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The Combined Effects of Atrazine and Tetracycline on Primary Producers and Zooplankton in Freshwater Microcosms

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The Combined Effects of Atrazine and Tetracycline on Primary Producers and Zooplankton in Freshwater Microcosms

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ABSTRACT

Widespread use of agrochemicals such as herbicides and antibiotics increases their likelihood of entering aquatic systems in mixture. Despite different modes of action, atrazine (herbicide) and tetracycline (antibiotic) adversely affect non-target photosynthetic organisms, such algae and macrophytes, with the potential to reduce food availability to higher trophic levels. However, the effects of simultaneous exposure to both contaminants have yet to be determined. I hypothesized that a mixture of atrazine and tetracycline affects freshwater communities differently than each compound alone. A microcosm experiment was conducted to test effects of environmentally relevant concentrations of atrazine (1 μg L^{-1}) and tetracycline (1 μg L^{-1}), alone and together, on the green microalga *Chlorella* sp., the duckweed *Lemna minor*, and the microscopic crustacean *Daphnia magna* in a greenhouse over a period of 10 days. The endpoints measured were *Chlorella* sp. cell density and chlorophyll *a* concentration, *L. minor* growth and tissue condition, and mortality and reproduction of *D. magna*. Atrazine appeared to decrease *Chlorella* sp. abundance but not enough to reduce food availability to *D. magna*, whose reproduction and mortality were unaffected. Tetracycline and atrazine decreased *L. minor* abundance individually and in combination. Furthermore, the reduction in the combination treatment appeared to be additive. Growth inhibition was highest in the combination treatment. The greater adverse effects associated with mixtures of atrazine and tetracycline on *L. minor* compared to the individual compounds suggests increased potential for population decline over the long term. Losses of aquatic plants in turn may alter aquatic community composition and species interactions.

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Introduction

The widespread use of agrochemicals such as herbicides and antibiotics increases their likelihood of entering aquatic systems via surface runoff (Fuhrer, 1999; Boxall et al., 2003). Although previous studies investigated the toxicity of many of these compounds to microscopic plants (phytoplankton) and animals (zooplankton) living in lakes and streams, typically the effects of only one compound was examined at a time (DeNoyelles et al., 1982; Quinlan et al., 2011; Weiner et al., 2004; Yang et al., 2013). While single compound toxicity tests provide important information on chemical toxicity to an organism under controlled conditions, in reality, many different compounds are introduced to aquatic ecosystems at the same time (Boxall et al., 2003) and may interact with unanticipated outcomes (DeLorenzo and Serrano, 2003). Frequently used herbicides, such as atrazine, and antibiotics, like tetracycline, have very high potential to enter surface waters together near agricultural sites (Boxall et al., 2003). Therefore, studying the effects of chemical mixtures on non-target aquatic microorganisms is essential.

Atrazine is a water-soluble herbicide used to control broadleaf and grassy weeds associated with crops by inhibiting photosynthesis via blockage of the electron transport in photosystem II (Knauert et al., 2008). Its solubility in water gives atrazine high capacity to contaminate surface runoff near application sites and leach into groundwater (DeLorenzo et al., 2001; Graymore et al., 2001). Not only is atrazine frequently used in agriculture, but it is also used in many other countries. Atrazine use is ~30-40,000 tons yr^{-1} in the US and 70-90,000 tons yr^{-1} worldwide (Graymore et al., 2001; Sass and Colangelo, 2006). Concentrations of atrazine measured in streams, ponds, and lakes in Canada and the United States average 1-5 $\mu g\ L^{-1}$ (maximum range = 0.1-30 $\mu g\ L^{-1}$) (DeNoyelles et al., 1982; DeLorenzo and Serrano, 2003). The common use of atrazine as an herbicide on crops means that it enters streams and waterways near agricultural land and has high potential to adversely affect non-target aquatic microalgae and macrophytes living there.

Aquatic primary producers experience adverse effects from atrazine (Boxall et al., 2003). Concentrations as low as 1 $\mu g\ L^{-1}$ of atrazine inhibited chlorophyll production of green microalgae by 16-93% depending on the species, with a 50% decrease for *Chlorella vulgaris* (Torres and O'Flaherty, 1976). Atrazine removed sensitive phytoplankton taxa (chlorophytes and cyanobacteria) in freshwater ponds, thereby changing the structure of the aquatic community; green algae that *Daphnia magna* preferentially consume were replaced by less palatable taxa such as diatoms and flagellates (DeNoyelles et al., 1982; Tang et al., 1997; Seguin et al., 2002). Atrazine also inhibits photosynthesis in aquatic macrophytes. Forney and Davis (1991) found six different macrophytes sampled from Chesapeake Bay, including duckweed (*Lemna* sp.),

were sensitive to atrazine concentrations ≥10 µg L^{-1}. This potential for chemical-induced losses of primary producers in the field may not only adversely affect community composition, but also species interactions.

Loss of primary producers from direct atrazine toxicity alters feeding relationships with taxa in higher trophic levels (DeNoyelles et al., 1982; Graymore et al., 2001). Green algae are an important food source for grazing zooplankton but are more susceptible to atrazine toxicity than other phytoplankton taxa (Tang et al., 1997; Seguin et al., 2001; Work and Havens, 2003). Therefore it is not surprising that atrazine (500 µg L^{-1}) decreased *Daphnia pulex* abundance by 75% due to food limitation rather than by direct toxicity (which occurs at much higher concentrations ≥3.6 mg L^{-1}) (DeNoyelles et al., 1982). Reproduction rates of *Daphnia magna* declined at atrazine concentrations ≥250 µg L^{-1} due to direct toxicity (Marshall, 2009), however, reproduction rates may decline at lower concentrations as a result of lessened food availability. Furthermore, floating aquatic plants, such as the duckweed (*Lemna* sp.) provide food, habitat, and oxygen to consumers and remove excess nutrients from the water in pond communities (Okomoda et al., 2012). Atrazine decreased the abundance of a macrophyte community, including *L. gibba*, of a Kansas pond by 60% (Solomon et al., 1996), while another study found *Lemna gibba* abundance to have a half maximal effective concentration, or EC50, of 22 µg L^{-1} (Hoberg, 1991). Clearly the presence of atrazine alone can change freshwater community structure, but how the addition of other chemicals such as antibiotics might influence these responses is unknown.

Antibiotics enter aquatic systems via discharge from wastewater treatment plants and aquaculture, runoff associated with topical treatments to livestock and spreading of

manure, leaking septic tanks, and emissions during manufacturing (Fuhrer, 1999). Tetracycline is the most widely used broad-spectrum antibiotic on livestock in the US (>6,600,000 kg in 2014) (Chopra and Roberts, 2001; Yang et al., 2004; FDA, 2014), resulting in surface water concentrations up to ~1 μg L^{-1} in the US (Halling- Sørensen et al., 1998). The mode of action for tetracycline is inhibition of bacterial protein synthesis by preventing aminoacyl tRNA from binding to the ribosomal acceptor site (Chopra and Roberts, 2001). Even though the target organisms for tetracycline are bacteria, organisms with similar ribosomal structure, such as the chloroplasts in aquatic primary producers, are susceptible to tetracycline toxicity (Yang et al., 2013).

Aquatic photosynthetic organisms demonstrate decreased photosynthesis, cell growth, protein synthesis, and enhanced oxidative stress in the presence of tetracycline (Quinlan et al., 2011; Yang et al., 2013; Shang et al., 2015). Tetracycline inhibited photosynthetic activity in the cyanobacterium *Microcystis aeruginosa,* and the green microalga *Selenastrum capricornutum,* at concentrations of 100 μg L^{-1} (Yang et al., 2013). Tetracycline can affect other photosynthetic organisms, such as aquatic macrophytes, albeit at much higher concentrations. Concentrations of 1000 μg L^{-1} tetracycline inhibited frond growth in the aquatic macrophyte *Lemna minor* by 43%, while concentrations between 1 and 100 μg L^{-1} stimulated frond growth up to 26%, but the cause of these opposing responses was unknown (Pomati et al., 2004). Recent evidence suggests these concentration-dependent responses may result from removal of either growth-promoting or growth-inhibiting bacterial communities from the plant rhizosphere (Ishizawa et al., 2017). If tetracycline inhibits growth-promoting bacteria

present on the root and frond area of duckweed rhizosphere, then adverse effects on abundance and growth would be expected.

Tetracycline also affects abundance and reproduction in zooplankton, such as *Daphnia magna* (Halling-Sørenson et al., 1998; Wollenberger et al., 2000; Kim et al., 2012). Multigenerational exposure of *D. magna* to tetracycline at concentrations of 0.1 and 5.0 mg L^{-1} decreased population growth rate by 30 and 60%, respectively (Kim et al., 2012). Number of offspring of *D. magna* decreased at tetracycline concentrations of 29.4 mg L^{-1} or higher after 21 days of exposure (Wollenberger et al., 2000). That atrazine and tetracycline each have adverse effects on multiple interacting organisms further supports the need for examining mixture effects in aquatic communities.

The combined effects of herbicides and pesticides or antibiotic mixtures on phytoplankton have been examined, but not interactions between atrazine and tetracycline (Torres and O'Flaherty, 1976; Seguin et al., 2001; DeLorenzo and Serrano, 2003; Pomati et al., 2004; Knauert et al., 2008; González-Pleiter et al., 2013). Phytoplankton responses to the mixtures tested were additive, synergistic or antagonistic. For example, three photosystem II inhibitors (atrazine, isoproturon, and diuron) decreased phytoplankton photosynthetic activity in an additive fashion (Knauert et al. 2008). When atrazine was mixed with the organophosphate insecticide, chlorpyrifos or the fungicide, chlorothalonil, the insecticide mixture displayed additive toxicity while the fungicide mixture exhibited synergistic toxicity to marine phytoplankton *Dunaliella tertiolecta* growth (DeLorenzo and Serrano, 2003). Depending on the composition, antibiotic mixtures yield synergistic, additive, or antagonistic effects on growth inhibition of green alga and cyanobacteria, but synergism was most common (González-Pleiter et

al., 2013). The effects of chemical mixtures can often be complex and unpredictable, therefore, it is important to investigate the effects of atrazine and tetracycline mixtures on non-target organisms.

The goal of this study was to investigate the effects of atrazine and tetracycline, alone and combined, on freshwater communities. I hypothesized that the mixture of atrazine and tetracycline affects freshwater communities differently than each compound alone. Specifically, I predicted the combination treatment would have additive effects on abundance and growth inhibition of primary producers. I also anticipated higher mortality of *D. magna* in the mixture due to reduced food availability. Using microcosms, I exposed the microscopic algae *Chlorella* sp., the zooplankton *Daphnia magna*, and the floating macrophyte *Lemna minor* to environmentally relevant concentrations of atrazine and tetracycline over a period of 10 days. Results from this experiment will be useful in understanding potential interactive effects of chemical mixtures on non-target organisms.

Methods

To test the hypothesis that effects of atrazine and tetracycline in mixture differ from those of each compound alone on freshwater communities, a series of microcosm experiments was conducted to determine appropriate experimental conditions, followed by testing the effects of ecologically relevant treatments of tetracycline, atrazine or both.

1. *Water Flow Experiment*

A pilot experiment was performed to test the response of *Daphnia magna* to water flow to determine whether flowing or standing water experimental conditions should be

used. The experiment was performed from 28 November to 2 December 2016. In the Department of Biology greenhouse at Georgia Southern University (Statesboro, GA, USA), 20 *D. magna* were placed in artificial streams with 12 L of moderately hard water and a microalgal food source (*Chlorella* sp., cell density of ~50,000 cells ml^{-1}). Five of the streams were treated with 5 µg L^{-1} of tetracycline (Kim and Carlson, 2007), and five served as no-addition controls for a 7-day exposure period. None of the *D. magna* survived in either treatment, likely due to the presence of water flow. *Daphnia magna* are typically found in low to no flow waters ranging in size from small temporary pools to large lakes (Ebert, 2005), therefore all subsequent experiments were conducted in pond microcosms.

2. *Grazing Rate Experiment*

To determine an appropriate *Chlorella* sp. cell density to sustain *D. magna* grazing over one week, three algal cell densities prevalent in the literature ($1x10^4$, $5x10^4$, or $1x10^5$ cells ml^{-1}) were fed to five *D. magna* in 200 ml of spring water in 250 ml beakers (n=4) (Halling-Sørensen et al., 2000; DeLorenzo and Serrano, 2003; Pannard, 2009). No *D. magna* mortality occurred in any of the treatments, and all treatments had similar final *Chlorella* sp. cell densities (~$2x10^5$ cells ml^{-1}) (Figure 1) suggesting that a starting density of $1x10^4$ cells ml^{-1} is sufficient to sustain *D. magna* grazing for at least one week.

3. *Experimental Treatment Concentration and Duration Study*

To determine atrazine and tetracycline concentrations and exposure time for a freshwater community microcosm study, I examined the responses of *Chlorella* sp. and *D. magna* over seven days to one of six treatments (no-addition control, 1 μg L^{-1} tetracycline, 1 μg L^{-1} atrazine, 5 μg L^{-1} atrazine, 1 μg L^{-1} tetracycline with 1 μg L^{-1} of atrazine, and 1 μg L^{-1} tetracycline with 5 μg L^{-1}) (n=6). A time frame of seven days was selected (Tang et al., 1997). The two atrazine concentrations and one tetracycline concentration tested were ecologically relevant and known to affect the green microalga *Chlorella* sp. individually (Torres and O'Flaherty, 1976; DeNoyelles et al., 1982; Seguin et al., 2001).

Each experimental unit (250 ml beaker) contained 200 ml of spring water, *Chlorella* sp. at a density of $1x10^4$ cells ml^{-1} and five *D. magna*. This cell density sustained *D. magna* grazing for one week, and the *D. magna* density used is common in the literature (Wollenberger et al., 2000; Heckmann and Connon, 2007). Experimental units were placed under a light bank with daylight fluorescent lights at an irradiance of 80-100 μmol s^{-1} m^{-2} with a 16:8 light:dark regime (Pape-Lindstrom, 1997; DeLorenzo and Serrano, 2003; Weiner et al., 2004; Heckmann and Connon, 2007).

Cell density was measured before treatment application, after 24 hours of exposure, and then every 48 hours for one week using flow cytometry. Mortality of *D. magna* was also determined visually at each time point. Atrazine decreased cell density (two-way ANOVA, $F_{2,5} = 11.61$, p=0.0002). Both atrazine-only treatments resulted in >50% decrease in *Chlorella* sp. density compared to the control and tetracycline-only treatments (Figure 2). However, the combination of 1 μg L^{-1} of atrazine and tetracycline

was 59% lower than 1 μg L^{-1} of atrazine alone. Concentrations of atrazine in the field are more likely to be 1 than 5 μg L^{-1} (Boxall et al., 2003), therefore, the 1 μg L^{-1} of atrazine concentration was selected for the freshwater microcosm community study. Reproduction of *D. magna* is typically observed 8 days after spawning (Walton et al., 1982), therefore the microcosm study was conducted for 10 days to allow for measurement of reproductive effects on *D. magna*.

4. *Freshwater Community Study*

To determine the effects of atrazine and tetracycline alone and together on a freshwater community, a microcosm experiment was conducted at Georgia Southern University (Statesboro, GA, USA) in the Department of Biology greenhouse. Each 2 L glass microcosm contained primary producers (*Chlorella* sp. at a density of $1x10^4$ cells ml^{-1} and 10 colonies of *Lemna minor*), a zooplankton grazer (10 *Daphnia magna* individuals; Heckmann and Connon, 2007) and 1.5 L of treatment solution. The United States Environmental Protection Agency guidelines for *Lemna* spp. (1996) state that 3-5 colonies should be placed in 250-1000 ml glass beakers, therefore, 10 colonies placed in each 2 L vessel. The sides of the microcosms were covered with window screening to more closely resemble the light exposure in a pond (270.8 ± 25.7 μmol m^{-2} sec^{-1}). The experiment was conducted for 10 days to allow assessment of reproductive output and generation effects of chemical exposure on *D. magna* since the age at which *D. magna* begin reproduction by parthenogenesis is around 5-10 days (Ebert, 2005).

Treatments were a no-addition control, 1 μg L^{-1} of atrazine, 1 μg L^{-1} of tetracycline, and 1 μg L^{-1} of both atrazine and tetracycline (n=10). Treatment

concentrations were chosen to reflect those commonly found in surface waters in the United States (average 1-5 μg L^{-1} for atrazine and reach a maximum of 1 μg L^{-1} for tetracycline). An atrazine stock solution was prepared by dissolving 15 mg of atrazine (CAS 1912-24-9; 97% purity; Carbosynth Inc. Berkshire, UK) in 1L of deionized water from which aliquots were used to mix treatment solutions. Measured atrazine concentration of the starting solution was 1.2 ± 0 μg L^{-1}, determined by extraction of atrazine/herbicide from the water samples using ethyl acetate following a modified version of EPA Method 507 (Munch, 1995). The combined extract was then treated with muffled sodium sulfate to remove excess water, concentrated using Rotovap procedure and reconstituted to 5 ml with pesticide grade ethyl acetate. Samples were then analyzed using gas chromatography with a Nitrogen Phosphorous Detector (Dr. Teresita Ona, Agriculture and Environmental Services Lab, University of Georgia). A stock solution of tetracycline was prepared by dissolving 15 mg of tetracycline (CAS 64-75-5; Fisher Scientific; North Carolina, USA) in 1L of deionized water and diluted to make treatment solutions. Initial tetracycline concentration in the treatment solutions (0.76 ± 0.11 μg L^{-1}) was measured using high performance liquid chromatography. Briefly, the high-pressure liquid solvent used in the column as the mobile phase was acetonitrile. A detector recorded the absorption measurements and a standard curve was generated. The standard curve and absorption values of the stock and samples were used to determine the concentration of each (Munch, 1995).

Chlorella sp. cell density was measured initially and every 48 hours until completion of the experiment using an Accuri C6 flow cytometer (BD Biosciences, California, USA). Each sample was taken by gently stirring to microcosm and blasting

any algae build up on the bottom of the jar with a pipette. The samples were pipetted from the center of the jar into a microcentrifuge tube and a volume of 0.5 ml was taken. For each of the samples, microcentrifuge tubes were vortexed and then 27 µl was run through the flow cytometer. To measure chlorophyll *a* concentration, microcosms had to be homogenized by shaking and pipetting to resuspend algae that settled at the bottom before a 100 ml of sample was taken for analysis. Because of the destructive nature of this sampling, chlorophyll *a* concentration could only be measured on the final day of the experiment. Water samples were filtered through Whatman GF/F glass fiber filters (nominal pore size 0.7 µm). Pigments from cells collected on the filters were extracted in 90% acetone at -20°C for 24 hours, followed by measurement using a Trilogy fluorometer (Turner Designs, California, USA) according to EPA Method 445 (Arar and Collins 1997).

The number of *D. magna* per microcosm was determined visually every 48 hours for the duration of the experiment. Water quality (temperature, dissolved oxygen, conductivity, and pH) was also measured using a YSI Pro Plus (YSI Incorporated, Ohio, USA) to verify that water quality stayed within acceptable limits for *Chlorella* sp., *D. magna*, and *L. minor*. Colonies of *L. minor* were photographed every 48 hours. Using the digital images, abundance was calculated as frond number, while frond condition was measured as color (green, yellow, white, brown) using ImageJ (National Institutes of Health, MD, USA). Growth inhibition was calculated using the equation:

$$\frac{((\text{final-initial frond \#})-\overline{X}\ \text{control frond \#}))}{(\overline{X}\ \text{control frond \#})} * 100$$

Data were tested for normality and equal variances using the Shapiro Wilk *W* test and Levene's test, respectively. *Chlorella* sp. cell density and chlorophyll *a* content did

not meet the assumptions of parametric tests and were log transformed so the effects of treatments on the final day could be determined using two-way analysis of variance (ANOVA). Effects of time and treatment on *Chlorella* cell density and duckweed abundance and necrosis were elucidated with repeated measures ANOVA. *Daphnia magna* abundance and reproduction data could not be transformed and were therefore analyzed nonparametrically using the Scheirer Ray Hare extension of the Kruskal-Wallis test. Growth inhibition of duckweed was calculated relative to the control, and therefore analyzed using one-way ANOVA.

Results

Freshwater Community Study

Atrazine reduced chlorophyll *a* concentration (two-way ANOVA, $F_{1,39}$=5.29, p=0.03) (Table 1A) in *Chlorella* sp. by 35%, while tetracycline appeared to counteract atrazine effects because the combination treatment reduced chlorophyll *a* by 15% relative to the control (Figure 3a). No difference in cell density between treatments occurred, likely due to high within-treatment variability (Table 1B) (Figure 3b). However, the pattern in cell density was similar to that for chlorophyll *a* concentration; cell density in the combination treatment appeared to be less affected than cell density in the atrazine alone treatment (18% vs. 25% less than the control).

Reproduction of *D. magna* was unaffected by atrazine (Scheirer Ray Hare, x_3=0.17, 0.50<p<0.90), tetracycline (Scheirer Ray Hare, x_3=0.68, 0.10<p<0.50), or the combination (Scheirer Ray Hare, χ^2_1=0.41, 0.50<p<0.90) (Table 2A). Abundance was also unaffected by atrazine (Scheirer Ray Hare, χ^2_1=0.01, 0.90<p<0.98), tetracycline

(Scheirer Ray Hare, x_3=0.24, 0.50<p<0.90), or the combination (Scheirer Ray Hare, χ^2_1=1.24, 0.10<p<0.50) (Table 2B), although there appeared to be a ~32% decrease in abundance of *D. magna* in the atrazine only treatment (Figure 4a). Furthermore, the pattern in *D. magna* abundance seemed to follow that of *Chlorella* sp. cell density (Figure 3b). No difference in *D. magna* abundance due to atrazine (rmANOVA, $F_{6,239}$=0.14, p=0.78), tetracycline (rmANOVA, $F_{6,239}$=0.91, p=0.37), or the combination (rmANOVA, $F_{6,239}$=1.20, p=0.29) over time occurred (Table 3) (Figure 4b), although the treatments showed similar patterns in *Daphnia* abundance and *Chlorella* cell density Days 8 and 10 showed a slightly higher abundance of both organisms in the combination treatment while the other three treatments did not differ.

Tetracycline decreased *L. minor* abundance (two-way ANOVA, $F_{1,39}$ =4.59, p=0.04) and atrazine showed a strong trend toward reduction of abundance (two-way ANOVA, $F_{1,39}$=3.66, p=0.06) (Table 4A, Figure 5). The reduction of *L. minor* abundance in the combination treatment (19%), while not statistically significant, appeared to be approximately the sum of the reduction in the tetracycline-only (~8%) and atrazine-only (~7%) treatments. An interaction between atrazine and tetracycline on final *L. minor* necrosis appeared to be a result of intermediate effects of the mixture compared each compound alone (two-way ANOVA, $F_{1,39}$=9.46, p=0.04) (Table 4B). Plants necrosis in the combination treatment was 20% compared to 24% in the atrazine-only treatment and 17% in the presence of tetracycline-only (Figure 6a). Atrazine and tetracycline had an adverse effect on necrosis over time (Table 5). All of the treatments experienced more necrosis than the control by day 10, but the combination treatment also appeared to have the most necrosis between days 8-10 (Figure 6b). By the end of the experiment, growth

inhibition increased in the combination treatment relative to the single-compound treatments (one-way ANOVA, $F_{3,39}$=4.87, p=0.02) (Tukey-Kramer HSD). Furthermore, growth inhibition in the mixture (69%) appeared to be approximately the sum of the inhibition in the atrazine- (34%) and tetracycline-only (29%) treatments (Figure 7).

Discussion

I hypothesized that a mixture of atrazine and tetracycline affects freshwater communities differently than each compound alone. Atrazine alone reduced *Chlorella* chlorophyll *a* content by ~35%. Other studies reported adverse effects of atrazine of similar magnitude on *Chlorella* species, although at differing concentrations (Torres and O'Flaherty, 1976; Seguin et al., 2001). In a similar study, after a 7-day exposure to 1 µg L^{-1} of atrazine, *C. vulgaris* chlorophyll content was inhibited by 50% (Torres and O'Flaherty, 1976). A microplate assay of atrazine found *C. vulgaris* cell density decreased by 50% at 4.3 µg $L^{-1}$of atrazine (Seguin et al., 2001). The non-significant ~25% reduction in cell density I observed was likely due to high within treatment variability caused by *D. magna* grazing and stimulation of algal growth from excreted nitrogenous waste (DeNoyelles et al., 1982).

The concentration of atrazine used in this experiment (1 µg L^{-1}) was three orders of magnitude lower than a concentration known for direct toxicity to *D. magna* (~3.6 mg L^{-1}) (DeNoyelles et al., 1982; Solomon et al., 1996), but abundance appeared to mirror that of the *Chlorella* sp. food resource. Both *Chlorella* and *D. magna* showed patterns of reduction in the atrazine only treatment (Figure 3a and 4a), but the exposure time may not have been long enough to see significant changes. Over a period of 136 days, community composition of zooplankton shifted from cladoceran to rotifers following a 95% decrease

in abundance of phytoplankton due to atrazine (DeNoyelles et al., 1982).Therefore, future study on the mixture effects of atrazine and tetracycline should last more than 10 days.

Toxic effects of tetracycline on green microalgae have been reported, but generally at much higher concentrations, such as 0.2 mg L^{-1} for *P. subcapitata*, and 2.0 mg L^{-1} for *S. capricornutum* (González-Pleiter et al., 2013; Yang et al., 2013). Given that *Chlorella* sp. was unaffected, *D. magna* was not expected to experience decreased abundance or reproduction due to indirect or direct toxicity of tetracycline. Concentrations of tetracycline chosen were too low for direct toxicity to *D. magna*, which typically occur at or above 0.1 mg L^{-1} (Kim et al., 2012). Furthermore, reproductive effects on *D. magna* occur at concentrations of 29.4 mg L^{-1} of tetracycline.

Because atrazine is a photosystem II inhibitor (Knauert et al., 2008), our observation of decreased *Lemna minor* abundance and growth was expected. Solomon et al. (1996) similarly reported that atrazine decreased abundance of aquatic macrophytes, including *Lemna minor*, however, effects typically occurred at concentrations above 10 µg L^{-1} (Solomon et al., 1996). In this study, atrazine also increased duckweed necrosis which was unlike the results from other studies that recorded necrosis or color change (Mohammad et al., 2010; Brain et al., 2012). *Lemna gibba* growth rate was inhibited at concentrations ranging from 200-16000 µg L^{-1}, yet none of the treatments showed observable necrosis (Mohammad et al., 2010). After 9 days of exposure to atrazine, growth inhibition was observed in the 80 and 160 µg L^{-1} treatments, but without necrosis (Brain et al., 2012). I used much lower concentrations in my study that may have allowed enough time to observe necrosis before death and total frond disintegration of *L. minor*.

Tetracycline significantly reduced duckweed abundance, unlike Pomati et al. (2004) who found a slightly stimulatory effect at 1 µg L^{-1} that increased growth by 18%. Their stimulatory effect was attributed to the removal of growth inhibiting bacteria associated with duckweed. Growth inhibiting and growth promoting bacteria are rhizobacteria that can be found on duckweed fronds and roots and can either help or hinder plant growth (Ishizawa et al., 2017). Therefore, the inhibitory effect on duckweed growth in the present experiment may have been due to a reduction in plant growth-promoting bacteria (Ishizawa et al., 2017). Similar to this experiment, an inhibition of growth rate of *L. minor* occurred at 19 µM (665 µg L^{-1}) of tetracycline after 7 days of exposure (Baciak et al., 2016).

Atrazine and tetracycline have different modes of action yet can adversely affect nontarget photosynthetic organisms in similar ways. I predicted the combination would show additive toxicity on each of the *Chlorella* sp. and *Lemna minor* end points measured. However, tetracycline appeared to mitigate the effects of atrazine on *Chlorella* sp. chlorophyll *a* content possibly due to tetracycline slowing the growth of aquatic bacteria, and releasing *Chlorella* sp. from competition for nutrients (Faust et al., 2000). The interaction between tetracycline and atrazine also suggested that tetracycline lessened adverse effects of atrazine on *L. minor* necrosis. However, the decrease in *L. minor* abundance and increase in growth inhibition appeared to be additive in the mixture, as predicted. That two different aquatic primary producers responded in different ways to the same mixtures and concentrations highlights the importance of not only investigating mixture effects, but also multiple species.

Conclusion

Atrazine and tetracycline have similar pathways into aquatic systems and known individual adverse effects, underscoring the importance of investigating the combined potential of both compounds to influence aquatic communities. Atrazine adversely affected both primary producers by decreasing *Chlorella* sp. abundance and increasing *L. minor* necrosis. The concentration of atrazine used was too low for direct toxicity to *D. magna*, but abundance of *D. magna* appeared to parallel that of *Chlorella* sp. Tetracycline negatively affected *L. minor* abundance and growth, but did not have an effect on *Chlorella* sp. In the combination treatment, tetracycline appeared to mitigate effects of atrazine on *Chlorella* sp. Additive effects of atrazine and tetracycline on *L. minor* abundance and growth inhibition indicate potential for loss of an organism that serves as food, habitat, and has an important role in removal of excess nutrients from water. Decreased abundance of nontarget photosynthetic organisms in the presence of atrazine and tetracycline may affect higher trophic levels, particularly over longer time frames (DeNoyelles et al., 1982; Tang et al., 1997; Seguin et al., 2002). Long-term exposure has the potential to elicit a change in community structure and species interactions due to reduced food resources or competition. These findings show how compounds with different modes of action can have similar and additive adverse effects, such as a reduction in phytoplankton abundance and chlorophyll *a* concentration or macrophyte abundance and growth. The results highlight the importance of studying the effects of chemical mixtures and multiple interacting species to elucidate possible community level effects.

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Table 1. Analysis of *Chlorella* chlorophyll *a* content (A) and cell density (B) (two-way ANOVA).

A. Chlorophyll *a* concentration

Treatment	df (effect, total)	F ratio	p value
Atrazine	1, 39	5.29	**0.03**
Tetracycline	1, 39	0.07	0.79
Atrazine x Tetracycline	1, 39	0.65	0.43

B. Cell density

Treatment	df (Effect, Total)	F ratio	p value
Atrazine	1,39	0.83	0.36
Tetracycline	1,39	1.45	0.24
Atrazine x Tetracycline	1,39	0.50	0.48

Table 2. Analysis of *D. magna* reproduction (A) and abundance (B) (Scheirer Ray Hare).

A. Reproduction

Treatment	df (Effect, Total)	F ratio	p value
Atrazine	1,39	0.17	0.50<p<0.90
Tetracycline	1,39	0.68	0.10<p<0.50
Atrazine x Tetracycline	1,39	0.41	0.50<p<0.90

B. Abundance

Treatment	df (Effect, Total)	F ratio	p value
Atrazine	1,39	0.01	0.90<p<0.98
Tetracycline	1,39	0.24	0.5<p<0.90
Atrazine x Tetracycline	1,39	01.24	0.10<p<0.50

Table 3. Analysis of *D. magna* abundance over time (rmANOVA).

Factor	df (Effect, Total)	F ratio	p value
Time	5, 239	6.69	0.0080
Time x Atrazine	5, 239	0.14	0.78
Time x Tetracycline	5, 239	0.91	0.37
Time x Atrazine x Tetracycline	5, 239	1.20	0.30

Table 4. Analysis of duckweed abundance, in number of fronds, (A) and necrosis (B) (two-way ANOVA).

A. Abundance

Treatment	df (Effect, Total)	F ratio	p value
Atrazine	1,39	3.66	0.06
Tetracycline	1,39	4.59	**0.04**
Atrazine x Tetracycline	1,39	0.28	0.60

B. Necrosis

Treatment	df (Effect, Total)	F ratio	p value
Atrazine	1,39	20.28	**<0.0001**
Tetracycline	1,39	1.20	0.28
Atrazine x Tetracycline	1,39	9.46	**0.004**

Table 5. Repeated measures ANOVA (rmANOVA) of duckweed necrosis over time.

Factor	df (Effect, Total)	F ratio	p value
Time	6, 279	252.97	**<0.0001**
Time x Atrazine	6, 279	9.09	**<0.0001**
Time x Tetracycline	6, 279	9.09	**<0.0001**
Time x Atrazine x Tetracycline	6, 279	3.38	**<0.0001**

Figure 1. Average cell density of *Chlorella* sp. in three different starting densities (10, 50 or 100 $*10^3$ cells ml^{-1}) after 5-days of exposure to *D. magna* grazing. Error bars are ± one standard error of the mean (SEM) and n=4.

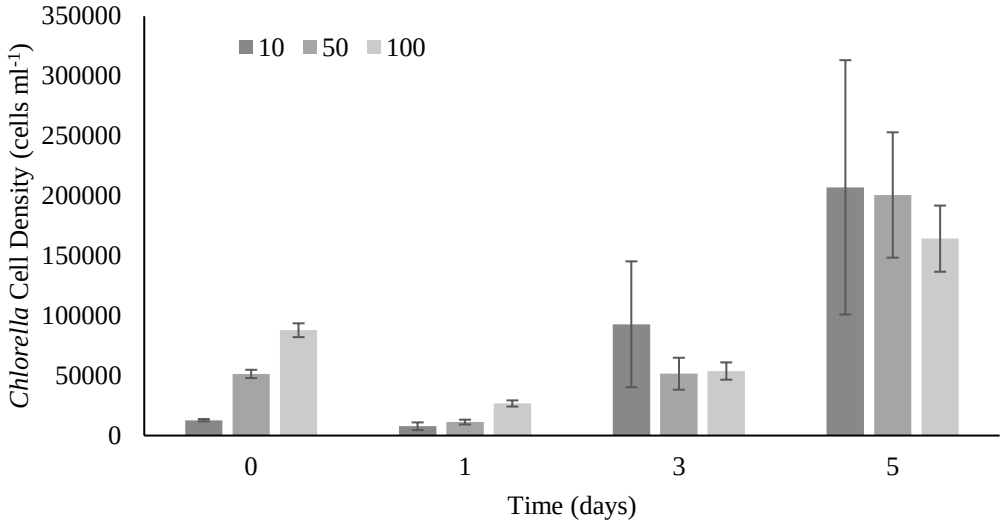


Figure 2. Average *Chlorella* sp. cell density after 7 days of exposure to atrazine (1 or 5 μg L^{-1}), tetracycline (1 μg L^{-1}), both, or a no-addition control. Error bars are ± one SEM and n=6. Significance indicated by an asterisk (*).

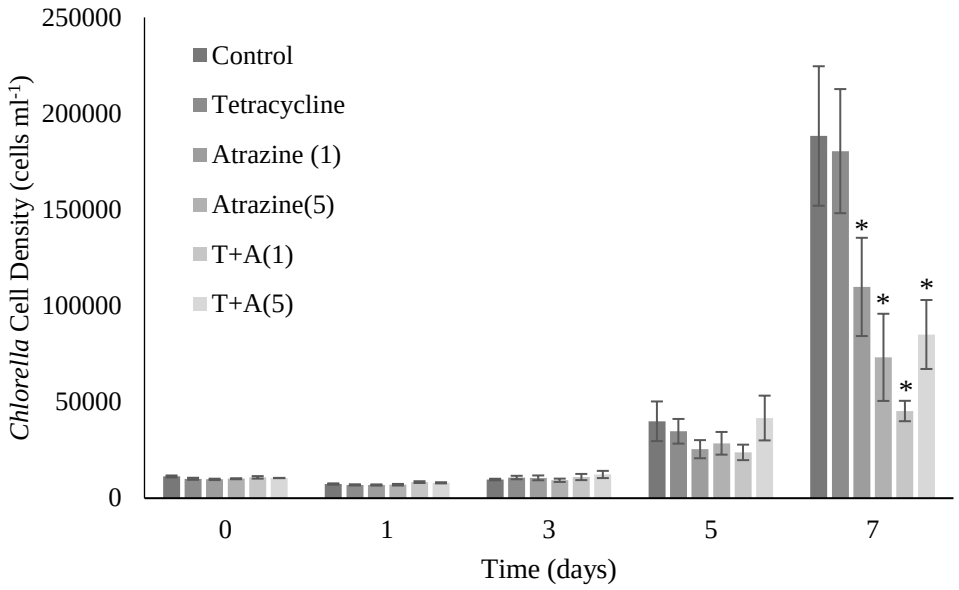
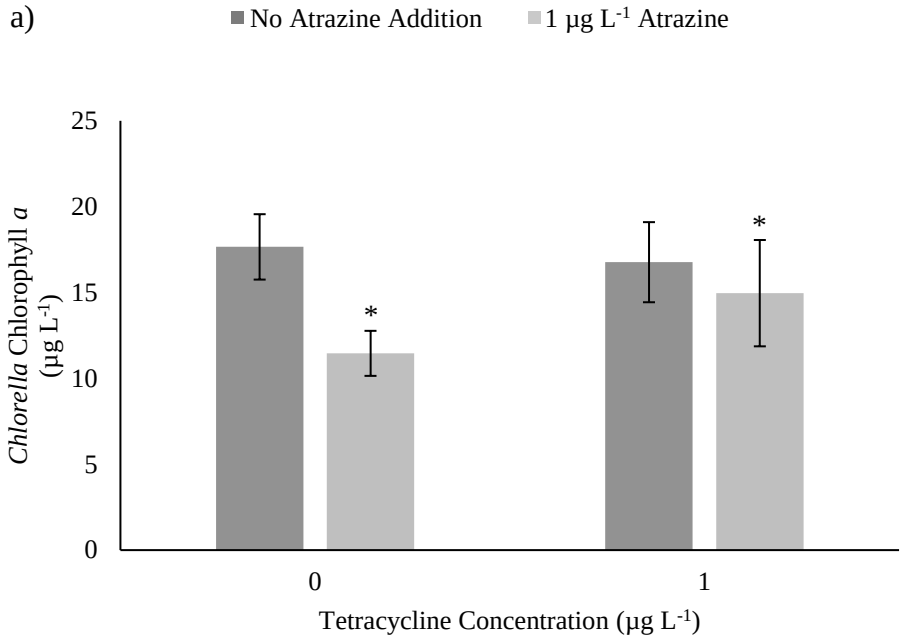


Figure 3. Average a) chlorophyll *a* content (µg L^{-1}) and b) cell density $x10^3$ (cells ml^{-1}) in *Chlorella* sp. after 10 days of exposure to 1 µg L^{-1} of atrazine, 1 µg L^{-1} of tetracycline, 1 µg L^{-1} of both, or a no-addition control. Error bars are ± one SEM and n=10. Significance indicated by an asterisk (*).



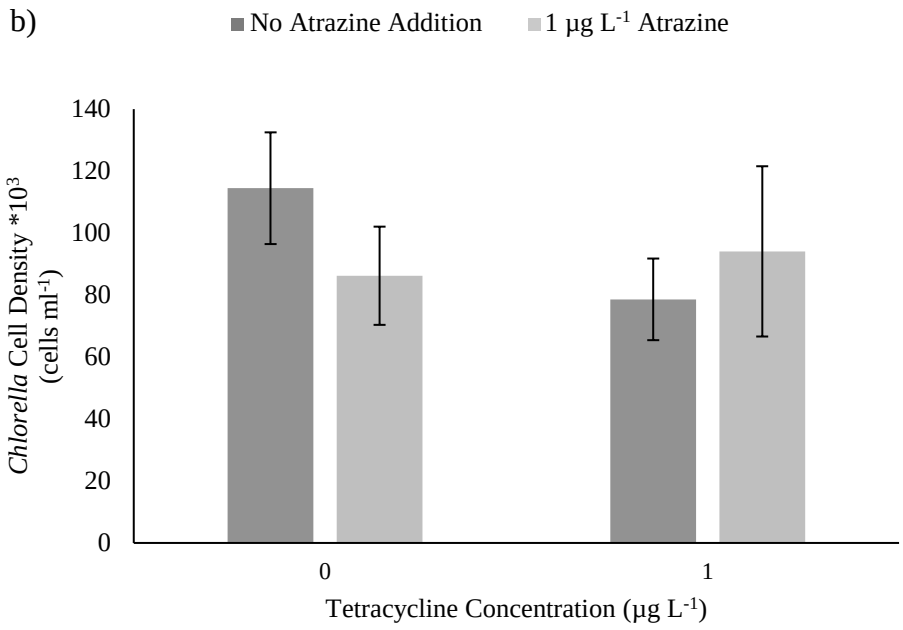
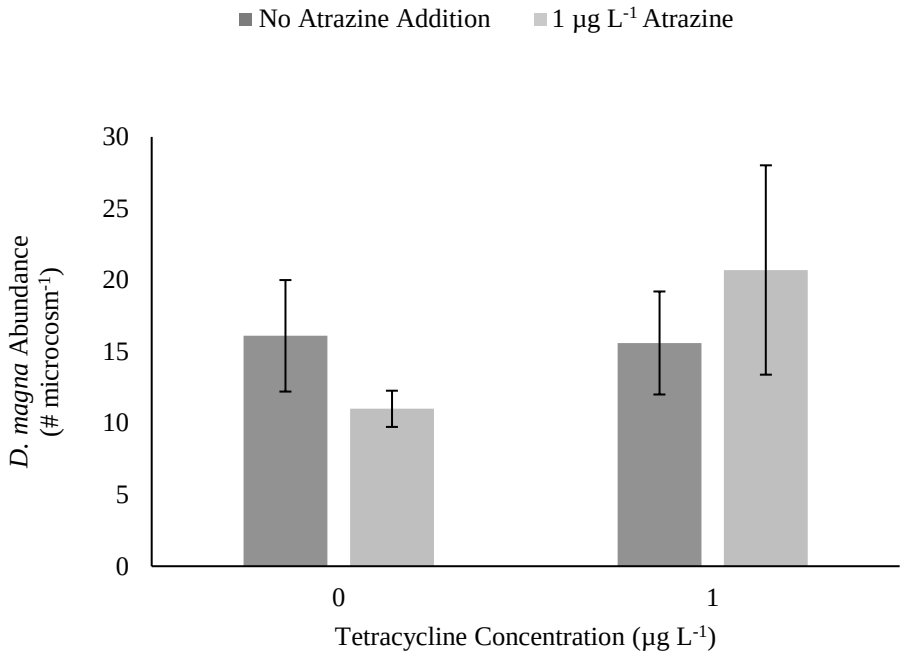


Figure 4. Average adult *D. magna* abundance a) after and b) during 10 days of exposure to 1 µg L^{-1} of atrazine (A), 1 µg L^{-1} of tetracycline (T), 1 µg L^{-1} of both (AT), or a no-addition control (C). Error bars are ± one SEM and n=10.

a)



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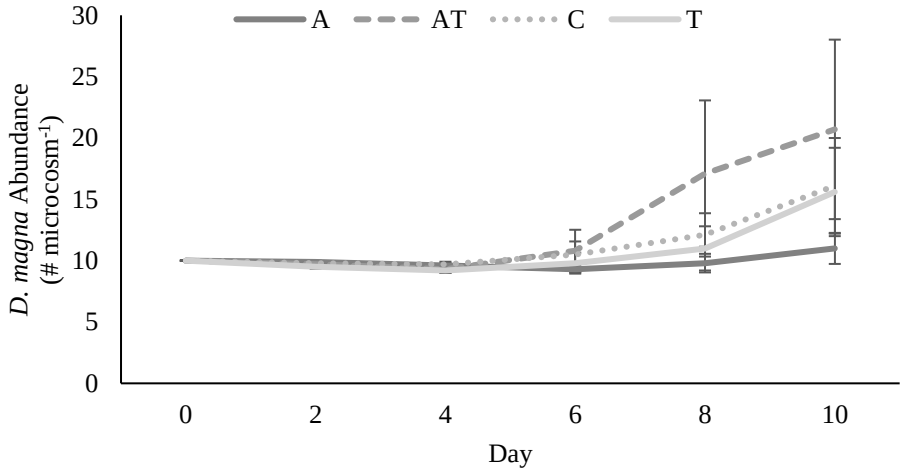


Figure 5. Average *L. minor* abundance after 10 days of exposure to 1 μg L^{-1} of atrazine, 1 μg L^{-1} of tetracycline, 1 μg L^{-1} of both, or a no-addition control. Error bars are ± one SEM and n=10. Significance indicated by an asterisk (*).

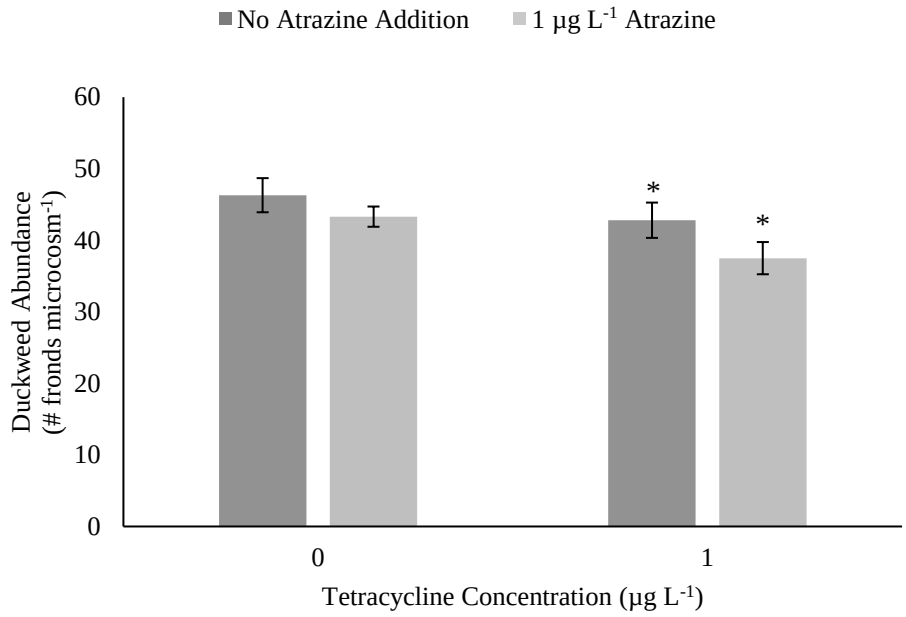
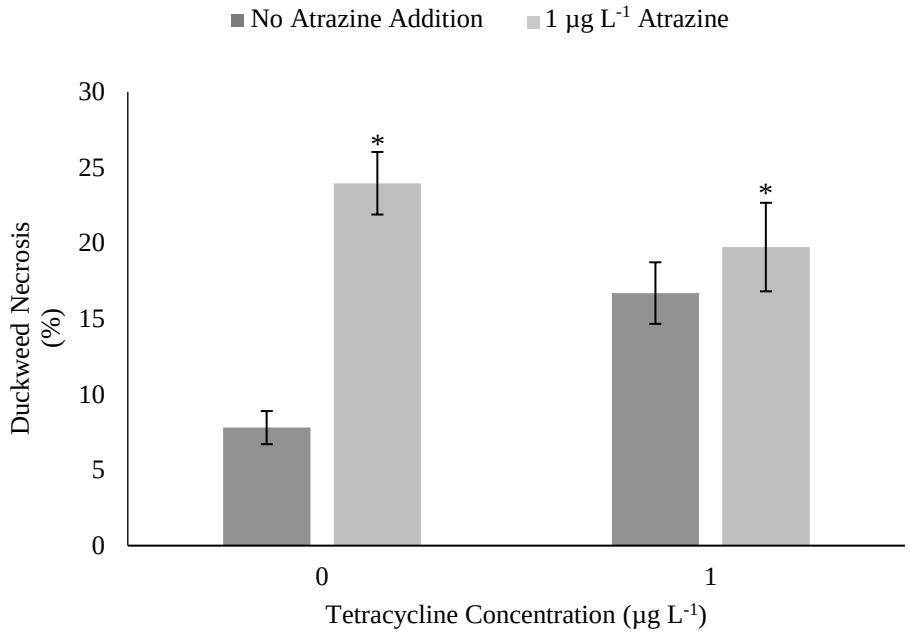


Figure 6. Average *L. minor* necrosis a) after and b) during 10 days of exposure to 1 µg L^{-1} of atrazine (A), 1 µg L^{-1} of tetracycline (T), 1 µg L^{-1} of both (AT), or a no-addition control (C). Error bars are ± one SEM and n=10. Significance indicated by an asterisk (*).

a)



b)

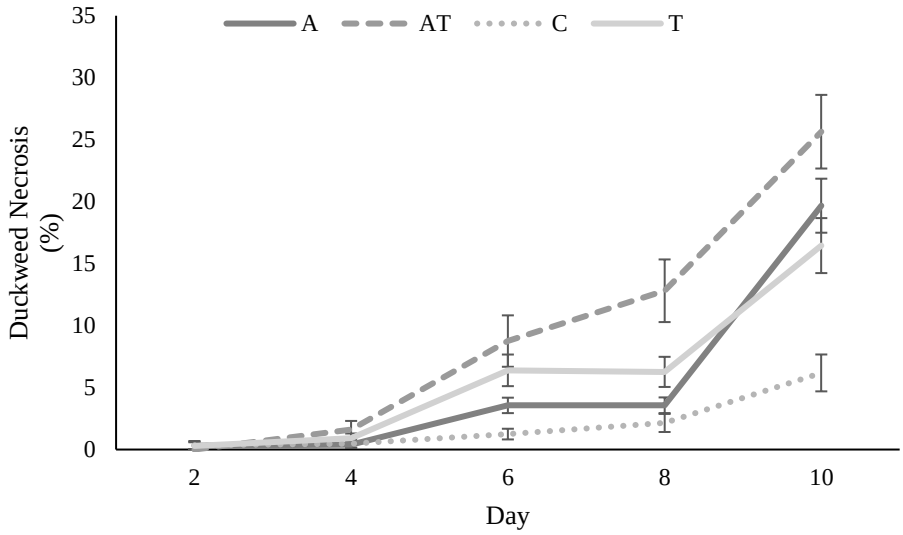


Figure 7. Growth inhibition in *L. minor* after 10 days of exposure to atrazine (A), tetracycline (T), both (AT), and a no-addition control (C). Error bars are ± one SEM and n=10. Significance is indicated by an asterisk (*).