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Toxicity of Agricultural Chemicals in *Daphnia magna*

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Abstract

Background

Daphnia magna is a useful aquatic organism for testing ecological toxicities of environmental pollutants. However, there were only a few studies on agricultural chemicals using these organisms.

Methods

We investigated acute and subchronic toxicities of 30 agricultural chemicals commonly used in Japan in *D. magna*. Acute toxicity of the agricultural chemicals was determined using the concentrations yielding 50% immobility of *D. magna* after 24 hr and 48 hr exposure as end points. *D. magna* was cultivated with the chemical and algae until the first brood production. Lethal toxicity and the number of survival broods were determined within 13 days.

Results

All insecticides among the agricultural chemicals exhibited the strongest acute toxicity (LC_{50} from 0.00053 to 0.037 mg/L). More than 50% of the herbicides and fungicides did not exhibit acute toxicity at 10 mg/L. Chlornitrofen, pencycuron, and fenitrothion showed significantly lower LC_{50} values at 8 days than at 24 hr and 48 hr. Isoprothiolane, flutolanil, and thiophanate-methyl significantly delayed the first brood at concentrations less than half of those for LC_{50} (8 days). Thiobencarb, iprodione, flutolanil, mepronil, and thiophanatemethyl significantly reduced the size of the first brood at concentrations less than half of those for LC_{50} (8 days).

Conclusions

In this study, chlornitrofen, pencycuron, and fenitrothion were suggested to have slow-acting toxicity. Also, thiobencarb, iprodione, flutolanil, mepronil, and thiophanatemethyl were suggested to have parthenogenetic toxicity.

Key Words: Agricultural chemical; *Daphnia magna*; Immobilization; Brood; LC_{50}

Introduction

The World Health Organization (WHO) has established guidelines for agricultural chemicals

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in drinking water to ensure human health[1]. However, the numerical values in these guidelines are not necessarily appropriate for the protection of aquatic organisms or the environment[1]. Protecting aquatic organisms requires examination of the effects of agricultural chemicals on ecological systems. The Organization for Economic Co-operation and Development (OECD) guidelines on the ecological effects of chemicals have adopted acute immobilization and reproductive tests for the small aquatic crustacean *Daphnia* sp.[2] and reproductive tests involving *D. magna*[3]. That *D. magna* is in an important position in the food chains is found worldwide and is sensitive to toxicants[4]. In addition, *D. magna* is mainly parthenogenesis, producing a brood every two days, and several generations can be easily reared in the laboratory. Thus, this species is useful for testing aquatic reproductive toxicity[3]. Results of acute toxicity testing of agricultural chemicals (EC_{50} or LC_{50} after 24 hr and/or 48 hr) in *D. magna* have been reported previously[5-8]. However, few studies have examined the chronic toxicity of agricultural chemicals in *D. magna*, and few have performed the reproductive tests on this organism. The OECD criteria for assessment of chronic toxicity include prolongation of broods and reduced total number of broods, measured as parameters of the survival rate. Based on chronic toxicity assessments, it may be possible to predict the adverse effects of low concentrations and/or long-term exposure of agricultural chemicals on aquatic ecosystems. Here, we examined the acute and subchronic toxicities of 30 agricultural chemicals commonly used in Japan in *D. magna*.

Materials and Methods

Thirty agricultural chemicals (Table 1) and acetone were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The chemicals were diluted with acetone to obtain desired concentrations.

The *D. magna* used in this study were obtained from the National Institute for Environmental Studies, Tsukuba, Japan, and reared in our laboratory for about ten years[6]. Powdered activated carbon (Wako special class; about 300 mesh or less) was used for treatment of tap water. For subchronic testing, 0.5 mM of $CaCO_3 \cdot 2\ H_2O$, 0.3 mM of $MgSO_4 \cdot 7\ H_2O$, 0.05 mM of KCl, 0.5 mM of $NaHCO_3$, 1 μg/L of selenium dioxide, and 0.1 μg/L of vitamin B_{12} were added to the treated tap water[6]. *D. magna* were reared in this culture water in an incubator (20±1℃), with a light/dark cycle of 16 hr light (range 550-720 lux) and 8 hr dark. Mixed algae (*Chlamydomonas* sp., *Chlorella* sp., *etc*.) were planted in same culture water under sunlight at room temperature for one week[6], with 30 minutes of aeration twice each day. The algae were collected by centrifugation at 3000 rpm for 15 minutes, washed twice with the treated water, and diluted to suitable density for use as *D. magna* food.

Acute toxicity tests

Toxicity tests were performed according to the OECD guidelines for acute immobilization tests[2] and reproductive tests[3]. To the activated carbon treated water, 0.1 μg/L of vitamin B_{12} and 1 μg/L of selenium dioxide were added. This culture water was then kept in an incubator (20±1℃) prior to use during acute toxicity testing of *D. magna*. Ten mL of the treated water, and 5-50 μL of the agricultural chemical solutions were added to four or five flat bottom 20 mL tubes. Treated water was added to make a total volume of 15 mL. Four or five concentrations of chemicals, determined using a geometric concentration ratio of two, were tested. Five *D. magna* of the third to eighth broods at less than 24 hr after birth were placed in each tube. The tubes

Table 1. Agricultural chemicals used in the study

Chemicals	Composition	CAS No.	Regulation level for drinking water in Japan[15] and (WHO[1]) (mg/L)
Herbicide			
Thiobencarb	$C_{12}H_{16}ClNOS$	28249-77-6	0.02
Propyzamide	$C_{12}H_{11}Cl_2NO$	23950-58-5	0.05
Chlornitrofen	$C_{12}H_6Cl_3NO_3$	1836-77-7	0.0001
2,4-D (2,4-PA)	$C_8H_6Cl_2O_3$	94-75-7	0.03 (0.03)
Asulam	$C_8H_{10}N_2O_4S$	3337-71-1	0.2
Terbucarb	$C_{17}H_{27}NO_2$	1918-11-2	0.02
Napropamide	$C_{17}H_{21}NO_2$	15299-99-7	0.03
Butamifos	$C_{13}H_{21}N_2O_4PS$	36335-67-8	0.01
Bensulide	$C_{14}H_{24}NO_4PS_3$	741-58-2	0.1
Pendimethalin	$C_{13}H_{19}N_3O_4$	40487-42-1	0.1 (0.02)
Mecoprop	$C_{10}H_{11}ClO_3$	7085-19-0	0.005 (0.01)
Fungicide			
Isoprothiolane	$C_{12}H_{18}O_4S_2$	50512-35-1	0.04 (0.009)
Chlorothalonil	$C_8Cl_4N_2$	1897-45-6	0.05
Iprofenfos	$C_{13}H_{21}O_3PS$	26087-47-8	0.008
Iprodione	$C_{13}H_{13}Cl_2N_3O_3$	36734-19-7	0.3
Etridiazole	$C_5H_5Cl_3N_2OS$	2593-15-9	0.004
Chloroneb	$C_8H_8Cl_2O_2$	2675-77-6	0.05
Flutolanil	$C_{17}H_{16}F_3NO_2$	66332-96-5	0.2
Pencycuron	$C_{19}H_{21}ClN_2O$	66063-05-6	0.04
Mepronil	$C_{17}H_{19}NO_2$	55814-41-0	0.1
Thiophanatemethyl	$C_{12}H_{14}N_4O_4S_2$	23564-05-8	0.3
Insecticide			
Isoxathion	$C_{13}H_{16}NO_4PS$	18854-01-8	0.008
Diazinon	$C_{12}H_{21}N_2O_3PS$	333-41-5	0.005
Fenitrothion	$C_9H_{12}NO_5PS$	122-14-5	0.003
Dichlorvos	$C_4H_7Cl_2O_4P$	62-73-7	0.008
Fenobucarb	$C_{12}H_{17}NO_2$	3766-81-2	0.03
EPN	$C_{14}H_{14}NO_4PS$	2104-64-5	0.006
Isofenphos	$C_{15}H_{24}NO_4PS$	25311-71-1	0.001
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	2921-88-2	0.03
Trichlorfon	$C_4H_8Cl_3O_4P$	52-68-6	0.03

were placed in an incubator and the *D. magna* were reared under fasting conditions. The supplied water was not changed during the study period. The LC_{50} (24 hr) and LC_{50} (48 hr) values were determined using the concentrations yielding 50% immobility of *D. magna*[6], and were calculated by recurrence straight line of concentrations and mortality rates observed after 24 hr and 48 hr exposures.

Subchronic toxicity tests

The initial chemical concentrations were determined from acute toxicity testing, and four or five chemical solutions were prepared by two-fold dilution (geometric concentration ratio of 2 with acetone). Culture water, the chemical solution , and mixed algae were added to 20 mL-flat bottom tubes to make a total volume of 15 mL.

To examine growth immobilization, an individual *D. magna* from the third to eighth broods,

and less than 24 hr after birth was placed in each tube, and reared for ten days until a first brood was produced. Lethal toxicity levels and the number of survival broods were determined after 8 days. During these tests, *D. magna* were transferred to other tubes containing fresh chemical solutions and new algae on alternate days because the chemical and algal concentrations in the original tubes decreased with time.

The LC_{50} (8 days) values were calculated by recurrence straight line of the concentrations and mortality rates observed after 8 days. Concentrations resulting in significantly prolonged days of broods were determined by t test. When broods were not observed for 13 days, the brood day period was set as 13 days. Concentrations resulting in significant reduction of broods were determined by t test. The concentrations resulting in significantly prolonged days of broods and significant reduction of brood numbers are shown as ranges or as minimum values.

Results

Acute toxicity

Neither acute nor chronic toxicity was observed in *D. magna* at the highest concentration of acetone in culture water (50 μL/15 mL) in the control group. Results of acute toxicity tests of 11 herbicides are shown in Table 2 and Figure 1. Immobilization of *D. magna* was not observed after 48 hr at maximum concentration (10 mg/L) of six herbicides: propyzamide, 2, 4-D, asulam, terbucarb, napropamide, and mecoprop. However, in the other five herbicides: thiobencarb, chlornitrofen, butamifos, bensulide, and pendimethalin, immobilization of *D. magna* was observed. The concentrations of these herbicides that immobilized half of the *D. magna* (LC_{50}) ranged from 1 to 10 mg/L (Fig. 1). Among the immobilizing herbicides, pendimethalin exhibited the strongest toxicity. In addition, its toxicity after 48 hr was greater than after 24 hr, but less than twice that at 24 hr (Table 2).

Results from the 10 fungicides tests are shown in Table 3 and Figure 2. *D. magna* did not exhibit immobilization at 48 hr in the maximum concentrations (10 mg/L) of six fungicides, isoprothiolane, chloroneb, flutolanil, pencycuron, mepronil and thiophanatemethyl. The immobilization LC_{50} (48 hr) of iprofenfos, iprodione, and etridiazole ranged from 1-10 mg/L

Table 2. Lethal concentration 50% of herbicides and their toxicity effects on broods (mg/L)

Agricultural chemicals	LC_{50}			Concentration for significantly prolonged days of broods	Concentration for significant reduction of broods
	24 hr	48 hr	8 days		
Thiobencarb	2.72	2.33	1.16	0.50-1.00	0.20
Propyzamide	10<	10<	10<	10<	10
Chlornitrofen	10<	8.69	0.13	0.10	0.10
2,4-D (2,4-PA)	10<	10<	10<	10<	10
Asulam	10<	10<	10<	10<	10<
Terbucarb	10<	10<	10<	10<	10<
Napropamide	10<	10<	10<	10<	10<
Butamifos	2.98	1.56	0.35	0.50-1.00	0.20
Bensulide	2.00	1.75	0.35	0.50-1.00	0.50
Pendimethalin	1.96	1.21	0.40	0.20-0.50	0.20-0.50
Mecoprop	10<	10<	10<	10<	10<

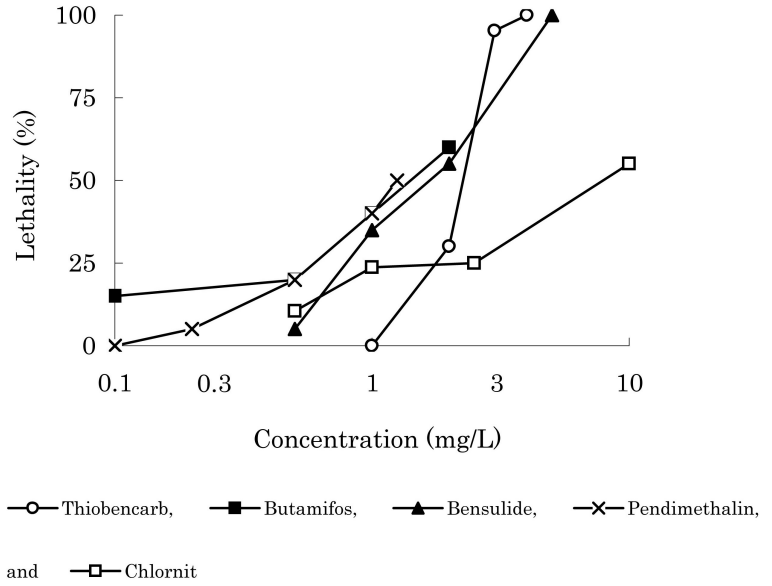

Figure 1. Acute toxicity LC_{50} (48 hr) of herbicides.

Table 3. Lethal concentration 50% of fungicides and their toxicity effects on broods (mg/L)

Agricultural chemicals	LC_{50}			Concentration for significantly prolonged days of broods	Concentration for significant reduction of broods
	24 hr	48 hr	8 days		
Isoprothiolane	10<	10<	10<	2.0	5.0-10.0
Chlorothalonil	0.17	0.13	0.16	0.08-0.20	0.20
Iprofenfos	5.81	4.20	0.50	0.50-1.00	0.50
Iprodione	4.70	3.93	5.0	5.0	1.0
Etridiazole	10<	9.15	5.0	5.0-10.0	5.0
Chloroneb	10<	10<	10<	10	10<
Flutolanil	10<	10<	10<	5.0	5.0
Pencycuron	10<	10<	0.19	0.125-0.250	0.125-0.250
Mepronil	10<	10<	5.0	5.0	2.0
Thiophanete-methyl	10<	10<	2.0	0.50	0.50

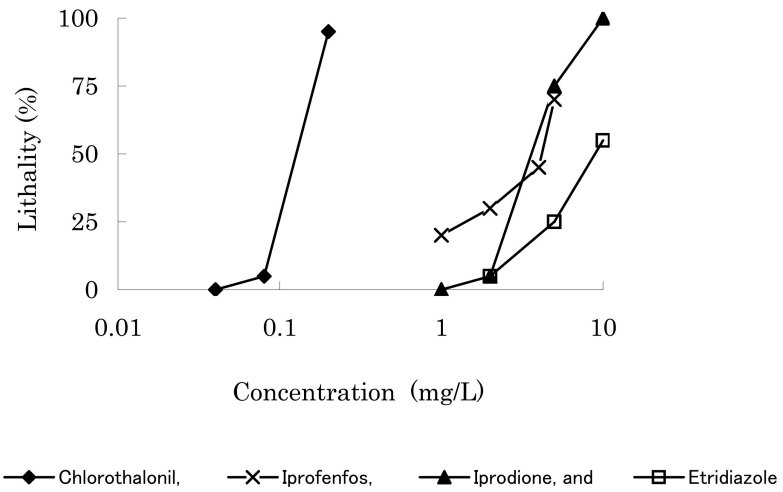

Figure 2. Acute toxicity LC_{50} (48 hr) of fungicides.

Table 4. Lethal concentration 50% of insecticides and their toxicity effects on broods (mg/L)

Agricultural chemicals	LC_{50}			Concentration for significantly prolonged days of broods	Concentration for significant reduction of broods
	24 hr	48 hr	8 days		
Isoxathion	0.0009	0.00057	0.0007	0.0004-0.0010	0.0004-0.0010
Diazinon	0.0053	0.0032	0.005	0.005-0.010	0.002
Fenitrothion	0.015	0.01	0.00050	0.0005-0.0010	0.0005-0.0010
Dichlorvos	0.00063	0.00053	0.00088	0.00050-0.00125	0.0005
Fenobucarb	0.037	0.035	0.0375	0.025-0.050	0.025-0.050
EPN	0.0028	0.0017	0.002	0.002-0.005	0.002
Isofenphos	0.0079	0.0052	0.0075	0.005-0.010	0.005-0.010
Chlorpyrifos	0.0014	0.0009	0.0006	0.0006-0.0008	0.0006
Trichlorfon	0.0019	0.0009	0.0013	0.0010	0.001-0.002

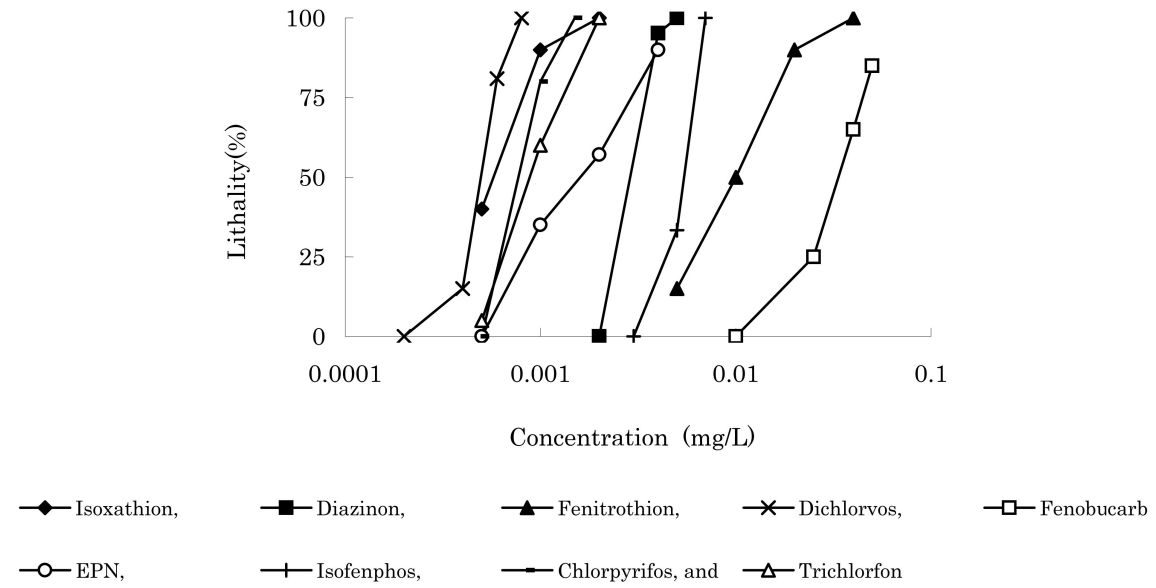

Figure 3. Acute toxicity LC_{50} (48 hr) of insectiicides.

(Fig. 2), while the chlorothalonil toxicity LC_{50} (48 hr) of 0.13 mg/L was 30-70 times stronger than those of the other immobilizing fungicides. For all immobilizing fungicides, toxicity after 48 hr was greater than after 24 hr, but less than twice that at 24 hr.

Results for the nine insecticides are shown in Table 4 and Figure 3. The LC_{50} (48 hr) values of all insecticides were lower than 0.1 mg/L. Of the insecticides, dichlorvos exhibited the strongest toxicity (0.00053 mg/L) and fenobucarb the weakest (0.035 mg/L).

Among all of the tested agricultural chemicals, the LC_{50} (48 hr) values for butamifos and trichlorfon were approximately half of the LC_{50} (24 hr) values. The LC_{50} (48 hr) values of diazinon, EPN, and chlorpyrifos, insecticides, were about 0.6 of the value at LC_{50} (24 hr).

Subchronic toxicity

The results of subchronic toxicity testing of the 11 herbicides are shown in Table 2. Significant reduction of broods by propyzamide and 2, 4-D was observed at 10 mg/L. Significant reduction of broods as the toxic effect of thiobencarb was less than about one-fifth of these at

LC_{50} (8 days). The LC_{50} (8 days) values of chlornitrofen, butamifos, bensulide, and pendimethalin were equivalent to the level showing the significant reduction of broods. These herbicides thus did not exhibit chronic toxicity. Thiobencarb, butamifos, bensulide, and pendimethalin were observed the significantly prolonged days of broods depending on the range of concentrations. Also, pendimethalin was observed the significant reduction of broods depending on the range of concentrations. The values of LC_{50} (8 days) for chronic toxicity, significantly prolonged days of broods, and significant reduction of broods were not observed at the maximum concentration (10 mg/L) of asulam, terbucarb, napropamide, and mecoprop.

The results of subchronic toxicity testing of the 10 fungicides are shown in Table 3. Significant reduction of broods was observed as a chronic toxic effect of iprodione. The other indices, LC_{50} (8 days) and significantly prolonged days of broods, except the significant reduction of broods due to the toxic effect of iprodione were not considered to have the chronic toxicity. The LC_{50} (8 days) for thiophanatemethyl was four times higher than the values for significantly prolonged days of broods and significant reduction of broods. The significantly prolonged days of broods due to the chronic toxic effect of isoprothiolane were observed at a concentration less than one-fifth of the LC_{50} (8 days). The significant reduction of broods due to the chronic toxic effect of iprodione was observed at a concentration less than one-fifth of the LC_{50} (8 days). Therefore, it was considered that iprodione significantly reduced the number of broods. Significantly prolonged days of broods was observed with chloroneb at 10 mg/L, but other indexes of LC_{50} (8 days), significantly prolonged days of broods, and significant reduction of broods were not found at the maximum concentration (10 mg/L).

The results of subchronic toxicity testing of the nine insecticides are shown in Table 4. The LC_{50} (8 days) values were notably lower than those observed for of the tested herbicides and fungicides. For all insecticides, LC_{50} (8 days) values and results of brood number and brood duration tests were similar. None of the insecticides exhibited chronic toxicity.

The LC_{50} (8 days) values in the subchronic toxicity tests were less than one half of the LC_{50} (48 hr) in the acute toxicity tests for four of the five acutely toxic herbicides (thiobencarb, butamifos, bensulide, and pendimethalin) and three of the seven acutely toxic fungicides (iprofenfos, mepronil, and thiophanatemethyl) in Table 3. The LC_{50} (8 days) for chlornitrofen, pencycuron, and fenitrothion (Table 4) were less than one-tenth of those at LC_{50} (48 hr).

Discussion

Since death in *D. magna* is not easily confirmed, immobilization of *D. magna* was used as an indicator of death in this study. The LC_{50} (48 hr) values in the acute toxicity tests were always lower than those for LC_{50} (24 hr) for all agricultural chemicals tested, indicating that using immobilization as a toxicity criterion was an effective indicator of fatal injury, and was analogous to death in *D. magna*. Previously reported LC_{50} (24 hr) values for thiobencarb[9] and chlorpyrifos[10] and LC_{50} (48 hr) for chlorothalonil[5], isoxathion, dichlorvos, fenobucarb, isofenfos, and trichlorfon[7] were comparable to those obtained in this study. However, the LC_{50} (24 hr) for diazinon in this study was 0.0053 mg/L, about 10 times higher than those reported in other studies[6,11]. Similarly, the LC_{50} (48 hr) value for fenitrothion, an insecticide, in this study was more than about 10 times those reported by Kikuchi et al[7] and Hosokawa et al[6], and was one-fifth of the value reported by Hatakeyama et al[12]. All agricultural chemicals examined in this

study were freshly dissolved in acetone immediately prior to testing and LC_{50} values were similar in repeated experiments (data not shown). The differences between the previously reported values and those determined in this study may be due to the differences in chemical lots or manufacturers.

Since the ratio of LC_{50} (48 hr) to LC_{50} (24 hr) for all chemicals was approximately 2:1, it appears to be difficult to detect chemicals manifesting toxic effects slowly by comparison of lethality at 24 hr and 48 hr. Of the agricultural chemicals, insecticides exhibited the strongest acute toxicity, LC_{50} (24 hr and 48 hr), 0.00053-0.037 mg/L, and herbicides the weakest, LC_{50} (24 hr and 48 hr), 1.21-10 mg/L. These differences agree with results previously reported by many researchers[5-7,9-12]. Significant differences in toxicity of the tested chemicals may be related to the numbers of P, S and Cl in the composition of each agricultural chemicals (see Table 1); however, that relationship was not assessed in this study. In our study, acute toxicity in *D. magna* was not been reported for eight herbicides: propyzamide, asulam, terbucarb, napropamide, butamifos, bensulide, pendimethalin, and mecoprop, seven fungicides: iprodione, etridiazole, chloroneb, flutolanil, pencycuron, mepronil, and thiophanatemethyl.

Chronic toxicity testing of agricultural chemicals is required to assess their long-term effects on the environment. However, there have been few studies of chronic toxicity of agricultural chemicals on *D. magna*, and the number of agricultural chemicals that have been tested is limited[10,11,14]. The LC_{50} (8 days) values of all the herbicides and fungicides, except chlorothalonil and iprodione, were much lower than the LC_{50} (24 hr) or LC_{50} (48 hr) values. However, the LC_{50} (8 days) values of all insecticides, except fenitrothion, were almost the same as those of LC_{50} (24 hr) or LC_{50} (48 hr). These findings indicate that long term environmental contamination by herbicides and fungicides might exert more serious effects than insecticides if water quality standards are produced solely on the basis of acute toxicity. The significant reduction of broods caused by thiobencarb and iprodione occurred at one-fifth, or less, the LC_{50} (8 days) value. In addition, in thiophanatemethyl, a fungicide, the significant reduction of broods caused by thiophanatemethyl was one-fourth of the LC_{50} (8 days) value. Thus, these chemicals appear to produce specific types of chronic toxicity. The LC_{50} (8 days) values in toxicity tests using insecticides, all of which prolonged days for broods and reduced for broods were markedly lower than those in herbicides and fungicides.

Chlornitrofen, pencycuron, and fenitrothion exhibited stronger chronic toxicity in the LC_{50} (8 days) test than in the acute toxicity LC_{50} (48 hr) test. These results suggest that these chemicals may have slow-acting toxicity. Naddy et al[13] reported that 7 days survival with short term chlorpyrifos exposure (12 hr pulse of 0.5 μg/L or 6 hr pulse of 1.0 μg/L) was lower with feeding than with fasting. There are no known reports suggesting a mechanism for such slow-acting toxicity. Acute/chronic toxicity ratios may play an important role in the development of water quality standards. Sanchez et al[11] and Ferrando et al[14] reported that it is quite possible to observe chronic effects on invertebrate orgamisms, growth inhibition or decrease in reproductive capacity at much lower levels in the acute toxicity test for fenitrothion or diazinon in *D. magna*. In our study, insecticides exhibited stronger acute toxicity than herbicides and fungicides. With the exception of fenitrothion, subchronic testing of insecticides did not reveal significantly stronger 8 days toxicity than that noted in the acute tests. These findings indicate that subchronic testing is as important as acute testing to estimate the effects of agricultural

chemicals on ecological systems.

References

1. WHO. Guidelines for drinking-water quality. 2nd ed. vol. 2. 1996;538-611.
2. OECD. OECD Guidelines for Testing of chemicals: *Daphnia* sp., Acute Immobilisation Test and Reproduction Test. 1984;202:1-12.
3. OECD. OECD Guidelines for Testing of chemicals: *Daphnia magna* Reproduction Test. 1998;211:1-21.
4. Bodar CWM, Van Leeuwen CJ, Voogt PA, Zandee DI. Effect of cadmium on the reproduction strategy of *Daphnia magna*. Aquatic Toxicol 1988;12:301-310.
5. Ernst W, Doe K, Jonah P, Young J, Julien G, Hennigar P. The toxicity of chlorothalonil to aquatic fauna and the impact of its operational use on a pond ecosystem. Arch Environ Contam Toxicol 1991;21:1-9.
6. Hosokawa M, Endo G, Kuroda K. Acute toxic effect of River Yodo water (Japan) on *Daphnia magna*. Bull Environ Contam Toxicol 1995;55:419-425.
7. Kikuchi M, Sasaki Y, Wakabayashi M. Screening of organophosphate insecticide pollution in water by using *Daphnia magna*. Ecotoxicol Environ Saf 2000;47:239-245.
8. Yoshimura H, Endoh YS. Acute toxicity to freshwater organisms of antiparasitic drugs for veterinary use. Environ Toxicol 2005;20:60-66.
9. Sancho E, Sánchez M, Ferrando MD, Andreu-Moliner E. Effects of thiobencarb herbicide to an alga (*Nannochloris oculata*) and the cladoceran (*Daphnia magna*). J Environ Sci Health B 2001;36:55-65.
10. Kersting K, Van Wijngaarden R. Effects of chlorpyrifos on a microecosystem. Environ Toxicol Chem 1992; 11:365-372.
11. Sánchez M, Ferrando MD, Sancho E, Andreu-Moliner E. Evaluation of a *Daphnia magna* renewal life cycle test method with diazinon. J Environ Sci Health B 1998;33:785-797.
12. Hatakeyama S, Sugaya Y. A freshwater shrimp (*Paratya compressa improvisa*) as a sensitive test organism to pesticides. Environ Pollut 1989;59:325-336.
13. Naddy RB, Klaine SJ. Effect of pulse frequency and interval on the toxicity of chlorpyrifos to *Daphnia magna*. Chemosphere 2001;45:497-506.
14. Ferrando MD, Sancho E, Andreu-Moliner E. Chronic toxicity of fenitrothion to an algae (*Nannochloris oculata*), a rotifer (*Brachionus calyciflorus*), and the cladoceran (*Daphnia magna*). Ecotoxicol Environ Saf 1996;35:112-120.
15. The Ministry of Health, Labour and Welfare, Japan. Paragraph 101 of public welfare labor ministerial ordinance. 2003.