

Effect of some pesticides on reproduction of rotifer *Brachionus plicatilis* Müller

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Abstract

Pesticides have been major contributors to environmental pollution and they are now widely distributed in aquatic environments. Zooplankters are frequently used as test animals to detect aquatic contaminants because of their sensitivity and ecological importance. We investigated the effect of a 7-day exposure to four commonly used pesticides (diazinon, fenitrothion, methoprene and isoprothiolane) on reproduction of the rotifer *Brachionus plicatilis*. Pesticide concentrations of 3–7 times lower than the 24-h 50% lethal concentration (24-h LC₅₀) were tested to determine the ‘no observed effect’ concentration (NOEC), ‘lowest observed effect’ concentration (LOEC), and the ‘50% effective’ concentration (EC₅₀) on specific growth rate (*r*), sexual reproduction, fertilization, resting egg production, and hatchability of resting eggs. Results showed that the lowest EC₅₀ value of *r*, mixis, fertilization, and resting egg production of 1.4 μM for diazinon was 63 times lower than its 24-h LC₅₀ of 88.4 μM, while for fenitrothion it was 66 times (3.5 and 229.8 μM, respectively). For isoprothiolane, the lowest EC₅₀ value of *r*, mixis, fertilization, and resting egg production of 8.9 μM was 25 times lower than its 24-h LC₅₀ of 220.7 μM, while for methoprene was 37 times (2.7 and 100.8 μM, respectively). In all pesticides, hatching rate of resting eggs consistently gave the lowest EC₅₀ values which is about 2–40 times lower than the lowest EC₅₀ of *r*, mixis, fertilization, and resting egg production. Hatchability of resting eggs therefore is the most sensitive parameter in detecting effects of pesticides exposure in rotifer *B. plicatilis*.

Introduction

The aquatic environment is often the final depository of most chemical contaminants. Among them, pesticides pose some of the most serious ecological problems because of their toxicity to both target and non-target organisms and their wide distribution. Some pesticides (e.g. atrazine, chlordane) are regarded as endocrine disruptors whereas others are persistent in the environment and bioaccumulate in food webs (e.g. organochlorine, carbamate). Several studies have shown that pesticides such as diazinon and fenitrothion have a severe impact in aquatic organisms (Ferrando et al., 1996; Sánchez et al., 1999; Marcial et al., 2002).

Zooplankton are frequently used to detect anthropogenic contamination because of their sensitivity to various toxicants and their important role in the ecosystem. Among the zooplankton species, rotifers are favored test animals because of their short generation time, ease of culture, and the commercial availability of their resting eggs (Snell & Janssen, 1995). In ecotoxicological studies using rotifers, end-points such as 24- or 48-h LC₅₀, swimming speed, filtration rate and enzyme activity have been used (reviewed by Snell & Janssen (1995)). Because only a portion of the rotifer life cycle is investigated in these studies, the true vulnerability of rotifer life cycles to toxicants is often underestimated (Preston & Snell, 2001). Hence,

several studies assessed the effects of pesticides, heavy metals, and endocrine disrupting chemicals on the entire life cycle of rotifers (Snell & Carmona, 1995; Preston et al., 2000; Preston & Snell, 2001; Radix et al., 2002). Results showed that sexual reproduction and resting egg production are among the most sensitive endpoints. All of the above-mentioned studies, however, investigate only up to the production of resting eggs. No study has examined the effects of toxicants on the viability of resting eggs produced by rotifers exposed to chemical contaminants. This study aimed to (1) determine the acute and chronic effects of some commonly used pesticides on the life history of the rotifer *Brachionus plicatilis*, and (2) determine the most susceptible endpoint of pesticide exposure.

Materials and methods

Test animals

B. plicatilis is a monogonont rotifer that reproduces via cyclical parthenogenesis, incorporating both asexual (amictic) and sexual (mictic) reproduction (Snell & Carmona, 1995). Most of the time, amictic females produce amictic eggs mitotically. However, upon receiving certain environmental stimuli, amictic females may produce both amictic and mictic daughters. The mictic females then produce haploid eggs that are smaller than the amictic eggs and, if fertilized, develop into resting eggs. If these eggs remain unfertilized, they develop into males.

B. plicatilis NH1L strain (Hagiwara et al., 1993), which originated from the University of Tokyo and has been cultured in our laboratory for 15 years, was used in this study. This strain has the highest mixis rate among the *B. plicatilis* stocks maintained in our laboratory. Stock cultures were maintained in diluted seawater at 22‰, stored in 25 ± 1 °C, and fed *Nannochloropsis oculata*. To obtain rotifers of similar age for the acute toxicity test, test animals were hatched from resting eggs. For the chronic toxicity test, amictic females were obtained by shaking egg-bearing females in a screw-capped bottle, and hatching the eggs in a 15 ml petri dish containing 10 ml of food solution. Amictic females were selected based on the morphology of their eggs (Hagiwara et al., 1988).

Test chemicals

Four pesticides were investigated: diazinon ($C_{12}H_{21}N_2O_3PS$), isoprothiolane ($C_{12}H_{18}O_4S_2$), fenitrothion ($C_9H_{12}NO_5PS$) and methoprene ($C_{19}H_{34}O_3$). Diazinon and fenitrothion are organophosphate insecticides known to inhibit acetylcholinesterase activity (Ecobichon & Joy, 1994), while methoprene is a juvenile hormone analogue known to mimic the action of juvenile hormones by disrupting the developmental processes of insects (Sehnal, 1983). These pesticides were purchased from WAKO Pure Chemical Industries Ltd., Japan. They were dissolved first in 100% dimethyl sulfoxide (DMSO), then in ultra-pure grade distilled water with the final test solution containing not more than 0.01% DMSO. Stock solutions and 10% DMSO were stored at 4 °C. Test solutions were prepared by the addition of appropriate aliquots of aqueous stock solution to filtered (Millipore 0.45 μ m) and autoclave-sterilized seawater diluted to 22‰.

Acute toxicity test

Using 24-h old rotifers hatched from the resting eggs, standard acute toxicity tests (ASTM, 1998) were carried out for each pesticide with seven concentrations each. Twenty rotifers were transferred to individual wells of a 6-well polystyrene plate, each containing 10 ml of a test solution, or a control solution (filter-sterilized seawater). The plates were incubated at 25 °C in darkness. Because of the short duration of the tests, the animals were not fed during the experiment. After 24 h, the rotifers were observed under the stereomicroscope. Rotifers were considered dead if no movement of the cilia and mastax was observed over a period of 30 s. The dead and alive rotifers were counted.

Chronic toxicity test

Range-finding tests consisting of five concentrations were conducted for 48 h. The concentration which did not result in mortality, was chosen to be the highest concentration in the definitive test. Final test concentrations were: diazinon (0.03, 0.3, 3.3, 16.4 and 32.8 μ M), fenitrothion (0.04, 0.4, 3.6, 9.0 and 18.0 μ M), isoprothiolane (0.3, 3.4, 17.2, 34.4 and 68.8 μ M), and methoprene (0.03, 0.3, 3.0, 8.0

and 16.0 μM). The lowest concentration of each chemical corresponds to 0.01 mg l^{-1} . Three replicates were carried out for each treatment. A solvent control containing 0.01% DMSO was also tested. In each treatment, 10 amictic females bearing one egg were added in a 20 ml screw-capped bottles containing 10 ml of 7×10^6 cells ml^{-1} *N. oculata* and toxicant solution. After the addition of rotifers, the bottles were incubated at 25 °C in darkness for 6 days. From days 2–6, the bottles were emptied everyday into a glass petri dish and the number of non-ovigerous females, ovigerous amictic females, ovigerous mictic females, and fertilized mictic females were counted (Hagiwara et al., 1988). After counting, the rotifers were returned to the bottles. About half of the culture water was changed every day using pre-conditioned culture media (water from a culture where rotifers had been raised) with 5×10^6 cells ml^{-1} *N. oculata* and test pesticides. Pre-conditioned culture media was used because it is known to induce mixis (Carmona et al., 1993). On day 7, all resting egg-bearing females and resting eggs were collected and transferred to a new bottle containing diluted sterilized-seawater (22%) and stored at 25° C in darkness. After 3 weeks, the period of obligated diapause (Hagiwara & Hino, 1989), the resting eggs were induced to hatch by placing them in a glass petri dish with 10 ml of diluted sterilized seawater (22%) and exposed to continuous light (4000–5000 lux) at 25 °C. After 48 h, the hatched and unhatched resting eggs were counted. The observation of possible hatching was continued until day 4.

Statistical analysis

The 24-h LC_{50} was calculated using Probit Analysis (Minitab, Ver. 13). For the chronic toxicity test, specific growth rate (r) was calculated for each bottle according to: $r = (\ln N_t - \ln N_0)/t$, where N_t = number of female rotifers in the bottle on t , N_0 = initial number of rotifers in each bottle (10), and t = 2, 3, 4, 5, or 6 days. One-way analysis of variance (ANOVA), with concentration as the independent variable and r , percent mixis, or percent fertilization as the dependent variable, followed by Dunnett's test was conducted for pairwise comparisons of each pesticide concentration relative to the control (Zar, 1999). From these results, NOEC, and LOEC were determined. In

addition, the concentration of the toxicant that reduces the test parameter to 50% (EC_{50}) was calculated using regression analysis (Stephan & Rogers, 1985). Regression lines were calculated for mean r , percent mixis, percent fertilization, total number of resting eggs produced, and percent hatching against log toxicant concentration per treatment. The total number of resting eggs produced at different pesticide concentrations were compared to the control using Dunnett's test and the percent hatching using Chi-square contingency test. A p value of 0.05 or less was regarded as being significant for all tests.

Results

Results from the 24-h acute toxicity tests showed that diazinon was the most toxic among the pesticides tested, having the lowest 24-h LC_{50} value. The second most toxic was followed by methoprene (Table 1). In contrast, DMSO concentrations as high as 640 μM were not toxic to *B. plicatilis* (Table 1). In the chronic toxicity test, 0.01% DMSO (solvent control) was tested in addition to the negative control (seawater only) only in the diazinon experiment. This was because results showed that in all parameters it was not significantly different from the negative control (Fig. 3).

Daily NOEC, LOEC and EC_{50} values for r , mixis, and fertilization endpoints for fenitrothion are shown in Figure 1. In 7 out of 12 cases, EC_{50} values lay between NOEC and LOEC. The LOEC and NOEC of r , mixis, and fertilization, tend to increase with exposure time, while EC_{50} is less dependent on the duration of exposure. A similar trend was observed with other pesticides.

Table 1. Twenty-four-hour medial lethal concentrations (LC_{50} s) of test pesticides to the rotifer *B. plicatilis* at 25 °C

	LC_{50} (μM)
Diazinon	88.39 (74.58–102.51)
Fenitrothion	229.76 (219.30–240.22)
Isoprothiolane	220.74 (215.92–225.56)
Methoprene	100.81 (88.89–112.40)
DMSO	> 640

Values in parenthesis are 95% confidence intervals.

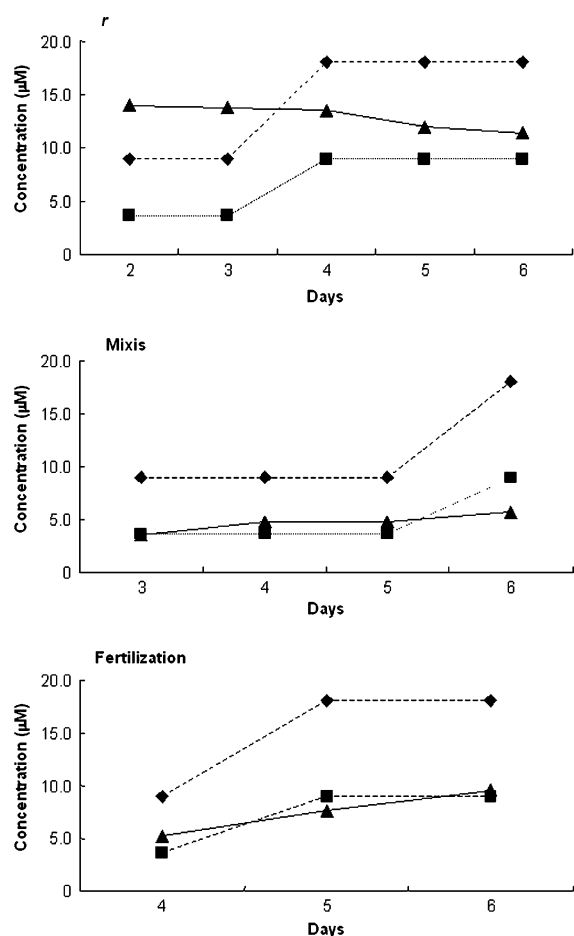


Figure 1. NOEC (■), LOEC (◆), and EC₅₀ (▲) daily values of *r*, mixis, and fertilization of *B. plicatilis* exposed to fenitrothion.

EC₅₀ values of *r*, mixis, fertilization, resting egg production (day 7) as well as percent hatching of resting egg of *B. plicatilis* exposed to the four pesticides are presented in Fig 2. In diazinon, EC₅₀ values of mixis were not calculated because the correlation coefficient was less than 0.5. EC₅₀ value of *r* was highest on day 2 and became stable from day 3 and onwards. Hatching rate of resting eggs gave the lowest EC₅₀ value (0.2 µM) of all the endpoints. In fenitrothion, mixis consistently gave low EC₅₀ values, however resting egg hatching rate gave the lowest EC₅₀ value (1.7 µM). In methoprene exposures, the endpoints *r*, mixis, and fertilization, gave similar EC₅₀s for days 2–6. The EC₅₀ value of resting egg production was highest (7.2 µM), however once again the hatching rate EC₅₀ was the lowest endpoint (0.06 µM). In

isoprothiolane, correlation coefficient of EC₅₀ values of mixis were lower than 0.5, therefore it was not included in the graph. Hatching rate of resting eggs again gave the lowest EC₅₀ value (2.7 µM).

The mean number of resting eggs produced in each treatment and their hatching rates are presented in Figure 3. Rotifers exposed to 16.4 µM and higher diazinon, 9.0 µM and higher fenitrothion, 8.0 µM and higher methoprene, and 68.8 µM isoprothiolane, produced significantly fewer resting eggs compared to the control.

The hatching rates of resting eggs in unexposed rotifers ranged from 56.7 to 67.9%. The hatching rate of the resting eggs from all exposed treatments was significantly lower than the control except for 0.3 and 17.2 µM isoprothiolane.

Discussion

Results from our chronic toxicity tests indicated that hatching rate of resting eggs produced by parents exposed to pesticides is the most sensitive endpoint. The resting egg hatchability EC₅₀ (0.2 µM) of diazinon exposed rotifers was seven times lower than the lowest EC₅₀ values of fertilization (1.4 µM), while that of fenitrothion was two times (1.7 and 3.5 µM, respectively), and more than three times in that of isoprothiolane (2.7 and 8.9 µM, respectively). Methoprene exposed rotifers gave a highest resting egg hatchability EC₅₀ ratio of more than 40 times lower than mixis EC₅₀ (0.06 and 2.7 µM). The EC₅₀ values for resting egg hatchability were more than 80 times lower than their 24-h acute toxic concentration. Among the reproductive endpoints (*r*, mixis, and fertilization), none of these was consistently the most sensitive for detecting toxicity in *B. plicatilis* among the pesticides tested. The study of Ferrando et al. (1996) on the freshwater rotifer *Brachionus calyciflorus* exposed to fenitrothion showed that net reproductive rate and *r* were more sensitive endpoints than generation time and life expectancy. However, in the study of Preston & Snell (2001), using the same species but exposed to pentachlorophenol and copper, fertilization and resting egg production were the most sensitive. In addition, Preston et al. (2000) and Radix et al. (2002) have shown that mixis and fertilization were sensitive parameters in *B. calyciflorus* exposed to

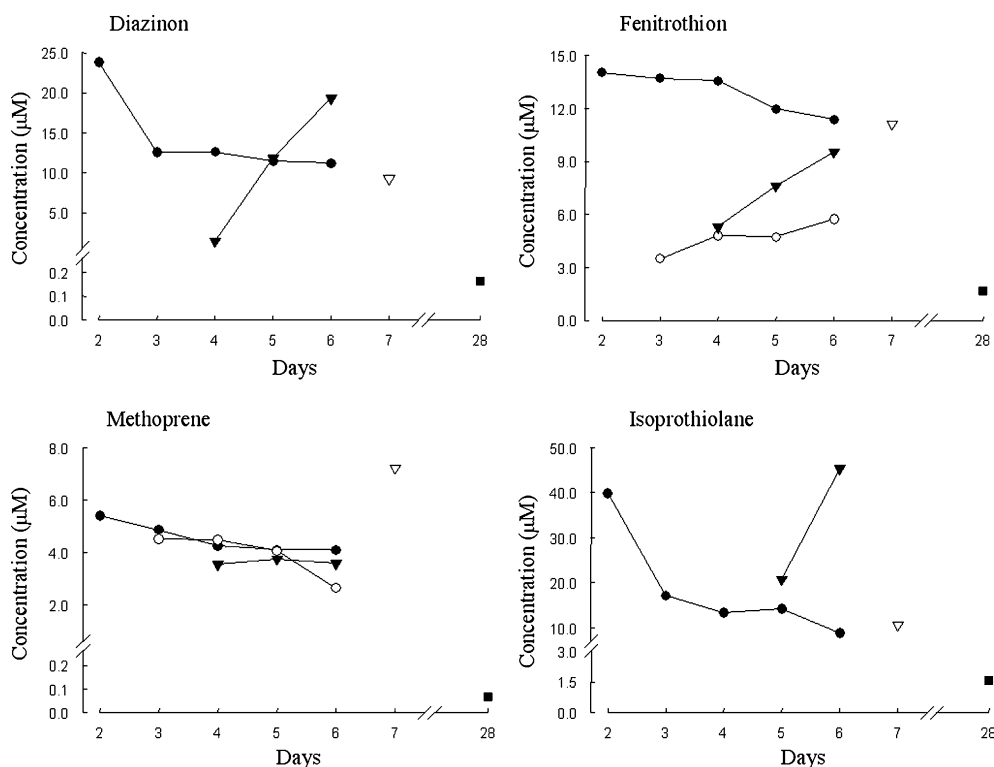


Figure 2. EC_{50} daily values of r (●), mixis (○), and fertilization (▼) of *B. plicatilis* exposed to diazinon, fenitrothion, methoprene and isoprothiolane. Resting egg production (▽) at day 7, and hatching rate of resting eggs (■) after 3 weeks are also indicated.

known and suspected endocrine disrupting chemicals. All of the above-mentioned studies however, did not investigate the hatchability of the resting eggs produced by the rotifers exposed to the test chemicals. The results of our study showed that rotifers exposed to 0.03–32.8 μM diazinon, 0.04–9.0 μM fenitrothion, 0.3–68.8 μM isoprothiolane, and 0.03–3.0 μM methoprene successfully produced resting eggs, however, their hatching rates were significantly lower than the control. Diazinon, fenitrothion, methoprene, and isoprothiolane affected the hatchability of the resting eggs at concentrations 2–40 times lower than r , mixis and fertilization EC_{50} s. The hatching rate of resting eggs therefore, is the most sensitive endpoint thus far described in rotifers for detecting the effects of pesticides.

The sensitivity of the hatching rate of resting eggs can be attributed to several factors. Resting eggs are the product of sexual reproduction, and sexual reproduction is induced by high asexual reproduction and under moderate environmental

conditions (Snell, 1986; Hagiwara et al., 1988). Resting egg production and hatching therefore encompasses the complete life-cycle of rotifers. In our experiments the parents (females and males) were continuously exposed to the toxicants and resting eggs were formed in the presence of the pesticides. Pesticides might affect the oogenesis of females, spermatogenesis of males, and developmental stages of the resting eggs.

Several factors influence the hatchability of the resting eggs of rotifers. Among them are light, temperature, salinity, and maternal diet (Hagiwara & Hino, 1989, 1990; Hagiwara et al., 1995). In the present study, rotifers were reared under optimal culture conditions for resting egg formation, including provision of optimal food and hatching conditions described in Hagiwara et al. (1995). Therefore, the reduced hatchability could not be due to the culture conditions. Temporary exposure to two organochlorine compounds reduced the hatchability of eggs of the calanoid copepods *Eurytemora affinis* and *Acartia biflosa* (Lindley et al.,

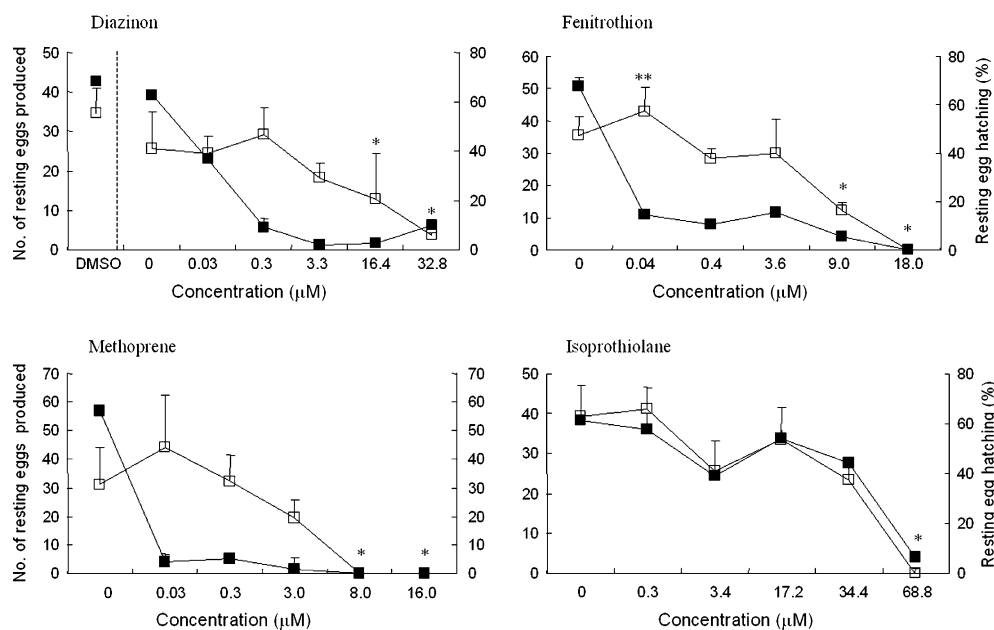


Figure 3. Mean number of resting eggs produced by *B. plicatilis* (□) and their hatching rates (■) exposed to different concentrations of diazinon, fenitrothion, methoprene, and isoprothiolane. *Significantly lower than the control ($p < 0.01$); **significantly higher than the control ($p < 0.05$) detected by ANOVA and Dunnett's test. The percent hatching of resting eggs of the exposed treatments were all significantly lower than the unexposed control except for 0.3 and 17.2 μM isoprothiolane. Vertical bars are standard error of the mean.

1999), and eggs laid in aqueous solutions of the two compounds did not yield viable nauplii. The same effects were observed by Naess (1991) in calanoid eggs exposed to the pesticide rotenone. This is the first report on the effect of toxicants on the hatchability of resting eggs in rotifers.

The effects of anthropogenic chemicals on the viability of resting eggs is an ecologically important parameter, because resting eggs enable rotifers to maintain their population during unsuitable environmental conditions. Modeling studies have indicated that a small decrease in r will reduce the number of resting eggs produced, resulting in loss of heterozygosity, depletion of the resting egg pool, and can lead to local population extinction (Snell et al., 1999; Snell & Serra, 2000).

Diazinon, fenitrothion and isoprothiolane have been detected in several rivers in Japan at 0.02–20 $\mu\text{g l}^{-1}$ (Fukushima et al., 1995; Sudo et al., 2002). Following field application of Altosid Liquid Larvicide (with 5% (S) methoprene) the expected environmental concentration of methoprene is 0.03 μM (Ross et al., 1994). The concentrations that affected r , mixis rate and fertilization rate are much higher than the concentrations found in the aquatic environments. However, the pesticides concentra-

tions affecting the hatchability of rotifer resting eggs are within the levels reported in some aquatic environments. Therefore, rotifer resting egg hatching rate could be useful as endpoints in ecotoxicology to detect the effects of pesticides.

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