The Effect of Valproate on the Phenotypic Appearance of *Paramecium tetraurelia*

Course Name and Lab Section

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Date: 12/05/2018

Introduction

Background Information

Paramecium tetraurelia, a unicellular, ciliated protist that thrives in fresh-water, relying on the defensive function of its trichocysts and cilia to protect itself from predators and other external threats. By changing the environment, or solution, to which it is exposed to, the overall defense mechanism of *P. tetraurelia* can be more clearly understood through close observation of resulting phenotypic changes. Moreover in doing so, the impacts of the solution itself on the organism can also be explored and thus we can gain deeper understanding of the compound in question.

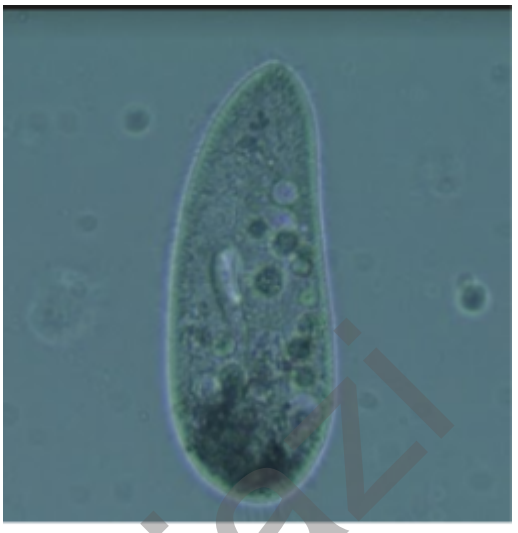

Figure 1: Image of Paramecium taken using EVOS microcope

P. tetraurelia is one of the twenty-six species in the genus of *Paramecium*. As a eukaryotic organism, it has membrane-bound organelles. Moreover, the size of a *P. tetraurelia* can range in size from 100-350 μm in length (Elwess, 2017). Any change in cell length (or width) to beyond the normal range is an abnormal phenotypic change and can thus serve as one of the few indicators of the *P. tetraurelia* undergoing a self-defense mechanism.

The cilia that cover the *P. tetraurelia* cell body surface serve two main purposes, in addition to general cell-to-cell communication. Firstly they serve the purpose of wafting small bacteria and algae into the gullet of the cell membrane. Bacteria, such as *Klebsiella pneunomiae* and algae typically serve as a primary food source for the *P. tetraurelia* and undergo endocytosis into the cell for further nutrient breakdown (Berger, 1981). Cellular waste is then excreted through the cell's anal pore. Secondly, the cilia also serve the purpose of aiding the movement of the *P. tetraurelia* – particularly by propelling the cell away from an unfavorable environmental condition (Elwess, 2017). Upon exposure to the unfavorable environment the Ca^{2+} channels open, which allows the rush of calcium ions into the cell (Nauli, 2016). This triggers the cell to reverse direction by propelling cilia in the opposite direction – an effective avoidance response. The ability of the *P. tetraurelia* to steer its way away from danger works hand-in-hand with the cell's trichocysts, which are located in an alternating pattern with the cilia on the cell's surface, in further protecting the cell and ensuring survival.

Trichocysts function as defensive organelles in *P. tetraurelia* (Miyake, 1996). Trichocysts in protozoans are ejected in response to external stimuli – particularly threatening ones. Much like with changing direction of movement using the cilia, with a rapid increase in calcium ion concentration within the cell (due to the opening of the Ca^{2+} channels), the rate of the discharge of trichocysts also increases (Elwess, 2017). While there are several types of trichocysts, *P. tetraurelia* has filamentous trichocysts. Filamentous trichocysts are spear-like structures that protrude from the cell. The discharge of the trichocysts thus causes a physical phenotypic change in the *P. tetraurelia* – another one of the few indicators that the *P. tetraurelia* is undergoing a self-defense mechanism.

Another structural change in *Paramecia* that result in visible phenotypic changes in the cell is the contractile vacuole activity of the *P. tetraurelia* (Bilinski, 1981). *P. tetraurelia* has contractile vacuoles located on the cell surface near the end of the cell. These vacuoles serve the purpose of water regulation within the cell and as excretory structures since metabolic waste is also expelled with the water. Vacuoles consist of canals that contain vacuole fluid with an osmolarity. This osmolarity is controlled by C^- and K^+ ions, which is higher than that of the cytoplasm. The difference in osmopolarity allows water to enter the canals via osmosis. With the entrance of water into the vacuole through the canal, the pore opens. The vacuole can then contract, expelling the contained water through the pore. *P. tetraurelia* cells are capable of regulated exocytosis when exposed to certain external stimuli as a form of self-defense (Wu, 2013). Thus, presence of regulated exocytosis is also another viable phenotype change to consider as an indicator that the *P. tetraurelia* is undergoing a self-defense mechanism.

P. tetraurelia can reproduce both by asexual fission as well as by sexual reproduction. Moreover, they divide by binary fission, which can also further be observed as a supporting factor of the impacts of a changing environment on *P. tetraurelia* cells. For example, during asexual reproduction the *P. tetraurelia*'s macronucleus and then cell split up (Elwess, 2017). Any changes to this process (or the rate of the process) can serve as a direct indication of the effects of the unfavorable environment on the *P. tetraurelia* cells. Furthermore, changes in the processes of any sexual reproduction in the *Paramecia*, such as cell alignment, cell fusion, micronuclei disintegration, etc., can further support any conclusions drawn from observations of any phenotypic changes in regards to the impact of a change in the surrounding environment on *P. tetraurelia*.

In order to better understand the impacts of exposing *P. tetraurelia* to an unfavorable environmental condition, the exposure of *Paramecia* to different concentrations of a Valproate solution is explored in this experiment. The source of the Valproate is Depakine Chrono 500 mg, which consists of sodium Valproate and Valproic Acid, a branched short-chain fatty acid made from Valeric Acid. Depakine, along with other medications that contain Valproate, is primarily used to treat epilepsy, bipolar disorder and migraines. While it is still unclear exactly how the Valproate mechanism works, research thus far has proposed a mechanism whereby gamma-aminobutyric acid (GABA) levels are affected, voltage-gated sodium channels are blocked, and histone deacetylases are inhibited (Ghodke-Puranik, et al, 2013).

According to a study conducted, the presence of GABA receptors on *Paramecia* was analyzed. Although the exact subtype of the GABA-A receptor was not determined, it was concluded that, just like typical mammalian neuronal cells, *Paramecia* do have a GABA receptor system, suggesting that this system developed early in excitable cells and remained conserved throughout evolution (Ramoino, et al, 2004). A follow-up study was then conducted to study the role of the GABA-A complex in the swimming control of the *Paramecia* that demonstrated that although *Paramecia* normally swim forward, the activation of GABA-A receptors induced a unique response whereby the *Paramecia* swam in an alternating period of whirling and forward swim (Bucci, et al, 2005).

It can thus be concluded that the exposure of the *Paramecia* to a Valproate solution could potentially affect the functionality of the cilia and swimming patterns

of the *Paramecia*, amongst other potential phenotypic changes due to the *Paramecia* defense mechanisms as a result of being exposed to a foreign substance. Due to the potential phenotypic impacts Valproate can cause to the *Paramecia*, it therefore makes it an ideal solution to explore. Not only does it give us insight and better understanding on the chemical and how it works, but also on the defense mechanisms of *P. tetraurelia* and the resulting phenotypic changes.

Purpose

The purpose of this experiment was to expose *Paramecium tetraurelia* to various concentrations of Valproate and observe any phenotypic changes that occurred to the *P. tetraurelia* cells.

In doing so, we were able to gain further understanding of the defense mechanisms of *P. tetraurelia* upon exposure to an unfavorable environmental condition, as well as the impacts of Valproate on a unicellular, ciliated organism..

Hypothesis

If the concentration (in mg/ml) of the Valproate solution exposed to the *Paramecia* increases, a change in the normal swimming activity and direction of the *Paramecia* will occur.

This will occur because the exposure of Valproate will increase the levels of GABA, thus activating the GABA-A receptors found on the cilia of the *Paramecium*.

Materials and Methods

Culturing *Paramecium*

Stock cultures of *Paramecium tetraurelia* were maintained at SUNY Plattsburgh, over a period of three weeks. Each week, sterile wheat medium was inoculated with a type of bacteria called *Klebsiella pneunomiae*, which served as a food source for the *P. tetraurelia* to live and grow. The cultured bacteria were grown overnight while shaking in an incubator at 37°C. The shaking incubator served the purpose of ensuring constant agitation, constant access of oxygen and nutrient supply for the bacteria, optimum bacterial reproduction, and the prevention of bacterial clump formation (Elwess, 2017).

The following day, after allowing the flask to cool to room temperature for five minutes, 1ml of *P. tetraurelia* was added to the inoculated wheat flask. This was repeated for the next two weeks; all experiments were done using three-week old *Paramecia*.

Stock solutions(s)

In order to determine the ideal stock solution concentration, a test of the Valproate stock solution on a sample of *P. tetraurelia* cells was done prior to the experiment. After observing the change in swimming patterns on the *P. tetraurelia* cells using the EVOS inverted microscope, a final 1 mg/ml stock solution of Valproate was used in all experiments. The Valproate was obtained from a Depakine Chrono 500 mg pill. Different volumes of water were added, using micropipettes, to different volumes of the 1 mg/ml to obtain the final three different concentrated solutions of Valproate. The final three different concentrations of the Valproate solutions used were: 0.33 mg/ml, 0.167 mg/ml, and 0.25 mg/ml.

These three concentrations were chosen because they were the most tolerable dilutions that the *P. tetraurelia* could be exposed to without immediate cell death. Moreover the exposure of these specific dilutions of Valproate allowed slow enough phenotypic changes with the *P. tetraurelia*, making observation of the respective changes easier to spot. Stronger solutions of the paraformaldehyde proved to be ineffective in exploring the effects of Valproate on the phenotypic changes of *P. tetraurelia*, and ultimately the overall purpose of this experiment. This is because with stronger solutions of Valproate, the *P. tetraurelia* mutated and blistered as a part of the *P. tetraurelia* defense mechanism, thus preventing accurate observation of the impact of Valproate on the cells -- particularly the cilia, and thus cell swimming patterns and speed.

The control solution for this experiment was water without any presence of Valproate, serving the purpose of to ensure that any phenotypic changes that were occurring to the *P. tetraurelia* were a direct result of the exposure to Valproate and not as a result of another external factor.

Calculations:

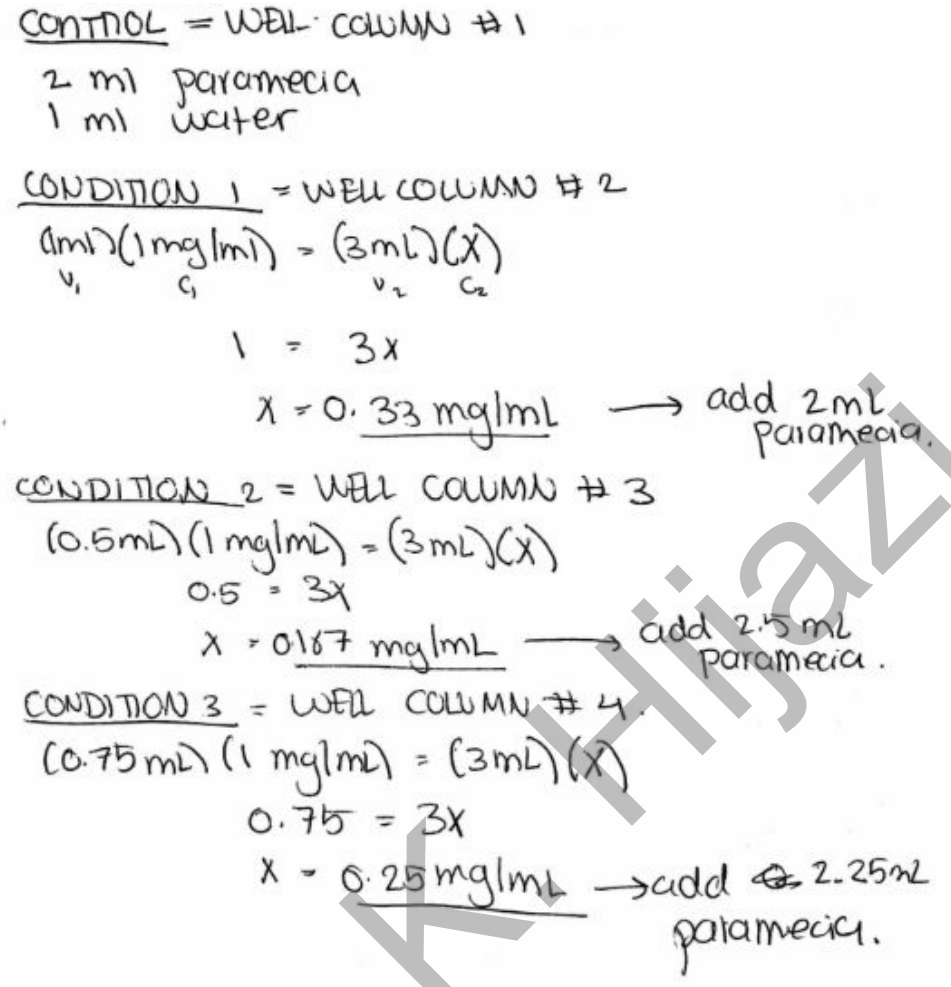

Figure 2. Calculations done to obtain final concentrations of different conditions

Experimental Set-Up

For each of the three solutions of diluted Valproate, three separate trials were done for increased accuracy. In addition, three trials of the exposure of the *Paramecium tetraurelia* to a water solution were done as the control setting. Thus, eight reaction-plate mixing wells were used, each containing previously cultured *Paramecium* and the respective diluted Valproate solution. The first condition setting of the Valproate concentration of 0.33 mg/ml had 2 ml of *Paramecium* added to 1 ml of the stock solution. The second condition setting of the Valproate concentration of 0.167 mg/ml had 2.5 ml of *Paramecium* added to 0.5 ml of the stock solution. The third condition setting of the Valproate concentration 0.25 mg/ml had 2.25 ml of *Paramecium* added to 0.75 ml of the stock solution. The control condition setting contained 2 ml of *Paramecium* without any Valproate.

The wells were then instantly placed under an EVOS inverted microscope for immediate observation of any potential phenotypic changes in the *Paramecia* upon exposure to the Valproate solution. Any visual observations were noted, and images of the *Paramecia* were captured and saved for further analysis using the EVOS imaging system.

Safety Precautions

When carrying out the different sections of the experiment, from culturing the *Paramecia*, to making the stock solution of Valproate, to the experimental set-up – safety precautions must be taken. When culturing the *Paramecia* it is important to be careful when dealing with the *Paramecia* itself however most importantly, when handling the bacteria *K. pneunomiae*. To ensure maximum safety, gloves must be worn at all times when handling the bacteria and as soon as the cell have be cultured, ensure that the conical flasks are covered in aluminum. Furthermore, anything that made contact with the ___ needed to be bleached and cleaned. Exposure to the bacteria could result in increased risk of pneumonia.

When making the stock solution of the Paraformaldehyde, it is also important to wear gloves at all times and avoid any skin or eye contact, inhalation and/or ingestion. However according to to the Material Safety Data Sheets (MSDS) of Valproic Acid, it has no reactivity or flammability and has a low health risk of 1 (Roche, 2009).

Analysis

Once data had been collected for the initial observation(s), the reaction-plate mixing well tray was covered, sealed and then left undisturbed for a week at constant temperature and exposure to light. These two variables were controlled to maintain accuracy and to ensure consistency of the variables, the tray was left in a controlled environment in the lab at Hudson Hall, SUNY Plattsburgh. Observations were then taken a week later under the EVOS inverted microscope. The cell lengths were measured both prior to and after exposure to the Valproate solutions using ImageJ. This was done to more clearly understand both the long-term and short-term effects of paraformaldehyde on the phenotypic appearance of *P. tetraurelia*.

Results

Although the three different conditions were organized chronologically -- i.e. condition 1, 2 then 3 -- for the duration of the experiment and when taking observations, the following data is organized from largest concentration of Valproate (condition 1) to the smallest concentration of Valproate (condition 2) instead of chronologically for ease in analyzing the results.

Raw Data:

Upon exposure to the different Valproate concentrations the activity level and swimming patterns were observed and recorded after a period of 30-40 minutes, 24 hours and then 72 hours. The activity levels were measured using a scale of 1 to 5 -- with 1 being low activity level and 5 being high activity level. The changes in swimming patterns of the *Paramecia* was also noted -- i.e. direction of swimming, repetitive behaviour, differences in comparison to "normal" swimming patterns found in the control setting, etc.

Table 1.: Activity Level of *Paramecia* in each condition at different times after exposure to Valproate solution

Concentration of Valporate Solution:	**Time from exposure to Valproate solution**		
	30-40 minutes	24 hours	72 hours
Control Condition: no added solution	4	3	3
Condition 1: 0.33 mg/mL	2	2	2
Condition 3: 0.25 mg/mL	3	3	3
Condition 2: 0.167 mg/mL	3	4	5

Table 2.: Observations of swimming patterns of *Paramecia* in each condition at different times after exposure to Valproate solution

Concentration of Valporate Solution:	**Time from exposure to Valproate solution**		
	30-40 minutes	24 hours	72 hours
Control Condition: no added solution	*Paramecia* swimming fast in multiple directions	*Paramecia* swimming fast in multiple directions	*Paramecia* swimming fast in multiple directions
Condition 1: 0.33 mg/mL	*Paramecia* spinning in circles fast	*Paramecia* spinning in circles fast	*Paramecia* spinning in circles fast
Condition 3: 0.25 mg/mL	*Paramecia* spinning in circles at a slow pace	*Paramecia* spinning in circles at a slow pace	*Paramecia* spinning in circles at a slow pace
Condition 2: 0.167 mg/mL	Reduced swimming control in *Paramecia*	*Paramecia* swimming fast in multiple directions	*Paramecia* swimming fast in multiple directions

Processed Data:
The above data was then processed using Excel Spreadsheet to find the Average activity level for each condition, and then the standard deviation to find how spread out from the average the data is.

Table 3.: Average Activity Level of *Paramecia* after exposure to Valproate Solution and Standard Deviation of Data

Concentration of Valporate Solution:	Average Activity Level of *Paramecia* after exposure to Valproate:	Standard Deviation:
Control Condition: no added solution	3.33	0.577
Condition 1: 0.33 mg/mL	2.00	0.000
Condition 3: 0.25 mg/mL	3.00	0.000
Condition 2: 0.167 mg/mL	4.00	1.000

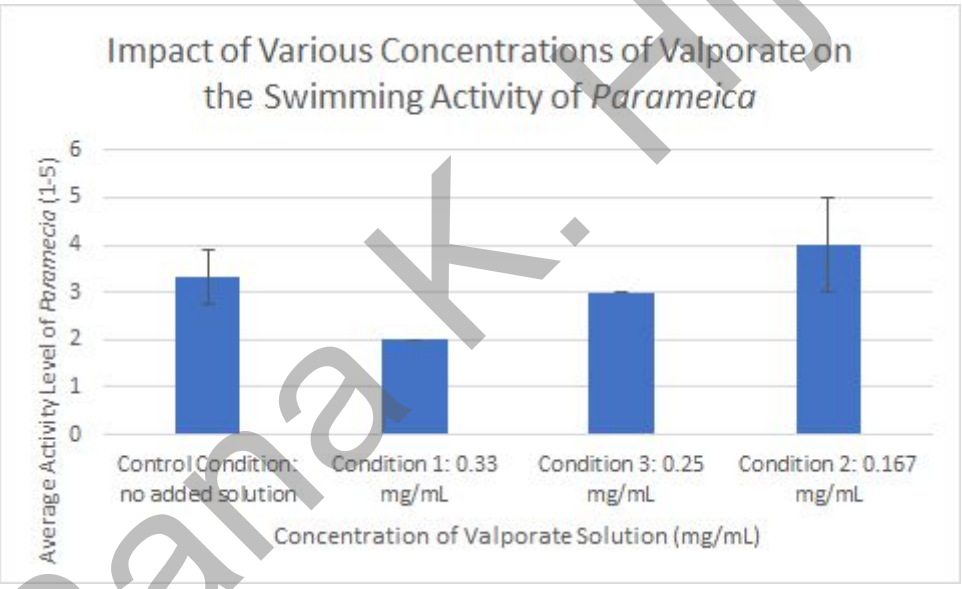

Figure 3: Graph of the effects of various Valproate solution concentrations on swimming activity level of *Paramecia* (on a scale of 1-5; 1 being slowest and 5 being fastest). Error bars represent standard deviation.

Table 3 shows the average *Paramecia* activity level over three days after exposure to the different Valproate solutions. Figure 3 shows the data plotted on a graph, with the standard deviation as error bars. Condition 1 and 3 had a standard deviation of zero, indicating that there was no change in the data over time and thus the activity level of the *Paramecia* stayed constant throughout the three days of observation. There was a significant overlap between the data of the control condition and condition 2, made evident by the overlap in the error bars.

Discussion

Hypothesis <u>SUPPORTED</u> by results.

If the concentration (in mg/ml) of the Valproate solution exposed to the *Paramecia* increases, a change in the normal swimming activity and direction of the *Paramecia* will occur.

As can be seen in Figure 3, the data supports the above hypothesis as there was a change in swimming activity levels in conditions 1 and 3, where the concentrations were highest. The activity levels of the *Paramecia* decreased with an increase in the concentration of the Valproate solution. The overlap of the data in the control condition and in condition 2, where the concentration of the Valproate solution was at its lowest, further supports the conclusion that the activity level of *Paramecia* and the concentration of the Valproate solution are inversely correlated.

It is also worth noting the changes in activity level in each condition over time, as well as the observations taken in swimming patterns. As can be seen in Table 1, the activity levels changed as time progressed and as can be see in Table 2, the swimming patterns also varied with time and per concentration condition. In the control condition, there was a slight decrease in swimming activity as time progressed, potentially due to the initial exposure of Kleb that cause the initial peak of activity in the cells. Additionally the decrease in activity level and the fast swimming patterns of the cells give us an idea as to what a “normal” Paramecium's cell activity level and pattern is expected to look like.

Condition 1, which had the highest concentration of Valproate of 0.33 mg/mL, had an average activity level of 2 -- as can be seen in Figure 3. Thus is less than the average of the control setting, indicating that the impact of a larger dose of Valproate reduced activity level of the Paramecia. Observations in Table 2 also indicated that the *Paramecia* began to spin which was another change in swimming patterns -- further supporting the hypothesis that Valproate affects swimming patterns of the cell.

Condition 3, which had the middle concentration of Valporate of 0.25 mg/mL, had an average activity level of 3 -- as can be seen in Figure 3. Much like in condition 2, the activity level remained consistent over time. Furthermore the activity level was faster than that of condition 1, and while initially it was slower than the control condition, the cells levelled out and matched the speed of the control setting by the third day of observations. This not only supports the hypothesis that Valproate reduces activity level of *Paramecia*, but also highlights that concentration of this condition was too low of be sustained for the three days and to remain impactful on swimming activity until the third day of observations. Finally as can be seen in Table 2, there was minimal spinning in terms of spinning patterns.

Condition 2, which had the lowest concentration of Valproate of 0.167 mg/mL, initially started with an activity level of 3. This, compared to that of the control, was slower but still not as slow as condition 1 -- which had the highest concentration. This, in addition to the overlap of the error bars on the control condition and on condition 2 in Figure 3, adds on to the conclusion that the less Valproate in the environment the faster the activity level of the *Paramecia*. Thus

this condition was closest and most similar to the control condition and had the least amount of an impact on both swimming patterns and activity. Table 2 shows an increase in activity level for condition 2 as time progressed from 3 to 4, and then finally 5. This supports the reasoning that the change in activity level occurred in both condition 2 and 3 was due to the low concentration of Valproate being used up by the cell by the third day of observations. A potential reason for why the activity level of condition 2 jumped to 5 by the end of the third day could be human error, as activity levels were taken by eye.

A potential explanation for this trend in data is that GABA-A receptors on the cilia of the cell were activated due to the increase in GABA neurotransmitters in the environment from the Valproate solution. An alternative hypothesis that could potentially explain the results is that the swimming patterns of the cells reduced upon exposure to the Valproate solution as a means to protect itself from a foreign substance. However, it is worth noting that there were no significant phenotypic changes in the cells, such as the presence of trichocysts, which would have been an indicator of the cell undergoing a defense mechanism to protect itself (Gerritsen, 2000). In order to obtain more accurate and precise results with minimal chance of error, the experiment could have been repeated several times with more concentrations of the Valproate solution. Additionally, a more reliable system of measuring activity level of the *Paramecia* could have been established as the numerical 1-5 scale could easily have resulted in an error -- particularly human error since the activity level was observed by human eye and not a sophisticated machine and/or software.

All in all, it can be concluded that the presence of Valproate in the environment of the *Paramecia* has an impact on the swimming activity, whereby an increase in Valproate solution causes a decrease in activity level, as well as the swimming patterns of the cell.

References

Berger, J. D., & Pollock, C. (1981). Kinetics of Food Vacuole Accumulation and Loss in Paramecium tetraurelia. Transactions of the American Microscopical Society,100(2), 120. doi:10.2307/3225795

Bilinski, M. (1981). Secretory protein decondensation as a distinct, Ca2 -mediated event during the final steps of exocytosis in Paramecium cells. The Journal of Cell Biology, 88(1), 179-188. doi:10.1083/jcb.88.1.179

Bucci, Giovanna, et al. "A Role for GABAA Receptors in the Modulation of Paramecium Swimming Behavior." *Neuroscience Letters*, vol. 386, no. 3, 2005, pp. 179–183., doi:10.1016/j.neulet.2005.06.006.

Elwess, N. L. (2017) Investigating Phenotypic Changes in Paramecium. In BIO305 General Genetics Laboratory Manual (Fall 2017, pp. 32-41). Plattsburgh, NY: Department of Biological Sciences at SUNY Plattsburgh.

Gerritsen, V. B. (2000). The Arsenal Of Paramecium. *Protein Spotlight*. Retrieved from https://web.expasy.org/spotlight/back_issues/003/.

Ghodke-Puranik, Y., Thorn, C.F., Lamba. J.K., Leeder, J.S., Song, W., Birnbaum, A.K., Altman, R.B., Klein, T.E. (April 2013). "Valproic acid pathway: pharmacokinetics and pharmacodynamics". *Pharmacogenet. Genomics*. **23** (4): 236–241. doi:10.1097/FPC.0b013e32835ea0b2. PMC 3696515. PMID 23407051.

Miyake, A., & Harumoto, T. (1996). Defensive function of trichocysts in Paramecium against the predatory ciliate Monodinium balbiani. European Journal of Protistology, 32(1), 128-133. doi:10.1016/s0932-4739(96)80048-4

Nauli, S. M., Pala, R., & Kleene, S. J. (2016). Calcium channels in primary cilia. Current Opinion in Nephrology and Hypertension, 25(5), 452-458. doi:10.1097/mnh.0000000000000251

Ramoino, Paola, et al. "GABAA Receptor Subunits Identified In Paramecium by Immunofluorescence Confocal Microscopy." *FEMS Microbiology Letters*, vol. 238, no. 2, 2004, pp. 449–453., doi:10.1111/j.1574-6968.2004.tb09788.x.

Roche. "MSDS for Valproic Acid." *Roche MSDS*, Roche, 18 Sept. 2009, uthsc.edu/eye/osha/documents/valproicacidmsds.pdf.

Wu, K., Wang, D., Ding, J., Yang, S., & Zhang, X. (2013). Whole-genome duplications contributed to the expansion of cytochrome b5 genes in Paramecium tetraurelia. Genetics and Molecular Research, 12(2), 1882-1896. doi:10.4238/2013.january.9.1