Effect of different concentrations of copper sulphate on the speed of *Caenorhabditis elegans*

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Abstract

The free-living parasitic nematode, *Caenorhabditis elegans,* is subject to a variety of chemicals in the wild, which have been shown to affect their behaviour (Bargmann *et al.* 1993). We exposed the roundworms to three different concentrations of a known repellent, copper sulfate (Troemel 1999, Wang and Xing 2008), and observed their response. We expected that the repellent would induce a negative chemotaxis such that the nematodes would move away from a repellent source at higher speeds when exposed to higher concentrations. We created a concentration gradient for our each of our experimental conditions by placing 100 uL of 5.308 mmol/L, 10.616 mmol/L and 15.925 mmol/L concentrations of $CuSO_4$ in the centre of each Petri dish and allowed it to diffuse for three hours. We then placed three nematodes equidistantly around the point source of $CuSO_4$ and recorded their movement for four minutes, taking note of their behaviours. From the video recordings, we measured the distance each worm travelled, and then calculated and compared the velocities of the nematodes. The nematodes moved an average speed of 0.24 ± 0.11 mm/s in water, 0.15 ± 0.05 mm/s in 5.308 mmol/L $CuSO_4$, 0.10 ± 0.01 mm/s in 10.616 mmol/L $CuSO_4$, and 0.06 ± 0.00 mm/s in 15.924 mmol/L $CuSO_4$. Contrary to our prediction, the data showed an apparent trend in which the nematodes moved slower as the concentration of $CuSO_4$ increased. The worms moved at a significantly slower speed at the highest concentration.

Introduction

Caenorhabditis elegans is a small and unsegmented roundworm, averaging 1mm in length that is found in various common environments (Blumenthal *et al.* 1997). The organism flourishes in nutrient rich environments, where food such as bacteria or decaying organic material is abundant. Although they are small in size, the internal anatomy of *C. elegans* contains similar organ systems to larger animals such as digestive and reproductive systems. More interestingly, it is one of the few simple organisms to contain a complex nervous system. The nervous system of the nematode, consisting of 302 neurons enables it to detect physical stimulation, olfaction, and chemosensation

(Prasad and Reed, 1999). This makes *C. elegans* an attractive choice for studies done on the chemotactic responses in biological organisms.

Our experiment focuses on the organism's motility in response to a chemical repellent. More specifically, we measured the speed of *C. elegans* when exposed to different concentrations of copper sulphate (5.308, 10.616, and 15.925 mmol/L). We chose $CuSO_4$ because it is one of the most effective repellents, as both ions are known to cause a negative chemotactic response in *C. elegans* (Blumenthal *et al.* 1997). The concentrations stated above were chosen in particular because $CuSO_4$ has been shown to act as a neurotoxin within a range from 0 to 16 mmol/L, since concentrations higher than this caused fatality after 14 hours of exposure (Hedges, 2010). Since we only exposed the nematodes to $CuSO_4$ for 4 minutes, we expected the chemical to act as a repellent rather than a neurotoxin. We formulated our hypothesis based on previous experiments that stated that when *C. elegans* was exposed to chemical repellent, it elicits a negative chemotactic response in which *C. elegans* reverses and changes direction when it encounters the chemical (Bargmann 2006). Building upon these findings, and assuming that the nematodes are attempting to move away from areas of higher repellent concentrations as quickly as possible, we hypothesized that the nematodes would increase their speed of movement as the concentration of the repellent increases (**Figure 1)**. With this in mind, we formed the following hypotheses:

H_o: *Caenorhabditis elegans* will decrease or display no change in speed of movement in response to increased concentrations of the repellent $CuSO_4$.

H_A: *Caenorhabditis elegans* will display an increase in speed of movement in response to increased concentrations of the repellent $CuSO_4$.

This study provides a broad-spectrum investigation on chemotactic behaviour of *C. elegans* in respect to the neurotoxin $CuSO_4$. As such, it may demonstrate important findings that can be used to supplement future studies on the defense mechanisms of *C. elegans* when exposed to chemical changes in the environment.

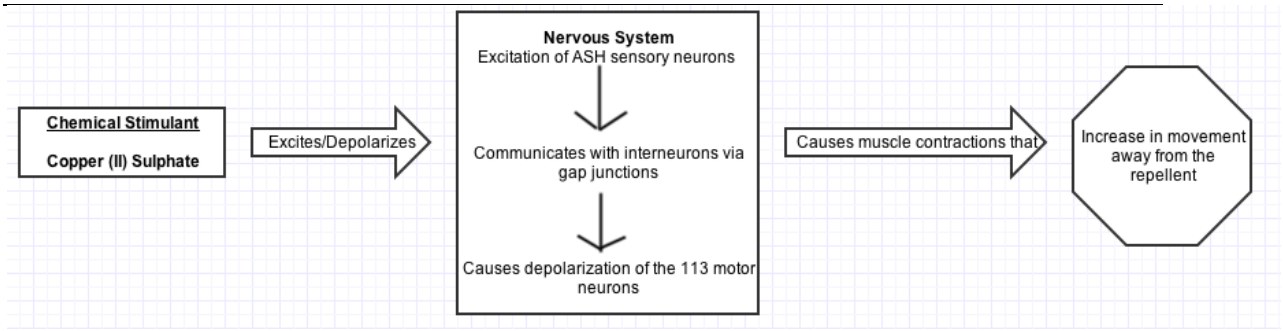

Figure 1. Proposed model for the experiment.

Methods

For our experimental study, we prepared $CuSO_4$ at three different concentrations by diluting a stock of 15.924 mmol/L by ⅓ and ⅔, for final concentrations of 5.308 mmol/L and 10.616 mmol/L respectively. We conducted the study by creating a diffusion gradient by placing 100 uL of the four treatments: sterile distilled water (control), 5.308 mmol/L, 10.616 mmol/L, and 15.925 mmol/L of $CuSO_4$, at the marked center of a 60 mm Petri dish containing agarose gel, and allowed it to diffuse over a three hour period. We replicated each treatment six times. It should be noted that the 100 uL of each treatment covered an initial 5 mm radius disk on each Petri dish.

$$C = a\,C_0 \int_0^{\infty} J_1(ua)\,J_0(ur)\,e^{-Dtu^2}\,du$$

Figure 2. The differential solution from Crank (1956) used to calculate the diffusion gradient of $CuSO_4$ in a 60 mm Petri dish. Where a = 30 mm, C_o = 1.0 mol/L (as a reference), J_1 = Bessel Function of the first kind at first order, J_o = Bessel Function of the first kind at zero order, r = 5 mm, radius of initial drop.

To determine whether or not we achieved a concentration gradient of $CuSO_4$, we used the standard diffusion coefficient of 5 $x10^{-5}$ cm^2s^{-1} and the differential solution from Crank (1956) shown in **Figure 2**, based on the diffusion of a circular disc source in a radial 2-dimensional manner over a region of a 60 mm Petri dish. In **Figure 3** we show the diffusion function that was produced from this:

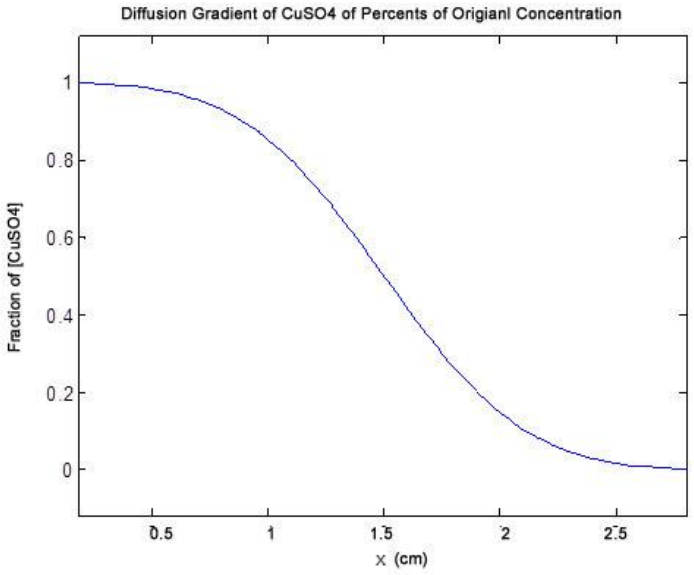

Figure 3. The diffusion gradient of $CuSO_4$ produced over a 60 mm Petri dish with a given initial concentration. The concentration of $CuSO_4$ is shown as a function of a distance away from the center of the Petri dish. X represents the distance away from the center of the Petri dish.

From stock plates of hermaphrodites of the N2 wild-type *C. elegans* strain growing on *E. coli* bacteria, we used platinum worm picks to transfer three adult

nematodes onto the concentrated agar plates and placed them within a parameterized 1x1 cm square boundary encasing the marked center of the Petri dish and at equidistant locations from each other (**Figure 4)**.

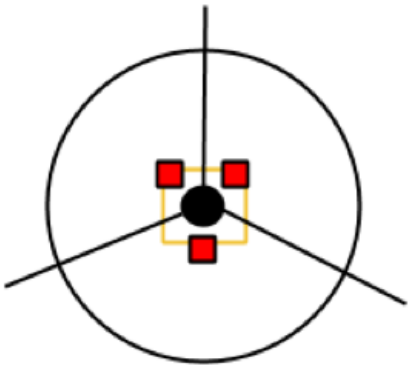

Figure 4. Placement of *C. elegans* (red square) equidistant from each other under experimental conditions. Yellow box indicates the 1x1 cm square field of view under the DinoXcope.

We recorded nematode activity for each replicate using the DinoXcope camera apparatus attached to a Kyowa dissecting microscope at 7x magnification for four minutes. The field of view captured by the DinoXcope was the parameterized 1x1 cm square box. After collecting the data, we reviewed the recordings and measured the distance traveled individually by each of the three nematodes in each replicate over a four minute period and then calculated the average speed of each of the six replicates and plotted them to produce **Figure 6**. Using the ImageJ program, we were able to calibrate and scale our videos to the field of view of the microscope. From these videos we traced the motion of the nematodes and the program measured and recorded the distance traveled (**Figure 5**). To standardize these measurements, we used the time the nematodes exited the 1x1 cm field of view to calculate the speed if they left before four minutes had passed. We also applied the statistics of 95% confidence intervals and t-tests to our data.

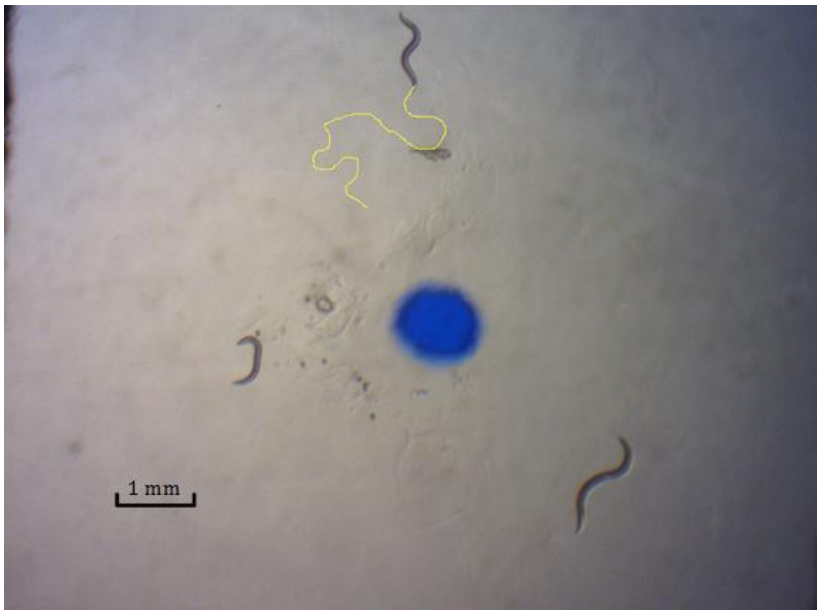

Figure 5. A screen capture of the DinoXcope recording at 15x magnification and the use of the ImageJ program to trace the movement and measure the distance traveled by the nematode (yellow line). The blue dot is the marked center of the Petri dish.

In addition to speed, we also observed the behaviour of the nematodes at the different concentrations. Specifically, we took note of the types and the frequency of body movements such as thrashing behavior, omega-turns (where the nematode's head curls back towards its tail as it moves forward), directionality of movement, and any biotic interactions that may have occurred between the three nematodes in each replicate (Bargmann and Mori 1997).

Results

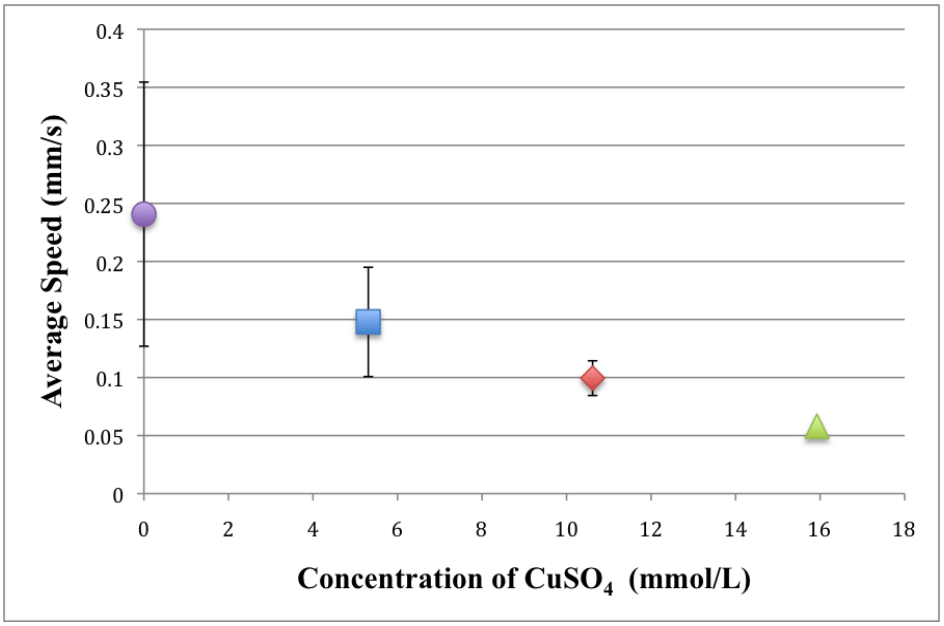

Figure 6. The effect of the concentration of $CuSO_4$ on the average speed (mm/s) of *Caenorhabditis elegans* at 5.308 mmol/L, 10.616 mmol/L, and 15.924 mmol/L. Maker label X is the distilled water control. Error bars represent 95% confidence interval, n=6.

Figure 6 displays a scatter plot comparing the mean speed (mm/s) of the six replicates under each of the environmental conditions, which consisted of sterile distilled water, and $CuSO_4$ concentrations of 5.308 mmol/L, 10.616 mmol/L, and 15.924 mmol/L, respectively. At 5.308 mmol/L of $CuSO_4$ the nematode moved an average of 0.15 ± 0.047 mm/s. At 10.616 mmol/L $CuSO_4$ the nematode moved an average of 0.099 ± 0.015 mm/s. At 15.924 mmol/L of $CuSO_4$ the nematode moved an average of 0.058 ± 0.00043 mm/s, and in water, the nematode moved at an average speed of 0.24 ± 0.11mm/s.

Overall, a negative linear trend can be observed in **Figure 6,** as there appears to be a decrease in the average speed of nematodes as the concentration of $CuSO_4$ increases. A 95% confidence interval was applied to these averages, and the error bars showed an overlap between the concentrations of 5.308 mmol/L and 10.616 mmol/L and 5.308 mmol/L and the water control.

We also applied a two-sided t-test to all the paired mean concentrations and found that the sample means of 5.803 mmol/L and 15.924 mmol/L are significantly different from each other: t(10) = 2.62 > theoretical value = 2.228.

Sample calculations (for concentration of 5.308 mmol/L):

1. Average speed of *C. elegans*:

$$\text{Speed} = \frac{\text{Average Distance}}{\text{Average Time}}$$

$$= \frac{32.817 \text{ mm}}{240 \text{ s}}$$

$$= 0.14 \text{ mm/s}$$

2. 95% confidence interval (s = standard deviation, n = sample size, $\bar{x}$ = mean of sample)

$$\text{C.I.} = \bar{x} \pm 1.96 * \frac{s}{\text{sqrt}(n)}$$

$$= 0.1479 \pm 1.96 * \frac{0.0588}{\text{sqrt}(6)}$$

$$= 0.1479 \pm 0.0471 \text{ mm/s}$$

3. Two-sided t-test ($\bar{x}$ = mean of each sample (x2 = mean of 15.924 mmol/L), s = combined standard deviation of the two samples, n = sample size)

$$t = \frac{\bar{x}1 - \bar{x}2}{s * \text{sqrt}(1/n1 + 1/n2)}$$

$$t = \frac{(0.1479 - 0.0581)}{(0.0588 + 0.0005) * \text{sqrt}(1/6 + 1/6)}$$

$$t = 2.62$$

Discussion

In the presence of a repellent, the roundworm *Caenorhabditis elegans* has been shown to detect the strongest concentration of the chemical and direct their movement away through a change in direction (Bargmann and Mori 1997). Troemel (1999) found that $CuSO_4$ is a repellent for nematodes. As such, we expected that increased concentrations of the chemical would elicit a negative chemotaxis, causing the nematodes to move faster in an effort to move away from the source. However, our results show a trend in which *C. elegans* appeared to move slower at higher concentrations of $CuSO_4$ (**Figure 6**). At the highest concentration, the nematodes showed a significant decrease in speed. In addition, they did not demonstrate directional movement away from the source. Thus, we are unable to reject the null hypothesis, which states that the roundworm *Caenorhabditis elegans* moves slower or shows no change in speed in response to higher concentrations of $CuSO_4$. We then fail to support our alternate hypothesis (**Figure 6**). However, the 95% confidence interval did not overlap between the control and 15.924 mmol/L, and between the 10.616 mmol/L and 15.924 mmol/L treatment groups. Upon closer examination of the treatments using a two-sided t-test, we determined that there was a significant difference between the samples of 5.308 mmol/L and 15.924 mmol/L as ($t(10) = 2.62 >$ theoretical value = 2.228).

This unexpected behaviour may be due to an unanticipated effect of copper sulfate on the roundworms. Nematodes can sense copper, and are repelled by it (Troemel 1999). However, at a higher concentration, they may be unable to move away from the source. Exposure to a low concentration of copper causes changes in locomotion, which is reflective to changes in the nervous system (Avila *et al.* 2012). A study done by Wang

and Xing (2008) demonstrated that the nematodes show defective movements at concentrations as low as 2.5 μmol/L over a 24-hour period. We used much higher concentrations (5.308 mmol/L, 10.616 mmol/L and 15.925 mmol/L), but since the worms were only exposed for 4 minutes, it was not enough to be lethal to the nematodes used in our study.

Changes in locomotive behaviour often imply altered neurological pathways (Wang and Xing 2008), and so the decreased movement in the presence of copper may be caused by a copper-induced neurological defect. Although *C. elegans* displays specific forms of movement when put under general stress (Wang and Xing 2008), locomotion is more sensitive to exposure to neurotoxins (Anderson *et al.* 2004). Copper acts as a neurotoxin for *C. elegans* since exposure to doses below lethal amounts causes neuronal damage, which induces paralysis (Hedges 2010). Nematodes decrease their rate of movement (Anderson *et al.* 2004), and suffer from defects in head thrashes (a mid-body bend), body bends (changing of direction), and forward turns (basic sinusoidal forward motions) when exposed to copper and other heavy metals (Wang and Xing 2008*).*

In addition, the decrease in motility at the higher concentration of $CuSO_4$ may have been caused by an overload of the ASH neurons (Bargmann 2006). These chemosensory neurons are analogous to pain sensing neurons in vertebrates, and are required to elicit avoidance and reversal responses in *C. elegans* (Bargmann 2006). Chemical cues cause a rapid and large increase in levels of calcium in ASH neurons, showing that neurotransmitter levels are strongly affected by repellents (Bargmann 2006). Constant chemical stimulation causes a reduction in the magnitude of sensory response in *C. elegans* (Hillard *et al.* 2005). Thus, the high concentrations of $CuSO_4$ may

have affected the nervous system of the nematodes such that they decreased their movement.

We also expected the nematodes to swim away from the point source of $CuSO_4$; however they often exhibited repeated motions during which they would move away from the source and then move towards it again. Similarly, in a study by Ward (1973), the nematodes moved up a gradient towards an attractant, change direction and move away from it, and then repeat the cycle. This may be due to the fact that the nematodes spatially compare the concentrations of the chemical in order to determine the concentration gradient (Ward 1973). *C. elegans* uses chemosensory neurons located on its head, in order to obtain information about its environment (Troemel 1999). The nematodes lack chemoreceptors on their tails, so they must perform a series of side-to-side thrashes, reversals, and turns in order to determine the chemical gradient (Ward 1973). These movements are consistent with those of the nematodes in our study.

Our results may show variation due to human error in certain procedural aspects of the experiment. The worms were obtained using worm picks and placed on the experimental Petri dishes using extreme care. Nevertheless, it is possible that the worms were stressed or injured, affecting their mobility. Additionally, there was error in the placement of the worms on the experimental dish, as it was impossible to position the worms perfectly equidistant from each other at exactly the same time.

There are a few variables that we were unable to control, which may have slightly affected our results. One such factor is the variation of age in each nematode. After a certain age, *C. elegans* species are less likely to be able to distinguish between differences in stimuli (Ardiel *et al.* 2013) and are therefore less likely to respond to the

different concentrations of repellent. To minimize this error, the nematodes were grown at the same time and used midway through their lifecycle in the experiment. We also used worms that appeared to be a standard adult size of around 1 mm (Riddle *et al.* 1997).

Finally, nematode social interactions may disrupt movement, direction, and speed of the replicates. The nematodes secrete pheromones that may alter their behavior (Edison 2010). We had three worms on each plate, and we observed that in some trials, two worms would come into contact and slide against each other. Nematodes are known to aggregate in social groups in response to pheromones of other individuals (Macosko *et al.* 2009). The response to pheromones that indicate the presence of other nematodes may overcome evasive reactions to the repellent, which would cause social behaviours rather than movement away from the repellent.

In order to further investigate the behaviour of the nematode *Caenorhabditis elegans* in the presence of the known neurotoxin $CuSO_4$, it may be interesting to repeat this experiment using lower concentrations of the repellent to prevent nervous damage. Additionally, fewer worms should be used in each replicate to reduce variation caused by transferring multiple worms and to reduce biotic interactions between worms. Other behaviours such as thrashes or reversals could be quantified and analyzed as well.

Conclusion

We analyzed the movement of *Caenorhabditis elegans* in the presence of different concentrations of copper sulfate repellent to investigate their chemotaxis and infer their responses to chemosensation *in vivo*. Contrary to our prediction, the nematodes did not increase their speed in response to higher concentrations of the repellent $CuSO_4$,

nor did they show directional movement away from the point source. Thus, we fail to reject the null hypothesis since the data show an opposite trend, in which the nematodes decreased their speed at higher levels of repellent. This may be due to the fact that copper is a neurotoxin (Hedges 2010) and can induce nerve damage, which causes defects in motility in the nematode *Caenorhabditis elegans* (Wang *et al.* 2008).

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