The effects of toluene on the ciliary function of *Tetrahymena thermophila*

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Abstract

Tetrahymena thermophila is a ciliated protist and a well-studied organism that is used as an experimental analogue for ciliary function in other eukaryotes. We studied it as a model for human endotracheal ciliary response to toluene, a compound found in both cigarettes and e-cigarettes. We performed three experiments with four replicates each: a motility assay to study the effects of toluene on movement, a food vacuole assay measuring the ability of *T. thermophila* to phagocytose Congo-red stained yeast using their oral cilia and a cell viability assay in order to determine whether cells without food vacuoles were still alive. We found significant differences in the percentage of moving cells in the first assay as we increased the concentration of toluene, as well as significant and similar differences regarding food vacuole formation in our second assay. Our last assay found no significant difference in the amount of viable cells without food vacuoles at each of three concentrations tested. Taken together, these data suggest that short-term exposure to toluene does not kill *T. thermophila,* but may inhibit ciliary function. The mechanism by which toluene acts on cilia is unknown, however previous studies have suggested that the toxicity of toluene is associated with its methyl side chain, as it can form hydrogen bonds with other molecules.

Introduction

Recently, e-cigarette use has become more prevalent globally, both among smokers and non-smokers (Grana, Benowitz and Glantz 2014). The aerosol produced from e-cigarettes contains a number of toxic compounds, including acrolein, acetaldehyde, formaldehyde and toluene (Grana, Benowitz and Glantz 2014). The aerosol is inhaled by individuals smoking e-cigarettes and interacts with the endotracheal cilia of the respiratory tract. These cilia play a role in clearing the airway by transporting particles collected in mucus towards the glottis (Bermbach et al. 2014). Non-functional respiratory cilia may lead to the presence of harmful bacteria, viruses or allergens in the respiratory tract (Bermbach et al. 2014), thus negatively impacting the health of the individual.

We were interested in exploring the effects of toluene, a toxicant found in e-cigarettes, on the function of respiratory cilia. Since the structure and mechanism of movement of cilia is conserved between ciliated protists and mammals (Satir and Christensen 2007), we used *Tetrahymena thermophila* as a model system for our studies. *T. thermophila* is a unicellular ciliated protist that uses somatic cilia to move and oral cilia to phagocytose its prey (Bozzone 2000). In order to understand the impact of toluene on the function of cilia, we examined *T. thermophila*'s mobility and ability to form food vacuoles, processes that necessitate functional cilia (Bozzone 2000).

Our first null hypothesis (H_{O1}) was that toluene has no effect on the movement pattern of *T. thermophila*. The alternate hypothesis (H_{A1}) was that toluene has an effect on the movement of *T. thermophila.* Our second null hypothesis (H_{O2}) was that toluene has no effect on the average number of food vacuoles formed by *T. thermophila*. Our corresponding alternate hypothesis (H_{A2}) was that toluene has an effect on the average number of food vacuoles formed by *T. thermophila*. Lastly, our third null hypothesis (H_{O3}) was that the effect of toluene on food vacuole formation is dependent on its effect on cell viability. The corresponding alternate hypothesis (H_{A3}) was that the effect of toluene on food vacuole formation is independent of its effect on cell viability.

Previous research has shown that toluene can inhibit the growth of *T. thermophila* and can be lethal in high concentrations (Li et al. 2009). It is hypothesized that the toxic effects of toluene are associated with the methyl side chain of the compound, as it can form hydrogen bonds with biomolecules (Li et al. 2009). Ciliary motion relies on dynein-tubulin interactions (Warner and Mitchell 1978); therefore hydrogen bonding of the methyl group to these proteins may interfere with their ability to interact with each other.

Toluene diisocyanate, a compound with toluene as its structural backbone, is also known to colocalize with tubulin of cilia and inhibit ciliary movement (Lange et al. 1999)**.** For this reason, we predict that the exposure of *T. thermophila* to toluene will have a negative impact on the function of cilia. We propose that toluene will bind to the tubulin of *T. thermophila*'s cilia, thus inhibiting ciliary function, and limiting the cell's ability to move and uptake food from the environment (Figure 1). We predict that we will observe abnormal movement in cells exposed to toluene, as well as a reduction in the number of food vacuoles formed over time; however, the cells should not die from this short-term exposure to the compound.

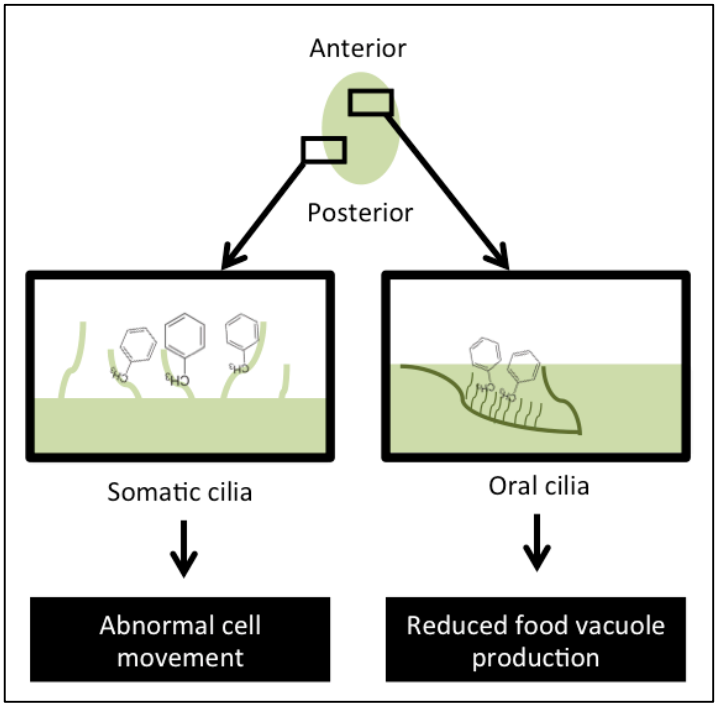

Figure 1: The predicted model that *T. thermophila* will follow when somatic and oral cilia are exposed to toluene.

Methods

Mobility Assay

We grew cells in SSP medium, composed of 2% proteose peptone, 0.1% yeast extract, 0.2% glucose and 33μM FeCl3, with an approximate concentration of

1.25×10^5cells/mL. We mixed the SSP medium with toluene to obtain concentrations of 1µg/mL, 5µg/mL, 10µg/mL, 30µg/mL, 70µg/mL and 95µg/mL of toluene in medium. We then centrifuged approximately 750µL of the cell stock solution at 13.2 rpm for 5 minutes to create a pellet, removed the supernatant and added 500µL of the appropriate toluene-containing medium to each replicate. Each experimental condition contained four independent replicates, and the addition of SSP media without toluene to *T. thermophila* served as a control. We incubated experimental and control cells at room temperature for 30 minutes in their respective media before we placed 20µL of cells on slides and examined them under a compound microscope (Figure 2). We counted a total of 20 cells in each replicate, and classified their movements as either normal or abnormal with respect to the control condition. Abnormal movement was described as cells moving at exceptionally slow rates or exhibiting a shaking behaviour, where cells were quivering or trembling, but not successfully moving in a particular direction. We then performed a one-way ANOVA to determine whether there were differences in the mean number of cells exhibiting normal behaviour between treatment levels.

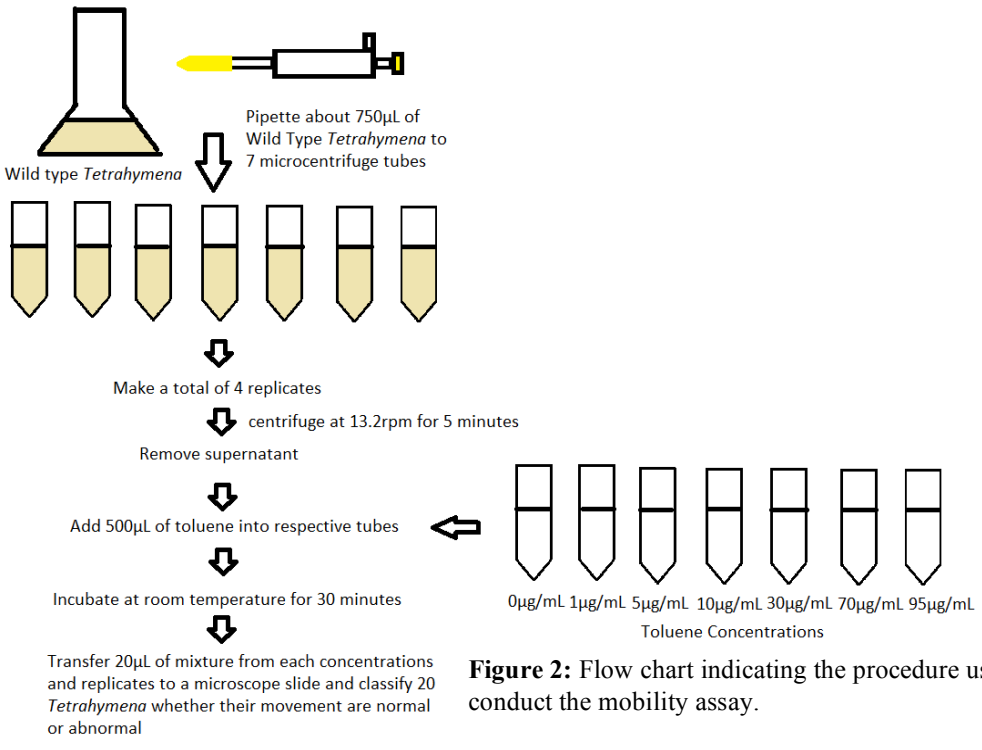

Figure 2: Flow chart indicating the procedure used to conduct the mobility assay.

Food Vacuole Assay

We prepared *T. thermophila* following the same procedure outlined in the cell mobility assay; the only differences were that the cell stock concentration was 6.375 x 10^4 cells/mL, and the volume of cells that were centrifuged into a pellet was 500µL. We incubated the experimental and control cells at room temperature for 30 minutes in their respective media before we added 500µL of Congo-red stained yeast to each replicate. We isolated 200µL of cells from each replicate and fixed them with 2% glutaraldehyde at 15, 30 and 45 minutes after the addition of Congo-red stained yeast (Figure 3). We determined the number of food vacuoles in approximately 10-15 cells from each replicate at each of the three times points by counting the number of vacuoles in fixed cells under a compound microscope. We then performed a one-way ANOVA to determine if there were significant differences in the mean number of food vacuoles formed among treatment levels.

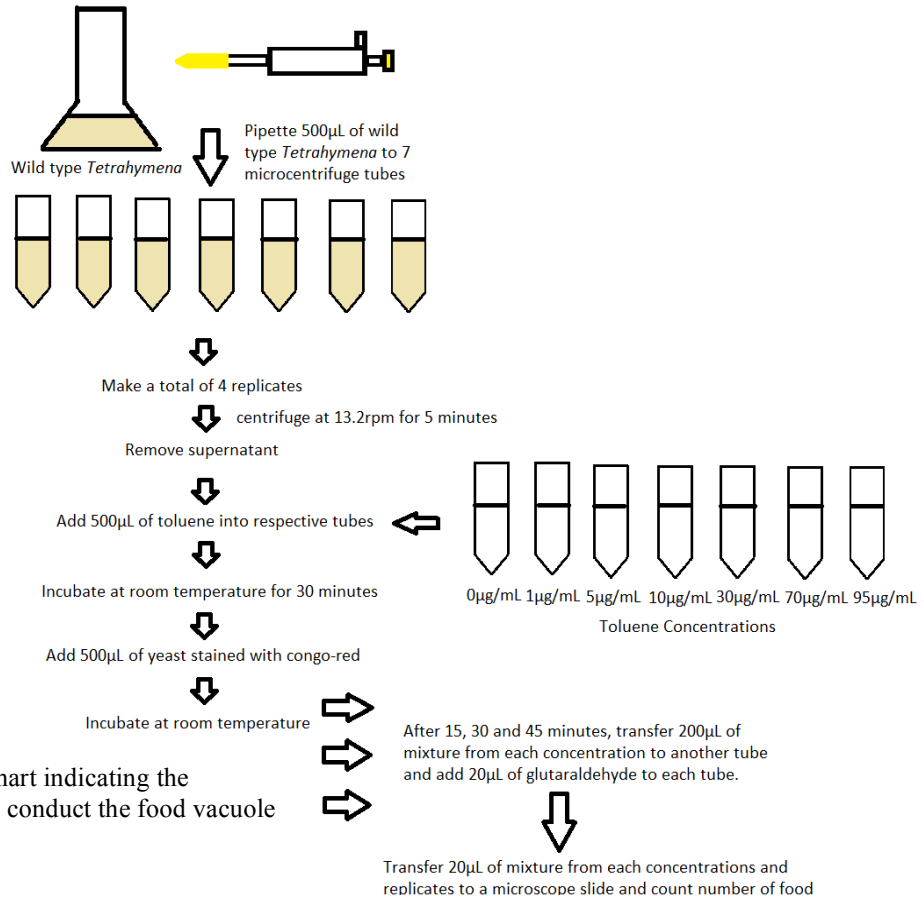

Figure 3: Flow chart indicating the procedure used to conduct the food vacuole assay.

Cell Viability Assay

We prepared *T. thermophila* following the same procedure outlined in the cell mobility assay; the only differences were that the cell stock concentration was 2.2×10^5cells/mL, and the volume of cells that were centrifuged into a pellet was 1000µL. We only prepared solutions with 95µg/mL, 70µg/mL and 10µg/mL of toluene in the media. We incubated the experimental and control cells at room temperature for 30 minutes in their respective media before we added 500µL of Congo-red stained yeast to each replicate. Forty-five minutes after the addition of Congo-red stained yeast, we placed 20µL of the cells on a slide for observation (Figure 4). We located cells that had not formed food vacuoles and examined them to assess cell viability. We examined a total of 10 cells per replicate. We then performed a one-way ANOVA to determine whether there were significant differences in the mean number of viable cells that contained no food vacuoles among treatment levels.

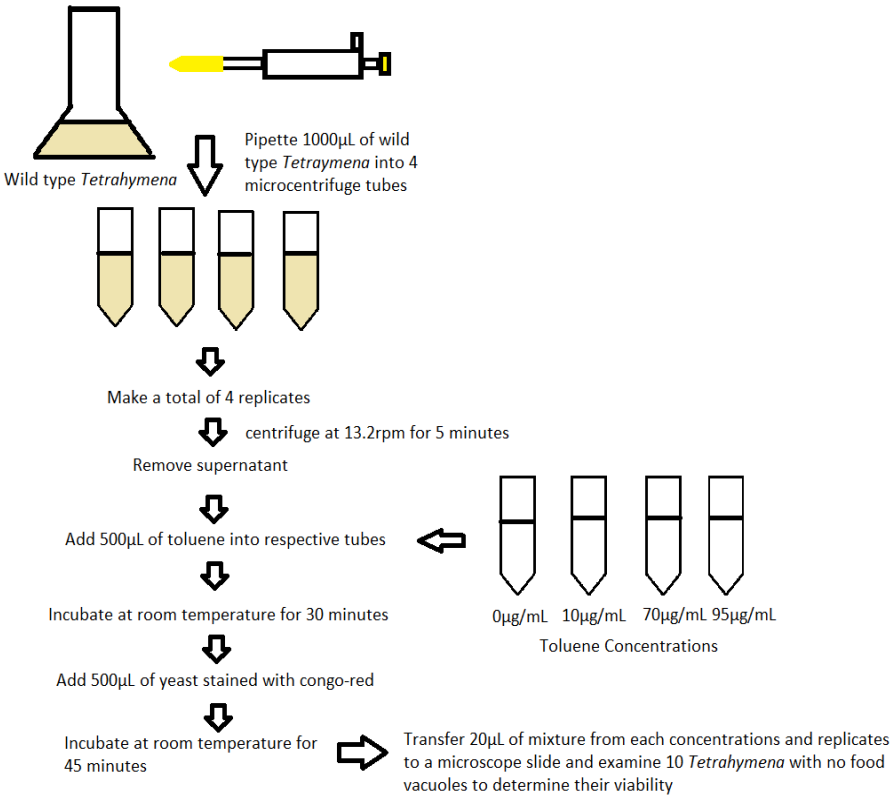

Figure 4: Flow chart illustrating the procedure used to conduct the cell viability assay.

Results

Mobility Assay

100 ± 0% cells incubated without toluene and with 1µg/mL of toluene displayed normal movement (Figure 5).). 99 ± 2% of cells incubated with 5µg/mL of toluene, 96 ± 5% of cells incubated with 10µg/mL of toluene, 89 ± 2% of cells incubated with 30 µg/mL of toluene, 68 ± 12% of cells incubated with 70µg/mL of toluene and 70 ± 11% of cells incubated with 95µg/mL of toluene displayed normal movement (Figure 5). Cells treated in 70µg/mL and 95µg/mL of toluene showed large variation among replicates in comparison to other treatment levels. A one-way ANOVA (df =6, F_{crit}=19.3) was conducted to compare the mean percentage of cells displaying normal movement when incubated for 30 minutes with varying toluene concentrations. There was a significant difference between the means, *p*-value<0.0001.

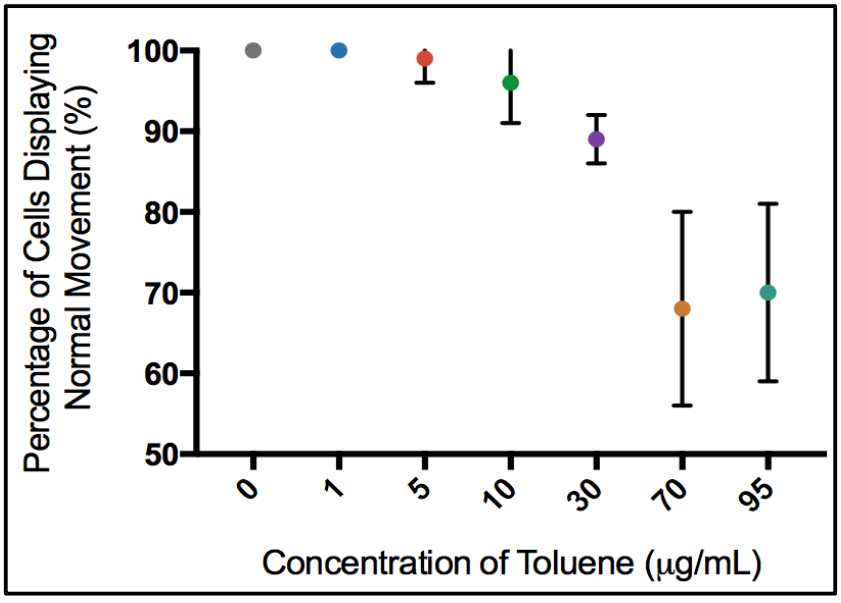

Figure 5: Percentage of *T. thermophila* cells displaying normal movement when incubated for 30 minutes with toluene concentrations of 95µg/mL, 70µg/mL, 30µg/mL, 10µg/mL, 5ug/mL, 1µg/mL and 0µg/mL. Error bars represent 95% confidence intervals (a= 0.05). (n=4) *p*-value<0.0001

Food Vacuole Assay

Cells incubated without toluene formed an average of 5.23 ± 1.04 food vacuoles per cell (Figure 6). Those incubated with 1µg/mL of formed an average of 6.59 ± 0.73 food vacuoles per cell (Figure 6). Cells in 5µg/mL of toluene formed an average of 7.87 ± 0.77 food vacuoles per cell (Figure 6), while those in 10µg/mL of toluene formed an average of 6.59 ± 1.06 food vacuoles per cell (Figure 6) and those incubated with 95µg/mL of toluene formed an average of 2.69 ± 0.42 food vacuoles per cell. A one-way ANOVA (df =6, F_{crit}=9.5262) was conducted to compare the mean number of food vacuoles formed when cells were incubated with varying toluene concentrations. There was a significant difference between the means, *p*-value <0.0001. Although there were significant differences among treatments, there were also large variations among replicates of certain treatments, such as the 70µg/mL and 30µg/mL of toluene.

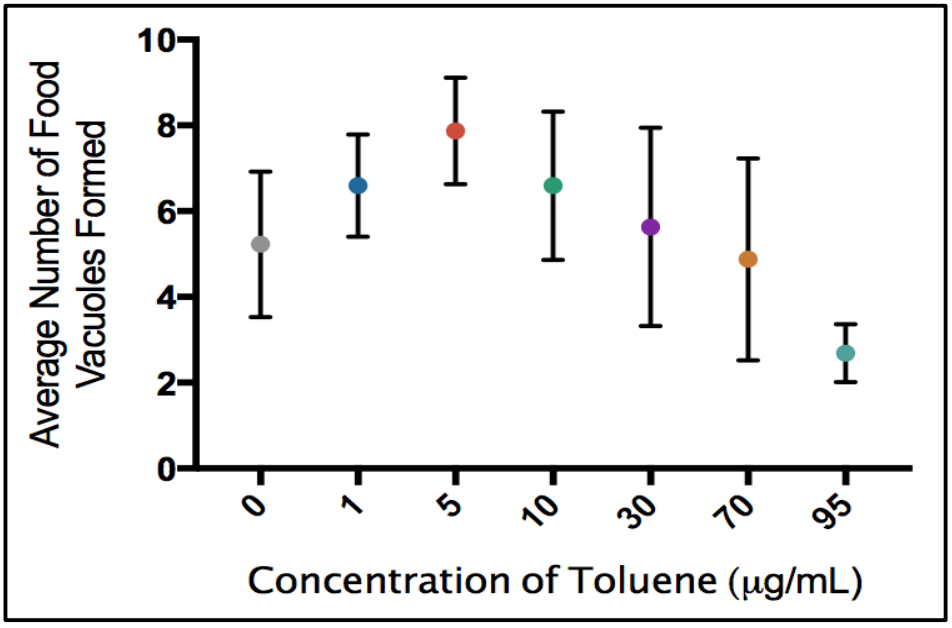

Figure 6: Average number of food vacuoles formed in *T. thermophila* cells when incubated for 30 minutes with toluene concentrations of 95µg/mL, 70µg/mL, 40µg/mL, 10µg/mL, 5µg/mL, 1µg/mL and 0µg/mL, and for 45 minutes with Congo-red stained yeast. Error bars represent 95% confidence intervals (a= 0.05). (n=4) *p-value*<0.0001

Cell Viability Assay

Many cells examined during the food vacuole assay were noted to contain zero food vacuoles. In order to determine whether cells without vacuoles were still viable, we performed a viability assay. Of the cells incubated without toluene, 100.00 ± 0% were viable (Figure 7). 95 ± 6% cells incubated with 10µg/mL of toluene, 95 ± 6% of cells incubated with 70µg/mL and 97.5 ± 5% of cells incubated with 95µg/mL were viable. A one-way ANOVA (df =3, F_{crit}=1.0000) was conducted to compare the mean percentage of viable cells without food vacuoles when cells were incubated with varying toluene concentrations. There was no significant difference among the means, *p*-value = 0.4262.

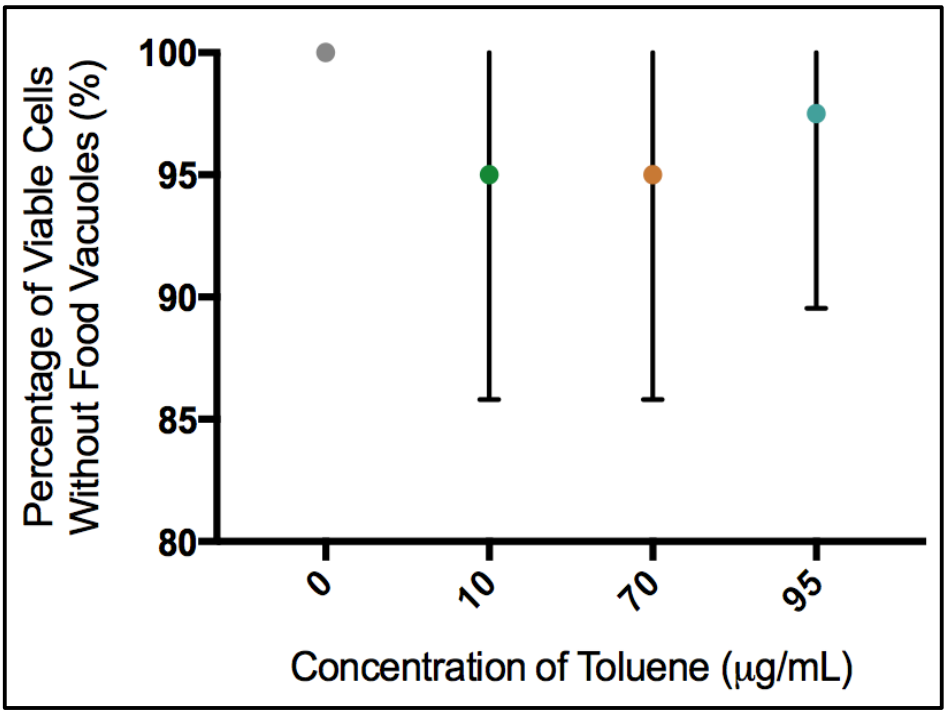

Figure 7: Percentage of *T. thermophila* cells without food vacuoles when incubated for 30 minutes with toluene concentrations of 95µg/mL, 70µg/mL, 10µg/mL and 0µg/mL, and 45 minutes with Congo-red stained yeast. Error bars represent 95% confidence intervals (a= 0.05). (n=4) *p-value* = 0.4262

Discussion

A toluene variant, toluene diisocyanate, has been found to bind to the cilia in human bronchi (Lange et al. 1999). To date, no reports have been made as to whether toluene has a similar effect as toluene diisocyanate on cilia. Since toluene is present in e-cigarette aerosol (Grana, Benowitz and Glantz 2014), we wanted to know whether e-cigarettes could negatively impact ciliary function. This paper presents an analysis of the effects of toluene on ciliary function in *T. thermophila* upon exposure to various concentrations of toluene. The upper limit of concentration was chosen as 95μg/mL because this is the concentration of toluene needed to inhibit the growth of 50% of *T. thermophila* cells (Li et al. 2009), so cells were likely to stay alive during the performed assays. The lower limit of 1μg/mL was determined by examining the average amount of toluene found in e-cigarette aerosol (Grana, Benowitz and Glantz 2014).

This study determined the mobility of *T. thermophila* when incubated in several different concentrations of toluene for 30 minutes. The mean percentages of cells displaying normal movement (Fig. 5), given $p < 0.0001$ by one-way ANOVA, were significantly different. Therefore, we are able to reject H_{O1} and provide support for H_{A1}, which states that toluene has an effect on the movement of *T. thermophila.* A visual analysis of the 95% confidence intervals suggests that the significance in this statistical test most likely arises from differences between the means at 30μg/mL, 70μg/mL and 95μg/mL with respect to those at 0μg/mL, 1μg/mL and 5μg/mL (Fig. 5). The cells that were incubated in higher toluene concentrations moved slower than the ones in lower concentrations, and also displayed shaking behavior. We speculate that toluene inhibits the somatic cilia that are used for movement (Figure 1), and causes some of the *T.*

thermophila to tremble or quiver instead of moving in a particular direction. The result supports our prediction that toluene has a negative effect on ciliary function.

Food vacuole formation in *T. thermophila* depends on the proper functioning of oral cilia that are necessary to phagocytose prey (Bozzone 2000). Therefore, the success of food vacuole formation can be used to quantify the effect of toluene on oral cilia. After performing a one-way ANOVA we found that the mean number of food vacuoles formed by *T. thermophila* when incubated in different toluene concentrations was significantly different. Since $p<0.0001$, we can reject the H_{O2} and provide support for the alternate hypothesis that toluene has an effect on the number of food vacuoles formed by *T. thermophila.* A visual analysis of the 95% confidence intervals suggests that the significance in this statistical test most likely arises from differences between the mean at 95µg/mL and those at 0µg/mL, 1µg/mL, 5µg/m and 10µg/mL (Fig. 6). This suggests that toluene has a negative effect on food vacuole formation, which is consistent with our initial prediction. It must be noted that many *T. thermophila* cells from the control sample were lysed and shrivelled, which may be associated with an extra two minutes they spent in the centrifuge.

Since many of the cells incubated at higher concentrations of toluene formed zero food vacuoles, they were further examined to ensure that the *T. thermophila* were not simply dead, and therefore incapable of phagocytosis. Since the *p*-value obtained from a one-way ANOVA was 0.4262, we fail to reject H_{O3}, and consequently support H_{A3}, as the mean percentage of viable cells without food vacuoles in different concentrations of toluene was not significantly different (Figure 7). This result suggests that the decrease in food vacuole formation we observed when incubating cells in high concentrations of

toluene was not due to cell death. This was expected, as cells were only incubated in toluene media for short periods of time during the experiments.

Although the results of our experiments suggest that toluene may inhibit ciliary function, it is worth mentioning that toluene could also have other physiological consequences on the cell. It has been found that toluene negatively impacts the plasma membranes of *E. coli*, as permease activity is lost and an efflux of cellular components, including proteins and RNA, is observed (Jackson and DeMoss 1965). Since toluene has a high affinity for lipids (Cruz, Rivera-Garcia and Woodward 2014), it is possible that treating *T. thermophila* with this compound may have disrupted cellular membranes. If the disruption is similar to what occurs in *E. coli,* important cytoplasmic molecules may have been lost to the environment. These losses could have led to the dysregulation of physiological processes in *T. thermophila,* which in turn may have resulted in a loss of movement and ability to phagocytose that was independent of ciliary function.

It is important to note that there was subjectivity in the quantification of food vacuoles. Each red spot was treated as one food vacuole in the study, however some of the food vacuoles were pale red and others were dark red. Since the SSP media contained yeast extract, some *T. thermophila* may have consumed more unstained yeast than stained yeast, leading to the formation of paler vacuoles. It was difficult to distinguish whether some of these pale spots were indeed food vacuoles, which could have led to an underestimation of the number of food vacuoles. To minimize differences in subjectivity across samples, a single group member performed the food vacuole counts and samples were blinded prior to analysis.

It is known that *T. thermophila* stops forming food vacuoles during cell division

(Suhr-Jessen 1978). Dividing *T. thermophila* may possibly have been present in the cell stock solution, which could account for the observation of no food vacuoles in some cells. Although we did not observe cells undergoing telophase, some *T. thermophila* may have been nearing metaphase during our experiments, which could have limited their ability to form food vacuoles. This may have resulted in an underestimation of the number of food vacuoles.

Lastly, it is advised that *T. thermophila* cells are pelleted at 600g-1000g for one minute (Cassidy-Hanley 2012), however our centrifuge was set to 13200 RPM for five minutes, which is approximately equivalent to 16000g (Eppendorf 2013). This may have resulted in reduced cell viability, and adverse effects (Cassidy-Hanley 2012). The control cells that were used in the food vacuole experiment were spun for an additional three minutes after the five minutes, as the supernatant was not immediately removed and cells had to be re-pelleted. This extra time spent in the centrifuge may have led to the reduced number of viable cells that were observed in the control replicates.

Conclusion

The null hypotheses that toluene has no effect on the movement pattern of *T. thermophila*, and that toluene has no effect on the average number of food vacuoles formed by *T. thermophila* were rejected, and the respective alternative hypotheses were supported by the data. The null hypothesis that toluene has no effect on the viability of *T. thermophila* could not be rejected, and the alternative hypothesis was not supported. Toluene has been found to decrease food vacuole formation in *T. thermophila*; it also caused abnormal movements in *T. thermophila*, such as shaking behavior and slower speed, when incubated in high concentrations of toluene. These results agree with our

predictions. There was no significant difference in the percentages of viable cells without food vacuoles found in the different toluene concentrations, suggesting that cell death by toluene exposure does not cause the decrease of food vacuoles observed during the food vacuole assay. Taken together, these findings suggest that toluene may inhibit the ciliary function of *T. thermophila*.

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