

Bioaccumulation of atrazine: effects on population growth and reproduction of the rotifer *Brachionus calyciflorus* fed atrazine-exposed microalgae

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Abstract

Little information is known about bioaccumulation of atrazine or the effects it has on consumers that ingest atrazine-contaminated prey. For that reason, freshwater microalgae *Chlorella vulgaris* cultures were exposed to different concentrations of atrazine for twenty-four hours and were then used as an exclusive food source for *Brachionus calyciflorus* for three days with the intent to evaluate the effects on rotifer population growth, reproduction, and survival. Considering a multivariate response, there was a deleterious effect of feeding on atrazine-exposed algae. Specifically, the rate of population growth, total ovigerous females and egg ratio (i.e. the total number of parthenogenic eggs divided by the total number of females in a population) in the rotifer populations significantly decreased as atrazine exposure concentrations of *C. vulgaris* increased. The rate of mixis in rotifer populations fed atrazine-exposed microalgal cells also decreased as herbicide concentration increased. The results of this study suggest that herbicides absorbed by microalgal cells can adversely affect higher trophic level organisms.

1. Introduction

Increased use of herbicides in agribusinesses has raised a general concern about how they are affecting aquatic environments. Herbicides pollute rivers, streams, and lakes either indirectly by leaching into groundwater or directly through runoff (Graymore et al., 2001; He et al., 2012). Originally introduced in the 1950's, atrazine—a class of triazine herbicide—is now one of the most heavily employed herbicides on the planet due to its relatively inexpensive cost (Graymore et al., 2001). Atrazine works by inhibiting the electron transport chain of photosystem II in plants, resulting in a disruption of photosynthesis (Corbett et al., 1984). It's primarily been used for pre- and post-emergence broadleaf weed control in both agricultural and nonagricultural areas (Girling et al., 2000).

In 2012, the USDA reported that 23 million kgs (~50.7 million lbs.) of atrazine was employed in corn production alone. In Florida, roughly 566,900 kgs (1.25 million lbs.) of atrazine were used between citrus, sugarcane, and vegetable crops, and other agricultural commodities such as field crops, ornamental plants, and turf grass, from 1999 to 2002 (Schuler & Rand, 2008). As a result of its high use, atrazine is one of the most frequently detected herbicides in contaminated ground soils and aquatic ecosystems (Vecchia et al., 2009; Chalifor & Juneau, 2011).

Ecosystems polluted by atrazine have been shown to impact non-target species such as copepods (Forget-Leray et al. 2005), fish (Bringolf et al., 2002; Tillitt et al., 2010), and amphibians (Hayes et al., 2002; Hayes et al. 2003; Hayes et al. 2010). Studies have found evidence that an accumulation of atrazine in aquatic systems generates harmful effects on growth

and reproduction (Dornelles & Oliveira, 2014) and induces alterations in behavior (McCallum et al., 2013) of non-target species inhabiting contaminated areas. Hayes et al. (2003) showed that exposure to atrazine-polluted waters with as little as 0.1 ppb caused gonadal dysgenesis (retarded gonadal development) and testicular oogenesis (hermaphroditism) in American Leopard Frogs, *Rana pipiens*. These findings held not only for laboratory experiments, but also for animals collected from atrazine-polluted waters around the US.

The literature on atrazine exposure has extensively demonstrated the effects that direct exposure has on non-target species. However, little information is known about bioaccumulation of atrazine or the effects it has on consumers that ingest atrazine-contaminated prey.

Zooplankton are among the most susceptible organisms to alterations within aquatic ecosystems (Chang et al., 2005). Since zooplankton are a basal part of aquatic food webs, changes in their population size, reproduction, behavior or physiology can potentially lead to significant ecosystem level impacts (Dodson and Hanazato, 1995), for instance via effects on higher trophic level taxa that are directly or indirectly associated with these basal trophic species.

To investigate the toxicological effects of ingesting atrazine-contaminated prey on consumers, I have chosen to study the monogonont rotifer, *Brachionus calyciflorus*. Its short generation time, complex reproductive biology, ubiquity in aquatic systems, and ease to maintain in laboratory settings (Janssen et al., 1993) make *B. calyciflorus* a prime candidate for this study.

The primary goal of this experimental study is to gain insight into the effects of ingestion of atrazine, specifically via atrazine-exposed prey. This investigation will use *Brachionus calyciflorus* as the primary consumer and the microalgae, *Chlorella vulgaris*—a commonly used species in toxicity testing—as the primary food source. *C. vulgaris* cultures were exposed to different concentrations of atrazine and were then used as an exclusive food source for *B.*

calyciflorus. We then evaluated the consequences of these exposed algae on rotifer population growth, reproduction, and survival.

2. Methods

2.1. Study Species

The heterogonic life cycle of *B. calyciflorus* (Figure 1) includes many generations of amictic (i.e. asexual) reproduction before mictic (i.e. sexual) reproduction takes place (Birky & Gilbert, 1971). Amictic females will mitotically reproduce diploid eggs that develop into genetically identical amictic females by means of parthenogenesis. If environmental conditions become unfavorable, amictic reproduction is disrupted and triggers amictic females to reproduce diploid eggs that mature into mictic females. Further, amictic females excrete a chemical into their surrounding environment when they reach a high population density that induces mictic reproduction (Fussmann et al., 2007). Once mictic reproduction is triggered, mictic females will meiotically produce haploid eggs that develop into males capable of fertilizing mictic females. At about one-fourth the size of female rotifers, males do not possess a gut, are short lived, are fast swimmers. Males only have roughly 30 sperm, of which only 2 to 3 are transferred during each copulation event (Wallace & Snell, 2001). After a male successfully inseminates a mictic female, the female will produce ovoid, thick-shelled embryos in diapause (called resting eggs or cysts), which can lie dormant for extended periods of time and endure harsh environmental conditions. Once environmental conditions become favorable (e.g. the optimal conditions in light, temperature, pH, salinity and oxygen; Wallace & Snell, 2001), a resting egg hatches and releases a diploid amictic female, beginning the asexual life cycle anew.

2.2. *Chlorella vulgaris* twenty-four hour exposure

The microalgal species, *Chlorella vulgaris*, was obtained from Carolina Biological Supply Company (Burlington, North Carolina). For the experiment and routine cultures, *C. vulgaris* was cultured in 250-mL Erlenmeyer flasks containing 100-mL of Bristol medium (Bold, 1949). Microalgae cultures were stored in a thermoregulated chamber at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ on top of a shaker table (100 ± 10 rpm) and illuminated under cool-white florescent lamps on a 16h:8h light:dark photoperiod. The microalgae cultures were maintained using semi-continuous conditions (i.e. preexisting algal cultures were partially harvested and topped with fresh media to the original volume, and harvested cells were added to a sterilized flask containing fresh media) with the intention to keep cells in logarithmic phase—a period during cell growth when essential nutrients are abundant and growth is not limited, driving cell populations to divide and proliferate exponentially.

Algal cell culture densities were determined spectrophotometrically at a wavelength of 685nm ([OD₆₈₅ i.e. optical density at 685nm]; Qian, 2008). The regression equation between the density of a cell culture (y[cells/mL]) and OD₆₈₅ (x) was determined to be: $y = 1.49195 \times 10^7(x) + 1.06092 \times 10^5$ ($R^2 = 99.23\%$).

Chlorella vulgaris cells were exposed to atrazine concentrations at 2, 5 and 10 mg/L for 24h prior to starting the experiment. 4.0×10^6 cells/mL of *C. vulgaris* were harvested via pipette and added into individual screw-cap tubes and centrifuged at 3300 rpm for 1hr. The supernatant was decanted and 5-mL of atrazine-contaminated EPA medium (96 mg NaHCO₃, 60 mg CaSO₄·2H₂O, 60 mg MgSO₄·7H₂O, and 4 mg KCl per liter of distilled water) of 2, 5, or 10 mg/L was introduced into randomly selected tubes containing centrifuged algae (see atrazine solution description below) Tubes were sealed to prevent external contamination, agitated and

resuspended algae, and stored in the same manner as describe above for the maintenance of algal cultures. Cell cultures not exposed to atrazine were used as a control. Cultures exposed to acetone were carried out as a complementary control, and showed no difference to the control without acetone. The maximal amount of acetone in any treatment did not exceed 1.0% (v/v), which has previously been shown to not significantly affect results when comparing to the no acetone control (Tang et al, 1997; DeLorenzo et al., 2007; Qian et al., 2008, Snell and DesRosiers-noac 2008; Lu et al., 2012). Prior to feeding rotifers, exposed *C. vulgaris* cultures (atrazine and acetone-only) were washed with EPA media to remove any unabsorbed atrazine and centrifuged for 0.5hr.

The amount of atrazine absorbed by exposed *C. vulgaris* cultures was not measured. However, published evidence suggests that *C. vulgaris* cells exposed to trizine herbicides for 24hr removed >90% of the surrounding toxicant from the media, and that viability of cells remained around 100% for concentrations as high as 500nM (Rioboo et al., 2007), which is approximately 0.120 mg/L.

2.3. *Brachionus calyciflorus* three-day test

The rotifer species, *Brachionus calyciflorus* Pallus, was obtained as resting eggs from Pentair Aquatic Eco-Systems (Apopka, Florida). Resting eggs were hatched by hydrating eggs in a petri dish containing 10-mL of EPA medium adjusted to a pH of 7.5 ± 0.02 . Hydrating resting eggs were stored in a thermoregulated chamber at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under continuous light of cool-white florescent lamps 24hr prior to the experiment.

All the experiments were conducted by introducing 4 neonates into sterilized 8-mL screw top glass chambers containing 2-mL of EPA medium with 4×10^6 cells/mL of non-exposed, acetone-exposed, or atrazine-exposed (2, 5 or 10 mg/mL) microalgal cultures with four replicates

of each treatment. Rotifers were cultured under static-nonrenewal conditions and stored in a thermoregulated chamber at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ on top of an shaker table (100 ± 10 rpm) and illuminated under cool-white florescent lamps on a 16h:8h light:dark photoperiod for 3d. Algae deposited at the bottom of a glass chamber was resuspended once every 12hr using a micropipette.

After 3 days, the number of living and dead rotifers were counted per chamber and classified by the morphology and type of their eggs, which are different for females, males, and resting eggs (Wallace and Snell, 2001). Rotifers were categorized as amictic female (AF), unfertilized mictic female (UMF), fertilized mictic female (FMF), nonovigerous female (NOF), or male (M). From these counts, the intrinsic rate of population increase (r) was calculated for each glass chamber using the following equation:

$$r = \frac{(\ln N_t - \ln N_0)}{t}$$

where N_t and N_0 are the final and initial population densities respectively and t is the time in days. The ratio of ovigerous females to nonovigerous (OF/NOF) females was calculated for each population. The sex ratio (M/F) was determined as the total number males divided by the total number of females within a population. The mictic rate (MR) was calculated by the number of fertilized and unfertilized females divided by the total number of females in each glass chamber. The egg ratio (ER) was calculated as the total number of parthenogenic eggs divided by the total number of females in a population.

2.4. Atrazine Exposure

Atrazine (1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine; purity > 98%) was obtained from Chem. Service, Inc (West Chester, PA). Atrazine stock solutions (1mg/mL) were

prepared by dissolving atrazine in acetone (99.5% purity). Test solutions were diluted to the appropriate concentrations by adding the atrazine stock solution to 100-mL EPA media prior to exposing algal cultures. Previous experimental results suggest that *B. calyciflorus* has an LD₅₀ value of 39.2 mg/L of atrazine (Lu et al., 2012). Based on this result, three nominal atrazine concentrations (2, 5, and 10 mg/L) were chosen as treatments for the experiment.

2.5. Data analysis

A multivariate analysis of variance (MANOVA) with the concentration as the explanatory value and the intrinsic rate of population increase (r), OF/NOF, M/F and MR as the response variables was conducted to assess the effect of the test compound on asexual and sexual reproduction.

3. Results

During the 3d exposure period, some of the treatment replicates (three acetone and one 2mg/L) were found with a profusion of unidentified organisms that contaminated the algae. These replicates were not used in the statistical analysis.

Overall, there was a significant effect of atrazine exposure on the response variables considered, (r), OF/NOF, MR, M/F, and EG (Wilk's Lambda: $F=9.815$; $df= 20, 24$; $P < 0.001$). All of the response variables show a consistent response, with the exception of M/F (Figure 6). There were no significant differences among the exposure treatments observed for the M/F. The experimental results for the control and treatments are listed in Table 1 and the specifics are discussed below.

The intrinsic rate of population increase (r) was adversely affected by the presence of atrazine in the microalgal cells used as food. Rotifer cultures fed with atrazine-contaminated *C. vulgaris* showed significant differences among the control and the treatments ($F = 89.907$; $df = 4, 11$; $P < 0.001$; Figure 2). The average (r) was lower for all treatments relative to the control. As the concentration increased from 2 to 5 mg/L and from 5 to 10 mg/L, there was steady decrease and increase, respectively, in the intrinsic rate of population growth after three days. The 10 mg/L treatment averaged higher than the other treatments, but was still lower than the control.

Atrazine-exposed microalgal cultures fed to rotifer cultures altered the ratio of ovigerous to nonovigerous females (OF/NOF) after three days. Rotifer cultures fed with atrazine-exposed *C. vulgaris* cultures showed significant differences among the control and the treatments ($F = 31.055$; $df = 4, 11$; $P < 0.001$; Figure 3). Compared to the control, the average OF/NOF was lower for all concentrations. The OF/NOF decreased and increased between 2 to 5 mg/L and 5 to 10 mg/L, respectively

C. vulgaris cultures contaminated with atrazine and then fed to rotifer cultures for three days had an impact on the mictic rate (MR). Cultures fed with atrazine-exposed microalgal cells showed statistical differences among the control and the treatments ($F = 3.587$; $df = 4, 11$; $P = 0.042$; Figure 4). The mean value was lower for all treatments when compared to the control. The MR decreased between 2 and 5 mg/L and moderately increased between 5 and 10 mg/L.

Finally, atrazine-contaminated algae cells fed to rotifer cultures for three days affected the egg ratio (ER). Cultures fed with atrazine-exposed *C. vulgaris* cells showed statistically significant differences when comparing the control to the treatments ($F = 150.069$; $df =$

4, 11; $P < 0.001$; Figure 5). Compared to the control, the ER decreased between 2 and 5mg/L and increased between 5 and 10mg/L.

Rotifers fed with atrazine-contaminated microalgal cells show no significant differences in sex ratio (M/F) after three days when comparing the control to the treatments ($F = 2.246$; $df = 4, 11$; $P = 0.130$; Figure 6). The 5 mg/L of atrazine had the highest average M/F ratio, while the control, 2 mg/L and 10 mg/L had decreased averages. It should be noted that one of the 5 mg/L replicates has an abnormally low number of total females when compared to all replicates, suggesting an explanation for the elevated average sex ratio compared to the other treatments.

4. Discussion

The primary goal of this study was to determine the toxic effects that atrazine bioaccumulated in algae have on consumer levels of the food chain. Utilizing zooplankton to assess the effects of atrazine bioaccumulation is important for a number of reasons (Hanazato T, 2001). Zooplankton are found in nearly all aquatic systems and are highly susceptible to a wide variety of toxicants (Snell & Janssen, 1995; Marcial et al., 2005). Studies can be performed using large population densities in a small volume of media and data can be collected quickly. Our results suggest that *C. vulgaris* cells have a propensity to absorb atrazine via water (bioconcentration) in as little as twenty-four hours, and that atrazine-exposed microalgae consumed by *Brachionus calyciflorus* alters a variety of biological parameters. The effect a toxicant has depends on the duration of exposure and the concentration absorbed by the microalgae. Although the effects direct exposure to toxicants like atrazine have been studied for several aquatic species, the current results suggest that the effects of herbicides ingested via food

by herbivores should be taken into account.

The concentration of a toxicant can decrease the size of a population without changing the survivability of individuals within an affected population (Snell and Janssen, 1995). Toxicity studies, for instance, have corroborated that a decrease in the rate of population growth caused by toxicity exposure is usually due to a decrease in egg production (Rioboo et al, 2007). In the current study, the intrinsic rate of population increase, OF/NOF, and ER in *B. calyciflorus* fed with atrazine-contaminated microalgae was significantly altered in all treatments when compared to the control. Even the lowest concentration reduced the population growth rate and decreased the OF/NOF and ER in rotifers fed with atrazine-contaminated microalgae.

Sexual reproduction is a necessary component in the life cycle of monogonont rotifers, which is triggered by environmental stimuli such as crowding, perennial photoperiods, and diet (Gilbert, 1975). Amictic reproduction rapidly increases a population of rotifers to a considerable size; however, it is the production of resting eggs via mictic reproduction that ensures the survival of rotifers from year to year (Snell & Serra, 2000) and promotes genetic variability via recombination. A population of rotifers affected by ingesting herbicide-contaminated microalgae will reduce the probability of resting egg production. This will consequently lower the number of resting eggs added to the egg bank and therefore decrease the chance for a population to recover after an environmental disturbance or even predictable season variation. In the current study, rotifers fed atrazine bioaccumulated *C. vulgaris* cells showed a significant decrease in the MR when comparing the treatments and the control; however, no significant differences were observed for the M/F. A drop in the rate of mixis (MR) leads to a decreased number of mictic females in a population (Lu et al., 2012), and therefore results in a decreased number of males capable of fertilizing mictic females. *Brachionus sp.* males have a copulation

success rate between 10 and 75% (Snell and Hawkinson, 1983; Watson and Snell, 2001). A lower number of males within a mictic population might negatively shift this percentage. Moreover, this would reduce the overall rate of recombination, and thus decrease the frequency of novel genotypes within the population.

The current study demonstrates the importance of evaluating the bioaccumulation of atrazine and the effects it has on consumers that ingest atrazine-contaminated prey. Increasing the sample size and number of replicates per treatment, including more intermediate concentration levels, and extending the duration rotifers are exposed to atrazine-polluted algae allowing female rotifers to developmentally mature could strengthen future studies on this subject. Additionally, incorporating further response variables such as resting egg production and the hatchability of resting eggs would enhance bioaccumulation studies.

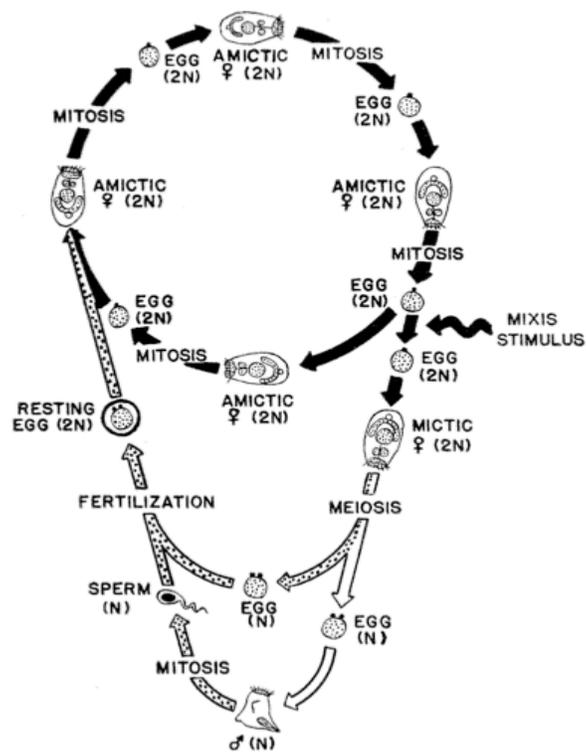


Figure 1. The bisexual life cycle of the monogonont rotifer *Brachionus calyciflorus* (taken from Birky & Gilbert, 1971). Read section 2.1 for information about the life cycle.

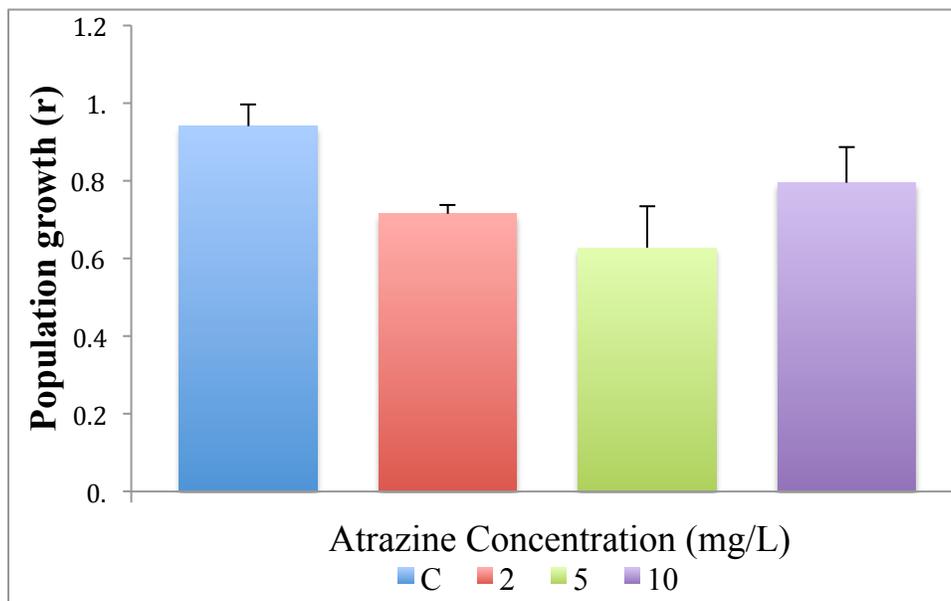


Figure 2. The effects of atrazine-exposed microalgae on the intrinsic rate of population growth (r) of *B. calyciflorus* after three days (mean \pm SE; $n=4$ for C, 5, and 10mg/L; $n=3$ for 2mg/L).

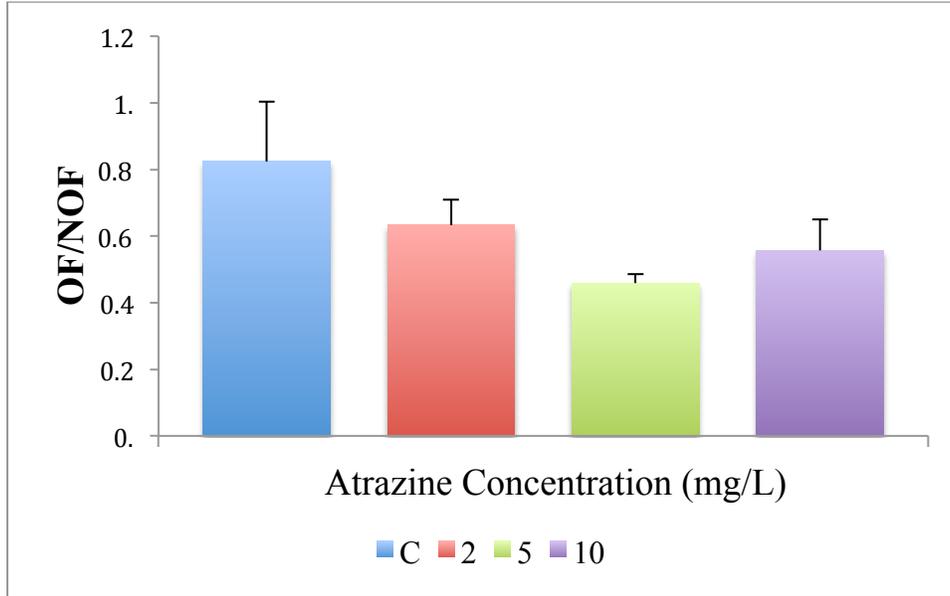


Figure 3. The effects of atrazine-exposed microalgae on the OF/NOF of *B. calyciflorus* after three days (mean±SE; n=4 for C, 5, and 10mg/L; n=3 for 2mg/L).

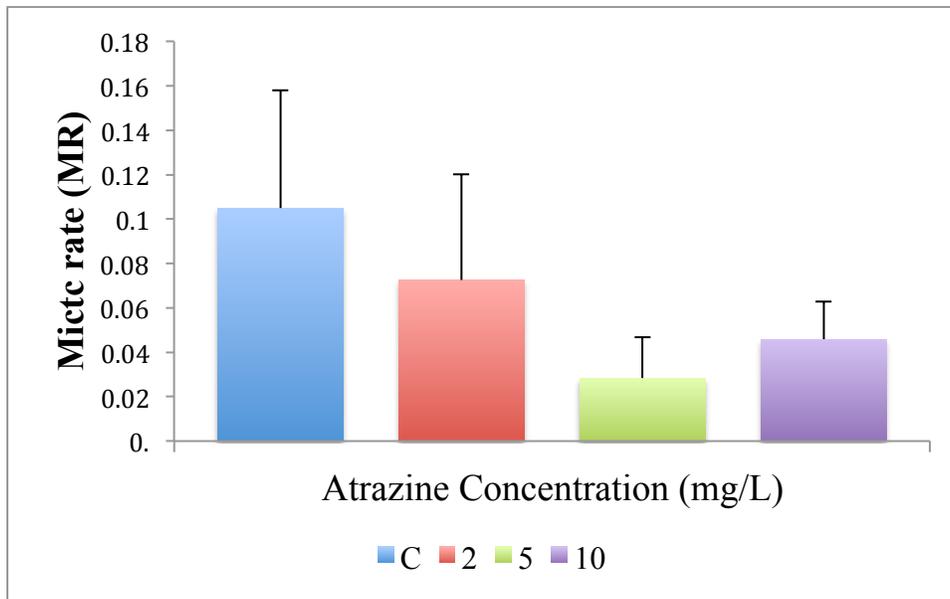


Figure 4. The effects of atrazine-exposed microalgae on the mictic rate (MR) of *B. calyciflorus* after three days (mean±SE; n=4 for C, 5, and 10mg/L; n=3 for 2mg/L).

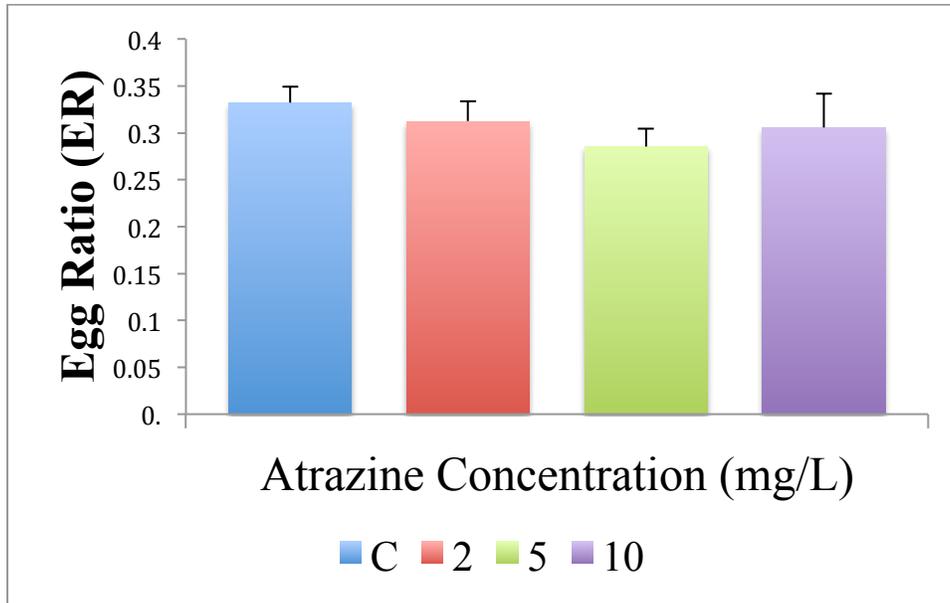


Figure 5. The effects of atrazine-exposed microalgae on the egg ratio (ER) of *B. calyciflorus* after three days (mean \pm SE; n=4 for C, 5, and 10mg/L; n=3 for 2mg/L).

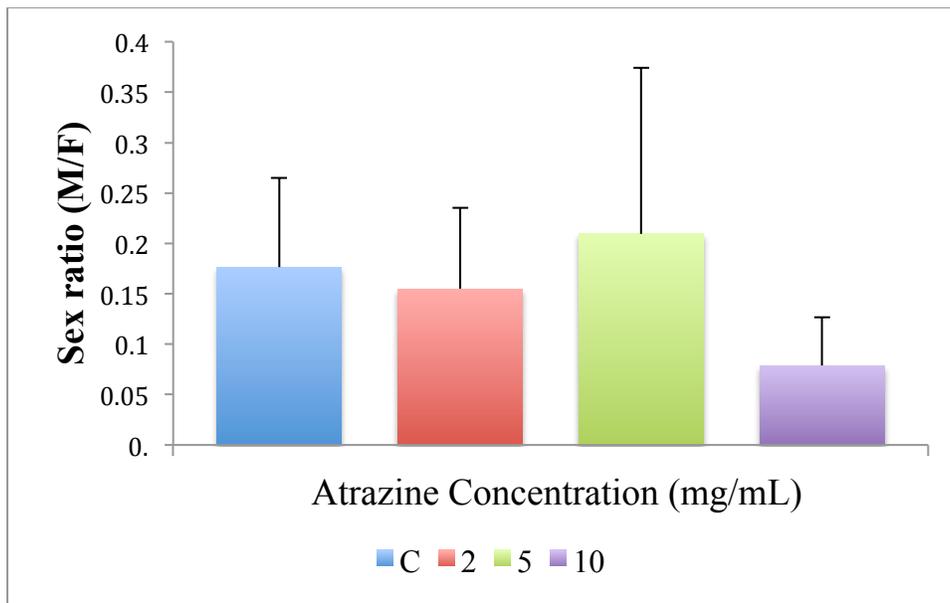


Figure 6. The effects of atrazine-exposed microalgae on the sex ratio (M/F) of *B. calyciflorus* after three days (mean \pm SE; n=4 for C, 5, and 10mg/L; n=3 for 2mg/L).

Table 1. Intrinsic rate of increase (r), ovigerous to non-ovigerous females ratio (OF/NOF), mictic rate (MR), and sex ratio (M/F) of *Brachionus calyciflorus* fed with atrazine-exposed microalgae to different concentrations calculated after the three day exposure period.

Treatments	(r)	OF/NOF	MR	ER	M/F
Control	0.9405±0.057	0.8241±0.179	0.1764±0.089	0.3322±0.017	0.1050±0.053
2 mg/L	0.7152±0.022	0.6328±0.076	0.1549±0.081	0.3123±0.021	0.0726±0.048
5 mg/L	0.6272±0.107	0.4584±0.027	0.2094±0.165	0.2853±0.019	0.0283±0.018
10 mg/L	0.7488±0.092	0.5571±0.093	0.0789±0.048	0.3057±0.036	0.0458±0.017

Data are given as mean values ± the standard errors of the mean

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