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Acute Toxicity of Four Agrochemicals on Larval and Juvenile Oriental Weatherfish (*Misgurnus anguillicaudatus*)

Dong-Hyuk Yeom※ **and Sung Kyu Lee**

Ecotoxicology Research Team, Korea Institute of Toxicology, P.O. Box 123, Yuseong, Daejeon 304-343, Korea

미꾸리 (*Misgurnus anguillicaudatus*)의 치어 및 자어에 대한 4가지 농약의 급성독성

염 동 혁※**, 이 성 규**

안전성평가연구소 생태독성연구팀

요 약

미꾸리 (*Misgurnus anguillicaudatus*)에 대한 4가지 농약(diazinon, carbaryl, fenpropathrin, myclobutanil)의 급성독성을 평가하기 위하여, 치어 및 자어 단계(postlarva I, postlarva II, 8 WPH (weeks posthatch), 16 WPH, 20 WPH, 32 WPH, 40 WPH)의 미꾸리를 사용하여 급성독성실험을 실시하였다. 각 생육단계 별로 구해진 각 실험농약의 96-hr LC_{50} 값을 비교하여 미꾸리에 대한 급성독성이 높은 순서를 정리한 결과는 Fenproparthrin > Diazinon > Carbaryl, Myclobutanil 순으로 나타났다. 또한, 본 연구에서 사용된 4가지 농약의 독성은 미꾸리의 생육단계에 따라 차이를 나타냈다. 농약에 대한 감수성이 민감한 미꾸리의 생육단계 순으로 정리하면 Postlarva I > Postlarva II, 8 WPH > 16 WPH, 20 WPH, 32 WPH, 40 WPH 나타났다. 그러므로 환경관리 및 규제목적으로 미꾸리 성체를 사용하여 독성실험을 할 경우에는 독성을 과소평가 할 수도 있음을 보여주었다.

Key words : oriental weatherfish, life stage, agrochemicals, acute toxicity

INTRODUCTION

A variety of agrochemicals are used widely throughout many countries of the world, including Korea. Standard ecotoxicological studies, based on the official test guidelines, have been one of the primary approaches in order to assess the effects of these agrochemicals on aquatic organisms (i.e., fish, crustaceans, and algae) and to classify these chemicals with respect to their potential hazard to the environment. According to the guidelines developed by the Organization for Economic Cooperation and Development (OECD), for example, acute toxicity tests on fish, using juvenile or adult stage, are to be carried out on only one of the eight recommended species. This would imply an equivalent susceptibility to the different species of fish to the chemicals, under the conditions described in the official guidelines (Vittozzi and De Angelis, 1991). However, Gallo *et al.* (1995) reported that the species-specific toxicity

※ To whom correspondence should be addressed.
Tel: +82-42-860-7347, Fax: +82-42-860-7399
E-mail: dhyeom@kitox.re.kr

shown by organosphothiates and carbamates indicates that toxicity testing with only one fish species may be inadequate to classify chemicals belonging to these classes for their environmental impact. In addition, Mckim (1977) and Phillips *et al.* (2002) reported that early life stages of fish generally are considered to be the most sensitive to waterborne toxicants. Therefore, knowledge on the difference in susceptibility to species and life stage of fish may help to improve the classification of chemicals, according to the regulations devoted to environmental protection.

The oriental weatherfish, *Misgurnus anguillicaudatus* (Cobitidae), is native to northeastern Asia southward to central China (Page and Burr, 1991; Kim, 1997). This species has been used in pesticides toxicity tests in Korea, Japan, and China (http:// preview. pesticideinfo.org) because a large number of this fish could be found in most paddy fields, agricultural waterways, ponds, and streams (Kim, 1977). However, currently the numbers of oriental weatherfish are decreasing due to environmental changes, such as the destruction of natural habitat of the fish (i.e., natural waterways and ponds) and the increased use of agricultural chemicals in paddy fields.

There is little information on not only the effects of short-term exposure to agricultural pesticides, but also changes of susceptibility caused by the change in life stage of oriental weatherfish. Therefore, the oriental weatherfish was selected for the bioassay experiments.

The objectives of our study were: (i) to assess the acute toxicity of four agrochemicals currently in use to oriental weather fish; and (ii) to determine the most sensitive life stage of oriental weatherfish exposed to these agrochemicals by comparing LC_{50} values of two larval stages and five early stages of juvenile oriental weatherfish.

MATERIALS AND METHODS

The broodfish were purchased from a commercial fish farm and kept at 24~26°C with a 16 : 8 hour light : dark photoperiod for two months. Oriental weatherfish eggs were obtained through ovulation caused by hormonal induction by the abdominal injection of Aqua Human Chorionic Gonadotrophin (Dae Sung Microbiological Labs Co. Ltd, Korea). Approximately twenty-four hours after injection the eggs were stripped into spawning plates and fertilized artificially with testicular homogenate taken from minced testes of males in normal saline solution. Immediately after fertilization, the fertilized eggs were placed in a glass tank with a volume of 40 L to incubate. Under the culture temperature of 24~25°C, the eggs began to hatch in 48 hours after fertilization and larvae were completely hatched out within 1 hour.

The newly hatched larvae were cultured in the same glass tank by supplying gentle circulation of water with gentle aeration. After hatching, the larvae were fed daily with live brine shrimp until reaching the juvenile stage within 2 months. Juvenile fish were fed daily with commercial weatherfish food and live brine shrimp until they were used in toxicity testing. Fish were cultured with tap water dechlorinated through membrane and charcoal (residual chlorine, <0.01 mg L^{-1}; hardness, 36~50 mg L^{-1} as $CaCO_3$; alkalinity, 19~39 mg L^{-1} as $CaCO_3$; pH 7.1~7.9). Fish were kept at kept at 24~26°C with a 16 : 8 hour light : dark photoperiod.

In this study, static acute toxicity tests were conducted according to standard methods (OECD, 1992). The concentration range of the agrochemicals was determined with a series of range finding tests. Thereafter, definitive acute toxicity tests were conducted by exposing fish to different concentrations of test chemicals. Each test consisted of exposing groups of 7 fish to a series of six or seven toxicant concentrations, one dilution water control, and one solvent control exposure. The four agrochemicals used in the present study had the specification listed in Table 1. Since all test substances have low solubility, a stock solution of the test chemicals was prepared by dissolving desired amounts in dimethylsulphoxide (DMSO, Merck, Germany) and then added to the same water

Table 1. Specification of the test chemicals

Chemicals	CAS No.	Purity (%)	Supplier
Diazinon	333-41-5	93.9	Syngenta Korea (Seoul, Korea)
Carbaryl	63-25-2	97.0	Syngenta Korea (Seoul, Korea)
Fenpropathrin	64257-84-7	91.3	Kyung Nong Corporation (Seoul, Korea)
Myclobutanil	88671-89-0	93.6	Kyung Nong Corporation (Seoul, Korea)

Table 2. Life stages, mean weight (g), total length (mm), and range (in parentheses) of oriental weatherfish tested with four pesticides

Life stage	Age[b]	Weight	Total length
Postlarvae I	10~12	0.005[c] (0.002~0.008)	10.6[c] (8.8~11.8)
Postlarvae II	24~28	0.015[c] (0.008~0.029)	15.5[c] (12.4~18.9)
8 WPH[a]	52~60	0.10[c] (0.06~0.19)	28.0[c] (23.1~29.8)
16 WPH[a]	108~112	0.21[c] (0.14~0.31)	36.3[c] (31.1~40.5)
20 WPH[a]	136~140	0.26[d] (0.20~0.41)	39.4[d] (35.8~45.4)
32 WPH[a]	220~224	0.70[e] (0.54~1.02)	55.0[e] (48.3~63.0)
40 WPH[a]	278~282	2.83[e] (1.89~5.02)	85.1[e] (74.8~92.1)

[a]Weeks posthatch (WPH) at test initiation, [b]Days posthatch at test initiation, [c]N=28, [d]N=21, [e]N=14

as that used for fish culture to give the desired exposure concentrations. The concentration of DMSO was less than 100 mg L^{-1}. Postlarvae I and II were tested in 1.5 L glass test vessels containing 1.0 L of test solution. 8 WPH (weeks posthatch), 16 WPH, 20 WPH, and 32 WPH juveniles were exposed in 2.5 L glass test vessels containing 2.0 L of test solution. 40 WPH juveniles were tested in 5.0 L glass test vessels containing 4.0 L of test solution.

Mortality and abnormal behavior were monitored and recorded at 24-hour intervals during the tests, and dead fish were removed at those intervals. Cessation of opercular movement was the criteria for mortality. At the termination of each test, all control fish were weighed and total length was measured. Mean weight and mean total length of life stages tested are given in Table 2. Dissolved oxygen and pH were measured and recorded in the control, solvent control, low, medium, and high concentrations of each test with live fish present at 0, 48, and 96 hour of exposure. Dissolved oxygen was measured with an YSI Model 57 dissolved oxygen meter and probe with a standard membrane. pH was measured with an Orion 720A pH meter with a Ross pH electrode and automatic temperature compensation electrode. The water temperature was maintained at 23±2°C and a 16 : 8 hour light : dark photoperiod was provided throughout the test.

The median lethal concentrations (LC_{50} values) with 95% confidence limits were calculated using either the Probit or Moving average angle methods as described by Stephan (1977). All LC_{50} values are expressed as nominal concentration of each test chemical. The Friedman test (Conover, 1980) was used the rank the life stages from most to least sensitive and the chemicals from most to least toxic. Significant differences ($P<0.05$) were determined with Friedman's multiple comparison tests. The standard error of the difference as described by Sprague and Fogels (1977) and Zar (1974) was used to determine statistical differences (P=0.05) between individual LC_{50} values.

RESULTS AND DISCUSSION

Although the concentrations of dissolved oxygen in most toxicity tests were maintained at greater than

Table 3. Acute toxicity (96-hr LC_{50} values with 95% confidence limits in μg L^{-1}) of four pesticides to larval and juvenile oriental weatherfish at different stages of development

Life stage	Diazinon	Carbaryl	Fenpropathrin	Myclobutanil
Postlarvae I	32^{a} (20~51)	5135^{a} (3933~6817)	3.9^{a} (2.3~5.9)	4417^{a} (3736~5126)
Postlarvae II	32^{a} (22~49)	8525^{b} (5777~12656)	$5.7^{a,b}$ (4.6~7.4)	11867^{b} (10496~13961)
8 WPH*	26^{a} (20~34)	11345^{b} (9229~14701)	7.1^{b} (4.9~10.2)	7790^{c} (5868~10171)
16 WPH*	71^{b} (49~102)	11042^{b} (9340~12815)	7.4^{b} (6.6~8.7)	11867^{b} (10496~13961)
20 WPH*	$49^{a,b}$ (37~64)	14834^{b} (13120~17451)	$9.7^{b,c}$ (7.3~12.7)	8635^{c} (6843~11332)
32 WPH*	93^{b} (71~117)	22691^{c} (18459~29402)	11.3^{c} (9.2~14.7)	7790^{c} (5868~10171)
40 WPH*	91^{b} (42~146)	29668^{c} (26240~34902)	$9.3^{b,c}$ (7.1~11.7)	9077^{c} (7384~11761)

Note. Letters in common denote no significant difference (P=0.05) among life stages within a test chemical.
*Weeks posthatch (WPH) at test initiation

60% saturation, as recommended by OECD test guidelines (1992), four tests had dissolved oxygen readings below 60% saturation at 96 hr (range, 18~45%). However, the live fish in the tests demonstrated no overt signs of oxygen deprivation due to their intestinal accessory organ which allows them to absorb atmospheric oxygen (McMahon and Burggren, 1987; Kim, 1997).

The 96-hr LC_{50} values with 95% confidence limits determined for the seven life stages tested with the four agrochemicals are presented in Table 3. There was no mortality in any solvent control or dilution water control exposures at any life stages. Normal behavior was observed for fish of the dilution water control and solvent control exposure. However, fish subjected to higher concentrations of four agrochemicals showed behavioral changes, such as less general activity and loss of equilibrium, when compared with the control group fish. Based on the Friedman test to the 96-hr LC_{50} values derived for each life stage, the rank order of the agrochemicals tested in this study from most toxic to least toxic was: fenpropathrin > diazinon > carbaryl, myclobutanil (Table 3) ($P < 0.05$).

The fenpropathrin, a pyrethroid insecticide, appeared to be the most toxic to oriental weatherfish with 96-hr LC_{50} values ranging from 3.9 to 11.3 μg L^{-1} among the agrochemicals tested (Table 3). The results of the present study were similar to other reports for fish exposed to fenpropathrin. For example, the 96-hr LC_{50} value was 2.2 μg L^{-1} for bluegill sunfish (*Lepomis macrochirus*), 2.3 μg L^{-1} for rainbow trout (*Oncorhynchus mykiss*), and 3.1 μg L^{-1} for sheepshead minnow (*Cyprinodon variegates*) (http://www.epa.gov/tri/chemical/hazard_enviro95.pdf). Pyrethroids have low toxicity in mammals, but they can be highly toxic to other animals, especially fish (Glickman and Lech, 1982). Yilmaz *et al.* (2004) reported the hypersensitivity of fish to pyrethroid intoxication is partly due to species' specific difference in pyrethroid metabolism, but principally to the increased sensitivity of the piscine nervous systems to these pesticides.

The 96-hr LC_{50} values of diazinon, an organophosphate insecticide, were reported as 1,530 μg L^{-1} for common carp (*Cyprinus carpio*) larvae (Aydin and

Köprücü, 2005), 3,910 μg L^{-1} for Japanese medaka (*Oryzias latipes*) (Oh *et al.*, 1991), 100 ~ 500 μg L^{-1} for fry bluegill sunfish (*L. macrochirus*), 1,470 μg L^{-1} for sheepshead minnow (*C. variegates*), 1,650 μg L^{-1} for fry rainbow trout (*O. mykiss*), and 7,800 μg L^{-1} for fathead minnow (*Pimephales promelas*) (Office of Pesticide Programs, 2000). In the present study, the 96-hr LC_{50} values of diazinon to postlarval and juvenile oriental weatherfish ranged from 26 to 93 μg L^{-1} (Table 3), and the oriental weatherfish were approximately19-300 times more sensitive than any other fish species reported above. Oh *et al.* (1991) reported that the selective toxicity of diazinon to fish was explained by significant contributions of acetylcholinesterase (AChE) inhibition, detoxification rate, and absorption. For example, Oh *et al.* (1991) found that: (i) initial absorption ration was 4.5 : 1 for Japanese medaka: oriental weatherfish; (ii) inhibition of AchE by diazoxon in oriental weatherfish was 22-fold more potent than in Japanese medaka; (iii) total radioactivity exposed to ethoxy-^{14}C labeled diazinon was decreasing in Japanese medaka with daily analysis, while increasing in oriental weatherfish. These results indicate that diazinon contamination is very dangerous to oriental weatherfish. Therefore, this fact should be taken into consideration when diazinon is used in agriculture.

Post and Schroeder (1971) reported the 96-hr LC_{50} values of carbaryl, a cabamate pesticide, on various fish species as follows: 1,470 μg L^{-1} for *Salmo gairdneri*, 1,500 ~ 2,169 μg L^{-1} for cutthroat trout (*S. clarki*), 1,070 ~ 1,450 μg L^{-1} for brook trout (*Salvelinus fontanalis*), and 1,300 μg L^{-1} for coho salmo (*Oncorhynchus kisutch*). Other 96-hr LC_{50} values reported were 2,500 μg L^{-1} for guppy (*Poecilia reticulate*) and 9,250 μg L^{-1} for zebrafish (*Brachydanio rerio*). In terms of relative sensitivity, the oriental weatherfish appeared generally resistant and their 96-hr LC_{50} values were higher than those in four salmonid fish species, guppy, and zebrafish. For example, the corresponding 96-hr LC_{50} values for the present study ranged from 5,135 to 29,668 μg L^{-1} in all life stages tested (Table 3). This suggests that carbaryl has greater margin of safety to oriental weatherfish in comparison to other fish.

The British Crop Protection Council (2003) reported the acute toxicity of myclobutanil, a triazole fungicide, on bluegill sunfish and rainbow trout as 2,400 μg L^{-1} and 2,000 μg L^{-1}, respectively. The 96-hr LC_{50} values (4,417 ~ 11,867 μg L^{-1}) reported in the present study for myclobutanil, however, were higher than those of British Crop Protection Council (Table 3). Since myclobutanil is rapidly excreted by animals, myclobutanil is classified as a moderately toxic compound (Athanasopoulos *et al.*, 2003). The toxicity difference of myclobutanil among fish species may be related to host metabolism. Therefore, additional study is needed to confirm the contribution of metabolism, such as absorption, detoxification rate, and excretion, to toxicity difference in the oriental weatherfish.

Life stage influenced the toxicity of the four agrochemicals. Based on the Friedman test, the rank order of the life stages tested from most sensitive to least sensitive was: postlarva I > postlarva II, 8 WPH > 16 WPH, 20 WPH, 32 WPH, 40 WPH. In all agrochemicals tests, the magnitude of difference in sensitivity between the most sensitive life stage and the least sensitive was 3-fold or more. The greatest difference was a 6-fold difference between postlarval I and 40-week-posthatch juvenile exposed to carbaryl (Table 3). In the present study, postlarval I oriental weatherfish was the most sensitive life stage. The sensitivity of this life stage is most likely caused by the change from endogenous to exogenous feeding. Gaikowski *et al.* (1996) reported that a possible change in metabolic processing from endogenous to exogenous resources may cause this life stage to be more prone to the stresses from the test chemicals. Another possibility of the increased sensitivity observed in the postlarval I stage may be related to gill development. The gills of this life stage have undergone various physiological changes in response to metabolic shift (Gaikowski *et al.*, 1996). Several studies on larval walleye reported an association between gill development and increased sensitivity to environmental stressors (Bergerhouse, 1992; Clayton and Summerfelt, 1996; Phillips *et al.*, 2002).

In conclusion, the data presented in this study suggest that certain stages of larval development are much more sensitive than juveniles or adults. The use of juveniles and adults, instead of larvae, may underestimate toxicity to the species in question. Therefore, the authors recommended the use of larval oriental weatherfish to evaluate toxicity of chemicals and environmental samples.

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