2], LI Shao-nan[1]
(1. *Institute of Pesticide and Environmental Toxicology*,
2. *Department of Environmental Sciences, Zhejiang University, Hangzhou* 310029, *China*)

Abstract: Investigations were carried to assess the sublethal effects of a herbicide clincher (100 EC) containing cyhalofop-butyl [100 g (*a. i*) · L^{-1}] as the active ingredient and the insecticide profurite-aminium [78% SP, 780 g (*a. i*) · kg^{-1}] on liver esterases and hepatic glutathione-*S*-transferase (GST) in goldfish (*Carassius auratus*) and topmouth gudgeon (*Pseudorasbora parva*). GST was found to be induced in both goldfish and topmouth gudgeon by the profurite-aminium at concentration of 0.234 mg · L^{-1}. Clincher both at concentration of 1 and 2 mg · L^{-1} caused GST induction in topmouth gudgeon. The mixture of clincher and profurite-aminium had the highest effect in topmouth gudgeon 722.3 nmol · (min^{-1} · mg^{-1} protein) with a possible indication of additive toxicity. Liver esterases were induced by both clincher (1 and 2 mg · L^{-1}) and profurite-aminium (0.117 and 0.234 mg · L^{-1}) in goldfish. In topmouth gudgeon liver esterases were induced by profurite-aminium but inhibited by clincher. It is suggested that the two biotransformation enzymes may be directly or indirectly affected by the tested chemicals and that there are differences between them.

Key words: cyhalotop-butyl; profurite-aminium; liver esterase; GST; sublethal effect
CLC number: S482.4; S482.3 **Document code**: A **Article ID**: 1008-7303(2002)03-0035-08

1 Introduction

Pesticides not only can control pests but also may have negative impact to the environment. Pesticide introduced into aquatic ecosystem can cause massive death or diseases of fish and shrimps[1], and health related injuries to human beings[2,3]. They can also reduce the reproductive potential of aquatic species[4] and may eventually have major impact on fishery resource, biological diversity and functional balance of the ecosystem.

Heavy use of agrochemicals especially pesticides has been identified as one of the main constraints for rice-fish culture in some Asian countries. A decline in paddy-cultured fish has

① **Biography**: ZHU Guo-nian, male, born in 1957, from Zhejiang, Ph. D, assoc. professor, environmental toxicology of pesticide.
Foundation item: Supported by ICAMA, P. R. China & Dow Elanco Co., USA.

been reported in Indonesia[5~7], Malaysia[8], Thailand, Philippines[9] and Vietnam[10]. The effects of a chemical on a biochemical parameter may be indirect or direct. The pesticide formulations normally contain other compounds apart from the parent compound. These together with the complex metabolic mechanisms of the organisms may have indirect or direct effect on glutathione-S-transferases (GST) or liver esterase or other enzymes, which may increase or decline level of the biotransformation enzymes.

Cyhalofop-butyl (XDE-537) is the active ingredient of clincher, a post-emergency herbicide used in most parts of Asia for controlling a wide range of grass weeds in paddy fields. Its efficiency as a graminicide has been well documented[11~13]. Profurite-aminium is mainly used in China for the control of a wide range of pest insects. It is one of those nereistoxin insecticides with stomach poisoning activity.

Glutathione-S-transferases and liver esterases are important biomarkers that can indicate the effect of xenobiotics on some exposed organisms including fish[14]. Since the two pesticides clincher and profurite-aminium are intended to be used in paddy fields where fish and other aquatic organisms can be reared, it is important to have full understanding on the possible indications of the water contamination by these pesticides.

2 Materials and methods

2.1 Chemicals

Clincher (100 EC) and profurite-aminium (78% SP) were purchased locally in collaboration with pesticide residue analysis laboratory, Zhejiang University, China. Reduced glutathione (GSH, 98%) was obtained from Zheng-Yu Chemical Institute (Shanghai, China). 1-chloro-2, 4-dinitrobenzene (CDNB, 98%) was a product of Sigma Chemical Company. According to Ramage and Nimmo[15], CDNB is a wide nonspecific reference substrate for all forms of fish liver GST. Alpha-naphthyl acetate was purchased from Shanghai First Chemical Factory; α-naphthol was obtained from Xin-Tin Chemical Factory (Shanghai); Fast blue B and sodium lauryl-sulphate were purchased from Shanghai Reagent Factory (P. R. China). Bovine serum albumin was product of Boehringer Company. All other chemicals were of analytical grade and obtained from local commercial sources in Hangzhou.

2.2 Fish species and rearing

Fishes belonging to cyprinidae family weighing 3~5 g were bought from Hangzhou pet market and reared in the laboratory where they were sacrificed after appropriate exposures to pesticides. Goldfish (*Carassius auratus*), which is an ornamental, was particularly chosen because of its closeness and resemblance to crucian carp (*Carrasius carassius*), which is reared for food by many Asian rice growers. It has been urged that goldfish is a hybrid of crucian carp. Other researchers have concluded that the goldfish is a subspecies of the crucian carp *Carassius carassius* (i.e., *C. auratus*). More recently, it has been reported that *C. auratus* is a tetraploid derivative of *C. carassius*. Topmouth gudgeon (*Pseudorasbora*

parva) was chosen because of its easy availability in study area and the fact that it also belongs to cyprinidae family.

2.3 Treatments

A toxicity experiment prior to the commencing of this study, showed LC_{50} values for goldfish (96 h), to be 7.5 mg·L^{-1} clincher and 7.19 mg·L^{-1} profurite-aminium. In another preliminary experiment, almost 50% of topmouth gudgeon was found dead after exposing the fish to 6~10 mg·L^{-1} clincher and up to 1.17 mg·L^{-1} profurite-aminium for 96 h. From the values for topmouth gudgeon, it was decided, to use 0.1 and 0.2 as factors for getting the doses to be used in biochemical test, that wouldn't kill both topmouth gudgeon and goldfish. The exposure doses used were 1 mg·L^{-1} as a lower dose of clincher (C1), 2 mg·L^{-1} as a higher dose of clincher (C2), 0.117 mg·L^{-1} as a lower dose of profurite-aminium (P1), and 0.234 mg·L^{-1} as a higher dose of profurite-aminium (P2). These doses were accordingly combined to get mixture treatments CP1 and CP2.

2.4 Experimental protocol for enzyme tests

Each fish species was reared in different set of 50 liters volume containers. Every treatment having four replications for each fish species. 20 L tap water was added and left for 7 days to de-chlorinate naturally. After that, a pump was set to aerate each container. Six fishes were introduced in each container. The fishes were then allowed to acclimatize for one week before the exposure to the chemicals. The fishes were daily fed with dry daphnids purchased from Hangzhou pet market. After one-week acclimatization the fishes were exposed to clincher and profurite-aminium. On the fourth day (96 h exposure), fishes were sampled and were sacrificed for enzymatic assays.

2.4.1 Liver homogenate preparation From each replication, four of each fish species were sacrificed by a sharp blow on the head followed by cervical dislocation. The homogenate was prepared according to the colorimetric method of Mao, *et al*.[16]. The liver was quickly removed using scissors and tweezers, cleaned from adhering tissues with 0.15 mol·L^{-1} potassium chloride (KCl) and blotted with filter paper. For GST, the liver was then homogenized in the presence of 0.25 mol·L^{-1} sucrose containing 50 mmol·L^{-1} phosphate buffer solution-PBS (pH = 6.5) [10 mL buffer per gram tissue] with a teflon coated hand-driven tissue homogenizer and pestle for about 1 min. The homogenates were filtered through four layers of cheesecloth and centrifuged at 10 000 g for 15 min in a refrigeraor at 4℃. The supernatants were taken and used as an enzyme source.

For liver esterase the tissue was homogenized in 0.04 mol·L^{-1} phosphate buffer (pH7.1) [10 mL buffer per gram tissue] with a teflon coated, hand-driven tissue homogenized and pestle, for about 1 min. And then centrifuged at 1 880 g for 10 min. The supernatant was taken as a source of enzyme and used for liver esterase activity determination.

2.4.2 Enzymatic assays

1) GST activity determination The enzyme activity was measured spectrophotometrically by

using the assay procedures modified by Habig *et al*[17]. The reaction mixture consisted of 2.9 mL of 0.2 mol·L^{-1} K-phosphate buffer-PBS, pH 6.5, 0.1 mL of 0.03 mol·L^{-1} CDNB (dissolved in ethanol) and 0.5 mL of 0.02 mol·L^{-1} GSH (dissolved in PBS). The enzymatic reaction was initiated with addition of 0.2 mL liver homogenate supernatant and carried out at 25℃ for 3 min. 0.3 mL of 33% TCA (dissolved in water) was added to precipitate excess protein and the mixture was centrifuged at 345 g for 5 min. The absorbance of the CDNB-glutathione conjugate formed was measured by Shimadzu double-beam spectrophotometer, at 340 nm. Non-enzymatic increase in absorbance was corrected by running the parallel blank in all experiments. 0.2 mL more of 0.2 mol·L^{-1} PBS (pH = 6.5) was added to the blank instead of the homogenate. Each assay was repeated two times.

2) Esterase activity determination The enzyme activity was measured spectrophotometrically by using the assay procedures modified by Van Asperen[18]. Assay mixture consisted of 2.7 mL of 0.03 mmol·L^{-1} α-NA and 0.2mL liver homogenate supernatant. The mixture was incubated at 25℃ in a water bath. After exactly 10 min, 0.1 mL of diazoblue-sodium lauryl sulphate (DBLS) (1% fast blue B and 5% sodium lauryl sulfate, 2:5, *v*/*v*) was added. A strong blue color reflecting the amount of naphthol developed quickly and its absorbance was measured by Shimadzu double-beam spectrophotometer, at 600 nm. Non-enzymatic increase in absorbance was corrected by running the parallel blank in assays. The blank received 0.2 mL more of 0.03 mmol·L^{-1} α-NA.

The total protein concentration of liver tissue from test fish was determined by the folin phenol reagent method of Lowry *et al*[19] using bovine serum albumin as standard. The activity of GST was expressed as μmol·min^{-1}·mg^{-1} of protein. The activity of liver esterase was expressed as nmol·min^{-1}·mg^{-1} of protein. Each assay was replicated two times and the results are presented as the mean ± *SD* (standard deviation). One-way ANOVA was performed using STATISTICA software (version 5.0). Percentage activities were calculated with the assumption that the activity of the control was 100%. Percentages higher than 100 meant that the enzyme was induced where as values less than 100 inferred inhibition.

3 Results and discussion

3.1 Glutathione-S-transferase

For goldfish results indicated that there were no significant differences in GST activity between the control and the test doses of clincher herbicide applied alone (Table 1). The same observation was for low dose of profurite-aminium, but treated with higher dose, significant difference ($\alpha = 0.05$) from the control was observed. This probably suggested that the detoxification of the nereistoxin involves GST mediating the reactions. The mixed doses (CP1 and CP2) were not significantly different from the control. However, the percentage activities had a general increasing trend above the control suggesting that both clincher and profurite-aminium induce GST activities.

Table 1 GST activity in goldfish (*Carassius auratus*) and topmouth gudgeon (*Pseudorobora parva*) after 96 h exposure to various pesticides* /μmol·min^{-1}·mg^{-1}

Treatment	Dose	Goldfish	%	Topmouth	%
Control		1681.4^{a}±37.0	100	78.3^{a}±4.3	100
C1	1 mg/L	2162.2^{a}±80.4	128	185.7^{b}±14.4	237
C2	2 mg/L	2091.2^{a}±21.7	124	285.2^{b}±19.8	364
P1	0.117 mg/L	1842.2^{a}±73.5	109	331.3^{bc}±10.7	423
P2	0.234 mg/L	4808.0^{b}±96.1	285	414.0^{bc}±18.9	528
CP1	C1+ P1	2665.5^{ab}±43.9	159	722.3^{c}±24.2	922
CP2	C2+ P2	2612.7^{ab}±71.2	155	311.9^{b}±12.3	398

*Activities are mean±*SD* of four experiments (n= 4). Values followed by different letters are significantly different (α= 0.05). C-clincher; P-profurite-aminium. The same as in the following table.

The GST activity of topmouth gudgeon exhibited significant difference (α= 0.05) between the exposed fish and the control. The effect seemed highest with the mixed treatment (722.3 nmol·min^{-1}·mg^{-1}) with a possible indication of additive toxicity (C alone had a maximum activity of 285.2 nmol·min^{-1}·mg^{-1} and P had an activity of 414.0 nmol·min^{-1}·mg^{-1}). Like in goldfish percentage GST activities were higher than 100 indicating that clincher and profurite-aminium induce rather than inhibit the enzyme. Under normal conditions, goldfish have higher GST activity than topmouth gudgeon. However, the induction of the enzyme by either clincher or profurite-aminium seems to be higher in topmouth gudgeon as revealed by percentage activities. This shows that interspecific difference relative to GST activity exists in the two species and that topmouth gudgeon can be used as an indicator of water contamination by the two pesticides better than goldfish. According to Jacoby and Keen[20], one of the actions of GST is binding covalently with more reactive agents in a so-called "sacrificial" reaction. This indicates that in such reactions GSTs are not recovered, i.e., they are permanently inactivated. This can be said to be the case when inhibition is observed. In this study, however, GST activities were observed to be induced and therefore not inactivated. This possibly indicates that the fish species involved can be more tolerant to the chemicals studied as the chemicals can easily be removed from the fish body system.

Although the method of enzyme preparation was different, the presence of hepatic GST activity and species difference has been shown to exist in channel catfish (*Ictarus punctutus*) and bullhead (*Ameriurus nebulosus*)[21]. Sorsa[22] reported interspecies difference in topmouth gudgeon and mosquitofish (*Gambusia affinis*) when he studied the effect of fenitrothion on biochemical parameters. However, in his case topmouth gudgeon had lower GST activity than mosquitofish. The control group was reported to have activity of 4 202.08 μmol·min^{-1}·mg^{-1}, which anyway, was much higher than the value obtained in the current experiment (78.3 μmol·min^{-1}·mg^{-1}). Differences in values of the control groups may be attributed to different experimental conditions. It is important though, to note that interspecies differences are exist among different fish species in regard to GST

activity and that such difference can make a landmark in deciding which species is to be used as environmental contamination biomarker. In a recent study, Oruc and Uner[23] indicated that sublethal exposure of *Oreochromis niloticus* to two herbicides, 2, 4-D and azinphos-methyl did not affect GST activity.

Since there are no works so far reported about the effect of cyhalofop-butyl (and or its metabolites) and nereistoxin derivatives such as profurite-aminium on GST, it is difficult to make comparisons of the results obtained in this study with others.

3.2 Liver esterases

Significant difference ($\alpha = 0.05$) in liver esterase activities of two fish species was observed between the pesticide treatments and the control (Table 2). For goldfish, both clincher and profurite-aminium induced liver esterase activity. For topmouth gudgeon liver esterase induction was for treatments that contained profurite-aminium. When applied alone, clincher seemed to inhibit liver esterases.

Table 2 Liver esterase activity in goldfish (*Carassius auratus*) and topmouth gudgeon (*Pseudorobora parva*) after 96 h exposure to various pesticides, /nmol·min^{-1}·mg^{-1}

Treatment	Dose	Goldfish	%	Topmouth	%
Control		259.8^{a}±19.1	100	395.8^{a}±27.4	100
C1	1 mg/L	685.7^{b}±58.6	264	151.9^{a}±15.7	38.4
C2	2 mg/L	695.6^{b}±44.6	268	267.4^{a}±16.6	67.6
P1	0.117 mg/L	708.4^{b}±52.5	273	726.7^{b}±25.8	184
P2	0.234 mg/L	557.8^{b}±35.8	215	844.7^{b}±56.9	213
CP1	C1+ P1	549.5^{b}±59.4	212	552.9^{b}±23.9	140
CP2	C2+ P2	597.3^{b}±44.8	230	711.8^{b}±49.4	180

Regarding the dose-effect relationship, there was no evidence that higher doses had higher effects on the activities of liver esterases in the two fish species. This probably meant that the metabolism of the two pesticides in the two fish species follows a simple detoxification mechanism. Liver esterases (also called Ali esterases) have been strongly implicated as detoxification mechanism of many xenobiotics, and especially organophosphorus compounds[24, 25]. Biotransformation of chemicals by liver esterases has been studied by some other researchers on tsetse fly[26], and beef[27]. None of these studies involved either pesticides or fish. Chambers *et al.*[25] investigated detoxification of the oxons of six phosphorothionate insecticides in rat and stated that the presence of AliE (Eserine insensitive B-esterase) in the liver, in close proximity to where much oxon would be generated by cytchrome P-450 mediating desulfuration, would allow them the opportunity to be readily phosphorylated and prevent much of the hepatically generated oxon from entering the circulation. Sorsa[22] reported biotransformation of fenitrothion by liver esterases in topmouth gudgeon and mosquitofish following 96 h exposure. Interestingly it was also

reported in his findings that the inherent specific activity of liver esterase varied among fish species. Li and Fan[28] reported the existence of isozymes of liver esterase of the goldfish and topmouth gudgeon. They also indicated that the isozymes were different in nature. The different responses of the two fish species to pesticide exposure shown in this study may be a reflection of that.

References

[1] Wolff M S, Toniolo P O. Environmental organochlorine exposure as potential etiologic factor in breast cancer [J]. *Environ Health Persp*, 1955, 103: 141-145.

[2] Capel P D, Giger W, Reichert P, *et al*. Accidental input of pesticides into the Rhine river [J]. *Environ Sci Technol*, 1988, 22(8): 992-997.

[3] Colborn T, Saal F S, Soto A M. Developmental effect of endocrine disrupting chemicals in wildlife and humans [J]. *Environ Health Persp*, 1993, 101: 378-384.

[4] Lieberg-Clark P, Bacon C E, Bruns S A, *et al*. DDT in California sea lions: A follow-up study after twenty years [J], *Mar Pollut Bull*, 1995, 30: 744-745.

[5] Hardjamulia A, Koesoemadinata S. Preliminary experiments on the effects of thiodan and endrin on fish culture in Indosnesia [A]. Proceedings of Indo-Pacific Fishery Countries [C]. 1972, 15(11): 56-64.

[6] Koesoemadinata S. Pesticide as a major constrant to integrated agriculture-aquaculture farming systems [A]. Pullin R S V, Shehadeh S H. Integrated agri-aqua farming systems [C], ICLARM conference proceedings No. 4 Searca. Los Banos, Laguna and ICLARM, Makati, Phillipines, 1980, 45-51.

[7] Saanin H. Toxicity of insecticides and herbicides to some Indonesian cultivable fish species [A]. Proceedings of the Fourth Pan-Indian Ocean Science Congress, Section B: Biological sciences New Delhi [C]. India, 1960, 293-303.

[8] Lim C S, Seng-Hock O. Environmental problems of pesticide usage in the Malyasian rice field: Perception and future consideration [A]. The Fourth International Meeting [C]. Thailand: Chiang Mai, 1985.

[9] Spiller G. Rice-cum-fish Production in Asia: Aspects of Rice and Fish Production in Asia [C]. Bangkok, Thailand: FAO Office for Asia and the Pacific, 1985.

[10] Vincke M M J. Aquaculture en riziere: situation et role future [A]. Pillay T V R, Dill W A, Advances in Aquaculture [C]. Rome: FAO, 1979.

[11] Yasafumi I. Cyhalofop-butyl: A prominent tool for the control of weeds in direct seeded rice [A]. Proc. APWSS Satellite Symposium, Akita [C]. Japan, 1995.

[12] Ito M. Selectivity of cyhalofop-butyl in poaceae species [J]. *Journal of Weed Science Technology*, 1998, 43(2): 122-128.

[13] Mendoza N S. Cyhalofop-butyl, a selective postemergence graminicide for weed control in direct seeded rice [A]. Proc. MACA/GIFAP. International Pesticides Conference [C]. Malaysia: Kuala Lumpur, 1996.

[14] Stein K, Perci P, Gnassis-Barelli M, *et al*. Evaluation of biomarkers in caged fish and mussels to assess the quality of waters in a bay of Mediterranean sea [J]. *Environ Pollut*, 1998, 99(3): 339-345.

[15] Ramage P IN, Nimmo IA. The substrate specifity and subunit compositions of the hepatic glutathione-*S*-transferase of rainbow trout (*Salmo gairdneri*) Comp [J]. *Biochm Physiol*, 1984, 78B: 189-194.

[16] Mao D S (**毛德寿**), Tong Z C (**同宗灿**), Wang Z Y (**王志远**), *et al*. *Environmental Biochemical Toxi-*

cology [M]. Liaoning University Press (in Chinese), 1986.

[17] Habig W H, Pabst M J, Jacoby W B. Glutathione-S-transferases: The first enzymatic step in mercapturic acid formation [J]. *J Biol Chem*, 1974, 249, 22: 7130-7139.

[18] Van Asperen K. A study of housefly esterase by means of a sensitive colorimeteric method [J]. *J Ins Physiol*, 1962, (8): 401-414.

[19] Lowry O H, Rosebrough N J, Farr A L, *et al*. Protein measurement with a Folin phenol reagent [J]. *Journal of Biological Chemistry*, 1951, 193: 265-275.

[20] Jakoby W B, Keen J H. A triple treat in detoxification: the glutathione-Stransferase [J]. *Trends Biochem Sci*, 1977, 2: 229-230.

[21] Hasspieler B M, Behar J V, Di Giulio R T. Glutathione-dependent defense in channel Catfish (*Ictalurus punctatus*) and brown bullhead (*Ameriurus nebulosus*) [J]. *Ecotoxicology and Environmental Safety*, 1994, 28(1): 82-90.

[22] Sorsa S S. Comparative toxicity and subleathal effect of fenitrothion on some biochemical parameters of selected fish species [D]. Hangzhou: Zhejiang University, 1999.

[23] Oruc E O, Uner N. Combined effects of 2, 4-D and azinphosmethyl on antioxidant enzyme and lipid peroxidation in liver of *Oreochromis niloticus* [J]. *Comp Biochem Physiol*, Part C, 2000, 291-296.

[24] Fonnum F, Sterri S H. Factors modifying the toxicity of organophosphorus compounds including sarin and soman [J]. *Fundam Appl Toxicol*, 1981, 1: 143-147.

[25] Chambers J E, Chambers H W. Time course of inhibition of acetylcholinesterase and aliesterase following parathion and paraoxon exposures in rat [J]. *Toxicol Appl Pharmacol*, 1990, 103(3): 420-429.

[26] Robert A, Strambi C, Strambi A, *et al*. Ecdysteroids during the development of the tsetse fly [J]. *Invertebrate Reproduction and Development*, 1991, 19(1): 71-81.

[27] Ackermann H, Engst R. Occurrence of organophosphorus insecticides in the foetus [J]. *Arch Toxicol*, 1970, 26: 17-22.

[28] L I S N. Fang D F. Activity of esterases from different tissues of freshwater fish and responses of their isoenzymes to inhibitions [J]. *J Toxicol Environ Health*, 1997, 51: 149-157.

氰氟草酯、杀虫安亚致死剂量对金鱼 Carrasius auratus 和麦穗鱼 Pseudorasbora parva 肝脏酯酶及谷胱甘肽-S-转移酶活性的影响

朱国念[1*], 吴慧明[1], Jilisa K. Mwalilino[2], 李少南[1]
(1. 浙江大学 农药与环境毒理研究所; 2. 浙江大学 环境科学系, 杭州 310029)

摘要: 研究了 10% 千金乳油(有效成分: 氰氟草酯)和 78% 杀虫安可溶性粉剂对金鱼和麦穗鱼肝脏酯酶及谷胱甘肽-*S*-转移酶(GST)活性的亚致死剂量效应。发现杀虫安(0.234 mg·L^{-1})对两种鱼的 GST 活性均具诱导作用, 而氰氟草酯(1.2 mg·L^{-1})仅诱导了麦穗鱼的 GST 活性; 当杀虫安与氰氟草酯混合处理时, 对麦穗鱼 GST 活性的诱导效应最为明显。在一定的浓度范围内, 氰氟草酯(1.2 mg·L^{-1})和杀虫安(0.117, 0.234 mg·L^{-1})均可诱导金鱼肝脏酯酶活性; 对麦穗鱼肝脏酯酶而言, 杀虫安为诱导作用, 氰氟草酯则抑制其活性。研究结果表明: 两种酶的活性直接或间接地受供试药剂的影响, 两种试鱼对氰氟草酯和杀虫安的生物反应存在差异。

关 键 词: 氰氟草酯; 杀虫安; 肝脏酯酶; 谷胱甘肽-*S*-转移酶; 亚致死剂量效应