J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-010-1693-1
ISSN 1672-5182, 2010 9 (3): 257-264
http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

The Toxic Mechanism of High Lethality of Herbicide Butachlor in Marine Flatfish Flounder, *Paralichthys olivaceus*

GUO Huarong*, YIN Licheng, ZHANG Shicui, and FENG Wenrong

Department of Marine Biology and Key Laboratory of Marine Genetics and Breeding, Ministry of Education, Ocean University of China, Qingdao 266003, *P. R. China*

(Received June 9, 2009; revised December 25, 2009; accepted January 30, 2010)

Abstract The toxic mechanism of herbicide butachlor to induce extremely high lethality in marine flatfish flounder, *Paralichthys Olivaceus,* was analyzed by histopathological examination, antioxidant enzymes activities and ATP content assay. Histopathological examination of gill, liver and kidney of exposed fishes showed that gill was a target organ of butachlor. The butachlor seriously impaired the respiration of gills by a series of lesions such as edema, lifting and detachment of lamellar epithelium, breakdown of pillar cells, and blood congestion. The dysfunction of gill respiration caused suffocation to the exposed flounder with extremely high acute lethality. Antioxidant enzyme activity assay of the *in vitro* cultured flounder gill (FG) cells exposed to butachlor indicated that butachlor markedly inhibited the antioxidant enzyme activities of Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Furthermore, along with the decline of antioxidant enzyme activities, ATP content in the exposed FG cells decreased, too. This infers that the oxidative stress induced by butachlor can inhibit the production of cellular ATP. Similar decrease of ATP content was also observed in the exposed flounder gill tissues. Taken together, as in FG cells, butachlor possibly induced a short supply of ATP in pillar cells by inhibiting the antioxidant enzyme activities and then affecting the contractibility of the pillar cells, which in turn resulted in the blood congestion and suffocation of exposed flounder.

Key words butachlor; flounder; herbicide; toxicity

1 Introduction

Butachlor, a chloroacetanilide herbicide, has been most commonly employed in China to control a wide range of annual grasses and some broadleaf weeds. It is applied in either pre-emergence or early post-emergence stage. Approximately 10 000 t of butachlor is used annually and an upward trend is expected in the future (Hu, 1998). Butachlor has a half-life of 1.65–2.48 d in field water and 2.67–5.33 d in soil (Yu *et al*., 1993). When applied in field, butachlor will be eventually released into aquatic environment by rain and soil outflow and exert adverse effects on aquatic organisms (Ohyama *et al*., 1987; Castaneda and Bhuiyan, 1996). Rajyalakshmi *et al*. (1996a, b) and Tantawy (2002) have reported the biological and biochemical toxicity of butachlor on freshwater snails of *Pila globosa* (Swainson) and *Biomphalaria alexandrina*. Toxic effects of butachlor on the reproduction of green alga *Scenedesmus vacuolatus* (Junghans *et al*., 2003) and on the survival of freshwater prawn *Paratya compressa improvisa* (Hatakeyama and Sugaya, 1989) have also been reported. The vast application of butachlor in paddy field can also be deleterious to the natural amphibian populations in the world. It has been shown that the high toxicity of butachlor has several adverse effects on the survival, gonad development and genetic stability of the tadpoles of *Bufo melanostictus*, *Fejervarya multistriata*, *Polypedates megacephalus*, and *Microhyla ornate* (Geng *et al*., 2005a), *Rhacophorus megacephalus* (Geng *et al*., 2005b), *Rana guentheri* (Geng *et al*., 2005c), and *Microhyla ornate* (Xue *et al*., 2005).

In freshwater fish, Ateeq *et al*. (2002, 2005 and 2006) have reported the micronuclei formation, DNA damage and apoptosis in the erythrocytes of catfish *Clarias batrachus* exposed to butachlor. High acute toxicity of butachlor on three species of catfish, *Heteropneustes fossilis*, *Clarias batrachus* and *Channa punctatus*, silver carp *Hypophthalmicthys molitrix* and rice-field eel *Monopterus albus* (Farah *et al*., 2004; Fan *et al*., 2005; Hu *et al*., 2005) has also been reported. However, its effects on seawater fish have not been studied thoroughly. Recently, butachlor in the coastal water of Bohai Bay, China is detectable although the concentration is still lower than the limit of quantification (Xu *et al*., 2007). Yin *et al*. (2007) reported

* Corresponding author. Tel: 0086-532-82031628
E-mail: huarongguo@ouc.edu.cn

the 96h-LC_{50} of butachlor on flatfish flounder *Paralichthys Olivaceus* (6.55 nmolL^{-1}, *i.e.* 2 μg L^{-1}) and the 24 h-IC_{50} of butachlor on cultured flounder gill (FG) cells (43.32–44.91μmolL^{-1}, *i.e.* 13–14μg L^{-1}), and also the genotoxicity of micronuclei formation in the erythrocytes of exposed flounder and the DNA damage in exposed FG cells. These data indicated that butachlor was highly toxic to marine benthonic teleost, too. However, the mechanism of the high lethality of butachlor to flounder is still unknown.

The aim of this study is to examine the histopathological changes in the gill, liver and kidney of flounder exposed to butachlor. The relationship between the histological damages and the high lethality of butachlor to flounder will be revealed by analyzing antioxidant enzyme activities and ATP content in cultured FG cells.

2 Materials and Methods

2.1 Fish and Cell line

Three-month-old flounders (*Paralichthys olivaceus*) of body length 10 cm ± 2 cm were purchased from a fish farm in Jiaonan, Qingdao, China and acclimatized in 10 L glass tanks containing aerated natural seawater (18℃± 2 ℃) under an ambient photoperiod for two weeks before used. Fish were fed twice daily with chopped fresh fish. They were starved 24 h before and during the experiments.

The continuous marine fish cell line FG, derived from gill tissue of *F. olivaceus* in 1993, was used in the experiments and maintained according to the method described by Tong *et al*. (1997). These FG cells were grown in Eagle's minimum essential medium (MEM; Gibco BRL, New York) supplemented with 10% bovine calf serum (Hyclone, Utah), 100 IU mL^{-1} penicillin, and 100 IU mL^{-1} streptomycin in plastic culture flasks (Corning) at 20℃.

2.2 Chemicals

Technical grade 2-chloro-2, 6-diethyl-N-(butoxymethyl) acetanilide (butachlor), with a purity of 98.6%, was purchased from Shenyang Research Institute of Chemical Industry (China). Stock solution of 100 mmolL^{-1} butachlor was prepared in dimethylsulfoxide (DMSO) and the maximum final concentration of DMSO in the treatment medium was below 0.1%.

2.3 Histopathological Examination

Based on the 96h- LC_{50} value of butachlor to a flounder, 6.55 nmolL^{-1}, reported by Yin *et al*. (2007), sublethal concentration of butachlor, 3.84 nmolL^{-1}, was selected in the fish treatment. A group of 12 fishes were exposed to 3.84 nmolL^{-1} butachlor in seawater, and fishes were sampled at intervals of 24, 48 and 96 h after exposure and used for the histological examinations (4 fishes for each sampling). Control fishes maintained in natural sea water were processed similarly. Fishes were anesthetized with 50 mgL^{-1} MS 222 (tricaine methane sulphonate) for 2–3 min, then length and weight were measured. The second and third gill arches, livers and kidneys were rapidly dissected out, cut into pieces of (0.5–1.0) cm×(0.5–1.0) cm×0.2 cm and fixed in Bouin's fixative for 12 h at room temperature. The fixed samples were washed with distilled water and dehydrated in ethanol, followed by clearing in xylene, and embedding in paraffin wax. Serial sections of 7 μm in thickness were made and stained by haematoxylin and eosin. Only representative ones with structural changes were photographed under a BH-2 Olympus microscope.

2.4 Antioxidant Enzyme Activity Assay

Based on the 24h-IC_{50} value of butachlor to FG cells, 43.32-44.91 μmolL^{-1}, reported by Yin *et al*. (2007), 30 μmolL^{-1} butachlor was selected in this treatment. Changes of the activities of antioxidant enzymes of Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) during the 48 h exposure period of FG cells to butachlor were examined. A total of 48 culture flasks (25 cm^2) were each seeded with 5×10^5 cells and incubated for 24 h. Then the medium was replaced by a new one containing 0 (control) or 30 μmolL^{-1} butachlor. At each interval of 0, 2, 4, 8, 12, 24, 36 and 48 h after exposure, the cells of 6 flasks were gathered and washed once with phosphate-buffered saline (PBS; 3.0 g $Na_2HPO_4.12H_2O$, 0.2 g KH_2PO_4, 8.0 g NaCl and 0.2 g KCl per liter water, pH 7.2) by centrifugation at 2000 g for 10 min at 4℃, and the cell pellets were re-suspended in PBS (5%) for ultrasonication. The homogenates were centrifuged at 6000 g for 10 min at 4℃ and the supernatants were pooled and used for the enzyme activity assays of SOD, CAT and GPX according to the instructions of the kits (Nanjing Bioengineering Institute, China).

All experiments were performed in triplicate. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as standard.

2.5 ATP Content Assay

FG cells were seeded into a 96-well plate and incubated overnight at 20℃ as indicated above. Then the medium was removed and the cells were exposed to 0 (control), 0.1, 1, 10, 20, 30 and 40 μmolL^{-1} of butachlor in media for 24 h, or exposed to 30 μmolL^{-1} butachlor for 0, 2, 4, 8, 12, 24 and 48 h, respectively. Then the medium in each well was removed and the cells were lysed and the ATP content was examined according to the instruction of ATP Assay Kit (Beyotime Institute of Biotechnology, China). The ATP content was calculated as nmol ATP per milligram protein.

Gill tissues were dissected out of the flounder exposed to 0 (control) and 3.84 nmolL^{-1} butachlor at intervals of 24, 48 and 96-h after exposure and homogenated in PBS (5%, w/v). The homogenates were centrifuged at 6000 g for 10 min at 4℃, and 100 μL of the supernatants were used to test the ATP content with the same kit.

2.6 Data Analysis

Each experiment was repeated at least three times. Data

obtained were expressed as mean±SD and evaluated by one-way ANOVA (Spss v11.5 for windows, tests: least significant difference, Tukey's honestly significant difference).

3 Results

3.1 Histopathological changes in butachlor-exposed flounder tissues

Histopathological examination of the gill tissues from the treated fishes indicated that gill was a target organ of butachlor and obvious histological damages were observed in the exposed gill in a time-dependent manner.

Fig.1 shows the normal gill structure of control fishes. Flounder has four gill arches on each side of buccal cavity. There are numerous gill filaments attached on one side of the arches. To each gill filament, two rows of secondary lamellae are arranged perpendicularly, projecting the above and below of the filament (Fig.1A). Secondary lamellae is made up of two sheets of epithelium, which are connected and lined by many pillar cells to form blood channels, through which blood flows in the secondary lamellae. One to three erythrocytes were usually recognized within each blood channel (Fig.1B). The filamental epithelium, much thicker than lamellar epithelium, was mainly composed of squamous pavement cells and chloride cells. Chloride cells were characterized as large epithelial cells with light cytoplasm, usually present at the base of secondary lamella and surface of gill filament. Cartilage cells, large quadrate and irregular polygon shaped, were also found in the gill arches, trunk and outward end of gill filament (Fig.1C).

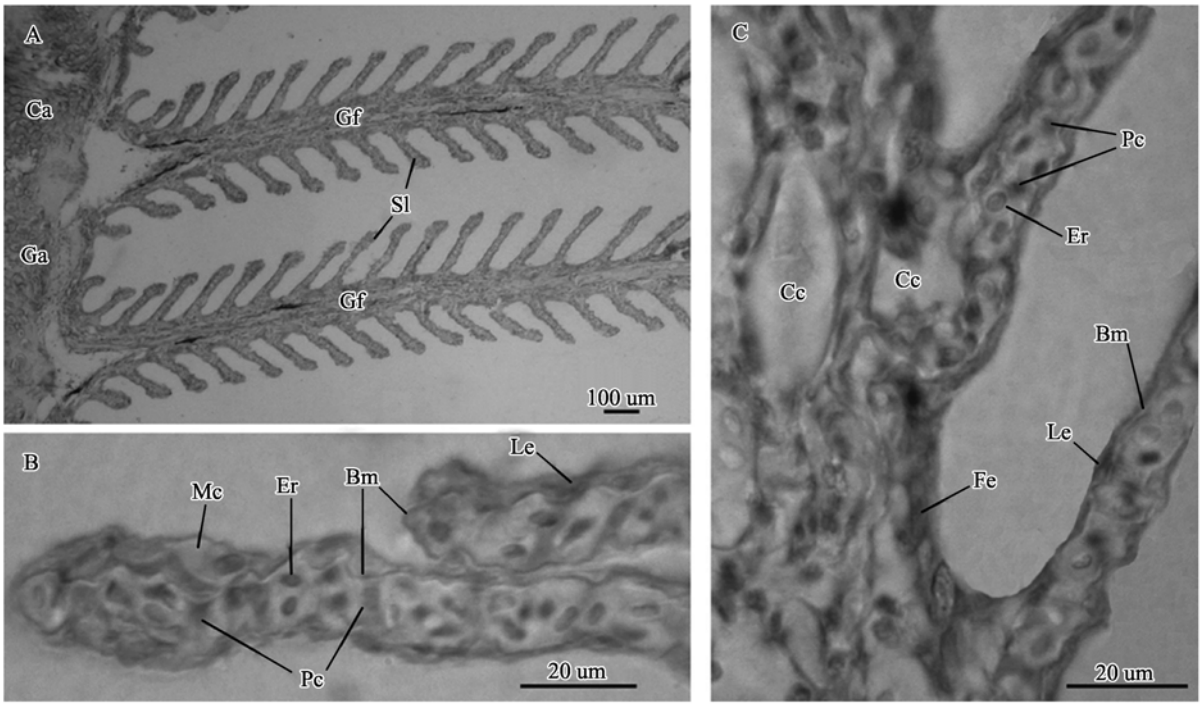

Fig.1 Normal histological structure of flounder (*Paralichthys olivaceus*) gill (control). A indicates the gill arch (Ga), gill filament (Gf), secondary lamellae (Sl) and cartilage cells (Ca). B and C show the mucus cells (Mc), lamellar epithelial cells (Le) and basement membrane (Bm), filamentary epithelial cells (Fe), chloride cells (Cc) and nucleated erythrocyte (Er) within the blood channels delimited by pillar cells (Pc).

Fig.2 shows the gill lesions of the fishes exposed to the sub-lethal dose of butachlor (3.84 $nmolL^{-1}$) for 24, 48 and 96 h respectively. At the first 24 h, most of the secondary lamellae were structurally normal (Fig.2-A1). A few pillar cells lost their contractility and orientation, leading to the blend and enlargement of neighboring blood channels (Fig.2-A2). After 48 h exposure, edema and lifting of lamellar epithelium were obvious in most secondary lamellae, resulting in the roughness of their surface. Pillar cell system was severely damaged and the two sheets of basement membranes became straight and smooth instead of showing the normal undulant morphology (Figs.2-B1 and B2). After 96-h exposure, the gill structure was further damaged. Extensive edema and lifting of lamellar epithelium led to the detachment of many epithelial cells. With the further breakdown of pillar cell system, blood congestion could be observed in the ends of some secondary lamellae (Figs.2-C1 and C2). In extreme cases, pillar cell system was completely destroyed, chloride cells degenerated, lamellar basement membranes ruptured and circulation of erythrocytes were blocked in the secondary lamellae and gill filaments (Figs.2-C3 and C4). In contrast, it is found that no obvious histological alterations were observed in kidney and liver tissues of the treated fishes even after 96-h exposure (data not shown).

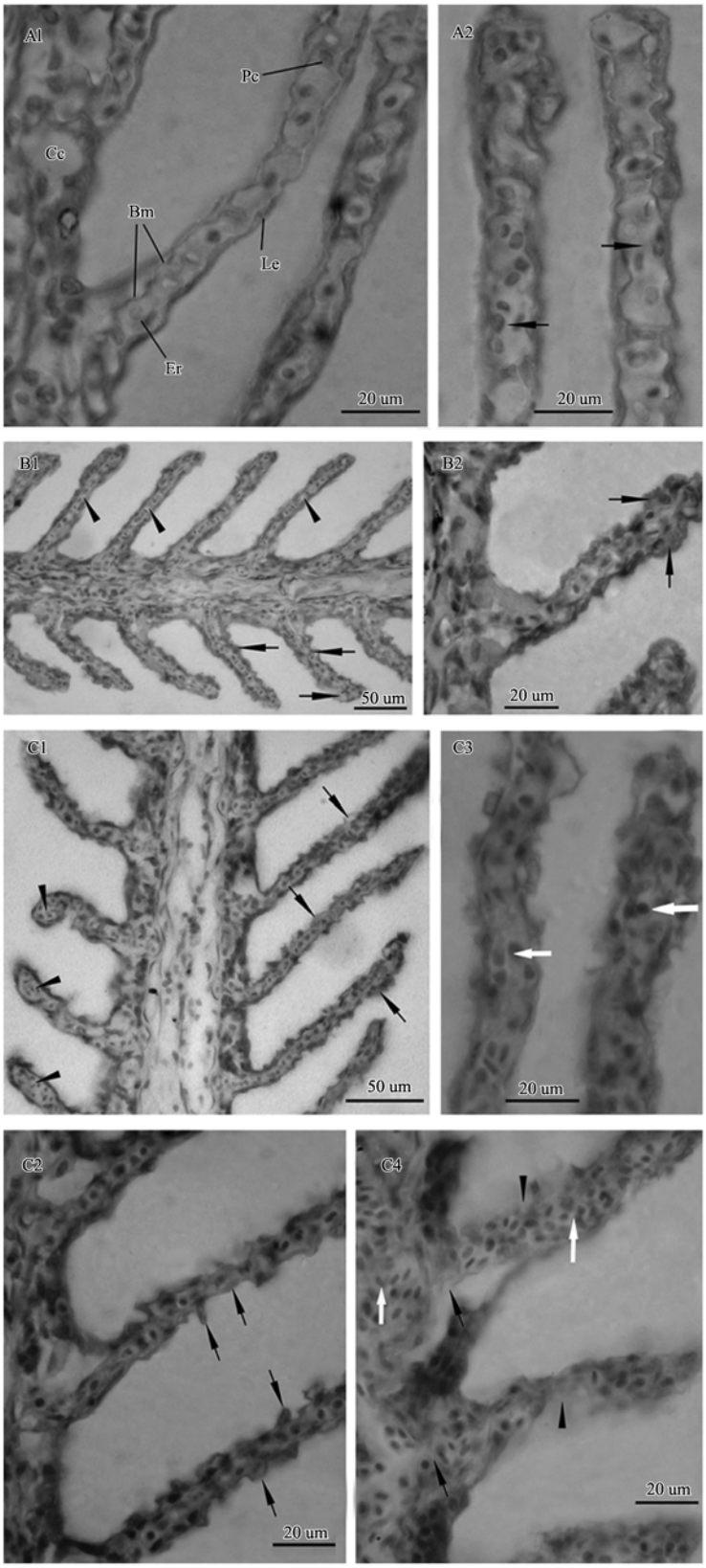

Fig.2 Histopathological alterations of the gills from the flounder (*Paralichthys olivaceus*) exposed to 3.84 nmolL^{-1} butachlor for 24 h (A1 and A2), 48 h (B1 and B2) and 96 h (C1–C4), respectively. A1 shows that most of the secondary lamellae are structurally normal after 24 h exposure. A2 shows the blend and enlargement of the neighboring blood channels due to the disfunction of pillar cells after 24 h exposure (arrows). B1 and B2 show the edema and lifting of the lamellar epithelium in most secondary lamellae (arrows), and the morphological changes of the lamellar basement membranes and blood channels due to the breakdown of pillar cell system (arrowheads) after 48 h exposure. C1 and C2 show the extensive edema, lifting and detachment of lamellar epithelium (arrows), and the blood congestion (arrowheads) after 96 h exposure. C3 and C4 show the structural alterations of the extremely damaged gill after 96 h exposure. The lesions

include the degeneration of chloride cells (arrows), rupture of lamellar basement membrane (arrowheads), breakdown of pillar cell system and the circulation blockage of erythrocytes or blood congestion in the secondary lamellae and gill filaments (open arrows). Cc, chloride cells. Bm, basement membrane. Er, erythrocyte. Pc, pillar cells. Le, lamellar epithelial cells.

3.2 Changes in antioxidant enzyme activities in butachlor-exposed FG cells

In the FG cells exposed to 30 μmolL^{-1} butachlor, all the activities of CAT, SOD and GPX were stimulated and showed significant increase during the first 6-h (for CAT) or 8-h (for SOD and GPX) exposure. Afterwards, the enzymes activities of treated cells began to decrease quickly and finally were lower than that of control ($P < 0.05$). The enzyme activities reached a relatively constant level after 12 h (for CAT) or 24 h (for SOD and GPX) exposure (Figs.3, 4 and 5).

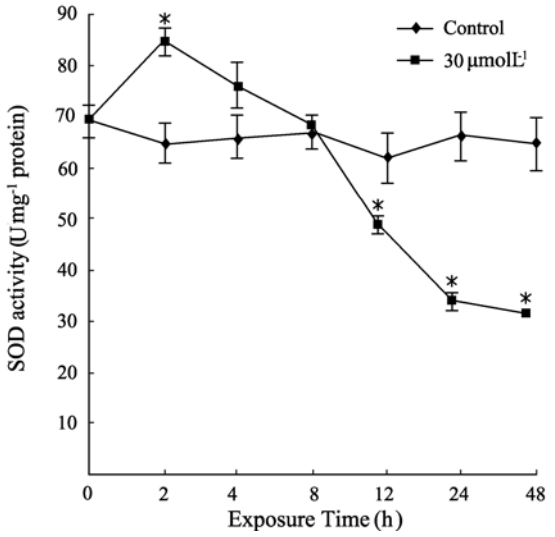

Fig.3 The change of SOD activity in FG cells exposed to 0 (control) and 30 μmolL^{-1} butachlor. The vertical bars show standard error of each data set. The symbol (*) denotes significant difference from control ($P < 0.05$).

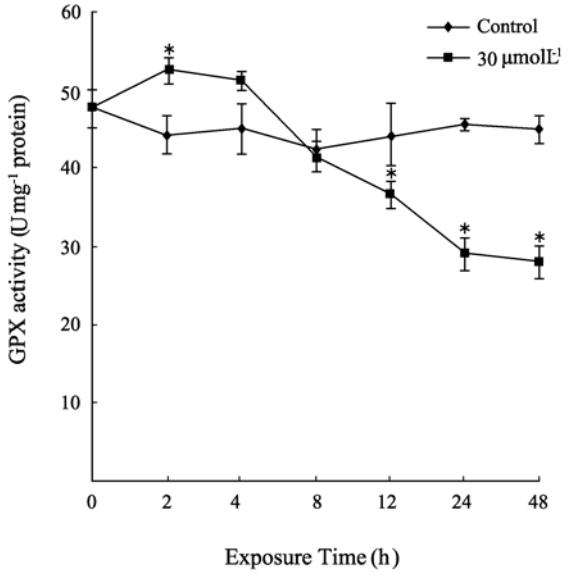

Fig.4 The change of GPX activity in FG cells exposed to 0 (control) and 30 μmolL^{-1} butachlor. The vertical bars show standard errors of each data set. The symbol (*) denotes significant difference from control ($P < 0.05$).

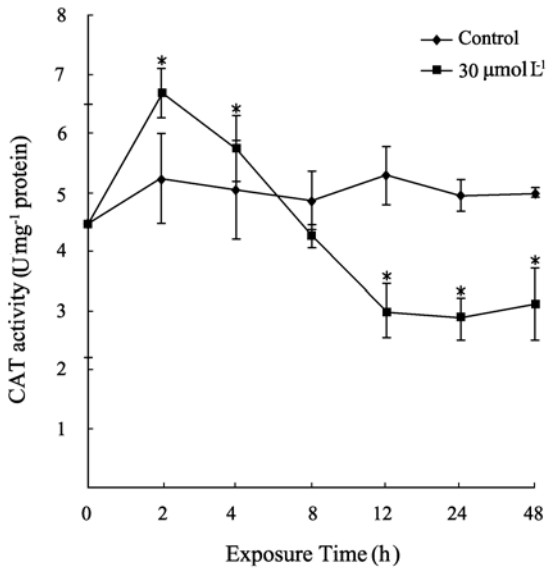

Fig.5 The change of CAT activity in FG cells exposed to 0 (control) and 30 μmolL^{-1} butachlor. The vertical bars show standard error of each data set. The symbol (*) denotes significant difference from control ($P < 0.05$).

3.3 Changes in ATP content in butachlor-exposed FG cells and gill tissues

As shown in Fig.6, compared with control, butachlor with a concentration from 0.1 to 40 μmolL^{-1} caused an obvious drop of ATP content in the 24 h exposed FG cells in a dose-dependent manner ($P < 0.05$). And the linear regression analysis data showed a good linear correlation between the ATP content and butachlor doses (R^2= 0.9353). In particular, there was about 50% drop of ATP level in the FG cells exposed to the highest concentration of 40 μmolL^{-1}. In the time course of experiments with the 30 μmolL^{-1} butachlor (Fig.7) in FG cells, obvious decrease

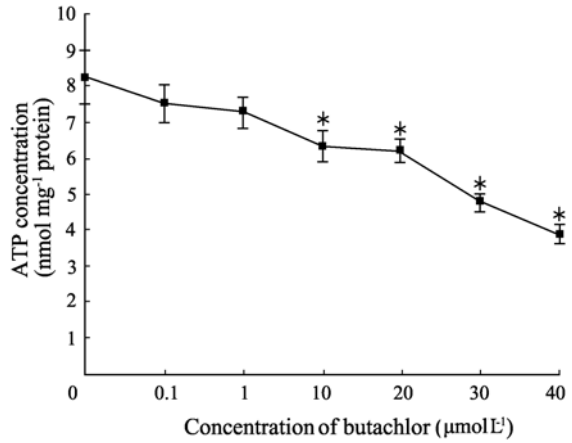

Fig.6 ATP contents in FG cells after 24 h exposure to 0 (control), 0.1, 1, 10, 20, 30 and 40 μmolL^{-1} butachlor, respectively. The vertical bars show standard error of each data set. The symbol (*) denotes significant difference from control ($P < 0.05$). Result of linear regression analysis: $Y = -0.7143X + 9.185$, $R^2 = 0.9353$.

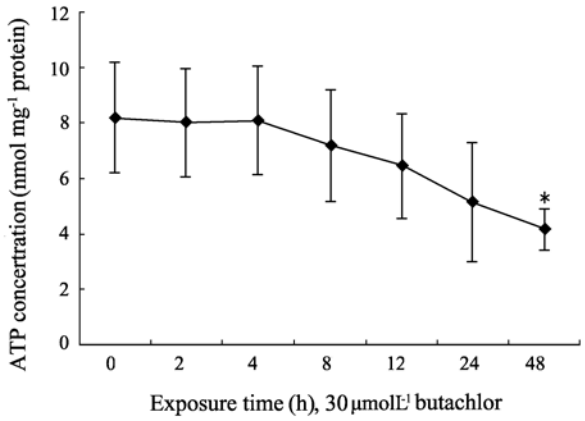

Fig.7 Changes of ATP contents in FG cells exposed to 30 μmolL^{-1} butachlor for 0, 2, 4, 8. 12, 24 and 48 h, respectively. The vertical bars show standard errors of each data set. The symbol (*) denotes significant difference from control ($P < 0.05$).

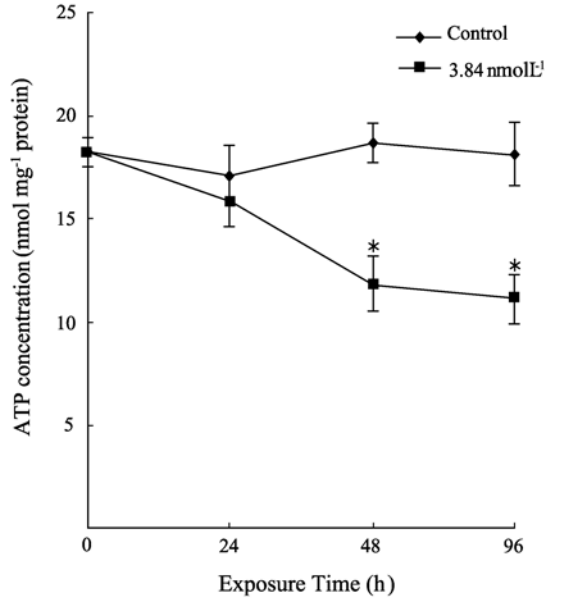

Fig.8 ATP contents of the gill tissues of flounder exposed to 0 (control) and 3.84 n molL^{-1} butachlor for intervals of 24, 48 and 96 h, respectively. The vertical bars show standard error of each data set. The symbol (*) denotes significant difference from control ($P < 0.05$).

of ATP level took place after 8 h post exposure.

Similar results were obtained in the gill tissues of treated flounders (Fig.8). ATP content in the gill tissues exposed to 3.84 nmolL^{-1} butachlor was markedly lower than that of control for all the three exposure periods of 24, 48 and 96 h ($P < 0.05$).

4 Discussion

As a popular herbicide, butachlor shows low toxicity to terrestrial animals following acute oral, dermal, and inhalation exposure (Wilson and Takei, 2000). The acute oral LD_{50} is 2 000 mg kg^{-1} for rats, >5 010 mg kg^{-1} for rabbits, >10 000 mg kg^{-1} for ducks and >100 mg kg^{-1} for bees, respectively (Bessen chemical company, 2006). But it is found to be highly toxic to aquatic organisms. The 96h-LC_{50} is 0.52 mgL^{-1} for rainbow trout, 0.44 mgL^{-1} for bluegill sunfish, 0.32 mgL^{-1} for carp and 0.14 mgL^{-1} for channel fish (Tomlin, 1994), 2.34–3.25 mgL^{-1} for three species of catfish (Farah *et al*., 2004) and 0.134 mgL^{-1} for silver carp (Fan *et al*., 2005). The 96h-LC_{50} is 2 μgL^{-1} for flounder (Yin *et al*., 2007) with an increasing sensitivity by three orders of magnitude. In the present study, histopathological examination of gill, liver and kidney of flounder indicates that acute exposure to butachlor can induce marked dysfunction of gill, but not the liver and kidney. In other previous experiments, acute exposure of butachlor to laboratory mammalian had no distinctive toxicity signs to liver and kidney, while subchronic and chronic exposure might lead to liver and kidney toxicity (Wilson and Takei, 2000). To some extent, this may account for the significant difference of butachlor toxicity to terrestrial animals and fish. Reasons for the notably different lethality of butachlor to flounder from that to other freshwater fishes need to be studied in detail, where age and size of fish as well as environment salinity should be considered.

CAT, SOD and GPX are three important antioxidant enzymes, defending the cells against oxidative stress. In our experiment, exposure of FG cells to butachlor with sublethal concentration induced the activities of these enzymes within the first 6 or 8 h. The results indicate that butachlor generated an oxidative stress in FG cells and the antioxidant enzymes were induced to maintain the homeostasis of cellular functions. However, the level of oxidative stress may overwhelm the antioxidant enzyme defenses, resulting in oxidative damage to biological molecules and cellular functions. After 6 or 8 h exposure, the enzyme activities of the treated cells began to decrease quickly and became much lower than that of control. A relatively constant level was reached after 12 or 24 h exposure. The inhibition of the antioxidant enzyme activities could lead to the accumulation of reactive oxygen species like superoxide (O_2^-) in cells, which in turn resulted in inactivation of enzymes, disruption of membranes, mutations, and ultimately cell deaths (Halliwell and Gutteridge, 1990). The result of inhibition of antioxidant enzymes can in particular damage mitochondria, the cellular energy generating plant, and inhibit the activities of NADH dehydrogenase, NADH oxidase and ATPase, which then causes a decline in energy production (Li and Zhang, 2002; Zhang *et al*., 1990). Similar results were observed in this study. During 48 h exposure to 30 μmolL^{-1} butachlor, the activities of the three antioxidant enzymes tested in FG cells increased in the first 6 or 8 h exposure and then decreased markedly in comparison with the control. The lowering of ATP level in FG cells was observed only after 8 h post exposure. The drop of ATP level in butachlor-exposed FG cells coincided with the decline of those antioxidant enzyme activities. It is of interest that obvious drop of ATP level was also observed in the gill tissues exposed to butachlor. Thus it can be concluded that butachlor may exert similar toxic effects on the exposed gill tissues by inhibiting antioxidant en-

zymes activities and decreasing the ATP level.

Fish gills are directly exposed to and are good indicators of aquatic pollutants. They serve as a major organ for respiration, osmoregulation, nitrogenous excretion and pH regulation. Failure of the gill's function during acute exposure to irritants can lead to the deaths of fish (Mallatt, 1985; Evans, 1987; Thophon *et al*., 2003). The respiratory function of gill is accomplished by blood circulation and diffusion of gas, water and ions in the secondary lamellae. Pillar cells connect the two sheets of lamellar epithelium and line most of the blood channels in the secondary lamellae. They are in direct contact with the blood and hence are readily influenced by reagents in the blood. Changes in the length of pillar cells or their contractibility will affect the cavity size of blood channels. Therefore, pillar cells play an important role in the lamellar blood circulation (Bettex-Galland, 1973).

It was found here that butachlor could affect the contractibility of the pillar cells in the first 24 h exposure, which resulted in the blend and enlargement of the neighboring blood channels. The obvious drop of ATP level in butachlor-exposed flounder gill might account for this damage, for ATP is the energy source for the contraction of the collagen columns in pillar cells. Then the ion and water exchange in the lamellar epithelium was affected, as edema and lifting of the lamellar epithelium were widely observed after 48 h exposure. With the extensive damage of lamellar epithelium and complete breakdown of pillar cell system, the velocity of blood circulation was lowered and blood cells were congested in the secondary lamellae. Then gas exchange and oxygen transport were blocked, eventually resulting in the suffocation and death of fishes.

In conclusion, gill is a target organ of butachlor. Butachlor induces the dysfunction of pillar cells possibly by the significant inhibition of the antioxidant enzyme activities and the ATP level followed by the disruption of gill respiration. Eventually it results in suffocation and death of fishes.

Acknowledgements

The authors would like to thank Dr. Siqing Chen, Yellow Sea Fisheries Research Institute, the Chinese Academy of Fishery Sciences for his kind help in supplying living fishes. We also appreciate Dr Yuyan Xu and Qin Xiao in our laboratory for the technical assistance. We are also very grateful to Dr. Milind S. Sawant, the College of Fisheries, Shirgaon, India for his kind help in the literal revision of this manuscript. This work was supported by the National Basic Research Program of China (Grant No. 2006CB101805) and Undergraduate Innovative Experiment Program of China (Grant Number 091042328).

References

Ateeq, B., Farah, M. A., Ali, N., and Ahmad, W., 2002. Induction of micronuclei and erythrocyte alterations in the catfish *Clarias batrachus* by 2, 4-dichlorophenoxyacetic acid and butachlor. *Mutat*. *Res*., **518**: 135-144.

Ateeq, B., Farah, M. A., and Ahmad, W., 2005. Detection of DNA damage by alkaline single cell gel electrophoresis in 2, 4-dichlorophenoxyacetic-acid- and butachlor-exposed erythrocytes of *Clarias batrachus*. *Ecotoxicol*. *Environ*. *Saf*., **62**: 348-354.

Ateeq, B., Farah, M. A., and Ahmad, W., 2006. Evidence of apoptotic effects of 2, 4-D and butachlor on walking catfish, *Clarias batrachus*, by transmission electron microscopy and DNA degradation studies. *Life Sci*., **78** (9): 977-986.

Bessen Chemical Company, 2006. Butachlor mammalian toxicity. http:// www.chinese-pesticide.com/herbicides/butachlor.htm.

Bettex-Galland, M., 1973. Contractile filamentous material in the pillar cells of fish gills. *J*. *Cell Sci*., **13**: 359-370.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal*. *Biochem*., **72**: 248-254.

Castaneda, A. R., and Bhuiyan, S. I., 1996. Groundwater contamination by ricefield pesticides and some influencing factors. *J*. *Environ*. *Sci*. *Health*, **31** (1): 83-99.

Evans, D. H., 1987. The fish gill: Site of action and model for toxic effects of environmental pollutants. *Environ*. *Health Perspect*., **71**: 47-58.

Fan, L. M., Ma, X. Y., Hu, G. D., and Chen, J. C., 2005. The studies on the acute toxicity of butachlor on two species of fish. *J*. *Zhanjiang Ocean Univ*., **24** (4): 377-379.

Farah, M. A., Ateeq, B., Ali, M. N., Sabir, R., and Ahmad, W., 2004. Studies on lethal concentrations and toxicity stress of some xenobiotics on aquatic organisms. *Chemosphere*, **55** (2): 257-265.

Geng, B. R., Yao, D., and Xue, Q. Q., 2005a. Acute toxicity of the pesticide dichlorvos and the herbicide butachlor to tadpoles of four anuran species. *Bull*. *Environ*. *Contam*. *Toxicol*., **75**: 343-349.

Geng, B. R., Yao, D., and Xue, Q. Q. 2005b. Genotoxicity of the pesticide dichlorvos and herbicide butachlor in *Rhacophorus megacephalus* tadpoles. *Acta Zool*. *Sin*., **51** (3): 447-454.

Geng, B. R., Yao, D., Zhang, Q. J., and Huang, H., 2005c. Toxicity influence of dichlorvos and butachlor on *Rana guentheri* tadpoles. *China Environ*. *Sci*., **25**: 118-121.

Halliwell, B., and Gutteridge, J. M. C., 1990. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol*., **186**: 1-85.

Hatakeyama, S., and Sugaya, Y., 1989. A freshwater shrimp (*Paratya compressa improvisa*) as a sensitive test organism to pesticides. *Environ*. *Pollut*., **59** (4): 325-336.

Hu, G. D., Chen, J. C., Wu, W., Huo, J. H., Fan, L. M., and Wu, J. C., 2005. Mutagenesis of butachlor to cells of *Monopterus albus*. *J*. *Zhanjiang Ocean Univ*., **25** (1): 43-46.

Hu, X. X., 1998. The current situation and developing trends of pesticide industry in China. *Pesticides*, **37**: 7-10.

Junghans, M., Backhaus, T., Faust, M., Scholze, M., and Grimme, L. H., 2003. Predictability of combined effects of eight chloroacetanilide herbicides on algal reproduction. *Pest Manag*. *Sci*., **59** (10): 1101-1110.

Li, H. Y., and Zhang, S. C., 2002. *In vitro* cytotoxicity of the organophosphorus insecticide methylparathion to FG-9307, the gill cell line of flounder (Paralichthys olivaceus). *Cell Biol*. *Toxicol*., **18** (4): 235-241.

Mallatt, J., 1985. Fish gill structural changes induced by toxicants and other irritants: A statistical review. *Can*. *J*. *Fish*.

Aquat. Sci., **42** (4): 630-648.

Ohyama, T., Jin, K., Katoh, Y., Chiba, Y., and Inoue, K., 1987. Fate and behavior of herbicides, butachlor, CNP, chlomethoxynil, and simetryne in river water, shellfish, and sediments of the Ishikari River. *Bull. Environ. Contam. Toxicol.*, **39**: 555-562.

Rajyalakshmi, T., Srinivas, T., Swamy, K. V., Prasad, N. S., and Mohan, P. M., 1996a. Action of the herbicide butachlor on cholinesterases in the freshwater snail *Pila globosa* (Swainson). *Drug Chem. Toxicol.*, **19** (4): 325-331.

Rajyalakshmi, T., Srinivas, T., Swamy, K. V., and Mohan, P. M., 1996b. Butachlor impact on protein, free amino acid and glutamine contents, and on activity levels of aminotransferases, glutamate dehydrogenase and glutamine synthetase in the fresh water snail, *Pila globosa* (Swainson). *Biochem. Mol. Biol. Int.*, **39** (5): 949-960.

Tantawy, A. A., 2002. Effect of two herbicides on some biological and biochemical parameters of Biomphalaria alexandrina. *J. Egypt. Soc. Parasitol.*, **32** (3): 837-847.

Thophon, S., Kruatrachue, M., Upatham, E. S., Pokethitiyook, P., Sahaphong, S., and Jaritkhuan, S., 2003. Histopathological alterations of white seabass, *Lates calcarifer,* in acute and subchronic cadmium exposure. *Environ. Pollut.*, **121**: 307-320.

Tomlin, C. D. S., 1994. *The Pesticide Manual*. 10th ed. Crop Protection Publications, British Crop Protection Council, Farnham, Surrey, UK, 37-50.

Tong, S. L., Li, H., and Miao, H. Z., 1997. The establishment and partial characterization of a continuous fish cell line FG-9307 from the gill of flounder *Paralichthys olivaceus*. *Aquaculture*, **156**: 327-333.

Wilson, A. G. E., and Takei, A. S., 2000. Summary of toxicology studies with butachlor. *J. Pestic. Sci.*, **25**: 75-83.

Xu, X. Q., Yang, H. H., Wang L., Han B., Wang X. R., and Lee F. S. C., 2007. Analysis of chloroacetanilide herbicides in water samples by solid-phase microextraction coupled with gas chromatography–mass spectrometry. *Anal. Chim. Acta.*, **591** (1): 87-96.

Xue, Q. Q., Yao, D., Huang, Z. Y., Ke, Q., Wen, X., and Geng, B. R., 2005. Acute toxicity of pesticide dichlorvos and herbicide butachlor on *Microhyla ornata* tadpoles. *Sichuan J. Zool.*, **24** (2): 209-212.

Yin, L. C., Guo, H. R., Zhang, S. C., and Wang, J., 2007. Study on the acute toxicity and genotoxicity of herbicide butachlor in flounder, *Paralichihys olivaceus*, and flounder gill (FG) cells. *J. Ocean Univ. China.*, **37** (4): 167-171.

Yu, K. N., Qi, C. J., and Tang, K., 1993. Relationship between degradation rate of butachlor and conditions of paddy fields. *Acta Sci. Circum*st, **13**: 169-173.

Zhang, Y., Marcillat, O., Giulivi, C., Ernsterg, L. and Davies, K. J. A., 1990. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *J. Biol. Chem.*, **265** (27): 16 330-16 336.

(Edited by Ji Dechun)