

REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF ATRAZINE ON THE ESTUARINE MEIOBENTHIC COPEPOD *AMPHIASCUS TENUIREMIS*

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**Abstract**—Atrazine is one of the most widely used herbicides in the United States. Atrazine concentrations in coastal environments chronically range from 90 ng/L to 46 µg/L, with rare but measured concentrations near 60 µg/L at edge-of-field conditions. Chronic atrazine effects on estuarine benthos exposed to environmentally relevant concentrations are unknown. The purpose of this research was to assess atrazine reproductive and developmental effects over multiple-generation exposures of the copepod *Amphiascus tenuiremis*. Copepods were chronically exposed to two environmentally relevant nominal atrazine concentrations (2.5 and 25 µg/L, and to an environmentally unrealistic concentration (250 µg/L). Chronic exposures were performed using a 96-well microplate life cycle bioassay. Individual stage I copepodites ( $C_1$ ,  $n = 60$ /treatment) were reared through two generations ( $F_0$  and  $F_1$ ) to sexual maturity and individually mated in microwells containing 200 µl of atrazine solution. Copepod survival across all treatments and generations was >95%. Atrazine did not affect development to reproductive maturity, time to egg extrusion, or time to egg hatch ( $p > 0.05$ ). However, reproductive failures increased across generations with increasing atrazine concentrations. Reproductive failures in the 0-, 2.5-, 25-, and 250-µg/L atrazine treatments were 11, 11, 20, and 24% for the  $F_0$  and 4, 9, 26, and 38% for the  $F_1$ , respectively. Compared to controls, total nauplii production per female was reduced by approximately 22% in  $F_0$  females exposed to 250 µg/L atrazine ( $p < 0.05$ ), and by approximately 23%, approximately 27%, and approximately 32% in  $F_1$  females exposed to 2.5-, 25-, and 250-µg/L atrazine treatments, respectively ( $p < 0.05$ ). The combined effect of reproductive failure and reduced offspring production significantly reduced total population growth in the  $F_1$  generation ( $p < 0.05$ ) even at atrazine concentrations lower than that considered safe for seawater chronic exposure (26 µg/L).

**Keywords**—Atrazine    Meiobenthic estuarine copepods    96-Well microplate bioassay

## INTRODUCTION

The triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine) is one of the most widely used weed control herbicides in U.S. agricultural crop production with an annual application ranging from 75 to 83 million pounds [1]. Since its registration in 1959, atrazine has been heavily used on corn and sorghum fields and in turfgrass and lawn care. Even though atrazine has a low potential for bioaccumulation and biomagnification by virtue of its low *n*-octanol:water partitioning coefficient ( $\log K_{OW} = 2.34$ ) and low carbon adsorption coefficient ( $\log K_{OC} = 2.36$ ), concerns have been raised that atrazine is a potential endocrine-disrupting chemical. Recent data suggest that the disruption of steroidogenesis in the amphibian *Xenopus laevis* exposed to a broad range of atrazine concentrations (0.1–200 µg/L) may be the cause of male demasculinization and an increase in hermaphroditic frogs [2]. These findings, however, are under debate since a similar study [3] found no consistent evidence of gonadal deformities in frogs resulting from exposures to low (0.1 µg/L) environmentally relevant atrazine concentrations.

Because of its high solubility (28 mg/L at 20°C) and persistence (half-life = 36–37 d [4]) in water, its high leaching potential (groundwater ubiquity score [GUS] = 4.5 [5]), and its widespread use, atrazine poses a latent chronic risk to exposed aquatic organisms. Studies in vertebrates have shown that exposure to concentrations as low as 5 µg/L or less cause necrotic and inflammatory damage to gill and kidney tissues

in the rainbow trout, *Onchorynchus mykiss* [6]. Similar studies indicate a reduction in growth of the fry of brook trout, *Salvelinus fontinalis*, exposed to 120 µg/L atrazine [7] and a potential disruption of olfactory response to reproductive stimuli in the Atlantic salmon, *Salmo salar* L. [8]. Studies of atrazine effects on chronically exposed estuarine invertebrates are scarce [9], and in most cases these have focused only on acute atrazine toxicity [10–12]. Chronic exposures, particularly at low (<4 µg/L) atrazine levels, may be important in sites near high-usage agricultural areas where atrazine residues can persist for extended periods (weeks to months) [13,14]. Information regarding effects of chronic atrazine exposure is needed since invertebrates, particularly meiobenthos, are important components of benthic faunal production in estuaries [15]. Our goal in this study was to assess multigenerational effects (reproductive, developmental, and population-level effects) of chronic exposures to atrazine using the culturable copepod model *Amphiascus tenuiremis* as a surrogate for other estuarine copepod species.

## MATERIALS AND METHODS

*Experimental organism*

The estuarine copepod *A. tenuiremis* is an amphi-Atlantic species [16], abundant in muddy sediments of intertidal and subtidal habitats of the Baltic and Black Seas [16]. This copepod has a life cycle consisting of six naupliar and six copepodite stages with sexually dimorphic adults clearly distinguished after the fifth copepodite stage (12th life stage) [17]. Gravid females produce multiple clutches (five to seven) in 10 to 14 d postinsemination. Clutches are extruded as dual

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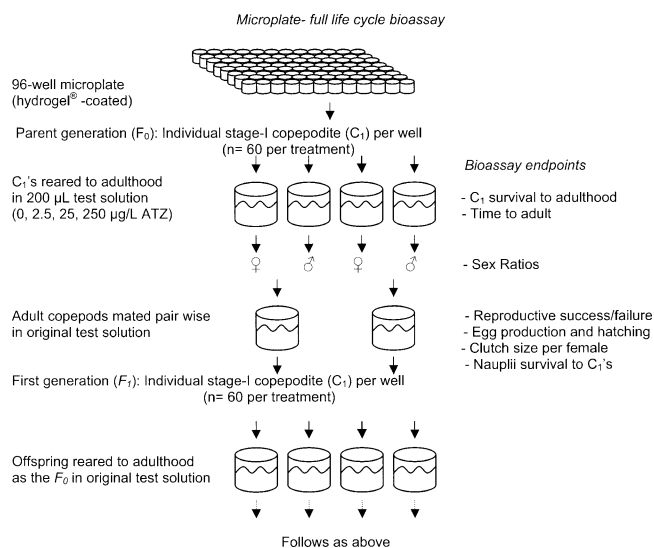


Fig. 1. Microplate full life cycle bioassay. This figure illustrates the experimental setup of the copepod *Amphiascus tenuiremis* exposed for two generations to several atrazine concentrations and a carrier control. Hydrogel (Corning Co-Star, Acton, MA, USA).

egg sacs each with six to nine embryos in a planar layout. *Amphiascus tenuiremis* has been monospecifically cultured in flow-through sediment microcosms [18] and used repetitively as a model for toxicological studies [19–21]. *Amphiascus tenuiremis* possesses a generation time of 21 d at 20°C [19], allowing for logistically simple and accurate assessment of reproductive, developmental, and endocrine endpoints.

#### Chronic bioassay

Atrazine (98  $\pm$  0.5% purity) was purchased from Chem Service (West Chester, PA, USA). Stock solutions were made in acetone and kept in the dark at  $-20^\circ\text{C}$ . Test solutions of atrazine were made by adding  $\mu$ L amounts of stock solutions to 100 ml filtered (0.2  $\mu$ m) and aerated (>90% dissolved oxygen [DO]) seawater (30 ppt). The atrazine concentrations used in this study included 2.5  $\mu$ g/L, a concentration below the average found in mid-Texas (USA) estuarine water samples (3.96  $\pm$  0.76  $\mu$ g/L) [14]; 25  $\mu$ g/L, a concentration near the U.S. Environmental Protection Agency seawater quality criterion (26  $\mu$ g/L) [22]; and 250  $\mu$ g/L, an unlikely concentration in estuarine areas. The highest concentration used in this assay was at least four times lower than the 96-h median lethal concentration (LC50 = >1 mg/L) for *A. tenuiremis* (A.C. Bejarano, unpublished data). A treatment containing a maximum of 0.02% volume/volume (v/v) acetone was used as a carrier control. Sublethal effects of atrazine were assessed using a 96-microwell microplate (Hydrogel<sup>®</sup>-coated, Corning Costar, Acton, MA, USA) life cycle bioassay (Fig. 1). Briefly, stage I copepodite juveniles ( $C_1$ ) were collected from stock cultures, sieved through a 90- and 75- $\mu$ m sieve onto a 63- $\mu$ m sieve, sorted out, and placed individually in microwells containing 200  $\mu$ L of test solutions ( $n = 60$ /treatment). The  $C_1$ s were reared to maturity and followed up to hatching of the second generation. Test solutions were removed (>90% water removal) and replaced every third day with fresh test solutions (>90% DO). The  $C_1$ s were fed every 6 d with 3  $\mu$ L of a 1:1 concentrated ( $10^7$  cells/ml) phytoplankton mixture of *Isochrysis galbana* Haines and *Dunaliella tertiolecta* Butcher. Microplates were held in an incubator (Cryo-fridge<sup>™</sup>, Baxter,

Thousand Oaks, CA, USA) at 25°C on a 12:12-h light:dark cycle. The  $C_1$ s were monitored daily via inverted stereomicroscopes and endpoints recorded (survival rates, time to successful maturation to reproductive adult, and sex ratios). On maturation, individual virgin female and male copepods were randomly mated pairwise in microwells containing original concentrations (0, 2.5, 25, and 250  $\mu$ g/L). Mating pairs were monitored daily, and the following endpoints were recorded: time to egg extrusion and time to hatch, embryonic development, number of nonviable embryos per clutch, first and second brood sizes, total viable offspring production, sex ratios, and reproductive success/failure. Within 24 h of molting from nauplii into the  $C_1$  juvenile stage, copepodites ( $F_1$ ) produced by the first clutch of starting copepods ( $F_0$ ) were collected from all treatments and individually placed into fresh control and treatment microwells ( $n = 60$ /treatment). These  $F_1$  offspring were reared to adulthood under identical conditions and concentrations as the parent copepods. After 7 d of mating, any  $F_1$  copepods unable to produce viable embryos were placed in new microwells containing their original test solutions but mated with new control unexposed males or females. Reproductive success for these original failures was then determined over a 4-d mating period.

#### Importance of copepod rearing history

To further explore reproductive effects of atrazine on individuals that had been reared in atrazine (atrazine reared), a final 15-d microplate bioassay was conducted on the third copepod generation ( $F_2$ ). Within 24 h of molting into the  $C_1$  stage,  $F_2$  copepodites produced from the first clutch of all  $F_1$  treatment and control females were collected and allocated individually into fresh atrazine treatment microwells ( $n = 35$ /treatment) under identical concentrations as the parent copepods. The  $F_2$  control  $C_1$  copepodites (non-atrazine reared) were also collected and assigned to each of the original treatments (0, 2.5, 25, and 250  $\mu$ g/L;  $n = 35$ /treatment). Copepodites ( $F_2$ ) were reared to adulthood and mated (as previously). Total offspring production ( $F_3$ ) was assessed after 7 d of mating, and comparisons were made between atrazine-reared and non-atrazine-reared copepods.

#### Stage-structured population growth model

Data from the  $F_1$  generation were used to derive a finite growth rate ( $\lambda$ ) per treatment and control to simulate population growth using a matriarchal stage-structured Leslie matrix model ([23]; RAMAS<sup>®</sup> EcoLab 2.0, Applied Biomathematics, Setauket, NY, USA). This model projects population size on the basis of stage-specific survival rates, the proportion of eggs hatching into nauplii, the proportion of nauplii developing into copepodites, the proportion of copepodites developing into females, the proportion of females able to successfully reproduce, and fecundity (viable offspring production per female). Population growth for each of the treatments and control was modeled through two generations beginning with 60  $C_1$ -stage individuals. Model constraints included logistic density dependence to account for within species crowding effects, demographic stochasticity (allowing for random differences in survival and reproduction among individuals), an arbitrary environmental carrying capacity of 10,000 individuals, and 50 replications of the simulated population growth model [24]. This model was used to estimate total population production per treatment ( $F_1$  and  $F_0$  and atrazine reared and

non-atrazine reared) on the basis of empirically observed female fecundity and reproductive success in each of the microplate bioassays.

#### Water chemistry

Aliquots (2 ml) of atrazine and carrier control fresh solutions were collected previous to each water change and stored at  $-70^{\circ}\text{C}$  for further analysis. Water samples ( $n = 5/\text{treatment}$ ) were analyzed for atrazine using an atrazine magnetic-particle enzyme immunoassay (Atrazine RaPID Assay<sup>®</sup>, Strategic Diagnostics, Newark, DE, USA), with immunoassay precision (coefficient of variation [%]) ranging from 4.4 to 7.6%. Samples were diluted on a 1- to 100-fold basis to fall within test detection range (0.04–5  $\mu\text{g/L}$ ).

#### Statistical analysis

All variables were tested for normality and homogeneity of variance using the Shapiro–Wilk goodness-of-fit test and the Levene test, respectively. Variables failing normality were transformed to meet normality ( $\log_{10}[x + 1]$ ). Differences in first and second brood sizes and viable offspring production between consecutive generations of copepods across treatments were determined by a two-way analysis of variance (ANOVA; PROC GLM, SAS Institute, Cary, NC, USA) using the Bonferroni adjustment for all possible multiple comparisons. All data following a binomial distribution (sex ratios, reproductive failure, and females producing viable/nonviable embryos) were analyzed using Fisher's exact test ( $2 \times 2$  contingency tables and when expected frequencies of one or more cells was less than 5) and Pearson's chi-square goodness-of-fit test (row by column [ $R \times C$ ] contingency tables) [25].

Statistical analysis of modeled population projections (using the  $F_1$  microplate endpoints) and estimated total population production (for the  $F_0$  and  $F_1$  and atrazine reared and non-atrazine reared) per treatment was accomplished by repeating individual stage-structured Leslie matrix simulations 15 times each per treatment. Projected population sizes in atrazine and control exposures were analyzed by a one-way ANOVA using Dunnett's procedure for multiple comparisons between atrazine-exposed and control mean model population projections. Estimated total population production for the  $F_0$  versus  $F_1$  in atrazine and control exposures and atrazine reared versus non-atrazine reared across atrazine treatments were analyzed by a two-way ANOVA using the Bonferroni adjustment for all possible multiple comparisons on mean model population production. All tests for significance were performed using an alpha level of 0.05 ( $\alpha = 0.05$ ).

## RESULTS

#### Water chemistry

The following values represent average ( $\pm 1$  standard deviation) physicochemical measurements just previous to all water changes: pH =  $8.25 \pm 0.02$ , salinity =  $30 \pm 1$  ppt, and DO =  $97 \pm 1\%$  saturation. Measured atrazine concentrations in fresh test solutions (Table 1) were 40 and 21% above the 2.5- and 25- $\mu\text{g/L}$  nominal concentrations, respectively, and 1.2% below the 250- $\mu\text{g/L}$  nominal concentration. A low, <4%, coefficient of variation (%) among water samples within treatments over time suggests that copepods were exposed to nearly constant atrazine concentrations over the bioassay period.

Table 1. Nominal and measured atrazine concentrations ( $\mu\text{g/L}$ ) in fresh seawater solutions. Measured concentrations and assay precision (% coefficient of variation [CV]) values represent the average ( $\pm 1$  standard deviation) of five water samples per treatment. CC = carrier control; NA = not applicable

Nominal concn.	Measured concn.	Precision (%CV)
0 (CC)	<LLOD <sup>a</sup>	NA
2.5	$3.5 \pm 0.2$	$3.8 \pm 0.2$
25	$30.3 \pm 0.7$	$4.1 \pm 2.7$
250	$246.6 \pm 9.2$	$2.6 \pm 2$

<sup>a</sup> Lower limit of detection (LLOD) = 0.1  $\mu\text{g/L}$ .

#### Chronic bioassay

Atrazine microplates were run for a total of 2.5 generations or 41 d. Stage I copepodite juvenile ( $C_1$ ) survival rates across all controls, treatments, and generations were >95%. At all atrazine concentrations tested, no developmental delays to reproductive maturity were observed ( $7 \pm 1$  d from  $C_1$ s). Sex ratios of atrazine treatments were not statistically different from carrier controls ( $F_0$  and  $F_1$ ,  $R \times C$ ,  $p > 0.05$ ). Sex ratios in the first generation ( $F_0$ ) exposed to atrazine were nearly 50F:50M for the 0-, 25-, and 250- $\mu\text{g/L}$  treatments. In the 2.5- $\mu\text{g/L}$  treatment, the proportion of males was twice that of females (66M:33F). Sex ratios in the second generation ( $F_1$ ) reversed and exhibited a U-shaped dose response. Ratios were nearly 50F:50M for the 0- and the 250- $\mu\text{g/L}$  treatment but 60F:40M in the 2.5- and 25- $\mu\text{g/L}$  treatment. At all atrazine concentrations tested, no significant differences (ANOVA,  $p > 0.05$ ) were seen relative to carrier controls for time to first ( $28 \pm 15$  h postmating) or second ( $45 \pm 11$  h postmating) egg extrusion and time to first ( $43 \pm 13$  h after extrusion) or second ( $78 \pm 11$  h after extrusion) hatch.

Hatching success was computed as the percentage of embryos successfully hatching into nauplii from females able to produce two consecutive broods. We did not find any significant differences (ANOVA,  $p > 0.05$ ) in hatching success between copepods exposed to atrazine compared to carrier controls. Hatching success was  $96.5 \pm 1.7\%$  for the  $F_0$  and  $92.5 \pm 6.8\%$  for the  $F_1$  generation. The number of nonviable eggs for first and second clutches of the  $F_0$  and  $F_1$  generations ranged from one to five per female across all treatments. The proportion of  $F_0$  and  $F_1$  females producing any nonviable eggs was consistently higher for females in their first clutch. In the  $F_0$ , the proportion of females producing nonviable eggs in their first clutch was elevated by 32.7 and 28% in the 2.5- and 25- $\mu\text{g/L}$  treatments, respectively, compared to control females ( $R \times C$ ,  $p = 0.016$ ); in the  $F_1$ , the proportion was elevated (32.5%) only in the 25- $\mu\text{g/L}$  treatment compared to control females. For all second clutches across treatments and generations, the number of females producing nonviable eggs ranged from 10 to 14% ( $R \times C$ ,  $p > 0.05$ ).

Total viable offspring production was calculated as the total number of hatched nauplii produced over two consecutive broods. Total viable offspring production per  $F_0$  female in the 250- $\mu\text{g/L}$  treatment was reduced by approximately five nauplii (22%) on average compared to controls (ANOVA,  $p < 0.05$ ; Table 2). Viable offspring production by  $F_1$  females was significantly reduced across all treatments compared to controls (ANOVA,  $p = 0.003$ ) by an average of five (23%), six (27%), and seven (32%) nauplii per female in the 2.5-, 25-, and 250- $\mu\text{g/L}$  atrazine treatments, respectively. The atrazine-exposed

Table 2. Mean ( $\pm 1$  standard deviation) first and second clutch sizes (nauplii + unhatched embryos) in females exposed to atrazine during two consecutive generations ( $F_0$  and  $F_1$ ). Total viable offspring represents the total number of hatched nauplii produced per female over two consecutive broods. The  $n$  represents the number of females per treatment. Total population size was estimated using a stage-structured Leslie matrix model. The  $n_p$  represents the number of mating pairs. CC = carrier control

Atrazine concn. ( $\mu\text{g/L}$ )	$F_0$				$F_1$			
	First brood	Second brood	Total viable offspring	Estimated total population size	First brood	Second brood	Total viable offspring	Estimated total population size
0 (CC)	11.3 $\pm$ 2.1 ( $n = 25$ )	12.7 $\pm$ 3 ( $n = 23$ )	23.5 $\pm$ 4.2 ( $n = 23$ )	586 $\pm$ 17 ( $n_p = 29$ )	8.9 $\pm$ 3.1 ( $n = 23$ )	12.7 $\pm$ 4 ( $n = 21$ )	21.5 $\pm$ 6.5 ( $n = 21$ )	594 $\pm$ 28 ( $n_p = 29$ )
2.5	10.8 $\pm$ 4.1 ( $n = 17$ )	11.9 $\pm$ 2.9 ( $n = 15$ )	22.6 $\pm$ 4.2 ( $n = 14$ )	563 $\pm$ 19 ( $n_p = 29$ )	7.8 $\pm$ 2.2 ( $n = 21$ )	9.8 $\pm$ 4.4 ( $n = 21$ )	17.3 $\pm$ 4.5 ( $n = 21$ )	446 $\pm$ 18 ( $n_p = 29$ )
25	11.2 $\pm$ 3.6 ( $n = 20$ )	11.9 $\pm$ 2.4 ( $n = 20$ )	22.2 $\pm$ 4.3 ( $n = 20$ )	500 $\pm$ 10 ( $n_p = 29$ )	6.9 $\pm$ 2.5 ( $n = 18$ )	10.1 $\pm$ 3.6 ( $n = 16$ )	16.0 $\pm$ 4.2 ( $n = 13$ )	332 $\pm$ 15 ( $n_p = 29$ )
250	10 $\pm$ 2.7 ( $n = 19$ )	9.1 $\pm$ 3.8 ( $n = 19$ )	18.7 $\pm$ 4.2 ( $n = 19$ )	404 $\pm$ 12 ( $n_p = 29$ )	6.3 $\pm$ 2.7 ( $n = 16$ )	9.3 $\pm$ 3.8 ( $n = 15$ )	15.1 $\pm$ 4.4 ( $n = 14$ )	266 $\pm$ 14 ( $n_p = 29$ )

females from the  $F_1$  generation showed a significant overall decrease in nauplii production compared to that of the  $F_0$  (Fig. 2).

Reproductive failure was defined as those mating pairs unable to produce viable offspring after 7 d of mating plus females unable to extrude more than one brood over a 10-d mating period. Reproductive failure for  $F_0$  copepods exposed to atrazine increased with concentration. Reproductive failures were 11, 11, 20, and 24% for the 0-, 2.5-, 25-, and 250- $\mu\text{g/L}$  atrazine treatments, respectively ( $R \times C$ ,  $p > 0.05$ ). Reproductive failure in the following generation ( $F_1$ ) was significantly different in the 25- and 250- $\mu\text{g/L}$  atrazine treatments relative to controls ( $2 \times 2$ ,  $p = 0.04$  and  $p = 0.0051$ , respectively) and elevated compared to that of the  $F_0$ . Reproductive failures were 4, 9, 26, and 38% for the 0-, 2.5-, 25-, and 250- $\mu\text{g/L}$  treatments, respectively (Fig. 3). Most cases of reproductive failure (98–100%) were due to failed mating success. Adult copepods from all treatments (including carrier controls) that were unable to reproduce when mated with individuals from their same treatments were removed and remated with unexposed female or male copepods (as previously described). An elevated proportion ( $32 \pm 7\%$ ) of those copepods that failed to reproduce initially were still unable to produce viable offspring when remated individually with unexposed mates in atrazine. For those mating pairs that were able to reproduce, clutch sizes were approximately 50% smaller than controls ( $\sim 12$  embryos/clutch).

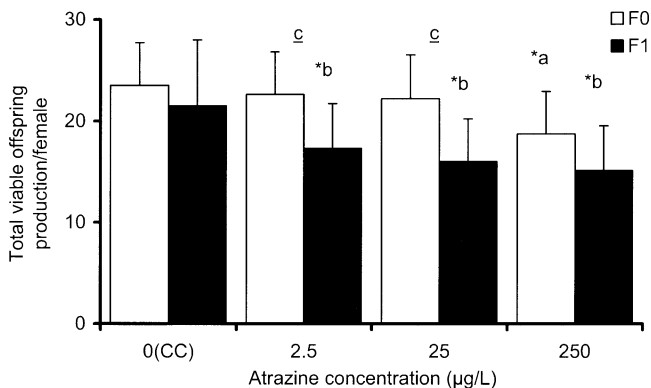


Fig. 2. Mean total viable offspring production ( $\pm 1$  standard deviation) of female copepods exposed over two generations ( $F_0$  and  $F_1$ ) to atrazine ( $n = 16$ –25 females/treatment/generation). \* represents significant differences compared to carrier control (0  $\mu\text{g/L}$ ) bar sharing same color; - represents significant differences across generations. CC = carrier control.

The cumulative effects of reduction in offspring production per female and reproductive failure in some copepod pairs chronically exposed to atrazine resulted in reduced final population sizes. Compared to controls, estimated  $F_0$  total offspring production by 29 mating pairs in the 25- and 250- $\mu\text{g/L}$  atrazine treatments was significantly reduced (ANOVA,  $p < 0.0001$ ) by 14% (87 nauplii) and 31% (183 nauplii), respectively. A more dramatic reduction was observed in the  $F_1$ , where relative to controls total offspring production was significantly reduced (ANOVA,  $p < 0.0001$ ) by 25% (148 nauplii), 44% (262 nauplii), and 55% (329 nauplii) in the 2.5-, 25-, and 250- $\mu\text{g/L}$  treatments, respectively.

#### Importance of copepod rearing history

After 7 d of mating, atrazine-reared and non-atrazine-reared  $F_2$  copepods mated individually in the 2.5- $\mu\text{g/L}$  treatment produced similar total viable offspring (Table 3). The atrazine-reared females in the 25- and 250- $\mu\text{g/L}$  treatments had on average a 30 and 38% offspring reduction compared to non-atrazine-reared females exposed to these same atrazine concentrations. Leslie matrix estimated total population production using data from the 15  $F_2$  mating pairs in the non-atrazine-reared treatments was similar across treatments ( $381 \pm 25$  nauplii). In contrast, offspring from atrazine-reared females exposed to 2.5, 25, and 250  $\mu\text{g/L}$  atrazine was reduced (ANOVA,  $p < 0.0001$ ) on average by 24% (91 nauplii), 24% (91 nauplii), and 38% (144 nauplii), respectively, compared to controls. Within treatments, atrazine-reared total offspring production

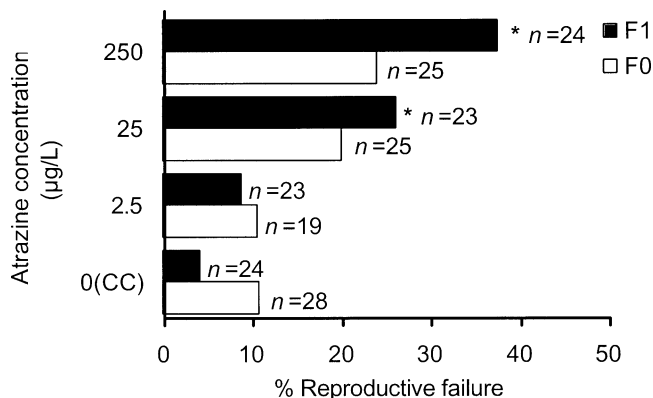


Fig. 3. Effects on reproduction in copepods exposed over two generations ( $F_0$  and  $F_1$ ) to atrazine. The  $n$  represents the total number of mating pairs per treatment; \* represents significant difference relative to carrier controls (CC; 0  $\mu\text{g/L}$ ) sharing bars.

Table 3. The importance of atrazine rearing history on second generation ( $F_2$ ) offspring production. Values represent mean ( $\pm 1$  standard deviation) of viable offspring production per female or Leslie matrix estimated total population size. The  $n$  represents the number of females per treatment, and asterisks represent a significant difference at alpha = 0.05. CC = carrier control; NA = not applicable

Endpoint	Atrazine concn. ( $\mu\text{g/L}$ )	Non-atrazine reared	Atrazine reared	$p$ value
Offspring production per female	0 (CC)	25.4 $\pm$ 7.7 ( $n = 10$ )	NA	NA
	2.5	22.3 $\pm$ 5.7 ( $n = 10$ )	19.6 $\pm$ 5.4 ( $n = 14$ )	>0.05
	25	27.1 $\pm$ 4 ( $n = 10$ )	19.3 $\pm$ 8 ( $n = 15$ )	0.03*
	250	26.1 $\pm$ 4 ( $n = 9$ )	16.1 $\pm$ 5 ( $n = 15$ )	0.003*
Estimated population size ( $n_p = 15$ mating pairs)	0 (CC)	385 $\pm$ 17	NA	NA
	2.5	339 $\pm$ 20	294 $\pm$ 13	<0.0001*
	25	406 $\pm$ 7	294 $\pm$ 11	<0.0001*
	250	393 $\pm$ 7	240 $\pm$ 184	<0.0001*

was reduced (ANOVA,  $p < 0.0001$ ) by 13% (45 nauplii), 28% (111 nauplii), and 39% (153 nauplii) compared to non-atrazine-reared females in the 2.5-, 25-, and 250- $\mu\text{g/L}$  treatments, respectively.

#### Stage-structured population growth modeling

As mentioned previously, atrazine had no significant effects on stage-specific survival. Thus, atrazine effects on population sizes at these concentrations were due mainly to reproductive and possibly sex ratio changes. The stage-structured Leslie matrix population growth model used here incorporated sex ratio changes as the proportion of copepodites (C) developing into females (F) (C-F), and reproductive failures were incorporated as the proportion of nongravid females (F) remaining in that state with time (F-F rather than F to gravid females). Using all empirical data from the  $F_1$  generation including sex ratio differences among treatments, the model showed that a reduction in clutch size (fecundity) and an increase in reproductive failure with increasing atrazine concentration might

be compensated by an increasing female-to-male ratio in the 2.5- and 25- $\mu\text{g/L}$  atrazine treatments (Table 4; note similar finite population growth rate [ $\lambda$ ] in control vs 2.5- and 25- $\mu\text{g/L}$  atrazine treatments). Projected relative population sizes modeled through two generations were significantly different (ANOVA,  $p < 0.0001$ ) between carrier and atrazine treatments. Relative to controls, modeled population size was reduced by 6% (362 nauplii), 15% (797 nauplii), and 64% (3,412 nauplii) in the 2.5-, 25-, and 250- $\mu\text{g/L}$  treatments, respectively. Furthermore, if one assumes that atrazine does not influence sex ratios (a 50:50 female-to-male ratio used for all population simulations), both  $\lambda$  and projected relative population sizes were even more strikingly lower in atrazine treatments compared to controls (ANOVA,  $p < 0.0001$ ). Compared to controls, projected mean population sizes were 41% (2,704 nauplii), 60% (3,906 nauplii), and 74% (4,816 nauplii) lower in the 2.5-, 25-, and 250- $\mu\text{g/L}$  atrazine treatments, respectively. For all model simulations, however, relative population sizes likely underestimate absolute production by approximately

Table 4. Simulated population growth for all atrazine treatments and controls based on fecundity pooled over two consecutive clutches (nauplii + unhatched embryos) and stage-specific survival in the  $F_1$  generation. Letters represent copepod stages: E = embryo, N = nauplius, C = copepodite, F = female, and GF = gravid female. Model outputs include: estimated finite population growth rate ( $\lambda$ ) and mean estimated population size ( $\pm 1$  standard deviation). CC = carrier control

Parameters	Atrazine concn. ( $\mu\text{g/L}$ )			
	0 (CC)	2.5	25	250
<b>Input</b>				
Fecundity	21.5 $\pm$ 6.5	17.3 $\pm$ 4.5	16.0 $\pm$ 4.2	15.1 $\pm$ 4.4
<b>Survival (proportion)</b>				
E-E	0.03	0.06	0.05	0.01
E-N	0.97	0.94	0.95	0.99
N-N	0	0	0	0.04
N-C	1	1	1	0.96
C-C	0	0.02	0.03	0.06
C-F <sup>a</sup>	0.47	0.55	0.61	0.46
C-F <sup>b</sup>	0.5	0.48	0.47	0.44
F-F	0.04	0.09	0.26	0.34
F-GF	0.96	0.91	0.74	0.62
<b>Output</b>				
$\lambda^a$	1.575	1.555	1.546	1.436
Population size <sup>a</sup>	5,352 $\pm$ 314	4,990 $\pm$ 236	4,555 $\pm$ 250	1,940 $\pm$ 104
$\lambda^b$	1.599	1.514	1.467	1.424
Population size <sup>b</sup>	6,532 $\pm$ 394	3,828 $\pm$ 197	2,626 $\pm$ 154	1,716 $\pm$ 80

<sup>a</sup> Using actual microplate sex ratios.

<sup>b</sup> Simulation assuming a 50:50 female-to-male ratio.

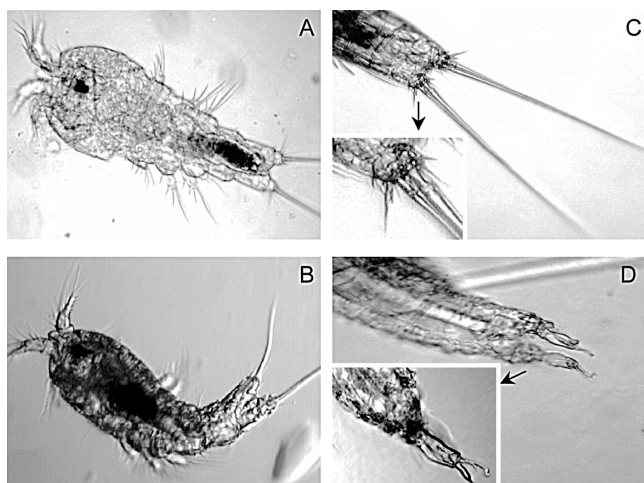


Fig. 4. Malformations in copepods exposed to atrazine. (A) Normal copepodite; (B) copepodite with sigmoidally deformed urosome; (C) normal caudal rami; (D) nondeveloping caudal rami.

60% since simulations were based on only two clutches per lifetime, and *A. tenuiremis* typically produces approximately five clutches per lifetime in microplate assays.

#### Developmental effects of atrazine

A small proportion of the  $F_1$  copepods exposed to atrazine (2, 2, and 6% for the 2.5-, 25-, and 250- $\mu\text{g/L}$  treatments, respectively) showed distinct developmental malformations of the urosome and/or caudal rami (Fig. 4). These malformations were also observed in the atrazine-reared  $F_2$  generation in the 2.5- and 25- $\mu\text{g/L}$  treatments (3 and 3%, respectively) but not in the 250- $\mu\text{g/L}$  treatment. None of these malformations were observed in the carrier control or in >1,000 carrier control copepods in other similar microplate bioassays (A.C. Bejarano, unpublished data; G.T. Chandler, personal observation). Individuals showing malformations were unable to swim, and the majority did not survive to sexual maturity. Those that survived to adulthood were unable to produce viable offspring. These copepod malformations may lack biological/ecological significance at the population level since they were present at a fairly low incidence in exposed individuals.

#### Summarized chronic effects of atrazine on *A. tenuiremis*

Combining all the information generated for both the parent ( $F_0$ ) and the first ( $F_1$ ) generation of copepods chronically exposed to atrazine, the concentration ( $\mu\text{g/L}$ ) where effects were observed (Table 5) was reported for each of the studied endpoints. Overall, few or no developmental effects were observed at any of the studied atrazine concentrations. However, reproductive effects (viable offspring production and reproductive failure) were detected in the  $F_1$  at as low as 25  $\mu\text{g/L}$  atrazine. The concentration where effects were observed for estimated population production in the  $F_0$  and  $F_1$  were  $\geq 25$  and  $\geq 2.5$   $\mu\text{g/L}$  atrazine, respectively. Likewise, effects on modeled population sizes (assuming parameters consistent to the  $F_1$ ) were found at atrazine concentrations  $\geq 2.5$   $\mu\text{g/L}$ .

### DISCUSSION

Several studies have reported acute toxicity of atrazine on different invertebrate species [9–12,26]. Standard 96-h LC50 values are reported as 1 mg/L for the American oyster *Crassostrea virginica*, 9 mg/L for the grass shrimp *Palaemonetes*

Table 5. Atrazine concentrations ( $\mu\text{g/L}$ ) where effects were observed for the developmental and reproductive endpoints of the copepod *Amphiascus tenuiremis* exposed for two generations to three atrazine concentrations. NE = no effects at any of the concentrations tested

Bioassay endpoints	Parent generation ( $F_0$ )	First generation ( $F_1$ )
$C_1$ survival to adulthood	NE	NE
Developmental delays	NE	NE
Sex ratios	NE	NE
Development time <sup>a</sup>	NE	NE
Hatching success	NE	NE
Viable offspring production per female	250	2.5, 25, 250
Reproductive failure	NE	25, 250
Copepod malformations	NE	2.5, 25, 250
Estimated population production <sup>b</sup>	25, 250	2.5, 25, 250

<sup>a</sup> Includes time (h) to first and second clutch extrusion; time (h) to first and second embryo hatch.

<sup>b</sup> Using the stage-structured Leslie matrix model.

*pugio*, >29 mg/L for the fiddler crab *Uca pugnator*, and 1,000 mg/L for the mud crab *Neopanope texana* [26]. These data suggest that atrazine is not likely to cause acute mortality on large estuarine invertebrates since these concentrations are highly unrealistic in the environment. Reported 96-h LC50 values for copepods include a 13.2-mg/L LC50 for the nauplii of the common planktonic copepod *Eurytemora affinis* [11,12] and a 153- $\mu\text{g/L}$  LC50 for the ovigerous female tide pool copepod *Tigriopus brevicornis* [10]. Based on LC50 values, *Acartia tonsa* is the most sensitive copepod to atrazine exposure with an LC50 at 94  $\mu\text{g/L}$  [9]. In our study, *A. tenuiremis* was highly insensitive to acute atrazine exposure (96-h LC50 > 1 mg/L). Studies involving the effects of multigenerational exposures of invertebrates to atrazine are limited. Dewey [7] and Kaushik et al. [27] exposed the cladocerans *Daphnia magna* to 250 and 200  $\mu\text{g/L}$  atrazine, respectively, over several generations. The former found a significant reduction in offspring production by the first generation but no effects in two subsequent generations, while the latter found reproductive effects only after a fourth generation. In contrast, our study found that consecutive generational exposure to atrazine had significant effects on overall population production at concentrations equivalent to and even much lower (10 $\times$ ) than that considered safe for seawater chronic exposure (26  $\mu\text{g/L}$  [22]). Although atrazine did not affect copepod survival or development rate, an increase in reproductive failure and a decrease in viable offspring production resulted in a reduction of 14 to 31% in the  $F_0$  and 25 to 55% in the  $F_1$  at concentrations  $\geq 2.5$   $\mu\text{g/L}$ .

In this study we presented the utility of employing a population growth model for prediction of potential population level effects based on individual-level empirical endpoints from a full life cycle culturing bioassay. Similar studies [28,29] have applied life table analysis using a modified Euler–Lotka equation to calculate changes in the intrinsic rate of natural increase ( $r_m$ ; an estimate of exponential population increase) resulting from exposures to contaminants. Full life cycle exposures of group cohorts of the harpacticoid copepod *Nitocra spinipes* to a 0.1-mg/L treatment of a synthetic nitro musk (musk ketone) [28] and to a 0.1-mg/L treatment of the polybrominated diphenyl ether (PBDE) BDE-99 [29] resulted in approximately a 90% and approximately an 80% reduction in  $r_m$ , respectively, relative to control copepods. For BDE-99, the

results were attributed to a reduction in the number of reproductive females and their fecundities. In our study, however, we were able to estimate both the finite population growth rate,  $\lambda$  (an analog of  $r_m$ ), and projected relative population sizes of *A. tenuiremis* under atrazine exposure. In particular, estimated population size was found to be very sensitive to sex ratio change, which is not surprising since this is a matriarchal model. Based on model results, even a two-generation atrazine exposure at the concentrations tested could result in a 6 to 64% population size reduction relative to controls. In contrast, assuming a more normal 50:50 male-to-female ratio, this population size reduction would range from 41 to 74%. Despite a U-shaped dose response in sex ratios of the  $F_1$ , insufficient evidence exists to suggest an atrazine induction of female gender above controls in the 2.5- $\mu\text{g/L}$  treatment. For species that can be cultured/tracked individually over short generation times, one of the strengths of the Leslie matrix model is the ability to incorporate the proportion of females successfully reproducing and their fecundities. Both of these endpoints were reduced in the  $F_1$  copepod generation in the nominal 25- and 250- $\mu\text{g/L}$  atrazine treatments. This model is not well suited for estimating population growth, however, in those species with long generation times (months to years), critical life stages that are hard to distinguish for stage-specific survival rates, and nonsexually dimorphic adults.

Estuarine copepods represent 10 to 40% of the meiobenthic community and are an important component of estuarine food webs [15,30]. Meiobenthos play an important role in carbon and nutrient cycling, and, although they comprise only approximately 20% of the benthic biomass in estuaries, their annual biomass production rates are often equivalent to that of the much larger macrobenthos [31]. In addition to a high abundance ( $>10^6$  meiofauna/ $\text{m}^2$ ) and biomass (0.75–2  $\text{g}/\text{m}^2$ ) [32], meiobenthos represent an important food source for shrimp and most juvenile fish species in estuaries [15,30]. Many estuarine juvenile fish undergo obligatory meiofaunal feeding stages where their primary food source is fatty-acid-rich benthic harpacticoid copepods (*A. tenuiremis*) [15].

Meiobenthic copepods, no longer considered strictly holobenthic, also spend short but frequent periods of time suspended in the water column [33,34]. For highly persistent [14] and soluble herbicides such as atrazine, this short but frequent exposure, equivalent to a chronic exposure, could potentially result in population declines (as demonstrated previously) since copepods have long been recognized as being sensitive to contaminants [35]. Hence, a decline in copepod densities resulting from chronic exposures to contaminants such as atrazine could potentially decrease available food sources for juvenile fish and thus potentially reduce fish densities/biomass.

Atrazine concentrations in estuarine systems and coastal environments have been reported from 90  $\text{ng/L}$  to 62.5  $\mu\text{g/L}$  during peak agricultural seasons [13,14,36]. Reported values for the half-life of atrazine under estuarine conditions range from 8 [4] to 120 d [37]. Thus, considering the short generation time of meiobenthic copepods (*A. tenuiremis*, 21 d at 35‰ and 20°C [19]; *Nitocra spinipes*, 15–19 d at 6‰ and 22°C [28]; and *Amphiascoides atopus*, 21–26 d at 30‰ and 24°C [38]), chronic exposures over several generations to low atrazine concentrations may be important under moderate to extreme cases of herbicide loading and persistence.

Some studies have evaluated community-level impacts of atrazine [39,40]. DeLorenzo et al. [39] examined effects of atrazine and its metabolite (deethylatrazine) on an estuarine

microbial food web. A 24-h exposure to 50 and 250  $\mu\text{g/L}$  atrazine and deethylatrazine produced severe effects on microbial primary production, biomass, and community structure. A similar study [40] with freshwater microbial communities showed that exposures to 32  $\mu\text{g/L}$  atrazine affected community metabolic status (dissolved oxygen and calcium and magnesium concentrations), while 337  $\mu\text{g/L}$  atrazine affected community structure and biomass.

Studies on a larger spatial scale (micro- and mesocosms) have assessed atrazine effects on several communities [4,41–44]. In freshwater microcosms dosed for several weeks with 5  $\mu\text{g/L}$  atrazine, this herbicide had no observed effects on phytoplankton, zooplankton, or macroinvertebrate communities [41]. These results were consistent with a previous freshwater microcosm study [42] in which exposures of mixed-biota communities produced negative effects only on primary producer communities at concentrations  $\geq 50$   $\mu\text{g/L}$  atrazine. The atrazine effects on aquatic communities were also evaluated in freshwater mesocosms dosed for six weeks at 5 to 360  $\mu\text{g/L}$  [43]. Phytoplankton effects were seen at  $\geq 182$   $\mu\text{g/L}$  and were probably linked to the negative observed effects on the invertebrates present in the system. In flow-through wetland mesocosms, periphyton productivity and *D. magna* survival were affected at 15 and 75  $\mu\text{g/L}$  atrazine, while no effects were observed in any of the other tested organisms (leopard frog tadpoles and fathead minnows) [4]. In tidal creek-simulated mesocosms, a 24-h exposure to 40 and 160  $\mu\text{g/L}$  atrazine caused significant effects on functional (reduced chlorophyll *a* and phototrophic biovolume and carbon assimilation) and structural (changes of algal assemblages) measures of community integrity in the microbial food web [44]. Although much is known about atrazine effects on microbial and phytoplankton communities, chronic atrazine effects on higher trophic levels, particularly in estuarine systems, are still poorly understood.

Finally, although increases in copepod reproductive failure and decreased reproductive output were observed in the  $F_1$  exposed to some of the atrazine treatments, the mechanisms by which atrazine may cause reproductive effects are unknown. Studies with vertebrates have shown potential atrazine-linked endocrine effects as it increases human aromatase activity in human adrenocortical carcinoma cells (in vitro atrazine exposures [45,46]) and also plasma levels of testosterone in male salmon exposed to 3.6  $\mu\text{g/L}$  atrazine [8]. Unfortunately, in comparison to vertebrate models, our knowledge of invertebrate endocrinology is still in its infancy. Further research should explore in more detail the mechanistic effects of atrazine on copepod reproduction with regard to, for example, sex determination, vitellogenesis, molting, and development.

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