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Effects of Thiophanate-Methyl and Glyphosate on Asexual and Sexual Reproduction in the Rotifer *Brachionus calyciflorus* Pallas

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Rotifers, especially *Brachionus calyciflorus* Pallas and *B. plicatilis* Muller, are particularly useful for aquatic toxicology because of their rapid reproduction, short generation time, cosmopolitan distribution and commercial availability of the resting eggs (Snell and Janssen 1995). Most planktonic rotifers have a cyclically parthnogenetic life cycle where asexual reproduction predominates, but there are periods where both asexual and sexual reproduction occur simultaneously. In monogonont rotifers, asexual reproduction in the absence of males is mixed with occasional bouts of sexual reproduction. Asexual (amictic) females are diploid and produce eggs mitotically, which develop into amictic females. Upon receiving the mictic stimulus, asexual females begin producing both sexual (mictic) and amictic daughters. Mictic females produce haploid eggs, if unfertilized, develop into haploid males. If fertilized, haploid eggs become diploid and develop into large, thick-walled resting eggs. After a period of dormancy that varies among species, resting eggs respond to species-specific hatching cues and hatch into amictic females, entering again into the asexual phase of the life cycle (Pourriot and Snell 1983, Snell and Carmona 1995).

Resting eggs of rotifers are the products of sexual reproduction, and have important ecological and adaptative functions. Resting eggs enable rotifers to escape adverse environmental conditions through diapause, and aid in dispersal. As products of recombination, the presence of resting eggs in the sediment provides a bank of genetic diversity from which a population can draw new genotypes for adaptation (Gilbert 1974, Pourriot and Snell 1983, King and Snell 1977, Mort 1991). However, since rotifers have been used as bioassay organisms for ecotoxicological studies, toxicity tests utilizing rotifers incorporate mainly the asexual phase of the life cycle. In 1995, Snell and Carmona compared the toxicant sensitivity of asexual and sexual reproduction in the freshwater rotifer *Brachionus calyciflorus*. Preston et al. (2000) investigated the effect of potential endocrine disruptors on the sexual reproduction of *B. calyciflorus*. More complete life-cycle tests should now be made to investigate the ecological impact of aquatic pollutants.

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Thiophanate-methyl and glyphosate are used widely in agriculture of China. The former is used to kill pathogenic bacteria living on the surface of or in plants, and the latter is used to remove weeds from farmland. Up to now, their toxic effects on asexual and sexual reproduction of rotifers have not been reported, except that the effect of thiophanate-methyl on the life history parameter of *B. calyciflorus* was studied by Xi and Hu (2003).

The main purpose of the present study was to assess the effects of different levels of thiophanate-methyl and glyphosate on asexual and sexual reproduction in the freshwater rotifer *B. calyciflorus*.

MATERIALS AND METHODS

The rotifer *Brachionus calyciflorus* used as the test species in the present study was obtained by hatching resting eggs collected from sediments of Lake Jinghu and thereafter clonal culturing. Stock rotifer cultures were kept at 25±1℃ on a 16hr: 8hr light: dark photoperiod at 130 lx provided by a fluorescent light in a illumination incubator. Rotifer cultures were daily fed on *Scenedesmus obliquus*. Before the experiment commenced, the rotifer cultures were fed on 4.0×10^6 cells/mL of *S. obliquus* for at least one week. For mass cultures as well as for experiments, reconstituted hardwater (EPA medium)(USEPA 1985) was used as the medium. Algae were grown in a semi-continuous culture using HB-4 medium (Li et al. 1959) renewed daily at 40%. Algae in exponential growth were centrifuged and resuspended in EPA medium.

The pesticides thiophanate-methyl (commercial grade, 71%, Nisson, Japan) and glyphosate (commercial grade, 41%, USA) were used as toxicants. Stock solutions of 1000 mg/L were prepared using distilled water immediately prior to each experiment. Before the experiments commenced, seven concentrations of thiophanate-methyl (1.2, 2.4, 3.6, 4.8, 6.0, 7.2 and 8.4 mg/L) and glyphosate (10, 15, 20, 25, 30, 35 and 40 mg/L) were prepared through serial dilution from the stock solutions using EPA medium with 4.0×10^6 cells/mL of *S. obliquus* to obtain median lethal concentration at 24 hr of assay in order to choose appropriate toxicant concentrations for experiments. LC_{50} tests were conducted in 3-mL of the medium in 4-mL containers each with 4.0×10^6 cells/mL of *S. obliquus* and 10 neonates (<2 hr old). Three replicates were used for each concentration of thiophanate –methyl and glyphosate. After 24 hr, the number of rotifers alive in each test container was counted, and the LC50 values were respectively derived following the probit method (Finley 1971).

Based on the LC50 values, four toxicant concentrations of thiophanate-methyl (0.05, 0.1, 0.2 and 0.4 mg/L) and glyphosate (2.0, 4.0, 6.0 and 8.0 mg/L) and a control were selected for the experiments, each consisting of three replicates. All the experiments were conducted in 12-mL glass test tubes and started by

introducing 10 neonates (<2 hr old) into each tube which contained 10 mL of test solution and 4.0×10^6 cells/mL of *S. obliquus*. During the experimental period, the algae depositing at the bottom of each tube were resuspended every 12 hr with a micropipette, and both the rotifers and their resting eggs were transferred daily to new test tubes with fresh test solutions and alga. After three days, the number of animals and resting eggs per tube was counted. Rotifers were classified as unfertilized and fertilized mictic females, amictic females by the size and morphology of their eggs, and non-ovigerous females. From these counts the mictic rate was calculated for each tube as the proportion of ovigerous mictic females in all the females. Meanwhile, the population growth rate ® for each tube was calculated according to the formula $r=(\ln N_t - \ln N_0)/t$, where N_t =density of females at time t, N_0=2 females/mL, and t=3 days.

RESULTS AND DISCUSSION

Based on the 24-hr acute toxicology assays for thiophanate-methyl and glyphosate, the LC50 values of *B. calyciflorus* were 5.02 and 28.0 mg/L, respectively.

Compared to the control, 0.05, 0.1 and 0.2 mg/L thiophanate-methyl did not influence significantly the population growth rate, however, 0.4 mg/L thiophanate-methyl decreased significantly the population growth rate ($P<0.01$). When the concentrations of glyphosate were 4.0, 6.0 and 8.0 mg/L, all the population growth rates were significantly larger than the control ($P<0.01$), but 2.0 mg/L glyphosate did not influence significantly the population growth rate (Fig. 1). The relationships between the population growth rates (Y, /d) and the concentrations (X, mg/L) of thiophanate-methyl and glyphosate could be respectively described as:

Thiophanate-methyl: $Y=0.4236-0.1719X-1.7639X^2$ $R^2=0.9190$, $P<0.01$
Glyphosate: $Y=0.5271+0.1021X-0.0063X^2$ $R^2=0.8578$, $P<0.01$

When the concentration of thiophanate-methyl was 0.05 mg/L, both the mictic rate and the resting egg production in the rotifer population were not significantly affected, but when the toxicant concentration was higher than 0.05 mg/L, no mictic females appeared, and no resting eggs were produced. Compared with thiophanate-methyl, 2.0-8.0 mg/L glyphosate increased significantly the mictic rate and the resting egg production in the rotifer population (Fig. 2, 3). The relationships between the mictic rate (Y_1, $\times 100$) and the resting egg production (Y_2, ind./10mL/3d), and the concentration of glyphosate could be respectively described as:

$Y_1=4.93+6.81X-0.70X^2$ $R^2=0.8681$, $P<0.01$
$Y_2=-5.43+18.70X-1.68X^2$ $R^2=0.8023$, $P<0.01$

Higher population density can induce the rotifer *Brachionus* to produce more

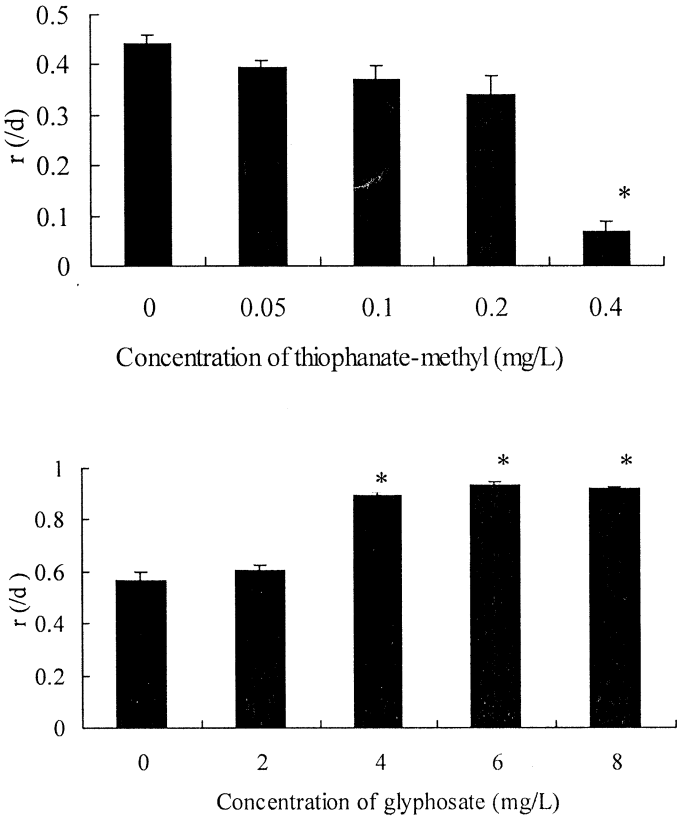

Figure 1. The effects of thiophanate-methyl and glyphosate on population growth rate (r) of *Brachionus calyciflorus* (* significantly different from the control, $P<0.05$)

mictic females (Gilbert 1974). In the present study, when the concentrations of glyphosate were 4.0, 6.0 and 8.0 mg/L, the population densities were significantly larger than the control, which might lead to higher mictic rates in the populations. Of course, whether higher concentrations of glyphosate can directly stimulate the rotifers to produce more mictic females needs to be further studied.

Because of the rotifer life cycle, the rate of female population growth estimates the amount of asexual reproduction occurring in the population (King and Snell 1977, Snell 1986). The mictic rate, in contrast, estimates the level of sexual reproduction in the population by comparing the number of amictic and mictic females produced (Snell and Carmona 1995). Hence, the results indicated that the higher concentrations of thiophanate-methyl inhibited asexual and sexual reproduction of the rotifer *B. calyciflorus*, but the higher concentrations of glyphosate enhanced them.

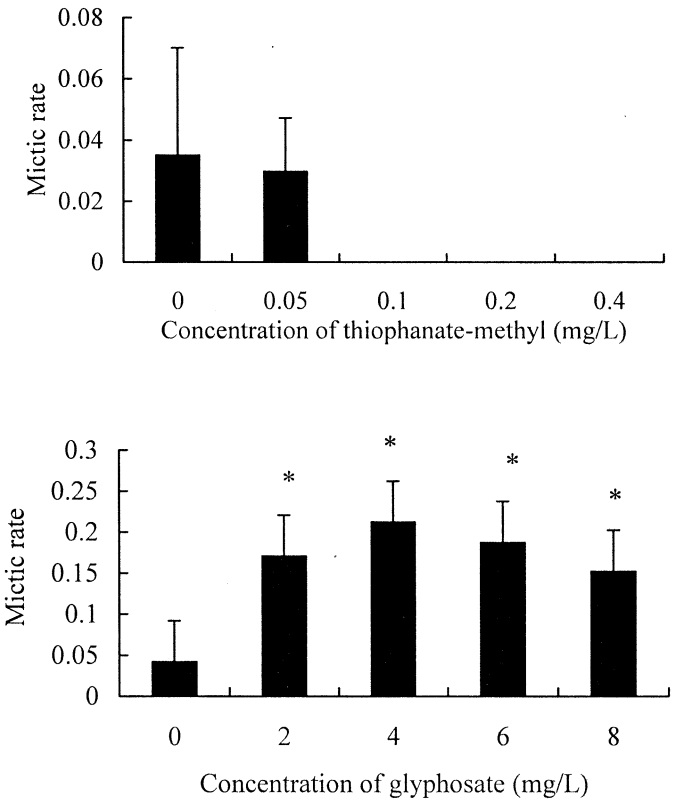

Figure 2. The effects of thiophanate-methyl and glyphosate on mictic rate of *Brachionus calyciflorus* (* significantly different from the control, $P<0.05$)

Compared to the controls, the population growth rates were significantly influenced by thiophanate-methyl and glyphosate when their concentrations were higher than 0.2 and 2.0 mg/L, respectively. However, the mictic rates in the populations were markedly affected just when the concentrations of thiophanate-methyl and glyphosate were higher than 0.05 and 0 mg/L, respectively. These results showed that sexual reproduction was more sensitive than asexual reproduction to thiophanate-methyl and glyphosate. Similar results were obtained by Snell and Carmona (1995).

Preston and Snell (2001) found that resting egg production was a more sensitive indicator of toxicity for pentachlorophenol than mictic rate. In the present study, when the concentrations of glyphosate were 2.0 to 4.0, 6.0 and 8.0 mg/L, respectively, the mictic rates were not significantly different, but the reverse was

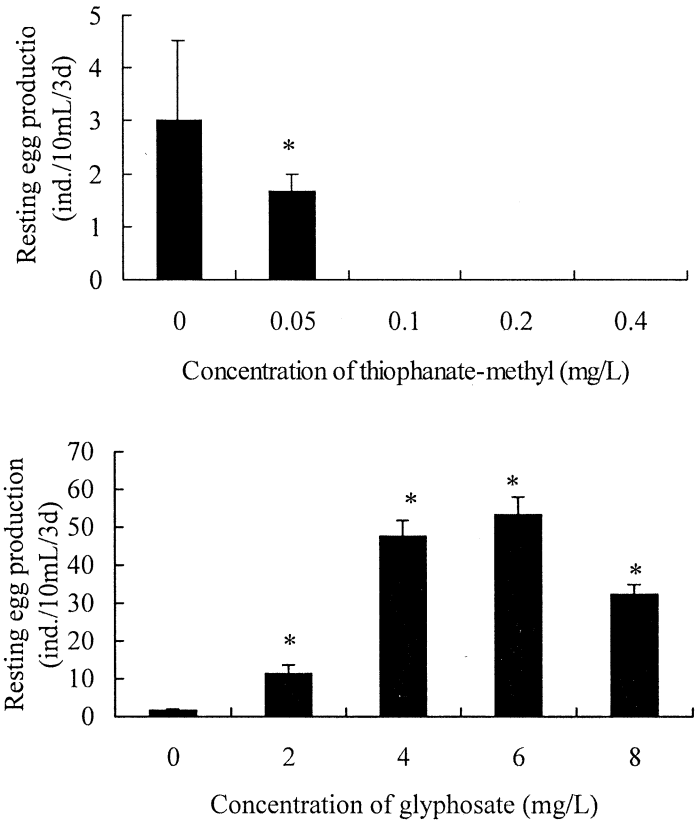

Figure 3. The effects of thiophanate-methyl and glyphosate on resting egg production of *Brachionus calyciflorus* (* significantly different from the control, $P<0.05$)

true for resting egg production, which was identical to the results obtained by Preston and Snell (2001)

Sexual reproduction of rotifers is initiated by the appearance of mictic females, followed by male production, fertilization, and resting egg formation. The resting egg production is determined by several factors, among the most important of these being the rate of sexual female production, male mate recognition and fertility, female susceptibility to fertilization, and fertilized female fecundity (Snell and Janssen 1995). In this study, the higher mictic rate caused by glyphosate might be one cause of higher resting egg production.

Rotifers are extremely important in freshwater ecosystem. They can convert primary (algal and bacterial) production into a form usable for second consumer (e.g., insect larvae, fish fry) and achieve this transformation with remarkable

efficiency, producing up to 30% of the total plankton biomass (Nogrady et al. 1993). Resting eggs are critical for rotifer survival from one year to the next. When environments at certain times are unsuitable for adult rotifer survival, dormancy is the only bridge across these unsuitable environments to the next period and, in monogonont rotifers, resting eggs are the sole means of dormancy (Gilbert 1974). Hence, the presence of the higher concentrations of thiophanate-methyl and glyphosate in freshwater bodies would change the structures and functions of freshwater ecosystems, and the presence of the higher concentrations of thiophanate-methyl would be unfavorable for the survival of the rotifer species at unsuitable environments. For large-scale agriculture use, the final concentration of less than 0.05 mg/L for thiophanate-methyl or 0.2 mg/L for glyphosate after they entered into freshwater bodies should be required.

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