Effects of Increasing Concentration of Isoamyl Acetate on Chemoattraction of *Tetrahymena*

Gagandeep Gill*, Jong J. Park, and Bryant Rathbone (*=Corresponding Author)

Abstract

This Research Study was conducted to determine how increasing concentrations of isoamyl acetate would affect the chemoattraction of *Tetrahymena* to the chemical. This is biologically relevant because isoamyl acetate is a compound produced by *E. coli*, a source of food for *Tetrahymena*. The data was obtained using a two-chambered system that was carved into wax on a glass slide. This method was designed and built by the researchers in this study. Data were obtained by measuring the proportion of *Tetrahymena* moving in and out of the chamber in which the chemoattractant was placed for 5 min. at varying concentrations. It was found that at concentrations of 0M, 10^{-6}M, 10^{-5}M, and 10^{-4}M the proportions of *Tetrahymena* entering the chemoattractant chamber were 50.0 +/- 1.7%, 56.7 +/- 4.4%, 57.6 +/- 1.5%, and 61.4 +/- 5.5% respectively. Statistical analysis indicates that there is a significant difference between the control (0M) and all three experimental conditions, but there is no significant difference between experimental conditions. This tells us that *Tetrahymena* do indeed have an attraction to isoamyl acetate but more research, especially using lower concentrations of chemoattractant, need to be done to determine the effects of concentration on chemoattraction in *Tetrahymena*.

Introduction

Tetrahymena is a free-swimming, ciliate protozoan. It is a heterotroph, meaning that it survives and grows by taking in organic molecules from its environment (Jost *et al.* 1973). Like many heterotrophs, *Tetrahymena* moves towards higher concentrations of certain nutrient and nutrient-associated molecules, a process known as chemotaxis (Làng *et al.* 2011). The direction of chemotaxis is driven by the concentration gradient of a chemical, that is, it is functions to move cells towards or away from a point source of the chemical. Whether a cell moves towards or away from the source depends on whether the chemical is a chemoattractant or a chemorepellant (Van Houten *et al.* 1975).

For *Tetrahymena,* some concentrations of certain chemicals cause a chemoattractant response, while other concentrations cause a chemorepellant response. Isoamyl acetate, a

common fruit ester, was used as the chemoattractant because it has previously been shown to act as a chemoattractant to *Tetrahymena* at all concentrations (Làng *et al.* 2011).

Tetrahymena have been used as a model organism when studying chemotaxis in the past, due to its sharing of some chemotactic responses with higher animals (Eliot 1974). Interestingly, humans and *Tetrahymena* share a chemoattractant response to isoamyl acetate. *Tetrahymena* respond to isoamyl acetate concentrations as low as 10^{-12}M and we informally observed humans smelling as little as a 10^{-5}M concentration (Lang *et al* 2011).

For *Tetrahymena* the evolutionary rational for this attraction is that isoamyl acetate is a metabolic byproduct of *E. coli* which is preyed upon by *Tetrahymena* (Ravishankar 2004, Jost 1973). For humans the evolutionary rational for attraction to isoamyl acetate is that it is present in fruits, such as banana and apples (Làng *et al.* 2011). Thinking of it this way, however, puts the cart before the horse, as plants produced these chemicals specifically as attractants in the first place. Isoamyl acetate also acts as a chemoattractant for *C. elegans* and *D. melanogaster*, meaning that plants may have evolved this attractant as a way to exploit an evolutionarily well conserved chemoattractant response in animals (Làng *et al.* 2011).

With this fundamental understanding of chemotaxis, we hypothesized that *Tetrahymena* would respond more strongly to a stronger concentration of chemoattractant at the source. The hypotheses we decided to test were:

Alternative Hypothesis: As the concentration of isoamyl acetate increases, the attraction of *Tetrahymena* to the chemoattractant will also increase, as measured by the proportion of *Tetrahymena* moving in and out of the chamber in which the chemoattractant is placed.

Null Hypothesis: As the concentration of isoamyl acetate increases, the attraction of *Tetrahymena*

to the chemoattractant will decrease or will not change as measured by the proportion of

Figure 1: Steps in our chemotaxis assay. T is the test chamber, C is the control chamber and FOV stands for *Field of view*.

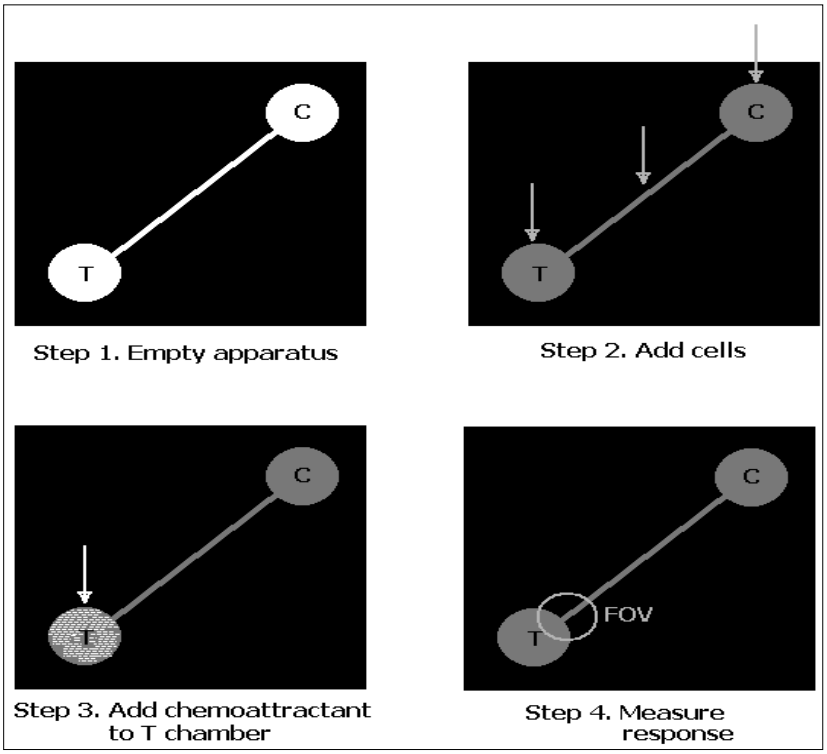
moving in and out of the chamber in which the chemoattractant is placed.

Methods

Concept

Testing our hypothesis required an assay determining the proportion of our organisms moving towards a chemoattractant. Multiple assays exist which would have allowed us to observe this responding variable (Levandowsky 1984, Kohidai 1995, Gronlein 2010). However, these assays either take too long, due to reliance on complete cell counts, or require specialized equipment, such as custom glassware, the production of which was outside our expertise and the procurement of which was outside our budget.

To this end, we designed a novel assay, similar in concept to a flat capillary assay but without the need for specially made glassware (Adler 1973).



Conceptually, our apparatus consisted of two chambers connected by a narrow channel (Figure 1). First, a solution (15 microliters each) containing a known concentration of cells was added, filling both chambers and the channel. Then, the chemoattractant (4 microliters each) was added to the test chamber. After this, response to the chemoattractant was measured by observing the juncture of the test chamber and the channel using a dissecting microscope for 5 minutes. The number of cells entering the chamber and the number exiting were counted and recorded. A higher proportion of cells entering would indicate a chemoattractant effect (Làng 2011).

The control was a solution of growth medium with no chemoattractant present. Since Lang and his colleagues in 2011 found strong chemoattraction of *Tetrahymena* to the chemoattractant (isoamyl acetate) at 10^{-6}M and higher, the experimental treatments were isoamyl acetate concentrations of 10^{-6}M, 10^{-5}M, and 10^{-4}M. The concentrations were achieved by diluting a 100% sample with growth medium. Also, 6 replicates for each experimental condition were conducted so that the effect of variation could be decreased. More replicates were not possible due to time constraints. Room temperature was also measured periodically to maintain consistency.

Chemoattractant

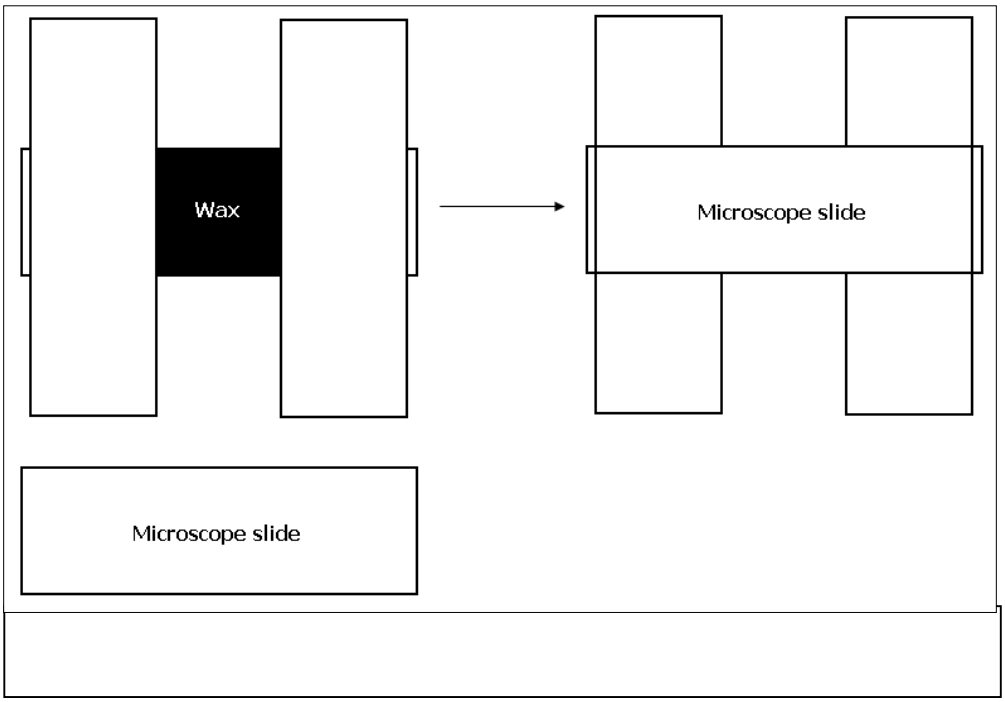
We selected isoamyl acetate, also known as banana oil, as our chemoattractant. This selection was based on the findings of Làng *et al*. (2011) and the work of Ravishankar *et al.* (2004). Làng found that isoamyl acetate was a chemoattractant to *Tetrahymena* at all tested concentrations. Ravishinkar's work provided us with an evolutionary rational for this finding; that isoamyl acetate is a metabolic byproduct of *E. coli,* one of *Tetrahymena's* many food sources (Jost 1973).

We synthesized our isoamyl acetate by the Fischer-Speier esterification of isoamyl alcohol and acetic acid. We purified the resulting ester (isoamyl acetate) by performing a liquid-liquid extraction and a simple distillation.

Construction of the Apparatus and Practical Considerations

We produced a working apparatus based on Adler's Conceptual model in 1973 by carving the chambers and channel into wax that had been sealed to the surface of a microscope slide. We chose to use black wax in order to maximize the visual contrast between empty spaces and walls. Before carving could begin, an even thickness of wax was adhered to the slide using the improvised mold depicted in Figure 2.

Figure 2. Mold for production of experimental apparatus



Once an even thickness of wax was achieved, the chambers and channel were carved using an etching needle. The size of the chambers was regulated using a stationary circular guide for the needle, while the size of the channel was limited to the thickness of the etching needle.

In some cases, bubbles developed under the wax that could have resulted in leakage of cell solution during the experiment. This problem was resolved by briefly reheating the test slides, allowing the wax to liquefy enough to reform to the glass without losing its shape.

Once the chambers and channels were carved, residual wax debris was rinsed from the channel and chambers. Although this debris did not limit flow of solution or movement of cells, it had the potential to distract the experimenter during cell counting or to obscure his view of the observed junction.

Addressing a similar issue, it was necessary to run the experiments using diluted stock solutions, as the full concentration produced so much visible cell movement that exact counts became difficult. The necessary dilution varied based on the stock solution and had to be determined empirically each time.

The ability of the chemoattractant to diffuse down the channel to the control chamber was confirmed using green dye in place of the chemoattractant. Also, tests were conducted at 28° C, which prevented an issue encountered by Levandowsky *et al.* (1984), who found no chemotactic response below 25° C.

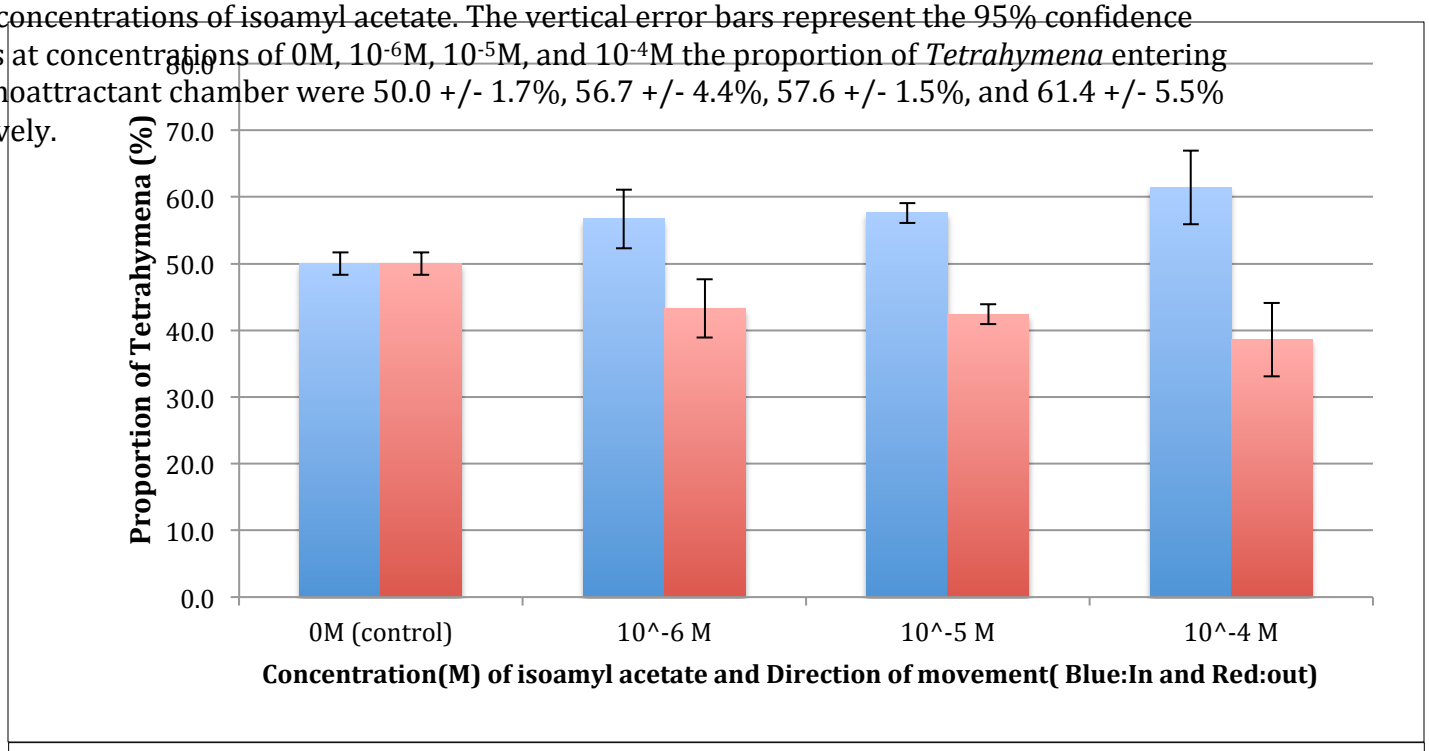
Once data was obtained, Microsoft Excel was used to calculate proportions, graph the data, and find the 95% confidence intervals. Sample calculations are shown in the results section.

Results

It was found that in the control condition, 50.0 +/- 1.7% of Tetrahymena moved into the chemoattractant chamber and the same proportion moved out. Similarly, the proportions of *Tetrahymena* moving in to the chamber at isoamyl acetate concentrations of 10^{-6}M, 10^{-5}M, and 10^{-4}M were 56.7 +/- 4.4%, 57.6 +/- 1.5%, and 61.4 +/- 5.5% respectively. The proportions of *Tetrahymena* moving out of the chamber at these concentrations were found to be 43.3 +/- 4.4%, 42.4 +/- 1.5%, and 38.6 +/- 5.5% respectively. As seen in Figure 3, there is a significant difference

between the proportions of *Tetrahymena* moving in and out of the chemoattractant chamber in all three experimental conditions compared to the control. Figure 3 also shows that due to overlapping error bars, there is no significant difference between the proportions of *Tetrahymena* moving in and out of the chemoattractant chamber between the treatment conditions, where the isoamyl acetate was present. Although there is no significant difference between the proportions, there is a trend: as the concentration of chemoattractant (isoamyl acetate) increases, the proportion of *Tetrahymena* entering the chamber increases and the proportion of *Tetrahymena* leaving the chamber decreases.

gure 3: The proportion of *Tetrahymena* moving in and out of the chemoattractant chamber at rying concentrations of isoamyl acetate. The vertical error bars represent the 95% confidence ervals at concentrations of 0M, 10^{-6}M, 10^{-5}M, and 10^{-4}M the proportion of *Tetrahymena* entering e chemoattractant chamber were 50.0 +/- 1.7%, 56.7 +/- 4.4%, 57.6 +/- 1.5%, and 61.4 +/- 5.5% spectively.



Sample calculations to demonstrate how the results were obtained are shown below.

$$\% \boldsymbol{In} = \frac{\#\ of\ Tetrahymena\ going\ in\ the\ chamber}{Total\ Tetrahymena\ displaying\ movement} x100\% = \frac{38}{75} x100\% = 50.67\%$$

$$\% \boldsymbol{Out} = \frac{\#\ of\ Tetrahymena\ leaving\ chamber}{Total\ Tetrahymena\ displaying\ movement} x100\% = \frac{37}{75} x100\% = 49.33\%$$

$$\boldsymbol{Average\ Proportion} = \frac{\sum(Proportions)}{Number\ of\ replicates} = \frac{(51.28 + 51.92 + 47.17 + 50.67 + 51.43 + 47.62)}{6}$$

$$= 50.01\%$$

$$\boldsymbol{Variance}\ (\boldsymbol{s^2}) = \frac{\sum(proportion - average)^2}{\#\ of\ replicates - 1}$$

$$= \frac{(51.28 - 50.01)^2 + (51.92 - 50.01)^2 + (47.17 - 50.01)^2 + (50.67 - 50.01)^2 + (51.43 - 50.01)^2 + (47.62 - 50.01)^2}{5}$$

$$= 4.30$$

$$\boldsymbol{Standard\ Deviation} = \sqrt{Variance} = \sqrt{4.30} = 2.07$$

$$\mathbf{95\%}\ \boldsymbol{Confidence\ Intervals} = 1.96\left(\frac{Standard\ Deviantion}{\sqrt{\#\ of\ replicates}}\right) = 1.96\left(\frac{2.07}{\sqrt{6}}\right) = 1.66 = \sim 1.7\%$$

Discussion

Although there was a significant difference between the proportions of *Tetrahymena* moving in and out of the chamber in the treatment conditions compared to the control, and even though there was an upward trend between experimental conditions as concentration of isoamyl acetate increased, due to overlapping error bars between experimental conditions we fail to provide support for the alternative hypothesis.

The observed trend, however, can be explained by the fact that the detection of chemoattractants in *Tetrahymena* occurs through receptors in the cell membrane (Lang *et al* 2011). Lang and his colleagues in 2011 found that *Tetrahymena* use a G-protein coupled receptor to detect chemoattractants and chemorepellents in their vicinity and that they use a pathway that involves cAMP to transduce the signal to other parts of the cell, where this information is processed. After the information has been processed, the *Tetrahymena* responds by opening ion

channels either to depolarize or hyperpolarize the cell (Tanabe *et al* 1980). The amount of depolarization indicates to the organism the strength of a chemoattractant and thus influences the magnitude of the organism's response (Tanabe *et al* 1980). The greater the depolarization: the larger the response that the *Tetrahymena* will have.

Tetrahymena uses a G-protein coupled receptor for detection. Since these type of receptors exhibit Mechaelis-Menten type saturation kinetics (Attie and Rains 1995). This means that as the concentration of ligand, in our case isoamyl acetate, increases, there will be an increasing number of receptors that will be activated by binding. This only occurs up to a certain point after which the concentration will not affect the receptors. At this point, the receptors are fully saturated. It should also be noted that this is not a linear relationship and that the effect of increasing concentration is strong at first but then slowly diminishes up to the saturation point. For our results, it is possible that the receptors in the *Tetrahymena* that detect chemoattractants became fully saturated or were very close to saturation at the first experimental condition, where the concentration of isoamyl acetate was 10^{-6}M. If this were to be true, there would be only minimal differences when concentrations of 10^{-5}M and 10^{-4}M were used because although there would a greater amount of chemoattractant present, there would be no receptors for the chemoattractant to bind to and stimulate the *Tetrahymena*. The small differences that did occur could be attributed to the biological variance in the *Tetrahymena* population because it is known that different *Tetrahymena* have different sensitivities to chemoattractants and that this trait can be modified and passed on to subsequent generations (Kohidal *et al*. 1994). Another source of error that could have led to these results is the ion concentrations in the solution and the chambers. This is because ultimately, it is the extent of depolarization of the *Tetrahymena* that determines the magnitude of the response. Since different amounts of the growth medium, of unknown ionic strength, was used

to dilute the isoamyl acetate, each experimental condition would have different amounts of ions. Since the *Tetrahymena* is such a small organism, even small differences in ion concentrations would lead to large response differences. If for example, there were a slight difference in sodium concentration between experimental conditions, the treatment that had the higher sodium concentration would depolarize more readily and cause a greater reaction. The dilution of the chemoattractant could have introduced extra variables, like ion concentration, which could impact our results. Also, varying calcium concentrations could also influence the motility of the *Tetrahymena*. A study conducted by Mori and Miki-Noumura in 1992 found that as the calcium ion concentrations around *Tetrahymena* increases, some cilia responsible for movement become inhibited. Since our mode of measurement for attraction was based on the organism's movement, this could be a factor that caused deviation from the expected results.

Biological variation could also be a major factor that could have influenced the results obtained from this study. Although great effort was made to compensate for biological variation by doing more replicates, this could still be contributing toward the obtained results. It has been found that older *Tetrahymena* tend to move both less and more slowly compared to younger ones (Cole 2000). The movement of *Tetrahymena* is also affected by hunger. *Tetrahymena* that are starving have been found to move much faster and to greater lengths (Cole 2000). This is partly due to the fact that when *Tetrahymena* are deprived of nutrients, the length and number of their cilia increases allowing them to propel themselves faster and for a greater amount of time (Cole 2000). Finally, the apparatus used in this experiment was homemade and has not been used in experiment before. Since it is a relatively new method, there may be other factors like the organic materials in the wax that could have influenced the results. This needs to be considered because different organic compounds have been found to reduce the chemotaxis properties of

Tetrahymena (Pauli and Berger 1997). Research done by Lang and his colleagues in 2011 found that isoamyl acetate is a strong chemoattractant for *Tetrahymena* at 10^{-6}M and higher but further research, especially that with lower concentrations needs to be conducted to support or refute the idea that concentration has a direct relationship with chemoattraction in *Tetrahymena*.

Conclusion

For the isoamyl acetate concentrations of 10^{-6}M, 10^{-5}M, and 10^{-4}M, the proportions of cells entering the test chamber were 56.7 +/- 4.4%, 57.6 +/- 1.5%, and 61.4 +/- 5.5% respectively. Our results, while not statistically significant between experimental conditions, show a trend that hints at the relationship between point source concentration and chemoattractant effect described by our alternate hypothesis. Although we cannot say for certain, it would seem that as the concentration of chemoattractant increases, the chemotactic response of the cells in the sample increases. What we can say for certain is that isoamyl acetate is indeed a chemoattractant for *Tetrahymena* as shown by the statistical difference between the control and experimental conditions.

Acknowledgments

We would like to thank Dr. Carol Pollock, Diana Rennison, Katelyn Tovey, and Mindy Chow for providing advice, technical assistance and suggestions on how to approach our study and helping us modify our design to obtain the most accurate results. We would also like to thank Dr. Christine Rogers for letting use the Organic Chemistry lab and helping us synthesize our sample of isoamyl acetate. The opportunity to conduct this study was given to us by the University of British Columbia.

Literature Cited:

Adler, J., 1973. A method for measuring chemotaxis and use of themethod to determine optimum conditions for chemotaxis by Escherichia coli. Genetic Microbiology **74**: 77–91.

Attie, A and Raines, R. Analysis of Receptor-Ligand Interactions. Chemical Education **72**(2): 119-124.

Cole, E. 2000. The *Tetrahymena* Conjugation Junction in Madame Curie Bioscience Database. Landes Bioscience, Austin, Texas.

Elliot, A.M. 1974. Biology of *Tetrahymena*. Dowden, Hutchinson & Ross Inc., Stroudsburg, Pennsylvania.

Gronlien, H.K., Ronnevig, AK, Hagen, B. 2010. Chemo-accumulation without changes in membrane potential in the microstome form of the ciliate Tetrahymena vorax. The Journal of Experimental Biology, **213**: 3980-3987

Jost, J.L, Drake, J.F, Fredrickson, A.G., and Tsuchyia, H.M. 1973. Interactions Of *Tetrahymena pyriformis, Escherichia coli, Azotobacter vinelandii*, and Glucose in a Minimal Medium. Journal of Bacteriology **113**(2): 834-840.

Köhidal, L, Karsa, J, and Csaba, G. 1994. Effects of hormones on chemotaxis of *Tetrahymena*: Investigation of receptor memory. The Faculty Press, Cambridge, Great Britain.

Kohidai, L. 1995. Method for Determination of Chemoattraction in *Tetrahymena pyriformis*. Current Microbiology, **30**: 251-253

Lang, J, Rakasz, V, Magyar, A, Pallinger, E, and Kohidai, L. 2011. Chemotactic effects of odorants and tastants on the ciliate *Tetrahymena pyriformis*. Journal of Receptors and Signal Transduction **31**(6): 423-433.

Levandowsky, M., Cheng, T. *et. al.*1984. Chemosensory Responses to Amino Acids and Certain Amines by the Ciliate *Tetrahymena*: a Flat Capillary Assay. Biological Bulletins, **167**: 322-330.

Mori, M and Miki-Noumura, T.1992. Inhibition of gliding movement by calcium in doublet Microtubules of *Tetrahymena* ciliary dyneins in vitro. Experimental Cell Research **2 03**(2): 383-487.

Pauli, J and Berger, S. 1997. Toxicological comparisons of *Tetrahymena* species, end points and growth media: Supplementary investigations to the pilot ring test.

Ravishankar V.V., Bennett G.B., San, K.Y. 2004. Enhanced Isoamyl Acetate Production upon Manipulation of the Acetyl-CoA Node in *Escherichia coli.* Biotechnology Progress, **20**: 692-697.

Tanabe, H., Kurihara, K and Kobatake, Y. 1980. Changes in membrane potential and membrane fluidity in *Tetrahymena pyriformis* in association with chemoreception of hydrophobic stimuli: fluorescence studies. Biochemistry **19**(23): 5339-5344.

Van Houten, J., Hansma, H., and Kung, C. 1975. Two quantitative assays for chemotaxis in paramecium. Comparative Physiology A. 104: 211-223.