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Effects of Co-existence of Dichlorvos and Phosalone on the Bioconcentration in Zebrafish (*Brachydanio rerio*)

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Dichlorvos와 Phosalone의 공존이 Zebrafish(*Brachydanio rerio*)의 생물농축성에 미치는 영향

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ABSTRACT – This study was performed to investigate the effect of co-existence of dichlorvos and phosalone on the bioconcentration in zebrafish (*Brachydanio rerio*). The bioconcentration of the pesticides was reached an equilibrium more rapidly in an exposure of the binary mixture than that in an individual exposure. The BCF values and depuration rate constants for dichlorvos and phosalone in the binary mixture in the zebrafish were not significantly different from that of single pesticide. The results suggest that the effect of co-existence of pesticides on bioconcentration and depuration in zebrafish can be evaluated with single pesticide datum.

Key words: Bioconcentration, Dichlorvos, Phosalone

Water pollution has been increasing due to industrialization and civilization. Pesticides are widespread pollutants of aquatic ecosystems and may have deleterious effects on living resources. Organophosphorus pesticides, carbamates, pyrethroids and triazines have largely replaced the organochlorine compounds in the agricultural practices.[1] The organophosphorus pesticides dichlorvos [2, 2-dichloroethenyl dimethyl phosphate] and phosalone [*S*-5-chloro-2, 3-dihydro-2-oxobenzoxazol-3-ylmethyl *O*, *O*- diethyl phosphorodithioate] are widely used in Korea for pest control such as caterpillar, beetle, scale and lygus. Recent studies have demonstrated that organophosphorus insecticides have both acute and chronic effects on survival of vertebrate, tissue accumulation, and on the physiological and reproductive processes of some organisms. Thus, the bioconcentration process of pesticides by aquatic organisms has been extensively studied.

The bioconcentration factor (BCF), which is generally used to estimate the propensity to accumulate chemicals in organisms, is defined as the ratio of the concentration of the chemical in whole fish at steady state to the concentration of the chemical in water during the exposure period. In the case of pesticide toxicity to fish, the influence of single and complex mixtures should be tested precisely to the aquatic ecosystem. On the basis of our earlier works,[2-6] this paper describes the results of the interaction of dichlorvos and phosalone on the bioconcentration in zebrafish(*Brachydanio rerio*) under flow through system.[7]

Materials and Methods

Materials

Dichlorvos (97% purity) and phosalone(98% purity) were obtained from Kyung Nong corporation in Korea and used without further purification. All solvents used were pesticide residue grade with no further treatment. Sep-Pak Florisil column(Waters, USA) was used for sample purification.

Zebrafish (Brachydanio rerio) were purchased from a commercial supplier in Korea, weighed 0.2 to 0.4 g and had an average length of 2.0 to 3.0 cm. All fish were acclimated in glass aquaria containing dechlorinated tap

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water for at least 4 weeks before use in experiments. Fish were maintained on a 8:16 hr. dark:light photoperiod. They were fed with commercial balanced fish feed at rate of 1 % body weight per day. Excrements and surplus food were removed daily. The characteristics of experimental water were: temperature, 23.5 ± 1°C, pH, 7.5 ± 0.1; DO, 7.1 ± 0.1 mg/*l*; hardness, 37 ± 2 mg $CaCO_3$/*l*. Dichlorvos and phosalone were not detected in zebrafish before exposure to these pesticides.

Acute Toxicity

A static acute toxicity test was performed according to OECD guideline 203 to determine the LC_{50} values of two pesticides.[8] To determine the LC_{50} values, ten fish in each tank were exposed in five serial concentrations of two pesticides. The fish were not fed for 24 hr. prior to or during the acute toxicity test. The concentrations tested were 40, 50, 60, 70 and 80 m*l*/*l* for dichlorvos, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/*l* for phosalone. Dead fish were counted and removed after every 3 hr. through 96 hr. of exposure. The LC_{50} values of each pesticide were determined using a logarithmic probability regression on actual concentrations.

Bioconcentration and Depuration Test

Bioconcentration and depuration tests were carried out according to OECD guideline 305 in a continuous flow-through system.[7] Bioconcentration factor(BCF) was calculated from the following equation:

$$BCF=\frac{\text{pestici(d)e concentration in whole fish study}(\mu g/g)}{\text{pesticide concentration inwate}(\mu g/ml)}$$

Steady state is a condition in which the amount of pesticide being taken up and depurated is equal at given water concentration. In the bioconcentration phase at the experiments, zebrafish were maintained at two concentrations of each pesticide for 72 hours. The stock solutions were prepared by dissolving acetone solution (2 m*l*) of dichlorvos (high exposure level 55 mg/*l*, low exposure level 5.5 mg/*l*) and phosalone (high exposure level 1 mg/*l*, low exposure level 0.1 mg/*l*) with dechlorinated tap water to 10 *l*, respectively.

The mixed stock solutions were prepared by dissolving acetone solution(2 m*l*) of dichlorvos(high exposure level 55 mg/*l*) and phosalone(high exposure level 1 mg/*l*) with dechlorinated tap water to 10 *l*, respectively.

The solutions were supplied to each of the four glass mixing chambers and connected to peristaltic pumps (Chunse BX-20, Korea) that generated constant solution flow of 3 m*l*/min diluting to the desired concentrations by constant dechlorinated tap water flow of 300 m*l*/min, the outlets were connected to each of the four 100 *l* glass aquaria containing 250 fish. In this way, the aqueous test solution was diluted 100 times continuously. The concentrations of the pesticides in each exposure tank were [mean SD(n=6)] 549.3 ± 13.2 μg/*l* for dichlorvos (high exposure level), 55.1 ± 1.6 μg/*l* for dichlorvos (low exposure level), 11.1 ± 1.2 μg/*l* for phosalone (high exposure level), 1.0 ± 0.1 μg/*l* for phosalone (low exposure level), 545.7 ± 15.3 μg/*l* for dichlorvos of mixtures and 10.5 ± 1.4 μg/*l* for phosalone of mixtures. Test aquaria renewed approximately 4.3 times a day. Zebrafish were exposed to each pesticide for 72 hours. After 6, 12, 24, 48 and 72 hours, twenty fish were removed, rinsed with distilled water, weighed and analyzed. After the exposure period, fish were transferred to clean water with same flow-through system but without each pesticide. Twenty fish were taken at 6, 12, 24 and 48 hr, respectively.

Analysis

Fish samples (ca. 5 g) were placed in a blender jar and anhydrous sodium sulfate (4 g) added. The contents were thoroughly mixed and acetonitrile (30 m*l*) was then added. The mixture was blended at high speed for 4 min. The homogenate was vacuum filtered through a GF/C glass filter. This operation was repeated and combined filtrate was then dried in a rotary evaporator under vacuum at 40°C The residue was redissolved in 5 m*l* of hexane and transferred to a preparative Sep-pak florisil column. The column was previously conditioned with 10 m*l* of hexane and eluted with 20 m*l* of acetone/hexane(8/2, v/v). The eluate was dried in a rotary evaporator under vacuum at 40°C and dissolved in 2 m*l* hexane and analyzed by GC-FPD. Average recoveries(n=3) were 86.1% for dichlorvos and 91% for phosalone at a spiked level of 10 μg/*l*.

To evaluate the concentration of each pesticide in the aquaria, 100 m*l* of test water were collected and extracted with 50 m*l* of ethyl ether/hexane(4/1, v/v). Extraction with ethyl ether+hexane was repeated and all extracts were combined and passed through glass column with anhydrous sodium sulfate. The eluate was dried in a rotary evaporator under vacuum at 40°C and dissolved in 2 m*l* hexane and analyzed by GC-FPD. Average recoveries(n=3) were 103.2% for dichlorvos and 98.7% for phosalone at a spiked level of 1 μg/g. The above GC analysis was performed on a Shimadzu GC-14A with a flame photometric detector. The fused silica capillary column(DB-17, 1 μm thickness, J&W Scientifics) was 30 m by 0.53 mm ID. Nitrogen was used as carrier gas at a flow rate of 1 kg/cm^2. The temperature of oven, injector and detector were 170, 180 and 300°C for dichlorvos, 270, 280 and 300°C for phosalone, respectively. Quantitation was carried out by means of external standard method.

Results and Discussion

The values of 24-hr LC_{50}, 48-hr LC_{50}, 72-hr LC_{50} and 96-hr LC_{50} were 28.34, 25.98, 24.43 and 22.03 mg/*l* for dichlorvos, 67.25, 61.37, 58.75 and 55.02 mg/*l* for phosalone, respectively (Table 1). The LC_{50} value in zebrafish showed that acute toxicity of phosalone was higher than that of dichlorvos.

Plots of bioconcentration and depuration of dichlorvos, phosalone and mixtures of two pesticides are shown in Fig. 1, 2, 3, 4 and 5.

In an individual exposure of dichlorvos, the concentration of dichlorvos in zebrafish reached an equilibrium in 12 hours at one-thousandth and one-hundredth concentration of 96-hr LC_{50} (low and high concentrations). The average BCF values of dichlorvos were 0.74(n=4, low concentrations) and 1.28(n=4, high concentrations) after 12~72 hours (Fig. 1a, 2a).

Depuration rate constants of dichlorvos were 0.16 h^{-1} and 0.13 h^{-1}, half-lives of dichlorvos were 4.3 and 5.3 at low and high concentrations, respectively. The concentrations of dichlorvos in zebrafish at low and high concentrations rapidly decreased after 12(0.010 μg/g) and 24 hours (0.016 μg/g) (Fig. 1b, 2b).

Table 1. Acute toxicity of dichlorvos and phosalone to zebrafish.

Pesticides	LC_{50}(mg/*l*)			
	24hr	48hr	72hr	96hr
Dichlorvos	67.25	61.37	58.75	55.02
Phosalone	3.76	2.43	1.86	1.05

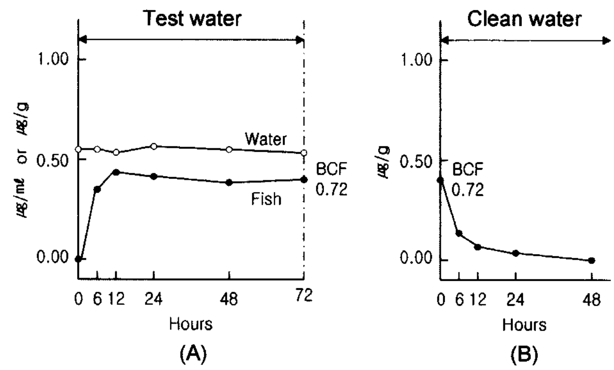

Fig. 1. Uptake(A) and depuration(B) of dichlorvos in zebrafish exposed renewally to the concentration of 0.55 mg/m*l* for 72 hours followed by 48-hour depuration in clean water.

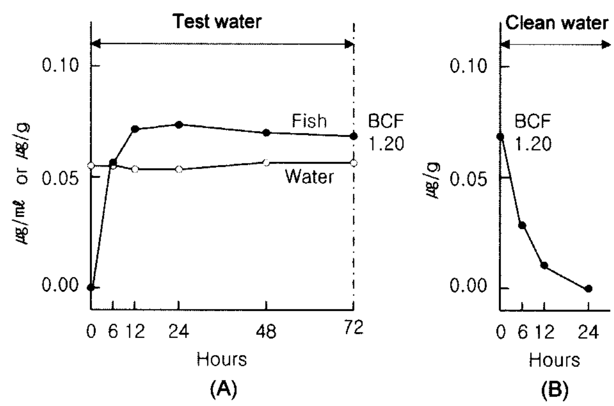

Fig. 2. Uptake(A) and depuration(B) of dichlorvos in zebrafish exposed renewally to the concentration of 0.055 mg/m*l* for 72 hours followed by 24-hour depuration in clean water.

In an individual exposure of phosalone, the concentrations of phosalone in zebrafish reached an equilibrium in 12 hours at one-hundredth concentration of 96-hr LC_{50} (high concentrations). The average BCF value of phosalone was 48.88 (n=4) at one-hundredth concentration of 96-hr LC_{50}(high concentrations) after 12~72 hours (Fig. 3a). However, phosalone was not detected throughout the experimental period at the low concentration (0.001 μg/m*l*). Depuration rate constant and half-life of phosalone were not estimated at low and high concentrations because the concentration of phosalone in zebrafish was under the detecting limit on GC (Fig. 3b).

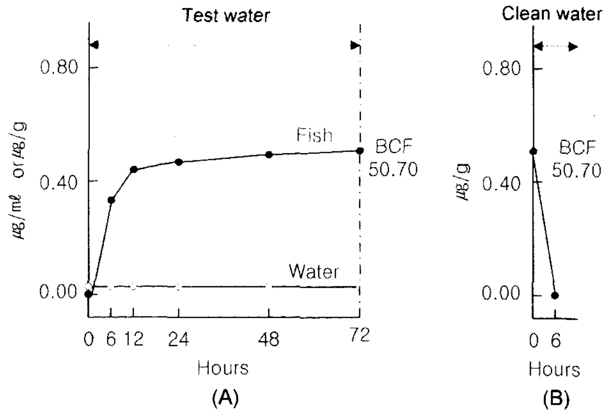

Fig. 3. Uptake(A) and depuration(B) of phosalone in zebrafish exposed renewally to the concentration of 0.01 mg/ml for 72 hours followed by 6-hour depuration in clean water.

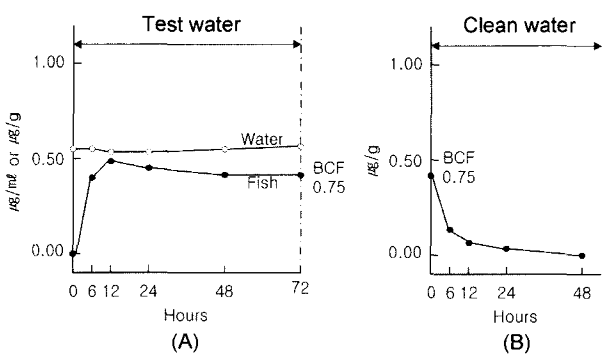

Fig. 4. Uptake(A) and depuration(B) of dichlorvos in zebrafish exposed renewally to the binary mixture of dichlorvos(0.55 mg/ml) and phosalone(0.01 mg/ml) for 72 hours followed by 48-hour depuration in clear water.

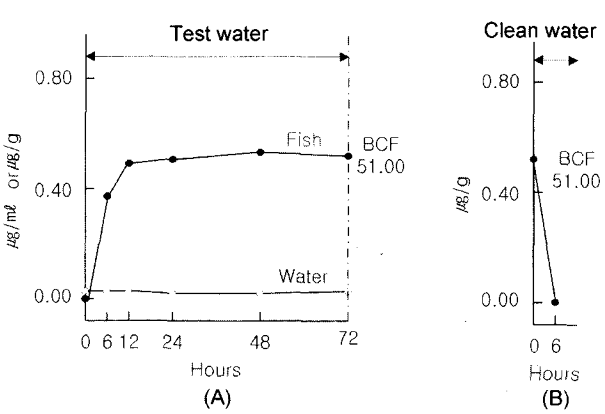

Fig. 5. Uptake(A) and depuration(B) of phosalone in zebrafish exposed renewally to the binary mixture of dichlorvos(0.55 mg/ml) and phosalone(0.01 mg/ml) for 72 hours followed by 6-hour depuration in clear water.

In an exposure of the binary mixture of dichlorvos (0.55 μg/ml) and phosalone (0.01 μg/ml), the concentration of dichlorvos in zebrafish reached an equilibrium in 6 hours. The average BCF values of dichlorvos were 0.80 (n=5) after 6~72 hours (Fig. 4a). Depuration rate constants of dichlorvos was 0.12 hh^{-1}, half-life of dichlorvos was 5.8, respectively. The concentrations of dichlorvos in zebrafish rapidly decreased after 24 hours (0.024 μg/g) (Fig. 4b).

In an exposure of the binary mixture of dichlorvos (0.55 μg/ml) and phosalone (0.01 μg/ml), the concentration of phosalone in zebrafish reached an equilibrium in 12 hours. The average BCF values of phosalone were 53.89(n=4) after 12~72 hours (Fig. 5a). Depuration rate constant and half-life of phosalone were not estimated, because the concentration of phosalone in zebrafish was under the detecting limit on GC (Fig. 5b).

In the present study, the values for BCF reached an equilibrium level more rapidly from the mixed pesticides. The average BCF values of dichlorvos(0.80, n=5) after 6~72 hours exposure and phosalone(53.89, n=4) after 12~72 hours exposure to the mixed pesticides were slightly higher than those of dichlorvos(0.74, n=4) and phosalone(49.88, n=4) after 12~72 hours exposure to the individual pesticides. Therefore, the fact that the equilibrium was reached more rapidly from the pesticide mixture was probably because the uptake rate of individual pesticide was increased by competition between the two pesticides.

Statistical analysis was carried out for each sampling time (6, 12, 24, 48 and 72 hours). In statistical analysis using a t-test, the bioconcentration factors (BCFs) for dichlorvos and phosalone at each sampling time in the binary mixture were not significantly different from those in an individual exposure except at 48 hours ($p<0.05$) (Table 2). These results indicate that there is no influence of mixed pesticides on the BCF of these individual organophosphorus pesticides. Min et al. concluded that there was little difference in the BCF of BPMC, carbaryl, carbofuran and chlorothalonil for goldfish between individual and mixed pesticides. A similar conclusion was, therefore, obtained for the organophosphorus pesticides.[5, 7-8)] Also, in an exposure of the binary mixture of dichlorvos and phosalone, the depuration rate constants and half-life was no difference in this study.

Table 2. Summary of BCFs for dichlorvos and phosalone in zebrafish exposed to the individual and to the binary mixture.

Exposure (hr)	Dichlorvos		Phosalone	
	single	mixture	single	mixture
6	0.64± 0.050	0.73± 0.056	33.00± 2.31	38.00± 2.08
12	0.80± 0.061	0.93± 0.056	45.50± 2.02	49.00± 2.08
24	0.75± 0.040	0.85± 0.049	49.40± 2.24	55.57± 2.28
48	0.69± 0.056	0.73± 0.060	49.90± 2.37	60.00± 1.52*
72	0.72± 0.056	0.75± 0.056	50.70± 2.27	51.00± 2.12

Each value represents an average BCF± S.E. of 3 experiments.
Exposure water concentration : dichlorvos(0.55 μg/m*l*), phosalone(0.01 μg/m*l*).
*Significantly different($p<0.05$) from the single exposure.

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국문요약

본 연구는 zebrafish를 실험어류로 하여 국내에서 혼합제로 사용되고 있는 dichlorvos와 phosalone을 선정하여 단독 및 혼합폭로시 생물농축계수와 배설속도상수를 측정함으로써, 두 농약의 공존이 개별농약의 생물농축성에 미치는 영향을 알아보고자 하였다. Dichlorvos와 phosalone의 혼합폭로시(dichlorvos : 0.55 μg/m*l*, phosalone : 0.01 μg/m*l*) zebrafish 체내에서 dichlorvos의 농축정도는 6시간에 정류상태에 도달하여 72시간까지 거의 일정하였으며 단독폭로시(12시간)보다 더 빠르게 정류상태에 도달하였다. 6시간에서 72시간 사이의 BCF평균값은 0.80(n=5)으로 단독폭로시의 12시간에서 72시간 사이의 BCF평균값 0.74(n=4)보다 더 높게 측정되었다. 배설속도상수는 $0.12h^{-1}$으로 단독폭로시와 차이가 거의 없었다. Dichlorvos와 phosalone의 혼합폭로시(dichlorvos : 0.55 μg/m*l*, phosalone : 0.01 μg/m*l*) zebrafish 체내에서 phosalone의 농축정도는 단독폭로시와 같이 12시간에 정류상태에 도달하여 72시간까지 거의 일정하였고, 12시간에서 72시간 사이의 BCF평균값은 53.89(n=4)로 단독폭로시의 BCF평균값 48.88(n=4)보다 더 높게 측정되었다. 배설속도상수는 단독폭로시와 같이 6시간 안에 어류체내에서 phosalone이 모두 배출되어 구하지 못했다. 두 농약(dichlorvos, phosalone)의 혼합폭로시의 BCF평균값이 단독폭로시의 BCF평균값보다 더 높게 나왔으나, 각 실험시간대(6, 12, 24, 48, 72시간)의 BCF실험값을 t-test로 분석한 결과 phosalone의 48시간을 제외하고는 두 농약의 단독폭로와 혼합폭로시의 BCF값에는 유의한 차이가 없었다($p<0.05$). 이상의 결과를 종합해 볼 때, dichlorvos와 phosalone을 zebrafish에 혼합폭로시 개개 농약의 생물농축성과 배설속도상수에는 유의한 영향을 미치지 않는 것으로 나타났다.

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