Acute toxic tests of rainwater samples using *Daphnia magna*

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Abstract

Rainwater samples were collected at Isogo Ward of Yokohama City, Japan, from 23 June to 31 July 2003. The toxic potency of pollutants present in 13 rainwater samples was tested using *Daphnia magna*. Most test animals died within 48 h in five test solutions that were prepared from rainwater samples. On the other hand, when nonpolar compounds such as pesticides were removed from rainwater samples before the toxic tests, mortalities in all test solutions were less than 10%. Eight kinds of pesticides were detected in rainwater samples. The highest concentration was of dichlorvos, at $0.74\,\mu g/L$. Results indicated that insecticides in rainwater sometimes lethally affected *D. magna* and that toxic potency of insecticides that are present in rainwater constitutes an important problem for environmental protection.
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Keywords: Rainwater; *Daphnia magna*; Toxic test; Mortality; Immobile; Pesticide; Dichlorvos

1. Introduction

Various pesticides are allowed for use in agricultural and public health purposes in Japan. Some of them are volatilized into air if their vapor pressures are relatively high (Bedos et al., 2002; Hochstedler et al., 2000; Suzuki et al., 2003; Van Dijk and Guicherit, 1999; Woodrow et al., 2001). Subsequently, these gaseous pesticides can be deposited with rain, snow, or particles after atmospheric transportation (Dubus et al., 2000; Grynkiewicz et al., 2001; Hamers et al., 2001; Majewski et al., 2000). For example, in Japan, six pesticides (dichlorvos, fenitrothion, chlorothalonil, etc.) were detected in rainwater sampled at Isogo Ward of Yokohama City in 2001–2002; the highest concentration was $0.33\,\mu g/L$ of dichlorvos (Sakai, 2003). Furthermore, Suzuki et al. (2003) studied pesticide contamination of rainwater in Utsunomiya, Japan. In their study, the annual deposition amount of fenitrothion was $546\,\mu g/m^2$, the highest value among 15 pesticides.

Pesticides are biologically active compounds that can harm animals and plants. In the case of river water contaminated by pesticides, toxic potency has often been examined using plankton (Hatakeyama, 1998; Hosokawa et al., 1995; Kikuchi et al., 2000; Sakai, 2002b). Rainwater often contained some amounts of pesticides. However, a few studies have addressed harmful effects in rainwater compared with those in river water. In a previous study (Sakai, 2002a), the 21-day reproduction test using *Daphnia magna* Straus (OECD, 1998) was applied to only two rainwater samples for examination of the toxicity. At that time, all animals died before reproduction in the test solution produced from rainwater collected on 14–15 June 2001. Therefore, this study investigated toxic effects of 13 rainwater samples using 48-h acute toxic tests with *D. magna*.

In addition, rainwater samples without nonpolar compounds, such as pesticides, were prepared using solid-phase extraction cartridges. Those samples were also applied to acute toxic tests. It was suggested that nonpolar compounds might contribute to harmful effects of rainwater if test animal mortalities were decreased after removal of nonpolar compounds.

Moreover, 54 pesticides in rainwater samples were determined using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame photometric detection (GC-FPD), similarly to a method used in a previous experiment (Sakai, 2003). Thereupon, pesticide concentrations were compared to results of acute toxic tests. The contribution of pesticides to harmful effects was discussed.

2. Materials and methods

2.1. Sample collection

Rainwater samples were collected using six glass dishes (i.d. 240–250 mm, depth 60–70 mm) on the roof of the Yokohama Environmental Research Institute (35°25′N, 139°37′E) located in Isogo Ward of Yokohama City from 23 June to 31 July 2003. That rainwater sample was poured into 1-L glass bottles when rain ceased or glass dishes were filled with rainwater. Samples were stored at 4 °C in a refrigerator and were applied for acute toxic tests within two days after sampling.

2.2. Test animal

D. magna (clone A), which were provided by the National Institute for Environmental Studies, Japan, were used as the test animal. Cultures were stored in reconstituted water (Elendt M7) (Elendt, 1990; OECD, 1998) with *Chlorella vulgaris* as a food source at 20 °C with a 16 h light and 8 h dark cycle (Sakai, 2002a).

2.3. Acute toxicity test

Acute toxic tests with *D. magna* were performed according to the OECD method (OECD, 1984). Average concentrations of calcium, magnesium, potassium, and sodium in rainwater in Yokohama were 0.28, 0.15, 0.05, and 1.3 mg/L, respectively (Kato and Umeda, 2004). Mineral concentrations in rainwater were very low. Therefore, appropriate amounts of minerals were added to rainwater and pH was adjusted to 6.5–6.8 before acute toxic testing. Amounts of minerals added to 400 mL of rainwater were as follows: calcium chloride dehydrate (118 mg), magnesium sulfate heptahydrate (49 mg), potassium chloride (2.3 mg), sodium hydrogen carbonate (26 mg), sodium metasilicate nonahydrate (4.0 mg), sodium nitrate (0.11 mg), potassium phosphate monobasic (0.057 mg), potassium phosphate dibasic (0.074 mg), and 20 mL of a stock solution of combined trace elements for Elendt M7 (Elendt, 1990; OECD, 1998). After adding of minerals, I measured pH using a pH meter (HM-16S; DKK-TOA Corp., Japan); it was adjusted to 6.5–6.8 with dilute hydrochloric acid

solution. Then its volume was adjusted to 450 mL with distilled water. This solution was poured into four vessels (each containing 100 mL of this solution). Excess solution (about 50 mL) was discarded. We divided 20 progenies of *D. magna* (less than 24 h old) into four groups of five progenies each. They were put into the vessels (each containing five progenies). These vessels were set in an incubator at 20 °C in a 16 h light–8 h dark cycle. Moreover, four control vessels (each containing 100 mL of Elendt M7 medium with five progenies) were prepared. They were set in the incubator along with testing samples. No foods were given during the acute toxic test. Surviving and mobile animals were counted after 24 h and again after 48 h.

Acute toxicity tests of diluted samples were conducted for rainwater collected on 23 June 2003. Initially, rainwater was diluted with the same volume of distilled water. Various minerals were added and pH was adjusted as-mentioned above. Then, acute toxic tests with *D. magna* were conducted for the diluted rainwater sample and for rainwater samples.

Acute toxic tests of rainwater without nonpolar compounds were performed as follows. The solid-phase extraction cartridge (Sep-pak PS-2; Waters Corp., USA) was washed with 5 mL of acetone and 100 mL of distilled water. Then an appropriate volume (400–500 mL) of rainwater was passed through the washed solid-phase extraction cartridge to remove nonpolar compounds from rainwater. After passage through the cartridge, various minerals were added to 400 mL of this solution; its pH was adjusted to 6.5–6.8 as-described above. Then its volume was adjusted to 450 mL and four vessels (each containing 100 mL of the testing solution with five progenies) were prepared for acute toxic tests in the same manner as rainwater samples. These were put into the incubator; the animals were observed.

2.4. Determination of pesticides

Pesticides used for identifications and determinations were purchased from Wako Pure Chemical Industries, Ltd. (Japan), Kanto Kagaku (Japan), and GL Sciences Co. Ltd. (Japan). Acetone was pesticide residue grade purchased from Wako Pure Chemical Industries, Ltd. (Japan) and Kanto Kagaku (Japan). The determination method for pesticides was almost identical to that of a previous experiment (Sakai, 2003). Solid-phase extraction cartridges (Sep-pak PS-2; Waters Corp., USA) were washed with 5 mL of acetone and 20 mL of distilled water. After washing, pesticides in the rainwater were extracted by the cartridge and pesticides were eluted with acetone. GC-MS (5973N; Agilent Technologies Inc., USA) and GC-FPD (6890N; Agilent Technologies Inc., USA) were used for determination. Quantitation limits of respective pesticides were set at 0.05 μg/L.

Determined pesticides were organophosphorus insecticides (dichlorvos, diazinon, disulfoton, fenitrothion, malathion, chlorpyrifos, fenthion, isoxathion, pyridaphenthion, EPN, parathion, methyl-parathion, phenthoate, prothiofos); chlorinated hydrocarbon insecticides (α-BHC, γ-BHC, α-endosulfan, β-endosulfan); carbamate insecticide (fenobucarb) and break down products (fenitrothion oxon, malaoxon); herbicides (molinate, trifluralin, simazine, swep, atrazine, propyzamide, bromobutide, terbucarb, simetryn, esprocarb, thiobencarb, pendimethalin, methyldymron, butachlor, butamifos, pretilachlor, oxadiazon, nitrofen, chlornitrofen, mefenacet, napropamide); and fungicides (chloroneb, pencycuron, PCNB, chlorothalonil, iprobenfos, tolclofosmethyl, fthalide, flutolanil, isoprothiolane, mepronil, and EDDP).

2.5. Rainfall data

The Yokohama Local Meteorological Observatory, which was located 4 km away from this Institute, provided rainfall data.

3. Results

3.1. Acute toxic test

Rainfall data for 23 June–31 July 2003 are shown in Fig. 1. Rainfall was high in 2003: total rainfall during that period was 281 mm and the average rainfall (1971–2000) for 23 June–31 July was 197 mm. Toxic tests were conducted for 13 rainwater samples indicated in Fig. 1.

Most animals were alive in the control solution: mortalities were less than 10% in control at the end of the test (guideline value of this test, OECD, 1984). Most animals were dead within 48 h in five test solutions prepared from rainwater samples (Table 1). The mortality of animals in the test solution prepared from rainwater sampled on 7 July reached 15% after 48 h (five animals in each of the four vessels initially; surviving animals were five, five, four, and three, respectively), which was slightly higher than the control value.

Percentages of immobilized animals are also listed in Table 1. They were higher than 10% in six test solutions (prepared from rainwater sampled on 23 June, 1 July, 6 July, 7 July, 25 July, and 29 July) after 48 h.

In addition, the mortality in the diluted rainwater sample (23 June 2003) became 70% after 24 h and 95% after 48 h.

On the other hand, when nonpolar compounds were removed from rainwater samples using a solid-phase extraction cartridge before acute toxic tests, most animals were alive and mobile in every test solution after 48 h (Table 1).

3.2. Determination of pesticides

Detected pesticides are indicated in Table 2 along with others that were not more than the detection limit (0.05 μg/L). Dichlorvos was the most frequently detected insecticide (69%); its highest concentration was 0.74 μg/L (23 June 2003). Guideline value of dichlorvos in rainwater was not specified in Japan. When determined concentrations of dichlorvos in rainwater samples were compared with the guideline value in river specified by Japan Ministry of the Environment (8 μg/L), all determined concentrations were less than this guideline value. Fenitrothion was the second most frequently detected insecticide; its highest concentration was 0.33 μg/L. Chlorothalonil was detected in three rainwater samples; its highest concentration was 0.14 μg/L. Moreover, malathion, fenitrothion oxon, fthalide, fenobucarb, and pyridaphenthion were detected in rainwater samples. However, their detection frequencies were less than 20% and their highest concentrations were less than 0.15 μg/L. Although herbicides (molinate, thiobencarb, simetryn, etc.) had often been detected in river water in Japan (Hatakeyama, 1998; Sakai, 2002b), no herbicides were detected from rainwater samples in this study.

4. Discussion

Previously, toxic potencies of two rainwater samples were tested using the reproduction test and one of two samples had a lethal effect (Sakai, 2002a). Rainwater with toxic potency is apparently not rare: 5 of 13 rainwater samples indicated apparent lethal effect on *D. magna* in this investigation. Additionally, the toxic potency of rainwater collected on 23 June 2003 did not disappear when rainwater was diluted with the same volume of distilled water before the test. This result indicated the possibility of a harmful effect of rainwater
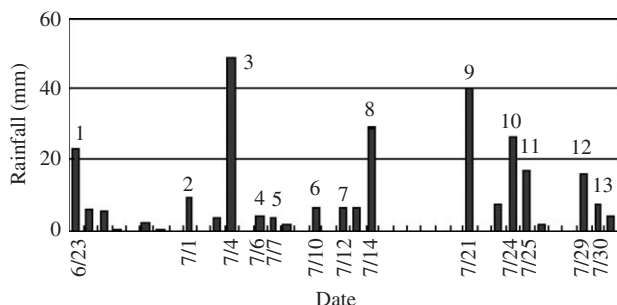

Fig. 1. Rainfall data and samples for the acute toxic test. The Yokohama Local Meteorological Observatory provided rainfall data. Acute toxic tests and pesticides determinations were conducted for the 13 samples indicated above.

Table 1
Mortalities and percentages of immobilized animals for testing solutions prepared from rainwater samples

No.	Date	Rainwater samples				Rainwater samples without non-polar compounds			
		24 h		48 h		24 h		48 h	
		Mortality	% immob.	Mortality	% immob.	Mortality	% immob.	Mortality	% immob.
1	6/23	90	100	100	100	0	0	0	0
1′[a]	6/23 (0.5)	70	100	95	100	0	0	0	0
2	7/1	0	0	70	90	0	0	0	0
3	7/4	0	0	0	0	0	0	0	0
4	7/6	95	100	95	100	0	10	5	10
5	7/7	10	15	15	15	0	0	0	0
6	7/10	0	0	0	0	0	0	0	0
7	7/12	0	0	0	0	0	0	0	0
8	7/14	0	0	0	0	0	0	0	0
9	7/21	0	0	0	0	0	0	0	0
10	7/24	0	0	0	0	0	0	0	0
11	7/25	0	0	90	100	0	0	0	0
12	7/29	5	5	100	100	0	0	0	0
13	7/30	0	0	0	0	0	0	0	0

Note: Mortalities and immobilization for controls were less than 10% in every case.
[a]6/23(0.5), rainwater collected on 23 June, 2003 was diluted to 1/2.

Table 2
Determined concentrations of pesticides in rainwater samples

No.	Sampling date	Concentrations of pesticides (µg/l)							
		DDVP	MEP	TPN	MAL	MEPOX	PYR	BPMC	FTH
1	6/23	0.74	0.33	0.14	0.06	0.05	0.05	N.D.	N.D.
2	7/1	0.16	0.06	0.10	N.D.	0.06	N.D.	N.D.	N.D.
3	7/4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4	7/6	0.36	0.06	N.D.	0.05	N.D.	N.D.	N.D.	N.D.
5	7/7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6	7/10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7	7/12	0.08	0.05	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8	7/14	0.07	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
9	7/21	0.08	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10	7/24	0.09	0.07	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
11	7/25	0.26	0.25	0.14	N.D.	N.D.	N.D.	0.14	0.07
12	7/29	0.23	0.14	N.D.	N.D.	N.D.	N.D.	N.D.	0.06
13	7/30	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Note: DDVP, dichlorvos; MEP, fenitrothion; TPN, chlorothalonil; MAL, malathion; MEPOX, fenitrothion oxon, PYR, pyridaphenthion; BPMC, fenobucarb; FTH, fthalide; N.D., not detected.

in ponds, rivers and seas even though rainwater is diluted many times over in such environments.

Harmful effects on *D. magna* by rainwater samples disappeared when nonpolar compounds were removed from rainwater samples with a solid-phase extraction cartridge (Sep-pak PS-2; Waters Corp.). This fact suggested that harmful effects were related to nonpolar compounds that were adsorbed by the solid-phase extraction cartridge.

Rainwater contained many nonpolar compounds such as pesticides and aromatic hydrocarbons. In general, *D. magna* was more sensitive to pesticides than aromatic hydrocarbons (Kühn et al., 1989). Especially,

organophosphorus insecticides lethally affected zooplankton during a short exposure period even if their concentrations were much less than 1 mg/L (Ferrando et al., 1996; Kikuchi et al., 2000; Kühn et al., 1989; Sanchez et al., 1998). Therefore, GC-MS and GC-FPD determined 54 pesticides in rainwater samples, but several pesticides (carbaryl, siduron, etc.) were indeterminate by this method.

Eight kinds of pesticides were detected as a result of this determination. Dichlorvos was the most frequently detected (69% of samples); its highest concentration was 0.74 µg/L (23 June). Suzuki et al. reported that pesticide concentrations in rainwater in Utsunomiya were related

to their vapor pressures (Suzuki et al., 2003). In the case of dichlorvos, its vapor pressure was $1.6\,Pa$ ($20\,^{\circ}C$) (WHO, 1989), the highest level among the 54 pesticides. Additionally, annual amount of dichlorvos used is considered to be $3350\,kg$ in Yokohama (area: $435\,km^2$) from PRTR data provided by the Kanagawa Environmental Research Center (Kanagawa Environmental Research Center, 2004), because it is effective against flies, aphids, mites, caterpillars, and other harmful insects in greenhouses, fruit and vegetable farms, and buildings. Thus, dichlorvos was frequently detected in air at the Isogo Ward of Yokohama City (N.D.$-4\,ng/m^3$; Sakai, 2004) and it was detected with high frequency in rainwater. Fenitrothion was the second most frequently agent detected in rainwater. It is also used popularly in Yokohama. Its annual amount of use is considered to $2690\,kg$ from PRTR data (Kanagawa Environmental Research Center, 2004). Atrazine was not detected at all in this investigation, but it is detected frequently in rainwater studies of other countries (Hamers et al., 2001; Hochstedler et al., 2000; Majewski et al., 2000). Atrazine is not used widely. The annual amount of its use is considered to be less than $100\,kg$ in Yokohama (Kanagawa Environmental Research Center, 2004). This fact might engender the low concentrations of atrazine in rainwater in this study.

After determination of pesticides, their concentrations were compared with the acute toxic test results. For concentrations of dichlorvos that were more than $0.20\,\mu g/L$, most test animals in those solutions were dead or immobile within $48\,h$. In addition, diluted rainwater samples (23 June 2003) lethally affected *D. magna*; the concentration was greater than $0.20\,\mu g/L$. These results were consistent with 48 h-EC50 that was reported at $0.20\,\mu g/L$ (Kikuchi et al., 2000). Most animals remained alive and mobile in the solution except for those in one sample prepared from rainwater on 7 July, for which no pesticides were detected in rainwater. It remains unclear why mortality in this sample was slightly higher than that in the control sample even though no pesticides were detected in the rainwater.

The results described above indicate that rainwater samples at Isogo Ward of Yokohama City often contain nonpolar toxic compounds such as insecticides and that they lethally affect *D. magna* in acute toxicity tests.

5. Conclusion

Toxic effects of rainwater were demonstrated in acute toxicity tests with *D. magna*. In addition, even though rainwater (23 June 2003) was diluted to 1/2 concentration before the test, the toxic effect did not disappear. On the other hand, the toxic effect disappeared after removal of nonpolar compounds, such as pesticides,

from rainwater. Eight kinds of pesticides were detected when pesticides in rainwater samples were determined. Concentrations of dichlorvos, an organophosphorus insecticide, were often more than 48 h-EC50. Its concentrations in rainwater samples were related to the mortality at the acute toxic tests. Therefore, insecticide contamination of rainwater is inferred to be an important problem for environmental protection.

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