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Effects of the Herbicide Atrazine on Members of the Freshwater Genus Hydra

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Introduction:

Atrazine is a commercial herbicide which most commonly used to combat broadleaf weeds in agriculture, typically with crops such as corn and sugarcane (Helfrich et al., 2009; Nwani et al., 2010). Atrazine garnered significant media attention when studies found that environmentally relevant concentrations of atrazine act as an endocrine disruptor in frogs (Hayes et al., 2002; Hayes et al., 2010). Atrazine alters the hormone levels of African clawed frogs, resulting in the feminization of male frogs due to high estrogen levels (Hayes et al., 2010). Atrazine is also a risk for humans, but the EPA has stated that environmental atrazine and atrazine levels of drinking water are not a significant concern to humans (Rinsky et al., 2012; Environmental Protection Agency, 2017). While atrazine presence in drinking water is not currently assumed to be prevalent to humans, much remains to be learned about the effect of atrazine on aquatic animals.

Atrazine is a triazine molecule that targets and disrupts photosystem II. It accomplishes this by interacting with plastoquinone-binding protein, preventing photosynthesis and leading to the death of photosynthesizing cells (Nwani et al., 2010). Atrazine is considered to be lipophilic and neutral in charge, indicating that it diffuses through the cell membrane via passive diffusion until it reaches equilibrium (Sterling, 1994).

Atrazine is not particularly stable in soil; it has a soil half-life of 60-150 days. This makes it unlikely that atrazine will be in high environmental concentrations for an extended period of time. However, atrazine has a half-life in water of around 6 months and can reach peaks of concentration following significant rainfall near farms (Agency for Toxic Substances & Disease Registry, 2003; Brent, Schofield, & Miller, 2001; Eisler 1989). Atrazine is known to have an effect treating land plants such as common weeds, but it also is known to have an effect on aquatic species such as algae (Nwani et al., 2010; Qian et al., 2008). For these reasons, aquatic

populations are most at risk for acute atrazine toxicity. Atrazine can be naturally degraded via hydrolysis, and this process can be catalyzed by the bacterial enzyme AtzA (Seffernick et al., 2007). The EPA cites the criterion maximum concentration (CMC) of atrazine to be 350 μg/L, while the criterion continuous concentration (CCC) is 12 μg/L (Brent et al., 2001). The CMC value indicates the highest concentration of a substance at which an aquatic community can be acutely exposed to without "an unacceptable effect", while the CCC is the highest concentration of a substance at which an aquatic community can be exposed to chronically (Environmental Protection Agency, 2017). Freshwater atrazine levels rarely exceed 20 μg/L, but can reach peaks following significant rainfall (Solomon et al., 1996).

Despite the mechanism for photosynthesis disruption being known, the precise mechanism for endocrine disruption is not as well categorized (Hayes et al., 2010; Plhalova et al., 2012; Vogel et al., 2015). Studies examining the short-term effect of high atrazine levels in freshwater animals have shown various growth and developmental defects (Plhalova et al., 2012; Hayes et al., 2010; Macek et al., 1976). Atrazine exposure has shown endocrine disruption effects in across multiples species of fish, amphibians, reptiles, and human cells. This was assumed to be caused by atrazine's ability induce aromatase production (Hayes et. al, 2010). Aromatase is known to cause estrogen production and to be involved in genetic differentiation between sexes, indicating that it is likely causing the feminization of the frogs (Stocco, 2012). Additionally, studies examining the short-term effect of high atrazine levels in freshwater animals have shown various growth and developmental defects (Plhalova et al., 2012; Hayes et al., 2010; Macek et. al, 1976). This is not the only theorized mechanism for the effects of atrazine in animals. Another study has found that higher atrazine levels are also linked to lower DNA methyltransferase activity, resulting in lower DNA methylation (Wirbisky-Hershberger et

al., 2007). Since the precise mechanism of atrazine's effect on animals has yet to be fully elucidated, it is unknown if a similar form of genetic alteration is present in *Hydra*.

Hydra are common freshwater organisms that can be subdivided in four main species groups: green, braureri, oligactis, and vulgaris (Martinez et al., 2010). The cell biology, molecular biology, and responses to toxins of members of the genus are well-studied (Quinn et al., 2012). *H. viridissima* are characterized by a symbiotic relationship with *Chlorella* algae, which are present within vacuoles in the *Hydra*'s endodermal epithelial cells (Kawaida et al., 2013). The algae provide host cells with carbohydrates, likely in the form of maltose, and the *Hydra* provide the *Chlorella* with glutamine (Hamada et al., 2018). The effects of the herbicide atrazine on *Hydra* and on the *Hydra-Chlorella* symbiosis have both yet to be studied. This study aimed to determine the effect of atrazine on *H. vulgaris*, *H. oligactis*, and *H. viridissima*. Effects on the photosynthetic symbionts of *H. viridissima* were also studied.

Methods:

Atrazine Solutions:

The commercial atrazine used was Hi-Yield Atrazine Weed Killer, diluted with *Hydra* medium (1 M $CaCl_2$, 30 mM KNO_3, 1 M $MgCl_2$, 80 mM $MgSO_4$, 0.5 M $NaHCO_3$). *Hydra* medium is composed of ultrapure water with salts to simulate pond water.

Hydra strains:

Animals used were from strains of genetically identical individuals initially generated from a single *Hydra* and expanded through asexual reproduction. The *H. vulgaris* strain used was derived from an individual collected from Lake Placida on the Elizabethtown College campus, Elizabethtown Pennsylvania, in 2015. Experiments with *H. oligactis* involved both a strain derived from an individual collected from Lake Placida in 2016, and the *H. oligactis* Swiss male

laboratory strain. Experiments with *H. viridissima* utilized the 1695C laboratory strain. Both the *H. oligactis* Swiss male strain and the *H. viridissima* strain were originally obtained from Dr. Daniel Martinez, Pomona College, Claremont California.

Experimental Design:

All experiments followed the same design, with variability in the species of *Hydra* used, the concentration of atrazine and the duration of the incubation. *Hydra* were examined at the conclusion of treatment for shortening of tentacles, thickening at the ends of tentacles, loss of tentacles, or disintegration. The traits are characteristic responses of *Hydra* when exposed to toxins (Quinn et al., 2012).

Six *Hydra* of the same species were added to each well of a 12-well plate. Six of the wells were filled with three mL of atrazine solution, and six of the wells were filled with three mL of *Hydra* medium. *Hydra* were incubated at room temperature with natural light. Animals were fed once weekly with 24 to 48-hour old brine shrimp nauplii, and the atrazine/*Hydra* medium solutions were replaced one to four hours after feeding.

Individual Experiments:

An initial experiment was conducted to determine if the EPA's criterion continuous concentration for atrazine would adversely affect *H. vulgaris*. *H. vulgaris* were incubated for three weeks in either 12 μg/L of atrazine or *Hydra* medium. At the conclusion of the incubation, *Hydra* morphology was examined and sperm motility was assessed by examining the transparent testes under a light microscope.

Next, effects of a higher concentration of atrazine, somewhat above the EPA criterion maximum concentration of 350 μg/L, were tested. *H. vulgaris* were incubated for 3 weeks in

either 400 μg/L of atrazine or *Hydra* medium. At the conclusion of the incubation, *Hydra* morphology was examined, and sperm motility was assessed.

The common species *H. oligactis* is more sensitive to both heavy metals and high temperatures than *H. vulgaris* (Bosch et al., 1988). To assess the effects of a high concentration of atrazine on *H. oligactis*, *H. oligactis* were incubated for three weeks in either 400 μg/L atrazine or *Hydra* medium. At the conclusion of the incubation, *Hydra* morphology was examined.

To determine whether even higher concentrations of atrazine might affect *H. oligactis* adversely, both the Lake Placida and the Swiss male strain of *H. oligactis* were incubated for 1 week in either 400 μg/L atrazine, 4000 μg/L atrazine, 10,000 μg/L atrazine, or *Hydra* medium. At the conclusion of the incubation, *Hydra* morphology was examined.

H. viridissima were incubated for 5 weeks in either 400 μg/L atrazine or *Hydra* medium. At the conclusion of the incubation, *Hydra* morphology was examined. In addition, the number of *Chlorella* cells per *Hydra* cell was measured. To do this, five *Hydra* from each well were disassociated into single cells using the protocol described in David (1973), except that animals in maceration solution were gently agitated for 20 minutes in tubes taped to the side of a vortexer set at a medium speed. *Chlorella* cells were counted from 15 randomly selected non-damaged cells per slide. In total, 12 slides of *H. viridissima* incubated in 400 μg/L atrazine and 12 slides of *H. viridissima* incubated in only *Hydra* medium were counted. Numbers of *Chlorella* cells per *Hydra* cell for the treatment and control were compared using a Student's t-test assuming equal variances.

Results:

Table 1: Qualitative results of *Hydra* atrazine incubations.

Species	*H. vulgaris*	*H. vulgaris*	*H. oligactis*	*H. oligactis*	*H. oligactis*	*H. viridissima*
Concentration of Atrazine	12 µg/L	400 µg/L	400 µg/L	4000 µg/L	10,000 µg/L	400 µg/L
Incubation Duration	21 days	21 days	21 days	7 days	7 days	35 days
Morphological Effect Present	No loss of tentacles, motile sperm present	No loss of tentacles, motile sperm present	No loss of tentacles	No loss of tentacles	No loss of tentacles	No loss of tentacles, decreased number of *Chlorella* per *Hydra* cell

No shortening of tentacles, thickening of the ends of tentacles, loss of tentacles, or disintegration were observed at any of the concentrations of atrazine tested for *H. vulgaris, H. oligactis,* or *H. viridissima.* Motile sperm were observed in the testes of *H. vulgaris* incubated for three weeks at 12 µg/L and at 400 µg/L atrazine (Table 1).

H. viridissima grown in 400 µg/L atrazine had significantly fewer *Chlorella* cells per *Hydra* cell than *H. viridissima* grown in *Hydra* medium ($p=2.48716 \times 10^{-15}$). It appeared that chlorella in the *Hydra* grown in atrazine were larger than normal, but measurements were not taken.

Future experiments regarding atrazine's effect on *Hydra* at environmentally relevant concentrations need to be conducted to confirm the potential negative effect of atrazine on *H. viridissima* with *Chlorella*.

Discussion:

Previous studies regarding atrazine have demonstrated effects in both vertebrates and invertebrates, including arthropods and mollusks (Eisler 1989; Hayes et al., 2002; Juhel et al., 2017; Yoon et al., 2019). It is unclear whether *Hydra*, as cnidarians, might also be impacted by

atrazine. However, no effect on *Hydra* morphology was observed across any of the *Hydra* strains used in the experiment. Some effects of atrazine on vertebrates are mediated by effects on aromatase expression (Hayes et al., 2010) and BLAST searches of the *H. vulgaris* genome indicate that no aromatase orthologue is present. Thus at least one mechanism through which atrazine affects vertebrates does not apply for *Hydra*.

While we detected no direct effect of atrazine on the three species *Hydra* tested, there was a significant decrease in the number of *Chlorella* within *H. viridissima* cells. The *Chlorella* provide the *Hydra* with carbohydrates (Kawaida et al., 2013; Hamada et al., 2018), so decreased *Chlorella* density or loss of the *Chlorella* symbiont have the potential to adversely affect wild *H. viridissima* populations. Further testing at environmentally relevant concentrations could confirm whether or not these results are applicable to *H. viridissima* in freshwater ecosystems.

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