Effect of Urea on the Growth of *Tetrahymena thermophila*

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I. Abstract

With the increase in fertilizer runoffs polluting water systems, we aimed to use *Tetrahymena thermophila* as a model organism to determine how increases in pollutants commonly found in fertilizers affect the health of water-based ecosystems. With urea being a common component of many fertilizers, we focused on how different concentrations of urea affect the growth of *T. thermophila*. The growth curves of *T. thermophila* cultures were obtained from four different treatments of urea concentrations: 0 μg/mL, 100 μg/mL, 200 μg/mL, and 400 μg/mL over the course of 25.5 hours. No significant differences in growth at these concentrations were measured, and thus further exploration would be required to determine the effects of urea in the context of fertilizer runoffs affecting our ecosystems.

II. Introduction

Tetrahymena thermophila is a unicellular eukaryote that lives in most freshwater and moist terrestrial environments (Collins & Gorovsky, 2005). Within these environments, it feeds on bacteria thereby connecting prokaryotes and eukaryotes in the food chain. Thus, not only does it play a vital role in the ecosystems it inhabits, but it also can act as a measure to quantify the health of an ecosystem (Maurya & Pandey, 2020).

Unfortunately, many of these freshwater habitats have succumbed to the effects of pollution, especially in the case of fertilizer run-off and soil leaching. Of the fertilizers that end up in the water, urea is one of the most frequently found, largely due to its high nitrogen content and relatively low price (Fortune Business Insights, n.d.). While *T. thermophila* is a quick-multiplying organism, its growth rate remains sensitive to small changes in the environment, such as those caused by fertilizer runoffs (Maurya & Pandey, 2020). Moreover, Bonnet et al. (2006) have shown that the growth of *Tetrahymena pyriformis*, a congener of *T. thermophila*, is hindered in the presence of large concentrations of herbicides.

When it comes to urea in particular, a previous study conducted by Dewey et al. (1952) on *Tetrahymena geleii*, another congener of *T. thermophila*, examined the effects of urea analogues on growth. It was found that urea analogues react with carbonyl groups by

disrupting transamidation reactions from occurring, which disrupts the production of certain amines such as ammonia (Dewey *et al., 1952*). More specifically, 412 µg/mL of urea was found to induce maximum inhibition of the growth rate of *T. geleii* (Dewey *et al.,* 1952). Furthermore, *T. thermophila* are ammonotelic organisms, and thus a build-up of nitrogen-containing compounds, such as urea, is likely to stop the cell cycle and prevent replication (Murata-Hori & Fujishima, 1996).

In light of the increasing pollution of fertilizers in water systems paired with the fact that *T. thermophila* can be used as a measure for how healthy ecosystems are, we aimed to determine how fertilizer concentrations in water systems affect the growth of *T. thermophila*. We decided to focus on urea and determine how differing concentrations of urea affect the growth rate of *T. thermophila*. We hypothesized that urea has an effect on the growth rate of *T. thermophila* and predicted that an increase in urea concentration in the media would decrease the growth of *T. thermophila*. Four treatment groups with varying concentrations of urea within the media (0 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL) were used. Growth curve data was then collected throughout the day and used to examine the effects of the treatments on growth rate.

III. Methods

Culture Preparation

Preparation began with an initial concentrated wild-type *T. thermophila* stock in *T. thermophila* growth media. 10 µL of fixative was added to 100 µL of this concentrated stock solution in an Eppendorf tube. 20 µL of the fixed sample was added to the hemocytometer and counted using a microscope to obtain an initial cell count. Using the count obtained, the concentrated stock solution was diluted in *T. thermophila* growth media to create 125 mL of $2 \cdot 10^4$ cell/mL initial working stock concentration cell culture.

Immediately after this stock cell culture was prepared, 50 mL of 4 different urea treatment groups (0 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL) were prepared in 125 mL Erlenmeyer flasks. Each of these treatment groups were prepared using an 800 µg/mL urea stock solution diluted to the correct concentration of each treatment, *T. thermophila* growth media, and 25 mL of our initial *T. thermophila* working cell culture ($2 \cdot 10^4$ cell/mL) (Fig. 1).

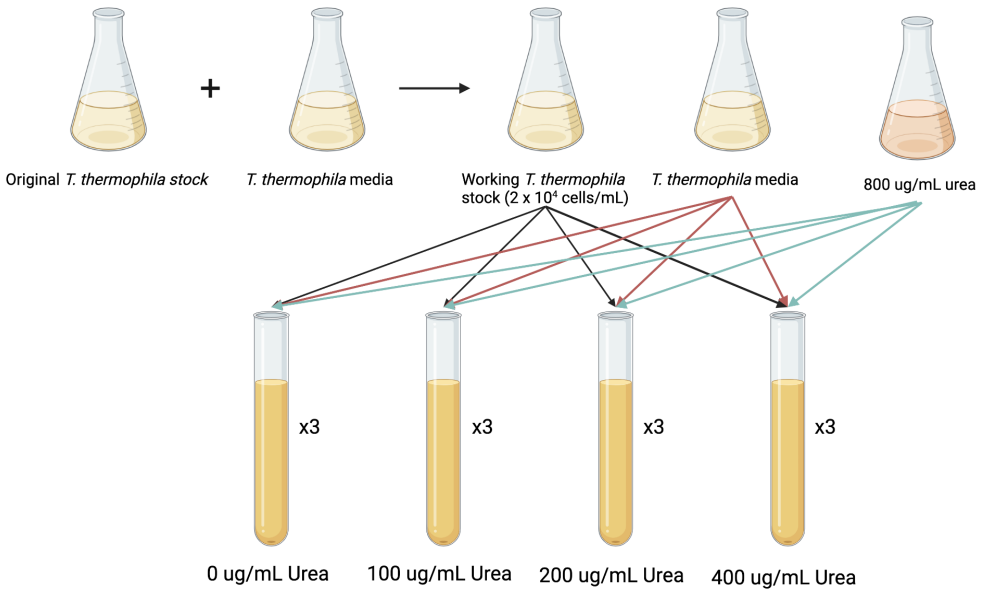

Figure 1. Preparing *T. thermophila* treatment cultures for growth.

Culturing

After preparing each of the treatments, 10 mL of each group was transferred into test tubes in triplicate (12 samples total). Tubes were placed into an incubator set at 30℃. During incubation, 100µL was taken out of each test tube and transferred into an Eppendorf tube containing 10 µL of fixative at the following time intervals following initial incubation: 2 hours, 4 hours, 6 hours, 23.5 hours, and 25.5 hours. The test tube tops were sterilized before and after the collection of cell cultures, using an open flame to minimize any contamination. Immediately after each sample was collected, the samples were placed into a refrigerator set at 4℃ until cell counting, and the test tubes were returned to the incubator.

Growth Curve Collection

After the last collection, 20 µL of each of the fixed samples was loaded onto a hemocytometer. The cells were counted using a tally counter clicker and a microscope using 10x magnification.

Data Analysis

Data from counting the cells was compiled. This included the number of cells counted for each treatment group as well as the number of boxes out of 16 from the haemocytometer grids to reach the number of cells counted. To analyze the growth curve of urea concentration over time, the average cell density (cell/mL) was calculated and compared. Further, the

logarithm of the cell density was taken, as well as the slope of these values per treatment. A one-way ANOVA was run using the resulting slopes to determine the significance of treatment groups.

IV. Results

To analyze the effects of different urea concentrations (0 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL) on the growth trends of *T. thermophila*, the average concentration of cells was plotted against the collection times (Fig. 2). In order to calculate the concentration of cells the following calculation was used:

$$\frac{number\ of\ cells\ counted}{number\ of\ boxes}(5 \times 10^{3})(1.1).$$

Across all four treatment groups, there was initially a drop in the concentration of cells between the first and third collection periods (Fig. 2). However, despite the initial decrease in cell concentration, there was positive exponential growth from the fourth collection onwards (Fig. 2).

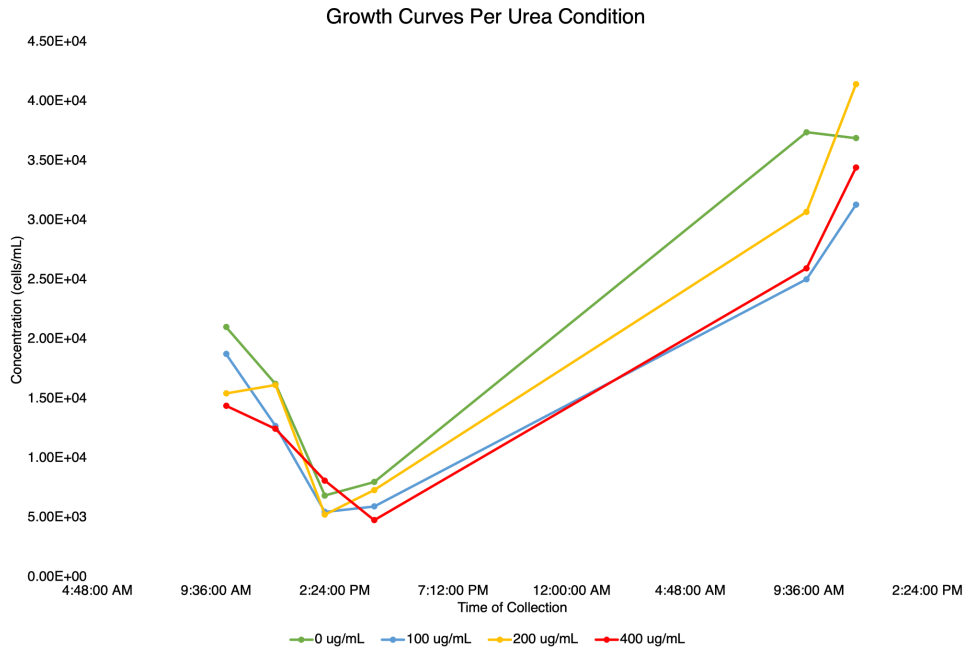

Figure 2. Number of cells per treatment group over collection times.

Because we are interested in the effect of urea concentration on growth, and growth only occurred following the fourth collection, the logarithm of the cell concentration for the fourth, fifth, and sixth collections was taken for all replicates across all treatment groups and the slope of these values was then determined. The average of the slopes per treatment with

standard deviation error bars was plotted (Fig. 3). Using these slopes, a one-way ANOVA was run, resulting in an F-statistic of 0.2958 and p-value of 0.8275. Furthermore, the Tukey HSD results for all treatment pairs had p-values larger than 0.05.

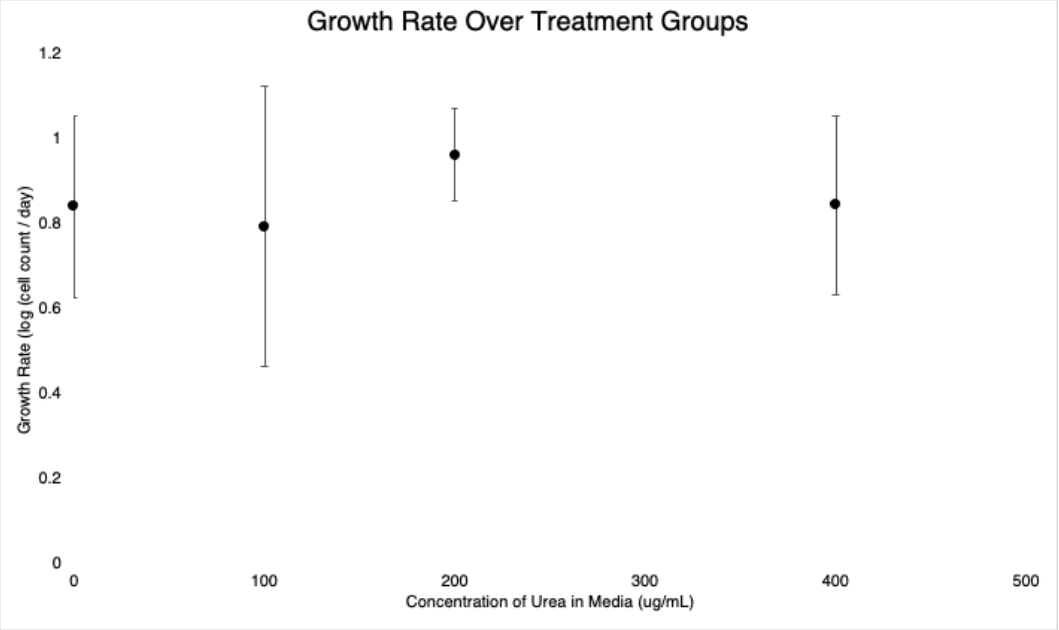

Figure 3. Average slope of logarithm of cell count for each treatment group for the fourth, fifth, and sixth collections with standard deviation error bars calculated from replicates.

V. Discussion

Based on statistical analysis from conducting a one-way ANOVA test, the p-value was determined to be 0.8275. Since this p-value is greater than 0.05, we fail to reject the null hypothesis, which was that urea has no significant effect on the growth rate of *T. thermophila.* The large p-value indicates that evidence is not strong enough to suggest that different urea concentrations have an effect on the growth rate. Furthermore, because the p-values between all pairs of treatments were also larger than 0.05, we also cannot conclude that any two treatments are significantly different from one another.

These results are inconsistent with our initial prediction that higher concentrations of urea would result in a decrease in the growth rate of *T. thermophila.* Instead, there was a decrease in growth rate with lower urea concentrations, and conversely with greater amounts of urea, there was an increase in growth (Fig. 2). Previous studies conducted on *Tetrahymena* species provide conflicting results on whether urea and urea analogues lead to an increase or decrease

in growth rates (Dewey *et al.,* 1952; Larsen *et al.*, 1988). However, the growth curves appear to trend in one single direction (Dewey *et al.,* 1952; Larsen *et al.*, 1988), whereas in the present study, both a decrease and an increase in growth rates of *T. thermophila* is observed. The decrease in the growth rate of *T. thermophila* when smaller amounts of urea were added is similar to findings conducted by Dewey et al. (1952), in which lower levels of urea contributed to the inhibition of the growth of a *T. thermophila* congener. This initial decrease in the growth rate may be explained by how urea appears to create inhibitory effects that prevent carbonyl groups from interacting with other substances, thus affecting *T. thermophila*'s metabolism (Dewey *et al.,* 1952). Furthermore, Dewey et al. (1952) state that an important component of cell growth is, however, dependent on substances that contain carbonyl groups. Contrarily, in another study conducted by Larsen et al. (1988), the increased addition of ammonia, which would occur with higher concentrations of urea, resulted in maximal cell growth of *Tetrahymena* despite unfavourable conditions. This is consistent with our results as all four treatment groups ranging from 0 μg/mL to 400 μg/mL led to exponential growth. A reason for this is likely because *Tetrahymena* are able to rapidly adapt to changes in environmental conditions, especially as more time elapses (Larsen *et al.*, 1988; Moerman *et al.*, 2021). In fact, this ability to adapt may explain the decrease followed by an increase in growth as seen across all treatment groups in this study.

Limitations

Though the results of this study do not align with previous results, this may have also been due to errors. A possible source of error could be due to insufficient mixing of the samples, which can account for variation in our data. When collecting the samples from the test tubes, it is possible that test tubes were not swirled sufficiently to ensure an accurate distribution of cells within the media. This would result in a smaller cell density count than in the samples. Along the same lines, as observed in Figure 2, the cells did not begin at the same cell density at the first collection where the cells were most evenly distributed. A difference at this time-point may have carried through to the rest of the collections.

Errors may have also resulted from not allowing for enough time between the initial culturing of cells and the collection points. This is most evident by observing that the cells did not enter an exponential growth phase until the fourth collection. Moreover, this could have resulted in a longer lag phase before entering the exponential growth phase.

A final source of error could be human differences. Different members of the group collected samples during data collection throughout the day. While the same techniques were practiced after collecting the first sample together, small differences could account for any possible errors or discrepancies in data. In addition, with different group members counting different samples, though the same protocol was used, there was room for error.

Recommendations for Future Research

In terms of future directions, firstly it would be interesting to reproduce the results of this research largely because it conflicts with previous findings of urea's inhibitory effects on *T. thermophila*. Reproducible results would solidify the conclusion drawn from this study. In addition, because *T. thermophila* is found to be sensitive to environmental changes and fertilizer pollution continues to be a problem, it is important to assess the effect of other key fertilizer components on the growth of *T. thermophila*. This could be studies involving compounds containing phosphorus or potassium, which are two other components present in fertilizers.

VI. Conclusion

We were unable to conclude that urea has an effect on the growth of *T. thermophila,* which is in disagreement with the results of previous studies. However, there is still room for exploration of other topics surrounding the effects of pollution on *T. thermophila* growth. It is important that we determine how fertilizer runoffs may affect the growth of *T. thermophila* as they remain a vital part of the ecosystems they are in, and those ecosystems are continuously and frequently polluted by fertilizer runoffs.

VII. Acknowledgements

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Musqueam, Squamish and Tsleil-Waututh nations for allowing us to learn on their ancestral, unceded and traditional territory.

VIII. References

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IX. Appendix:

Table A1: Number of Cells Per Urea Condition Over Six Collections

Concentration of Urea in Media	Replicate	Collection 1 10:00 AM March 15th, 2022		Collection 2 12:00 PM March 15th, 2022		Collection 3 2:00 PM March 15th, 2022		Collection 4 4:00 PM March 15th, 2022		Collection 5 9:30 AM March 16th, 2022		Collection 6 11:30 AM March 16th, 2022	
		Num Cells	Num Boxes	Num Cells	Num Boxes	Num Cells	Num Boxes	Num Cells	Num Boxes	Num Cells	Num Boxes	Num Cells	Num Boxes
0 ug/mL	Replicate 1	76	16	50	16	14	16	24	16	109	16	102	10

	Replicate 2	58	16	50	16	21	16	25	16	102	13	89	16
	Replicate 3	50	16	42	16	25	16	21	16	92	16	70	16
100 ug/mL	Replicate 1	55	16	36	16	16	16	21	16	113	16	110	16
	Replicate 2	54	16	39	16	16	16	11	16	60	16	97	12
	Replicate 3	55	16	36	16	22	16	20	16	46	16	34	16
200 ug/mL	Replicate 1	57	16	49	16	19	16	17	16	95	16	113	16
	Replicate 2	41	16	40	16	8	16	26	16	89	16	108	14
	Replicate 3	37	16	52	16	19	16	21	16	84	16	102	13
400 ug/mL	Replicate 1	52	16	29	16	17	16	9	16	89	16	110	16
	Replicate 2	39	16	32	16	22	16	22	16	69	16	97	16
	Replicate 3	35	16	48	16	32	16	11	16	69	16	94	16

Table A2: Calculated Cell Count Per Urea Condition Over Six Collections

		Collection 1	Collection 2	Collection 3	Collection 4	Collection 5	Collection 6
0 ug/mL	Replicate 1	2.61E+04	1.72E+04	4.81E+03	8.25E+03	3.75E+04	5.61E+04
	Replicate 2	1.99E+04	1.72E+04	7.22E+03	8.59E+03	4.32E+04	3.06E+04
	Replicate 3	1.72E+04	1.44E+04	8.59E+03	7.22E+03	3.16E+04	2.41E+04
100 ug/mL	Replicate 1	1.89E+04	1.24E+04	5.50E+03	7.22E+03	3.88E+04	3.78E+04
	Replicate 2	1.86E+04	1.34E+04	5.50E+03	3.78E+03	2.06E+04	4.45E+04
	Replicate 3	1.89E+04	1.24E+04	7.56E+03	6.88E+03	1.58E+04	1.17E+04
200 ug/mL	Replicate 1	1.96E+04	1.68E+04	6.53E+03	5.84E+03	3.27E+04	3.88E+04
	Replicate 2	1.41E+04	1.38E+04	2.75E+03	8.94E+03	3.06E+04	4.24E+04
	Replicate 3	1.27E+04	1.79E+04	6.53E+03	7.22E+03	2.89E+04	4.32E+04
400 ug/mL	Replicate 1	1.79E+04	9.97E+03	5.84E+03	3.09E+03	3.06E+04	3.78E+04
	Replicate 2	1.34E+04	1.10E+04	7.56E+03	7.56E+03	2.37E+04	3.33E+04
	Replicate 3	1.20E+04	1.65E+04	1.10E+04	3.78E+03	2.37E+04	3.23E+04

Table A3: Slopes of Logarithm of Growth Rates for 4th, 5th, and 6th Collections

	0 ug/mL	100 ug/mL	200 ug/mL	400 ug/mL
Replicate 1	1.075680483	0.96120016	0.9285083	1.08879268
Replicate 2	0.804493308	1.012107085	1.08974151	0.70355124

Replicate 3	0.651220915	0.417443838	0.8801638	0.74982124