Medical waste causing problems on a micro scale: The impact of antibiotics on the metabolic processes of *Daphnia pulicaria*

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Abstract

The introduction of antibiotics into our freshwater bodies through medical waste has probable negative effects on the freshwater biota. The antibiotic trimethoprim is known to have detrimental impacts on the living microbiota living inside the guts of *Daphnia*, but not much is known about the extent to which this antibiotic indirectly harm *Daphnia* hosting the microbes. It was hypothesized that *Daphnia* raised in higher antibiotic concentrations would have negatively affected metabolic processes. To test this, hatch rate and algal clearance was measured in *Daphnia pulicaria* under control conditions and trimethoprim concentrations of 0.25 mg L^{-1}, 0.75 mg L^{-1}, 1.25 mg L^{-1}, and 2.0 mg L^{-1}. Also, body lengths of *D. pulicaria* were measured for the control and the 2.0 mg L^{-1} treatments. To determine if the antibiotic treatment of *D. pulicaria* had an effect on higher trophic levels, *Chaoborus* were given control *D. pulicaria* and 2.0 mg L^{-1} treated *D. pulicaria* to see if they showed a preference for one over the other. Algal clearance, hatch rate, and body lengths of the *Daphnia* were all found to be significantly higher in the control treatments than in treatments with trimethoprim, supporting the hypothesis made. *Chaoborus* were found to prefer to feed on 2.0 mg L^{-1} treated *D. pulicaria* over the control *D. pulicaria*, which also supported the hypothesis. On a wide scale, the implications of this study have the potential to alter not only *Daphnia* population feeding and sizes, but higher trophic levels could also be negatively impacted.

Introduction

Host-symbiont relationships provide a stabilizing relationship between the organisms involved. Bacteria are often organisms that are involved in these symbioses, and the majority of these mutualistic relationships with bacteria and another organism occur within the guts of that organism (Gorokhova et al. 2015). Mutualisms between an organism and their gut microbiota play a vital role in the systems of both organisms. The biosynthetic capabilities of the bacteria are used as nutritional resources for the organism they are inhabiting (Gorokhova et al. 2015). The hosts of the bacteria benefit by physiological processes within them being heightened (Gorokhova et al. 2015; Mushegian and Ebert 2017). Gut microbiota can provide services, such as metabolic waste recycling and growth factor signaling (Mushegian and Ebert 2017).

Without these bacterial endosymbionts, there are associated negative consequences (Mushegian and Ebert 2017). The extent to which these organisms are able to perform their roles properly is dependent on various environmental factors. The environmental factors that are able

to influence the relationships alter the effectiveness of one or more of the organisms involved. These mutualisms have evolved to benefit the organisms, and when this relationship is impacted by different factors, there are associated direct, negative consequences. Antibiotics are a common environmental contaminant that are of high concern for the gut-microbe interaction. The antibiotics are both biologically active and have low biodegradability (Gorokhova et al. 2015). As a result, the antibiotics have the ability to destroy the microbiota living in the guts as well as stay in aquatic systems for a long period of time without breaking down.

Antibiotics have been shown to decrease the abundance and the structure of the bacterial communities within the cladoceran *Daphnia* (Gorokhova et al. 2015). Due to *Daphnia* being a good model organism for toxicity tests because of being highly sensitive to changes in the environment, gut mutualistic relationships are commonly studied between *Daphnia* and their bacterial endosymbionts (Manakul et al. 2017). *Daphnia* also make a great model organism because of their diverse host-microbe interactions (Mushegian and Ebert 2017). The microbiota endosymbionts help with catabolizing the algal cells that are consumed by the *Daphnia*. *Daphnia* without these microorganisms have a lesser ability to do so because both the allocation of energy and the digestive processes rely heavily on the bacteria associated with the gut of the *Daphnia* (Mushegian and Ebert 2017). The antibiotic trimethoprim, used in Gorokhova et al., is beneficial to study due to its common use as pharmaceutical to treat infections as well as the harms it can cause as waste in our waters (2015). Trimethoprim inhibits the production of tetrahydrofolic acid in microbes, which functions in the metabolism of amino acids and nucleic acids (Gorokhova et al. 2015). When trimethoprim is added, this function is lost in the *Daphnia*, and they lose the ability to make those essential substances.

The idea of bottom up effect on a trophic cascade is vital to this concept. The *Daphnia's* ability to process their food source is altered by the antibiotic, and that, in turn, alters the nutritional composition of the *Daphnia*. The concept of bottom up effect can be followed into higher trophic levels through organisms that feed on *Daphnia*. In this trophic cascade, the idea is that *Daphnia* are fed upon by a planktivorous species, and the composition of the food source is known to have an impact on that species. Factors like eutrophication and browning in lakes have been proven to have detrimental consequences on the nutritional quality and growth in higher trophic levels because not all of their nutritional requirements are being met (Taipale et al. 2017). *Daphnia* feeding on lesser quality food sources are often not adequate to support fish growth, development, and survival (Taipale et al. 2017). Other planktivorous species that feed on *Daphnia* are impacted in a similar way, and the organism being used for this study will be *Chaoborus*. *Chaoborus* is a predaceous, Dipteran larvae that has a larval lifespan of approximately a year that is broken into four instars (Berendonk 1999; Fedorenko 1974). *Chaoborus* have a diet made up of largely copepods and cladocerans (Castilho-Noll and Arcifa 2007). As a natural predator of *Daphnia*, *Chaoborus* could also be impacted by antibiotics through *Daphnia* consumption. The specific hypothesis being tested is that antibiotics will cause *Daphnia* to have decreased metabolic processes; higher antibiotic concentrations will result in lower *Daphnia* hatch rates, lower *Daphnia* body lengths, lower algal clearance, but a higher feeding preference by *Chaoborus*.

Materials and Methods

Daphnia Collection

Sampling was conducted at the University of Notre Dame Environmental Research Center (UNDERC). The UNDERC property is located in Northeastern Wisconsin and the Upper Peninsula of Michigan. All sampling was conducted on Tenderfoot Lake using a zooplankton net. Both vertical and horizontal tows were used. The majority of sampling occurred after 10:00 p.m., due to *Daphnia* being closer to the water surface at this time. After being collected, *Daphnia* were put into glass tanks and given time for populations to establish before experiments were started. The species of *Daphnia* used for this study, *D. pulicaria*, made up a high majority of the *Daphnia* collected from Tenderfoot Lake. *D. pulicaria* was used for all experimental setups for this reason.

Daphnia Hatch Rate

Using sterilized needles, the appropriate stage of diploid eggs (stage 0-3) were extracted from the female *D. pulicaria*, and five eggs were placed into a sterile 50 mL conical vial of autoclaved water from Tenderfoot Lake (Giardini et el. 2015). Adapted methods of Gorokhova et al. were used to determine the range of trimethoprim to be used for the antibiotic treatments of *Daphnia*: 0.25 mg L^{-1}- 2.0 mg L^{-1} (2015). The exact concentrations used were 0.25 mg L^{-1}, 0.75 mg L^{-1}, 1.25 mg L^{-1}, and 2.0 mg L^{-1}. Each vial of *Daphnia* eggs was treated with either one of the four concentrations of antibiotic treatments or was a control and given no trimethoprim. Feedings for the *Daphnia* were approximately 150,000 cells of desmid algae per mL (or 95 %T) at the time of setup, as well as every second day following. The vials were kept in front of a window and received natural light. 21 vials were set up for each of the four antibiotic treatments and the control. After three days, the vials were checked for hatched *Daphnia*. The number of hatched *Daphnia* per vial was recorded and then turned into a proportion hatched of the total number of eggs (105 eggs) for each treatment.

Daphnia Body Length

Body length measurements were taken of the control *D. pulicaria* hatchlings and the 2.0 mg L^{-1} hatchlings three days after setting up the vials with eggs (using the same setup methods as for hatch rate). To measure body length, *Daphnia* were put under a dissecting microscope and, using the Leica Application Suite EZ™ (LAS EZ™) version 1.5.0 computer program, a photo of the *Daphnia* was taken. Then, using the same program, a distance line was drawn from the top of the eye spot to the base of the tail spine and this length was recorded as the body length (Figure 1). Lengths were taken for 20 of the control hatchlings and 20 of the 2.0 mg L^{-1} hatchlings.

Algal Clearance

Sterile, 50 mL conical vials were blacked out using tin foil to avoid algae from using light to reproduce. For each of the four trimethoprim treatments and the control, 11 vials were set up. Each vial contained 50 mL of autoclaved Tenderfoot Lake, the designated amount of trimethoprim, and approximately 150,000 cell of algae per 1 mL. For each treatment, ten vials were given one *D. pulicaria* and one vial was left with no *Daphnia*. *D. pulicaria* with lengths of 1.5 mm ± 0.1 mm from the top of the eye spot to the base of the tail spine were selected. Lengths were determined using LAS EZ™. Using a spectrophotometer, percent transmittance was recorded three times from each vial before the introduction of *Daphnia* and three times 24 hours after the *Daphnia* were introduced. The vials were slightly mixed before each reading by pipetting 1 mL of the water from the vial up and then back into the water two times. The difference between the average starting and average ending percent transmittance was then calculated for each vial. The one vial without a *Daphnia* for each treatment was tested in the same manner to ensure that the increase in percent transmittance was a result of feeding and not due to the algae settling at the bottom of the vial.

Chaoborus Feeding Preference

Vials were set up with eggs in only the control and the 2.0 mg L^{-1} treatments. Three days after the vials were set up, half of the control *D. pulicaria* hatchlings and half of the 2.0 mg L^{-1} *D. pulicaria* hatchlings were dyed red using food coloring. To dye them, eight drops of red food coloring were put into the 50 mL conical vials containing the *Daphnia* hatchlings. The vials were left with the dye overnight for 16 hours. Following the 16 hour dye period, three control and three 2.0 mg L^{-1} *Daphnia* were put into a conical vial filled half way (25 mL) with autoclaved water from Tenderfoot Lake. Once the *Daphnia* were inside the vial, a *Chaoborus* was added. For half of the setups, three control *Daphnia* were dyed red and placed into a vial with three non-dyed 2.0 mg L^{-1} *Daphnia*, and for the other half, three 2.0 mg L^{-1} *Daphnia* were dyed red and placed into a vial with three non-dyed control *Daphnia*. This was to ensure that the *Chaoborus* preference, if any, would be based on the difference in antibiotic concentration upbringing and not color. *Chaoborus* used for this study measured between 6.5 and 8.5 mm from the middle of the eye spot to the base of their tail (Figure 2). The measurements were determined using the LAS EZ™ program. 14 vials were prepared in total. Once in the vials, *Chaoborus* were given one hour to feed on the *Daphnia*. After that hour, both the number of control and 2.0 mg L^{-1} *Daphnia* eaten from each vial was recorded.

Statistical Analysis

The program JMP® was used to analyze the collected data. Before running any statistical tests, the data was checked for normality. All data was normally distributed. For the hatch rate experiment, a chi-square test of proportions was performed. This compared the proportion of eggs hatched out of the total number of eggs set up for each treatment. A Bonferroni multiple testing correction was then completed on the hatch rate data to determine which proportions were

statistically significant from one another. To analyze the *Daphnia* body lengths, a one way ANOVA was completed to compare the mean lengths between the control and the 2.0 mg L^{-1} treatments. An ANOVA was completed to compare the means of algal clearance among the different treatments. The Tukey post-hoc test was then done to determine which means were statistically different. To analyze the feeding preference of *Chaoborus*, a chi-square test was performed to determine if the proportions of control *Daphnia* and the 2.0 mg L^{-1} treated *Daphnia* eaten were the same.

Results

The proportion of *D. pulicaria* that hatched differed among the treatments (DF=1, x^2=31.107, $p < 0.0001$; Figure 3). A Bonferroni multiple testing correction showed significant differences between the hatch rates of the control and the 2.0 mg L^{-1} and 1.25 mg L^{-1} treatments ($p = 0.0036$), the control and the 0.75 mg L^{-1} treatment ($p = 0.002$), and the control and the 0.25 mg L^{-1} treatment ($p < 0.0001$). Additionally the 0.25 mg L^{-1} treatment has a different hatch rate proportion than that of the 2.0 mg L^{-1} and 1.25 mg L^{-1} treatments ($p = 0.0081$). An ANOVA comparing the mean body lengths of control hatchlings and 2.0 mg L^{-1} hatchlings resulted in a significant difference [$F(1, 38) = 28.385$, $p < 0.0001$; Figure 4]. A significant result was found between the means of algal clearance between the different treatments [$F(4, 45) = 19.016$, $p < 0.0001$; Figure 5]. The Tukey post-hoc analysis of the algal clearance data showed a significant difference between control and 2.0 mg L^{-1} treatments ($p < 0.0001$), control and 0.75 mg L^{-1} treatments ($p < 0.0001$), control and 1.25 mg L^{-1} treatments ($p < 0.0001$), control and 0.25 mg L^{-1} treatments ($p = 0.0003$), and 0.25 mg L^{-1} and 2.0 mg L^{-1} treatments ($p < 0.0230$). *Chaoborus* ate

a significantly higher proportion of 2.0 mg L^{-1} *Daphnia* than control *Daphnia* (DF= 1, $x^2 = 9.355$, p = 0.0022; Figure 6).

Discussion

The results obtained support the hypothesis originally stated; antibiotics have a negative effect on the metabolic processes of *D. pulicaria*. Gorokhova et al. propose that trimethoprim has the ability to decrease the bacterial abundance within the guts of *Daphnia*, and the results of this research now suggest that this bacterial decrease results in a loss of metabolic activity in *Daphnia* (2015). The hatch rate, body lengths, and ability to process algal diets were all significantly lower for *Daphnia* in the antibiotic treatments than in the controls.

The majority of the obtained results followed the expected patterns of decreased metabolic activity with an increase in the trimethoprim concentration, but hatch rate veered from this pattern some. The control treatment did have the highest hatch rate, following the expected pattern, but the lowest concentration of antibiotic, 0.25 mg L^{-1}, had the lowest proportion of *Daphnia* hatch. This was a surprising result, because it would have been anticipated that the highest concentration, 2.0 mg L^{-1}, would result in the lowest hatch rate. Instead, the lowest concentration had the lowest hatch rate and the hatch rate increased slightly as the trimethoprim concentration increased. This is an area of question because the 0.25 mg L^{-1} treatment is the closest to the levels that have been found in natural water systems. If low concentrations of trimethoprim can cause such a dramatic decrease in the proportion of *Daphnia* eggs that hatch, there is the potential for the abundant organisms in a system to completely switch.

Not only did trimethoprim have a negative impact on the *Daphnia*, but the potentiality of this to become a problem in higher trophic levels was also demonstrated. *Chaoborus* showed a

preference for eating *Daphnia* that had been treated with 2.0 mg L^{-1} of trimethoprim. This could subsequently cause problems for *Chaoborus*. It has been shown that *Daphnia* raised in compromising conditions can cause lower growth and survival in the trophic level feeding off of them, and the fact the *Chaoborus* shows preference towards eating the antibiotically affected *Daphnia* could be potentially detrimental to both the growth and survival of that predator (Taipale et al. 2017). A reason that *Chaoborus* show preferential predation of the 2.0 mg L^{-1} treated *Daphnia* could be because the 2.0 mg L^{-1} antibiotic treatment resulted in significantly smaller sized *Daphnia*. Smaller *Daphnia* may be easier for the *Chaoborus* to eat, therefore making them a more likely target for predation.

The obtained results bring about a host of new potential areas of study. Most notably, growth and survival should be measured in *Chaoborus* feeding off of *Daphnia* raised in the different concentrations. It could also be beneficial to see if other predators of *Daphnia* would have the same preference for the antibiotically treated *Daphnia*. Other predators could also be measured for growth, survival, and nutrient transfer as well, to obtain a fuller picture of the impact of trimethoprim on more of the food web. It could also be interesting to see if trimethoprim would influence inducible defense in *Daphnia*. *Daphnia* produce defensive spines when exposed to kairomones of *Chaoborus* (Rissen 2012). Knowing this, along with the finding that *Chaoborus* prefer to eat *Daphnia* that were hatched in the 2.0 mg L^{-1} concentration of trimethoprim over the control, brings up whether or not the trimethoprim treatments would result in the formation of more inducible defenses. Another potential avenue of study to look into would be to look at the impact that trimethoprim would have on other zooplankton. It could be interesting to see if, for example, copepods, *Holopedium*, and *Bosmina* (all of which can be

found on the UNDERC property), show similar patterns of decreased metabolic activity when raised in trimethoprim.

Results found are indicative of the consequences of human action. Although the concentrations of trimethoprim used in this study are not the average concentrations found in our natural bodies of water (ng L^{-1} to 10 μg L^{-1}, but as high as 0.3 mg L^{-1}), this study depicts just a piece of the potential detrimental impacts that could enfold (Gorokhova et al. 2015). Even the lowest concentration had a significant impact on the hatch rate and algal clearance of *D. pulicaria*. *Daphnia* could likely experience a population wide impact from just trimethoprim alone, but when other human caused changes that are known to have a strong impact on the nutritional quality of *Daphnia*, like eutrophication and browning, are considered, this already bleak outlook is unsettling (Taipale et al. 2017). There is a strong likelihood that antibiotics can impact entire trophic cascades, meaning that this is a whole system problem that needs to be studied further before population dynamics become dramatically altered.

Figures

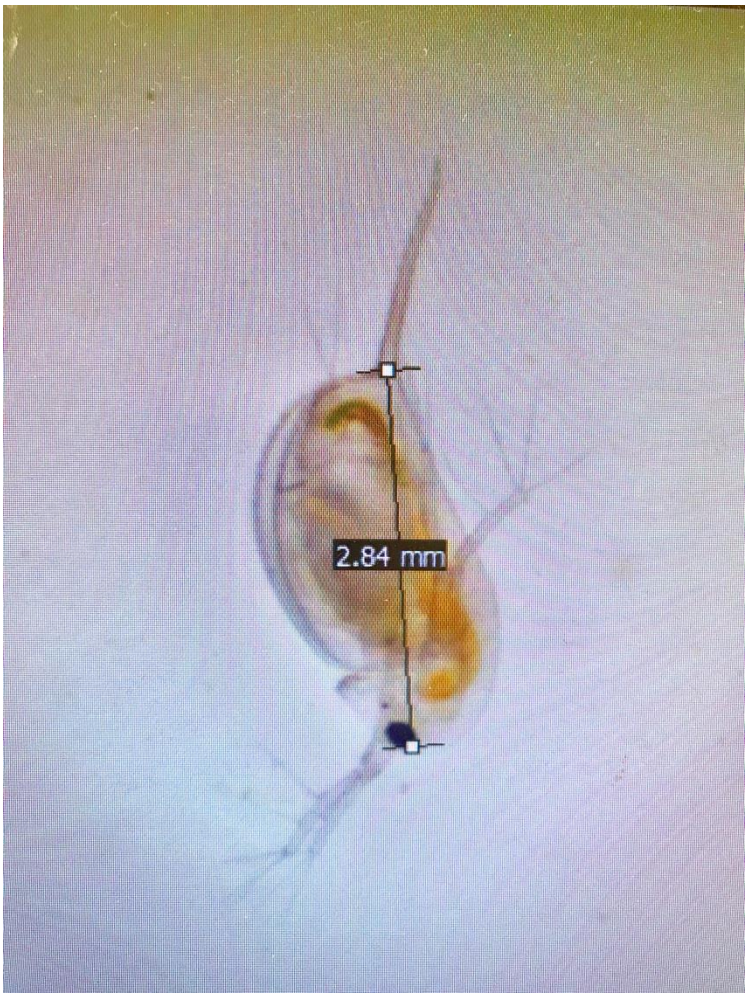

Figure 1. Measuring *Daphnia* body length. The *D. pulicaria* body lengths were measured from the top of the eye spot to the base of the tail spine. LAS EZ™ was used to both take the image of the *Daphnia* from the dissecting microscope and add the distance line.

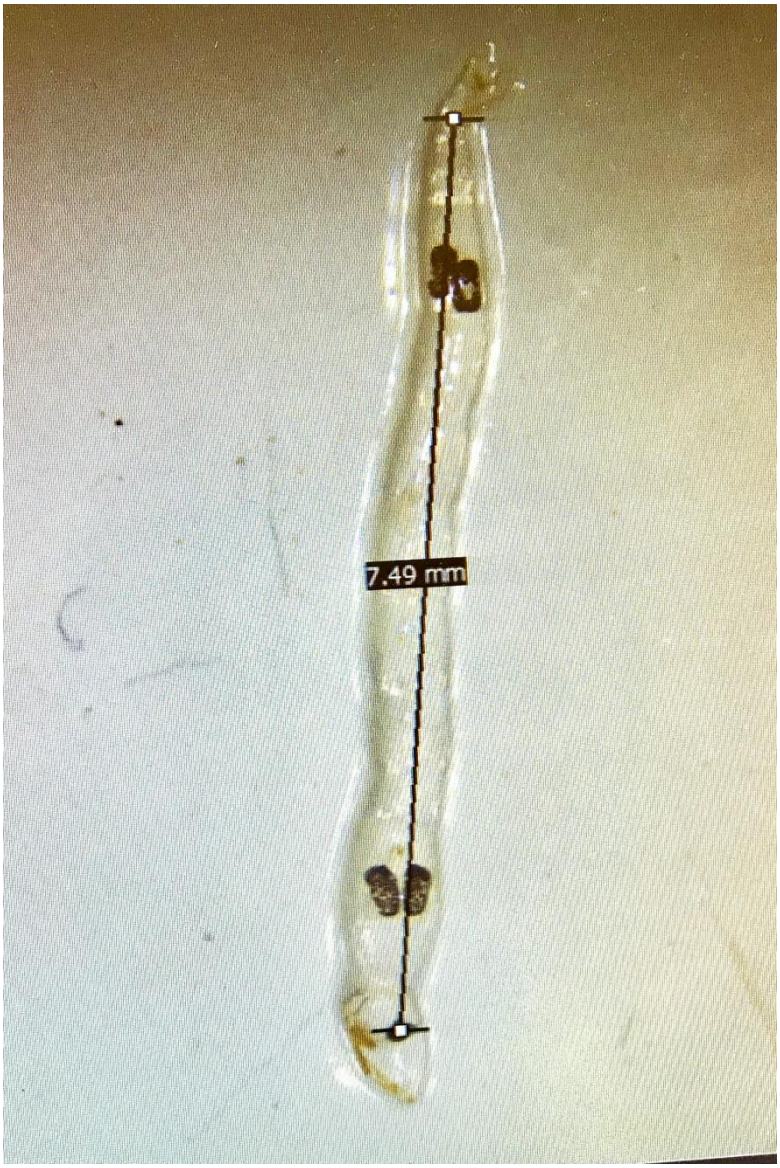

Figure 2. Measuring *Chaoborus* body length. The *Chaoborus* body lengths were measured from the center of the eye spot to the base of the tail. LAS EZ™ was used to both take the image of *Chaoborus* from the dissecting scope and add the distance line.

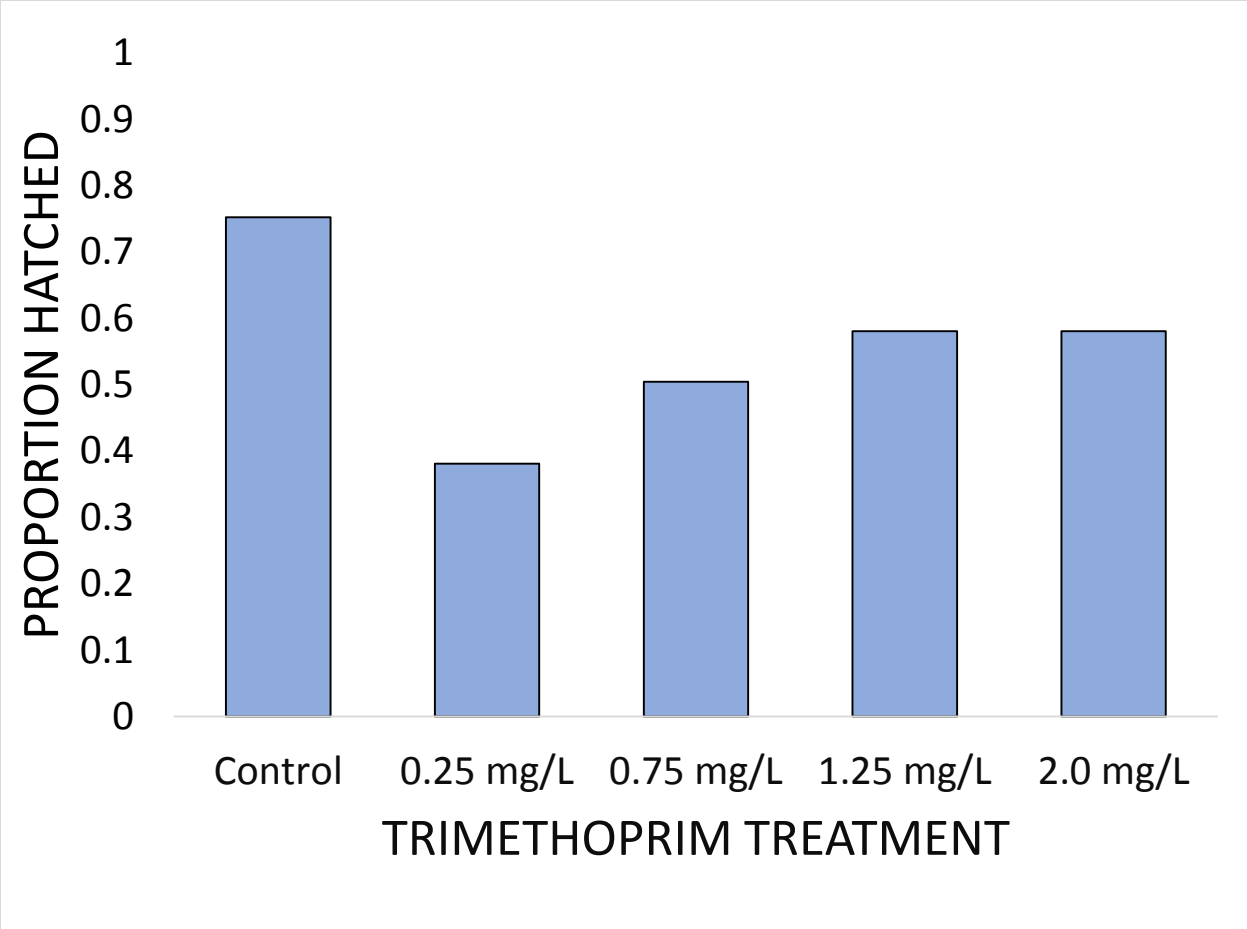

Figure 3. The proportions of *D. pulicaria* hatched in different trimethoprim treatments. Results of a chi-square test reveal a significant difference between the proportions (DF=1, x^2=31.107, $p < 0.0001$). A Bonferroni multiple testing correction showed significant differences between the hatch rates of the control and the 2.0 mg L^{-1} and 1.25 mg L^{-1} treatments ($p = 0.0036$), the control and the 0.75 mg L^{-1} treatment ($p = 0.002$), the control and the 0.25 mg L^{-1} treatment ($p < 0.0001$), and the 0.25 mg L^{-1} treatment and the 1.25 mg L^{-1} and 2.0 mg L^{-1} treatments ($p = 0.0081$).

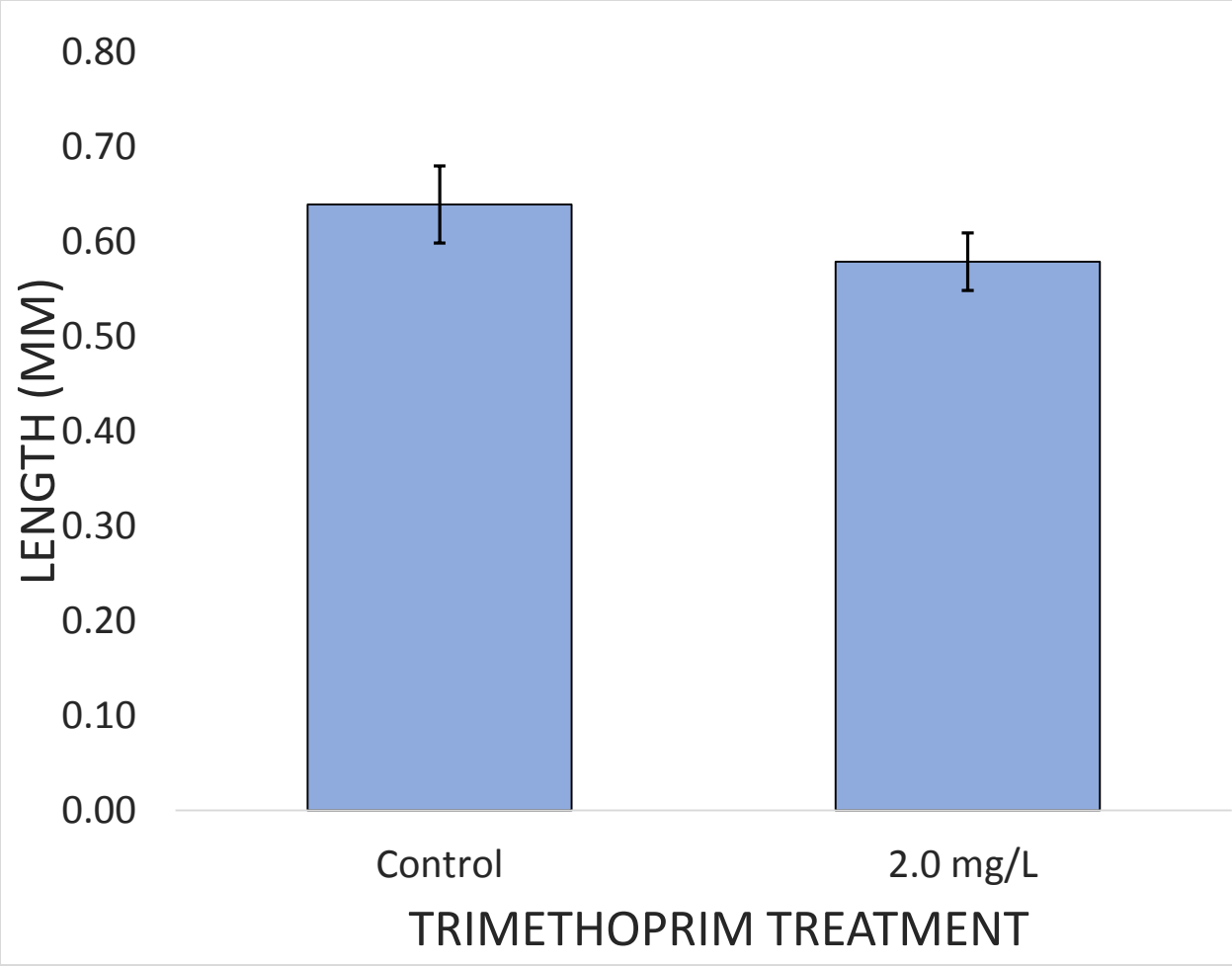

Figure 4. Differences in lengths (mm) of control and 2.0 mg L^{-1} treated *D. pulicaria* hatchlings. An ANOVA comparing the mean body lengths of control hatchlings and 2.0 mg L^{-1} hatchlings resulted in a significant difference [$F(1, 38) = 28.385$, $p < 0.0001$].

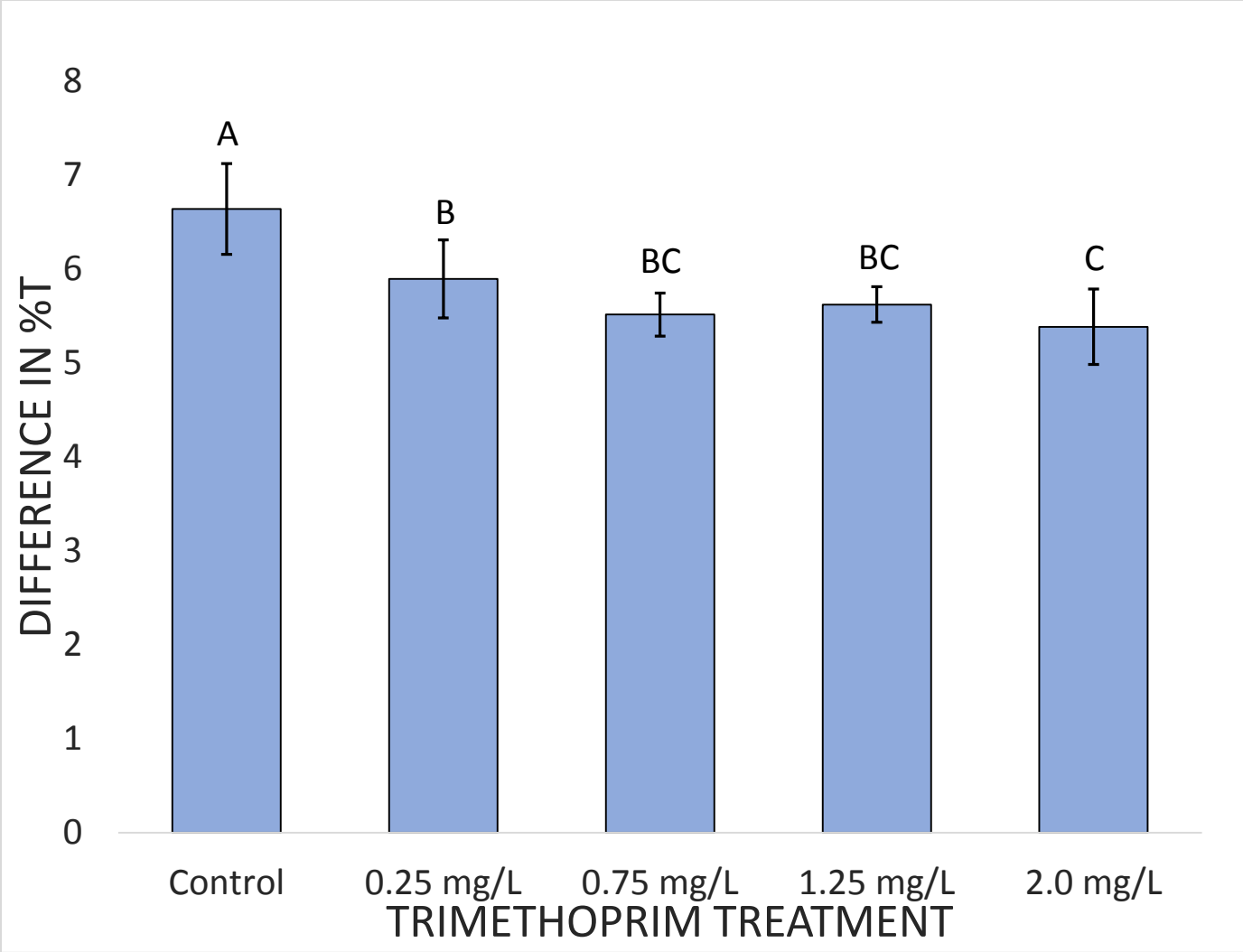

Figure 5. Difference in algal clearance between *D. pulicaria* treated different trimethoprim concentrations. An ANOVA resulted in a significant difference between the means of algal clearance between the treatments [$F(4, 45) = 19.016$, $p < 0.0001$). The Tukey post-hoc analysis of the algal clearance data showed a significant difference between control and 2.0 mg L^{-1} treatments ($p < 0.0001$), control and 0.75 mg L^{-1} treatments ($p < 0.0001$), control and 1.25 mg L^{-1} treatments ($p < 0.0001$), control and 2.0 mg L^{-1} treatments ($p = 0.0003$), and 0.25 mg L^{-1} and 2.0 mg L^{-1} treatments ($p < 0.0230$).

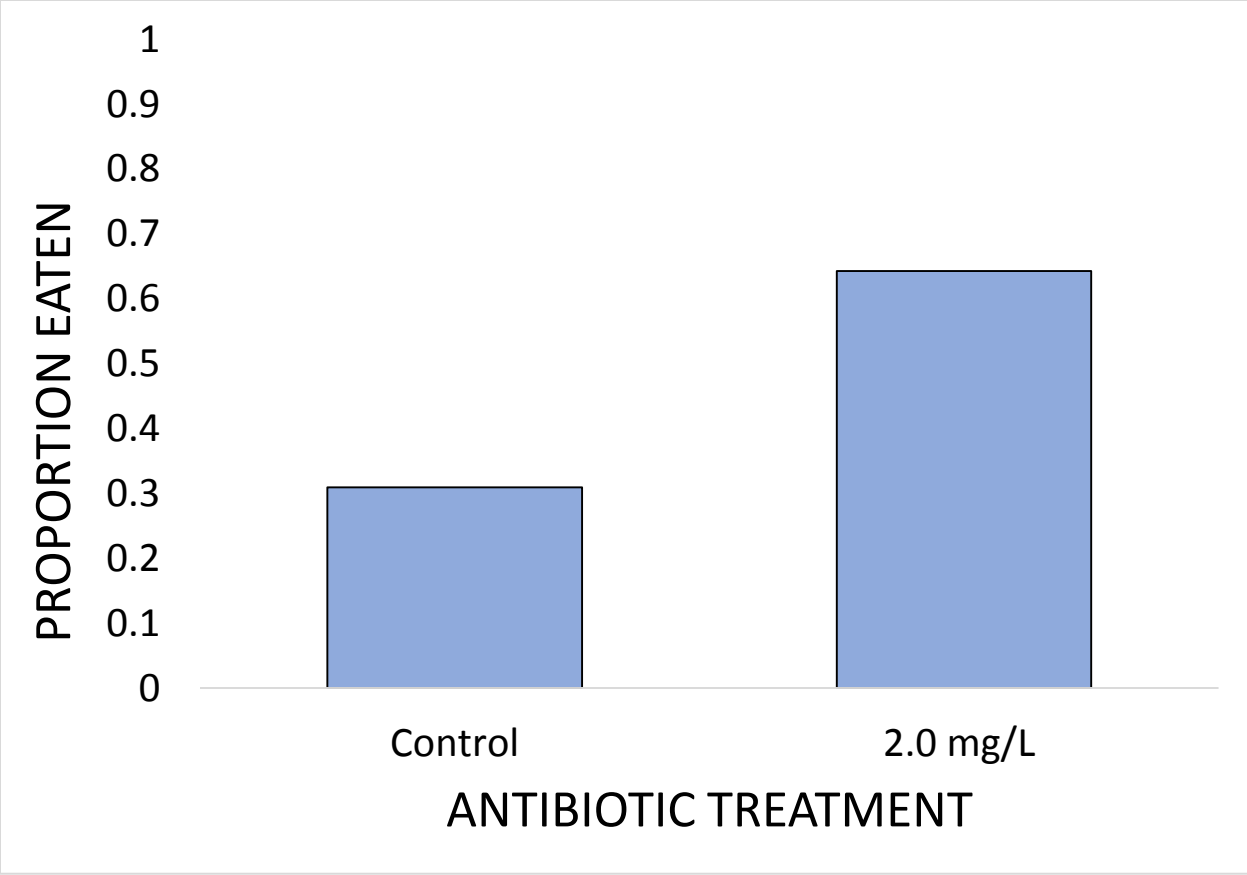

Figure 6. Feeding preference of *Chaoborus* between the control and 2.0 mg L^{-1} treatments. *Chaoborus* ate a significantly higher proportion of 2.0 mg L^{-1} *D. pulicaria* than control *D. pulicaria* (DF= 1, x^2= 9.355, p = 0.0022).

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