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Neurotoxicity of Two Related Organophosphates on Caenorhabditis Elegans

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NEUROTOXICITY OF TWO RELATED ORGANOPHOSPHATES ON CAENORHABDITIS ELEGANS

By

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ABSTRACT

Organophosphates are a class of toxicants that act by inhibiting the activity of acetylcholinesterase, an enzyme vital to normal neuronal activity. Dimethoate and omethoate are two organophosphates that are chemical “cousins” of one another. Omethoate is a metabolite, or byproduct of dimethoate decomposition, and is more toxicologically active than dimethoate. Both toxicants were applied to cultures of *Caenorhabditis elegans* to determine two qualities of the organophosphates: their relative toxicity and their cumulative effects. The toxicity of omethoate was found to be significantly higher than that of dimethoate. Omethoate was found to have a 144.4% more lethal toxicity than dimethoate, and was 132.3% more effective at causing flaccid paralysis. Neither compound exhibited notable cumulative effects.

Keywords: organophosphate, neurotoxicity, omethoate, dimethoate, toxicity, acetylcholinesterase

INTRODUCTION

The purpose of this study was to determine the toxicity and potency of dimethoate, and its metabolite, omethoate on neurotoxicity. These compounds belong to a class of insecticides called organophosphates, which, until they were banned in 2001, were administered to agricultural fields. Organophosphates were used in the development of chemical weapons in the 1940's. These concoctions, called nerve agents, had various codenames and were used to incapacitate and potentially kill large groups of targets at once (Gilbert, 2014). Cholinesterase inhibition activity is the method by which organophosphates are toxic.

A value for LC50 and EC50 must be determined to quantify and express data collected related to the toxicity of each organophosphate. An LC50 (Lethal Concentration 50) is the value of the dosage that will kill 50% of the sample population if administered. EC50 (Effective Concentration 50) is similar, but represents an endpoint other than death; for example, if the substance being tested is a pharmaceutical, the EC50 is the dosage that elicits the desired effect in 50% of test subjects. To determine these benchmarks, an endpoint must be established. According to previous toxicological research of *Caenorhabditis elegans* (*C. elegans*), the most useful endpoint is impairment of motility. The hypothesis of this study has two parts. Firstly that omethoate, being more active than dimethoate, will have a greater effect than dimethoate at a similar concentration. The second hypothesis regarding cumulative effects is that neither organophosphate will display a notable ability to accumulate inside of an organism because of the rate at which they decompose. Data gained from this experiment was used to estimate the toxicity of both compounds to humans, based on toxicological data relating *C. elegans* to *Homo sapiens.*

C. elegans is a roundworm that is about 1 mm in length which can be found in soil. In terms of value in toxicology, Leung states in *Caenorhabditis elegans: An Emerging Model in Biomedical and Environmental Toxicology*, written in 2008, that *C. elegans* is a prime organism for studying eukaryotic toxicology, especially in terms of neurotoxicity. Leung mentions that *C. elegans* is useful in studying several fields of neurotoxicity: metal toxicity, neurodegeneration caused by a toxicant, and, most importantly for our study, the effects of toxic pesticides. This study compares the toxicity of two pesticides, dimethoate and omethoate. These pesticides belong to a class of toxicants called organophosphates.

C. elegans is a suitable choice for a model organism for this study for two main reasons. First, it is easy to culture and grow. This roundworm can thrive at room temperature (recommended temperature is about 20^{o}C) as long as it has access to oxygen and food, which comes in the form of *Escherichia coli* bacteria applied directly to the culture plates of *C. elegans.* The second reason *C. elegans* is a desirable model organism for this study is the level of detail that is known about its nervous system, along with the fact that its nervous system is similar to that of a human's. For example, it is known that each organism has 302 neurons (Hobert, 2005).

REVIEW OF LITERATURE

C. elegans, being a living organism, requires some basic needs to be met in order to survive long enough to be of experimental use. First of all, a food source is required. This is achieved by introducing *C. elegans* to bacterial culture media which has been cultured with *E. coli,* a food source for *C. elegans*. It is important to prevent the organism from starvation, as this can impact toxicological findings (Boyd, 2003).

There are 959 cells in female organisms, and 1031 in male organisms. The growth patterns exhibited by these cells are generally uniform from organism to organism. *C. elegans* takes four days to go from egg to a mature, fertile adult. The organism has a lifespan of two to three weeks. These times are based on a constant environmental temperature of 20^{o}C (Felix, 2010). While most of the worms are hermaphroditic females, meaning that they can reproduce asexually, a few worms are male. If a worm self-fertilizes, it can produce up to 300 eggs. If the worm is fertilized by a male, the egg count more than triples to a possible 1000 eggs. It is important to understand the life cycle of this organism in order to reduce variability in testing and data collection.

When working in a lab setting, safety is always a concern. A focus on safety is even more critical when working with highly potent toxins like dimethoate and omethoate. For this reason, it is important to understand how these substances are to be safely handled and disposed of. Organophosphates such as dimethoate and its metabolite, omethoate, absorb easily into tissues. Because of this, protection such as gloves, goggles, and masks are recommended when dealing with them, especially in doses high enough to negatively impact humans. Dimethoate is a solid at room temperature, while omethoate is liquid, and both must be refrigerated to be kept stable. Both decompose quickly in soil and when exposed to direct sunlight for a prolonged

period of time. (Cornell, 1993) Dimethoate decomposes especially rapidly when exposed to high heat, and is subject to explosion. Finally, both compounds are most safely disposed of using "quick lime", and should not be drained into public water systems. (Kilford, 2014)

The predicted oral LD50 for dimethoate in humans is 30mg/kg (Uchida, 1996). No substantial data exists concerning omethoate; the only fatal exposure case on record was a suicide involving omethoate insecticides. However, in the 1991 article *Human toxicology of pesticides,* Simeonova found that the acceptable daily intake of omethoate is .0003 ppm. Previous studies suggest that LC50 values are expected to fall in the range of about 1.00 to 100. ppm (Cole). Our experimental range greatly exceeds this amount, but not all of the administered solution will make it into a single roundworm, instead it is shared by all the worms in the dish. Also, part of this study involves determining the acute toxicity of both compounds, which implies that the administered concentration should be higher than that of an "acceptable daily intake."

Organophosphates have distinct toxicological effects. They have been shown to negatively affect reproduction in adult organisms (mice), as well as the survival of offspring. These effects are observed in exposure to both compounds, but the potency of omethoate is higher than that of dimethoate. This means less omethoate is required to produce the same effects as a higher concentration of dimethoate. Cole found the EC50 of omethoate (4.47 mM) to be 9.44% of the EC50 of dimethoate (42.2 mM) (p. 250).

Organophosphates are toxic through all routes of exposure. They are most easily absorbed in lungs, but also pass through the epidermis and digestive tract. (McVey, p. 4) Symptoms may manifest some time (1-4 weeks in humans) after an acute exposure event. Chronic exposure results in the same symptoms of acute exposure. Dimethoate has also been

shown to have teratogenic, and mutagenic effects in mammals. Carcinogenic effects have been observed in some studies. Common symptoms of dimethoate overexposure are weakness, involuntary muscle spasms and loss of appetite. (Cornell, 1993) The root of the most common symptoms is the inhibition of cholinesterase in the synaptic cleft, resulting in nervous dysfunction.

Organophosphates are cholinesterase inhibitors. They prevent the enzyme cholinesterase from decomposing acetylcholine, an important neurotransmitter. This results in accumulation of acetylcholine in the synaptic cleft, causing the receptors that are specific for the ligand to be overstimulated. (Pope, p. 433) This overstimulation can cause permanent damage to a victim, especially a developing fetus. A study done by the Food and Agriculture Organization of the United Nations found that when rats were exposed to various levels of dimethoate by oral, dermal, or intravenous exposure, 0.2% to 3.7% of the initial dimethoate dose was metabolized into omethoate. The metabolite found in abundance was dimethyl hydrogen phosphorodithioate, a harmless waste product. (No, p. 385) This suggests that most of the dimethoate was transformed inside the body to be excreted.

All good toxicity studies have at least one endpoint besides death. A common endpoint, besides death, in studies involving *C. elegans* is impairment of motility (Anderson, 2003). The neurological effects of the organophosphate family often result in loss of movement in C.Elegans (Rajini, 2008). As more acetylcholine binds to neurotransmitters, the rate of muscle contraction increases. Opperman found that nematodes experienced a short burst of hyperactivity (likely because of the increased concentration of acetylcholine) followed by rigid, then flaccid paralysis (likely due to overstimulation of the nervous system). This finding was published in 1990 in *Plant-parasitic Nematode Acetylcholinesterase Inhibition by Carbamate and Organophosphate Nematicides.* Previous studies used an image based computer tracking system

to express changes in movement as a percent of movement of the control (Cole, 2003; Rajini, 2008). Lacking this sophisticated equipment requires the use of an alternative method of quantifying motility. *C. elegans* move in a unique manner. Instead of a smooth continuous movement, like that of a snake, they move in short "pulses." In order to quantify the motility of the nematode, the number of pulses made in a set amount of time is a suitable substitute.

MATERIALS AND METHODS

To achieve a large enough population of *C. elegans* to perform an experiment on, subcultures were made on 35mm diameter Petri dishes containing Nematode Growth Agar (NGA, purchased from Carolina Scientific). The agar was inoculated with an LB broth solution containing a strain of OP50 *E. coli* (purchased from Carolina Scientific), which served as a food source for the nematodes. Strain N2 *C. elegans* were transferred from the original plate (purchased from Carolina Scientific) by cutting the agar in the original plate into 1 cm by 1 cm squares, and then transferring these squares onto the plate designated for subculture. The squares of NGA containing live *C. elegans* must be applied face down to the new agar to ensure that the organisms come into direct contact with their new medium. The subculture plates were then wrapped in Parafilm M and stored at 20^{o}C for three days to allow any new eggs to hatch on the new medium. Parafilm M is permeable to oxygen and has no effect on the growth and development of *C. elegans*. (Rothman, p.75; Spica) Subcultures reserved for maintaining a viable population were kept for up to 10 days before a new subculture was made. Subcultures reserved for experimentation were only allowed to sit for three days after inoculation with *C. elegans* to ensure a young healthy population, as well as to reduce variability.

Solutions of organophosphate and water were prepared by making a stock solution of 100,000 ppm by adding 10mg of organophosphate per 1 mL of water. The various solutions used during experimentation were prepared from dilutions of this stock solution. When not in use, all solutions containing organophosphate were kept inside of a freezer to prevent the decomposition of the organophosphates. At the end of the experiment, the solutions were mixed with excess amounts of ”quick lime” to neutralize the toxicants.

This experiment consisted of two different trials. The first trial was an acute exposure trial, while the second was an extended, chronic exposure trial. The first trial involved working with higher concentrations of organophosphate, but only one exposure event. The second trial used significantly lower concentrations, but had multiple exposure events spread over a longer period of time.

To quantify movement, worms were observed on an individual basis for 20 seconds each. The nature of *C. elegans* movement causes them to move in short "pulses" instead of smooth movement. These pulses were counted during the 20 second duration and subsequently recorded in a data table. A sample size of 25 worms was used to achieve an average number of pulses per worm in each plate. This method of quantifying movement was used in both the acute and chronic toxicity trials. Worms exhibiting either rigid or flaccid paralysis were counted as "paralyzed." Rigid paralysis is best described as a straightening of the worms' usual wavy shape, with occasional twitches. Flaccid paralysis is characterized by a relaxation of the worms. At this point, their nervous systems "short circuit" from the overstimulation of acetylcholine that occurred during the rigid paralysis phase. Paralyzed worms are easy to differentiate from dead worms, as the dead *C. elegans* curl up upon passing.

The first trial began with two sets of five cultures (one set for each toxicant) and a control. Toxicants were administered in a dropwise manner at a volume of .5 ml, spread across the face of the medium. The concentrations used for both organophosphates were 50ppm, 100ppm, 500ppm, 1000ppm, and 5000ppm. Each plate was observed immediately after exposure, and the motility of the *C. elegans* inside was recorded. The motility of the nematodes was also observed 30 minutes after the initial exposure event. This data was translated into a probit plot to determine the LC50 and EC50 of each toxicant. The probit plot compares the proportion of the sample that elicits a positive response (death or loss of motility) to the

concentration of organophosphate used, represented on a probit-log scale. A probit transform is a way to represent sigmoid curve data in a linear fashion, making it easier to interpret.

The second trial began in the same manner as the acute toxicity trial, with two sets of five cultures, plus one culture to serve as a control. The concentrations used in this trial were 5ppm, 10ppm, 50ppm, 100ppm, and 500ppm. These concentrations were chosen based on the results of the first trial. This trial was carried out over the course of seven days, with a fresh dose of organophosphate in the amount of .5ml administered every 12 hours at the same time every day. Before each exposure event besides the first one, the nematodes were observed, and their motility levels recorded in the matter described above. The same area was observed each time, so that if any organisms died during the trial, they would always be counted, as they would stay in the designated area. The collected data was compiled into a probit plot, and LC50 and EC50 values were determined.

In the interest of accuracy, each trial was run in duplicate. The results of each run of each trial are represented separately on all figures. The acute toxicity trial was actually run three times, but the first run involved concentrations of organophosphate that were too low to achieve a lethal concentration. The concentrations used in the acute exposure trial were raised by an order of magnitude, and the trial was run two more times under the revised conditions.

RESULTS

Table 1 below lists the LC50 and EC50 values for each organophosphate calculated from each run of the acute toxicity trial. This table compares the averages of both values for each toxicant as a percentage.

Table 1: Comparison of LC50 and EC50 Values			
Dimethoate		Omethoate	
LC50		**LC50**	
Run #1 121.2 ppm	Run #2 119.6 ppm	Run #1 96.5 ppm	Run #2 70.8 ppm
Average LC50 120.4 ppm		**Average LC50** 83.4 ppm (144.4% more effective than dimethoate)	
EC50		**EC50**	
Run #1 29.9 ppm	Run #2 30.6 ppm	Run #1 19.4 ppm	Run #2 26.3 ppm
Average EC50 30.3 ppm		**Average EC50** 22.9 ppm (132.3% more effective than dimethoate)	

The following figures (Figures 1-4) display the probit plots obtained from run #1 of the acute toxicity trial. Tables 2-5 contain the raw data that went into Figures 1-4.

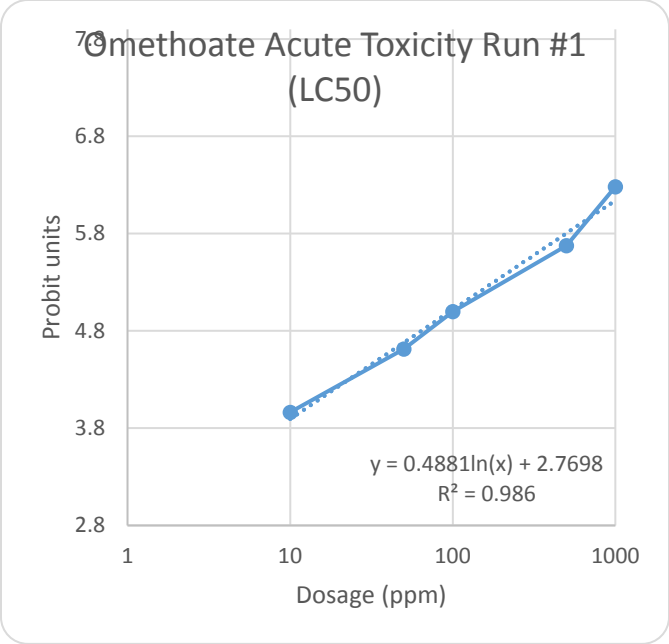

Figure 1: Probit analysis of lethal omethoate toxicity for run #1 of acute toxicity trial.

Table 2: Raw data of lethal omethoate toxicity for run #1 of acute toxicity trial.

Dosage	Number dead	probit units
0	0	0
10	3	3.96357
50	7	4.61468
100	10	5
500	15	5.67449
1000	18	6.28155

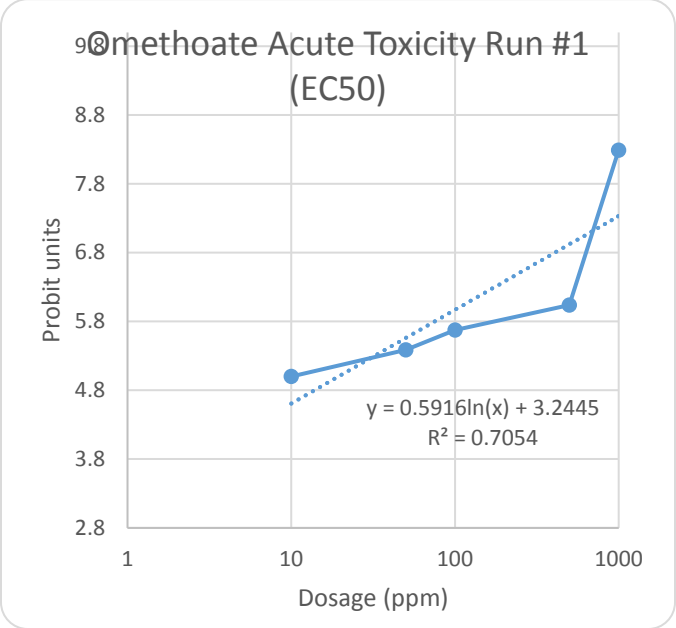

Figure 2: Probit analysis of effective omethoate toxicity for run #1 of acute toxicity trial.

Table 3: Raw data of effective omethoate toxicity for run #1 of acute toxicity trial.

Dosage	Number paralyzed	probit units
0	0	0
10	10	5
50	13	5.38532
100	15	5.67449
500	17	6.036433
1000	19.99	8.290527

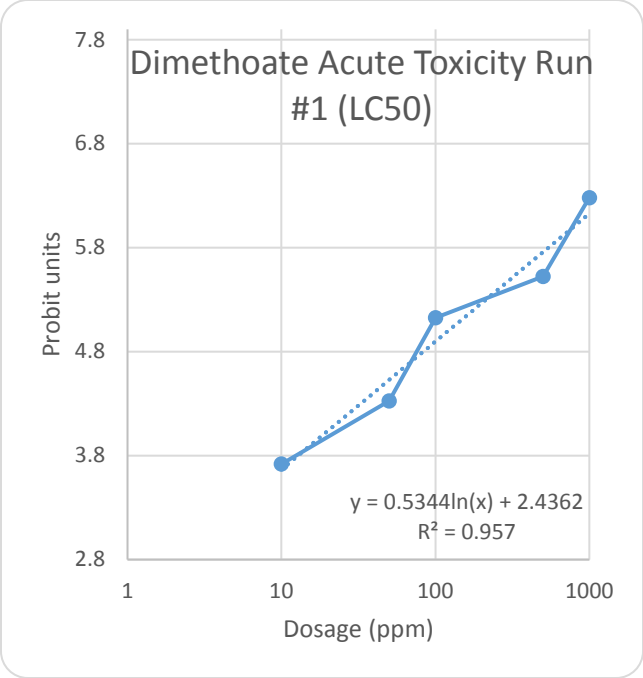

Figure 3: Probit analysis of lethal dimethoate toxicity for run #1 of acute toxicity trial.

Table 4: Raw data of lethal dimethoate toxicity for run #1 of acute toxicity trial.

Dosage	Number dead	probit units
0	0	0
10	2	3.718448
50	5	4.32551
100	11	5.125661
500	14	5.524401
1000	18	6.281552

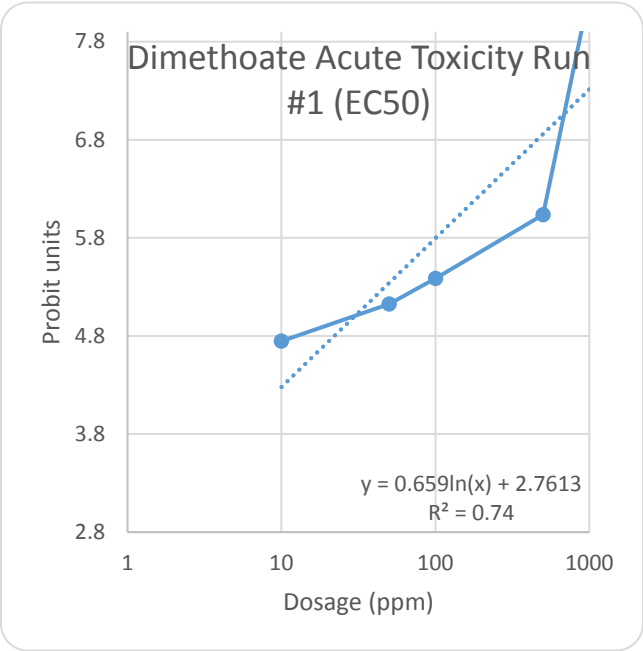

Figure 4: Probit analysis of effective dimethoate toxicity for run #1 of acute toxicity trial.

Table 5: Raw data of effective dimethoate toxicity for run #1 of acute toxicity trial.

Dosage	Number Paralyzed	probit units
0	0	0
10	8	4.746653
50	11	5.125661
100	13	5.38532
500	17	6.036433
1000	19.99	8.290527

The following figures (Figures 5-8) and their accompanying tables (Tables 6-9) represent data collected from run #2 of the acute toxicity trial.

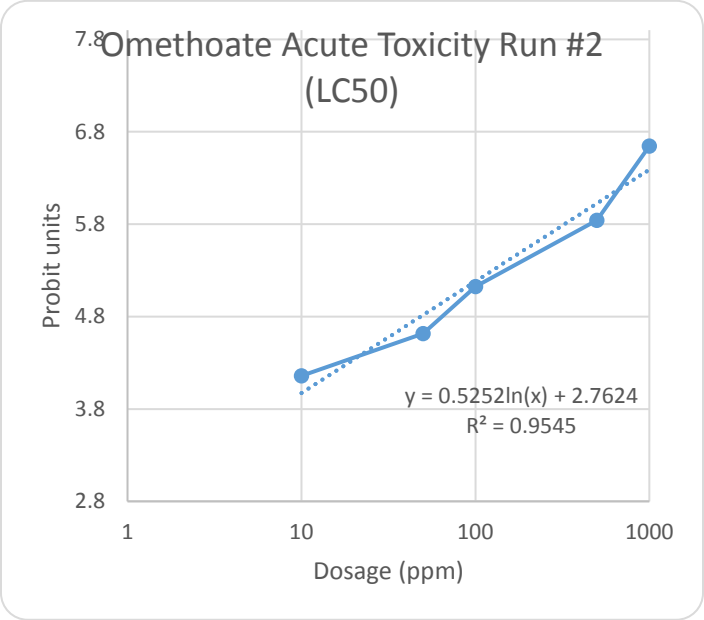

Figure 5: Probit analysis of lethal omethoate toxicity for run #2 of acute toxicity trial.

Table 6: Raw data of lethal omethoate toxicity for run #2 of acute toxicity trial.

Dosage	Number dead	probit units
0	0	0
10	4	4.158379
50	7	4.61468
100	11	5.125661
500	16	5.841621
1000	19	6.644854

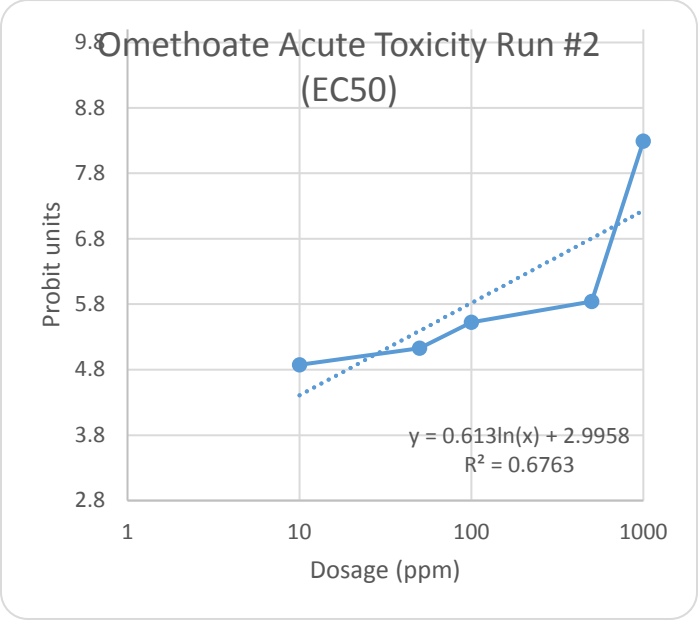

Figure 6: Probit analysis of effective omethoate toxicity for run #2 of acute toxicity trial.

Table 7: Raw data of effective omethoate toxicity for run #2 of acute toxicity trial.

Dosage	Number Paralyzed	probit units
0	0	0
10	9	4.874339
50	11	5.125661
100	14	5.524401
500	16	5.841621
1000	20	8.290527

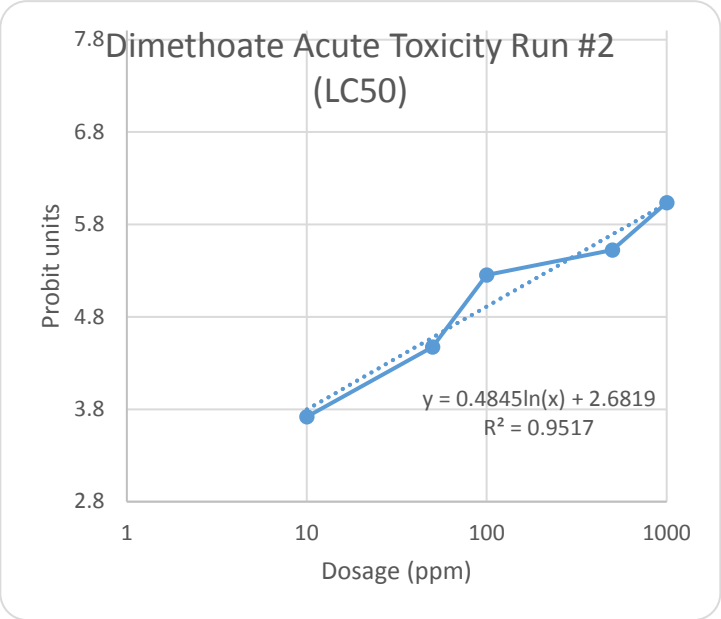

Figure 7: Probit analysis of lethal dimethoate toxicity for run #2 of acute toxicity trial.

Table 8: Raw data of lethal dimethoate toxicity for run #2 of acute toxicity trial.

Dosage	Number dead	probit units
0	0	0
10	2	3.718448
50	6	4.475599
100	12	5.253347
500	14	5.524400
1000	17	6.036433

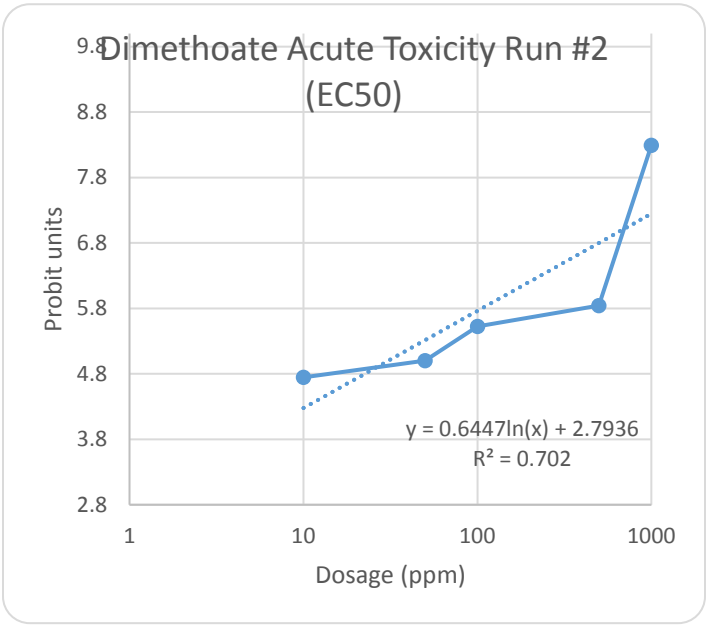

Figure 8: Probit analysis of effective dimethoate toxicity for run #2 of acute toxicity trial.

Table 9: Raw data of effective dimethoate toxicity for run #2 of acute toxicity trial.

Dosage	Number Paralyzed	probit units
0	0	0
10	8	4.746653
50	10	5
100	14	5.524401
500	16	5.841621
1000	20	8.290527

Figures 9-12 represent data taken from the chronic toxicity study.

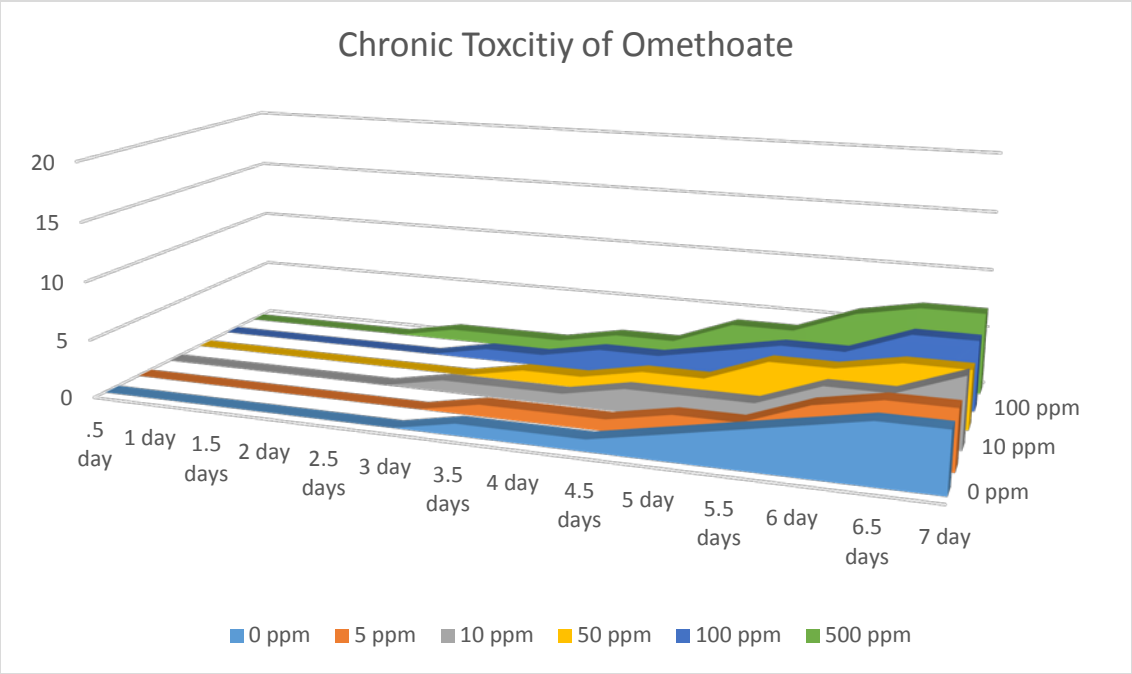

Figure 9: Graphical representation of Table 10. Dose of omethoate versus number of dead *C. elegans* over 7 days at 12 hour increments.

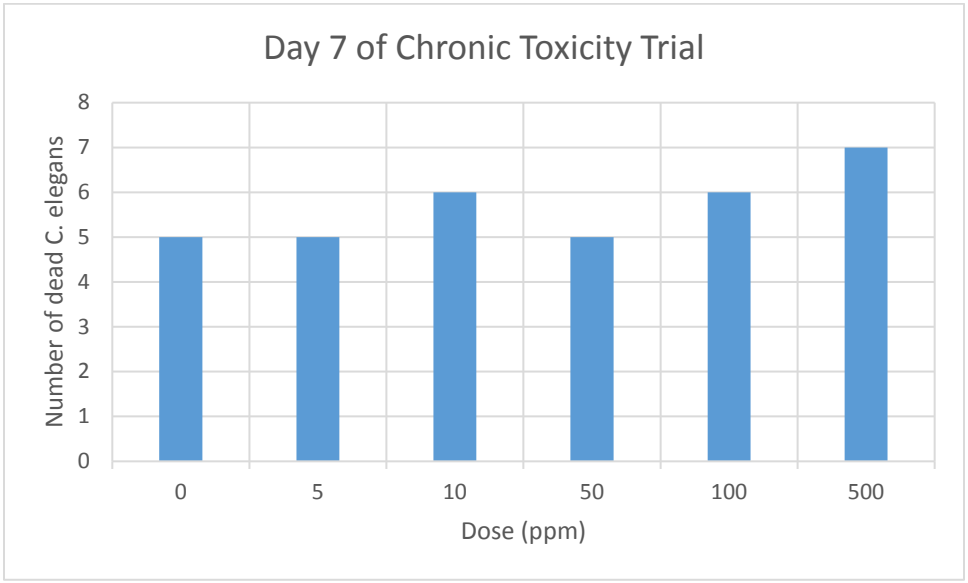

Figure 10: Bar graph comparing total number of dead *C. elegans* on day 7 of the omethoate chronic toxicity trial.

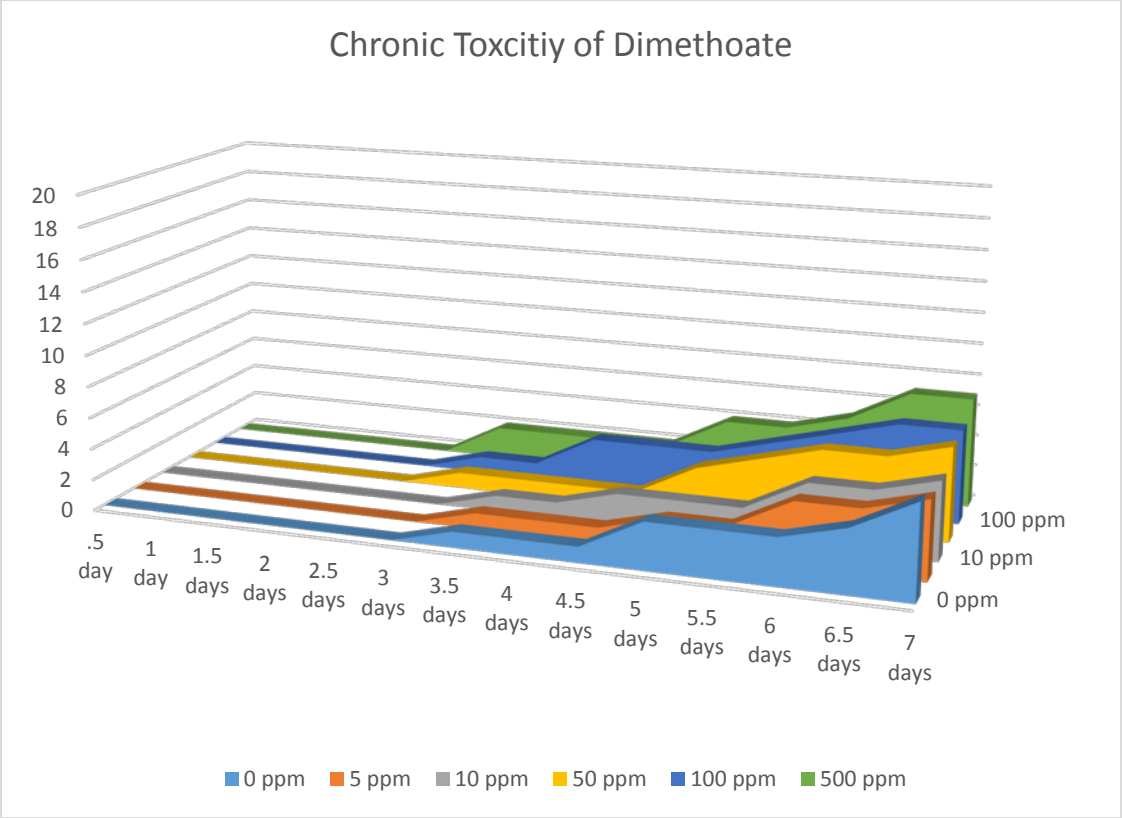

Figure 11: Graphical representation of Table 11. Dose of dimethoate versus number of dead *C. elegans* over 7 days at 12 hour increments.

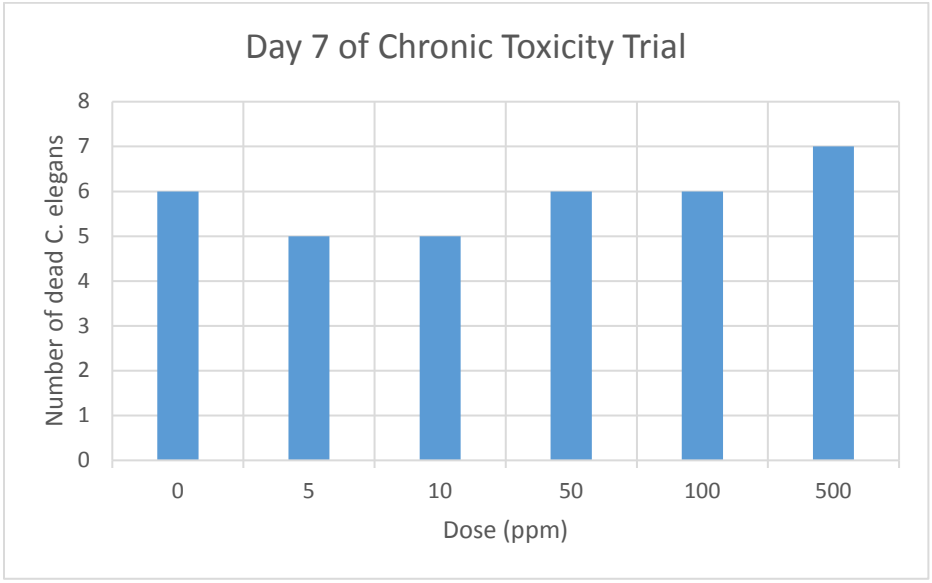

Figure 12: Bar graph comparing total number of dead *C. elegans* on day 7 of the dimethoate chronic toxicity trial.

DISCUSSION

The first hypothesis in this study states that omethoate is more active, and thus more potent than dimethoate. This hypothesis is supported based on the data collected in the acute toxicity trial. As seen in Table 1, the average LD50 of omethoate (83.4ppm) was 69.3% of the average LD50 for dimethoate (120.4ppm), which means that omethoate was 144.4% more effective at killing *C. elegans* than dimethoate. The average EC50 of omethoate (22.9ppm) was 75.6% of the average EC50 of dimethoate (30.3ppm), translating to a 132.3% increase in effectiveness over dimethoate. Qualitatively, this data is supported by findings made by Cole. Cole found that the EC50 of omethoate was a mere 9.44% of the EC50 of dimethoate (p. 250). While Cole's data supports our hypothesis of omethoate having a higher potency than dimethoate, it also suggests that the difference in potencies of the two organophophates in this study is very low. This could arise from cross contamination of the organophosphate solutions or another form of experimental error. Figures 1-8 and their associated tables (Tables 2-9) present probit plots that do not exhibit sharp peaks, which would indicate that the test plates were not exposed to the same conditions. Cole's study also suggests that LC50 values should fall between 1.00 and 100 ppm. The LD50 values found in this study are close to this range, and lean towards the upper end of the spectrum.

The second hypothesis states that neither organophosphate will display any definite ability to accumulate inside the organism. Figure 9 and 10 show that while plates exposed to a higher concentration of omethoate showed a higher amount of dead worms, this increase is only slight. The increase is small enough to suggest that omethoate has an insignificant amount of accumulation ability. Figures 11 and 12 suggest that the same is true for dimethoate as well. Keenland states that organophosphates rarely show bioaccumulation activity, supporting this

hypothesis further. Although organophosphates irreversibly inhibit acetylcholinesterase, new enzyme is produced to counter the effects of the toxicant. (Doctor, p. 168)

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APPENDIX

Appendix 1: Data tables

Both of the below tables contain data collected from the chronic toxicity study.

Dose of omethoate versus number of dead *C. elegans* over 7 days at 12 hour increments														
Dose (ppm)	.5 day	1 day	1.5 days	2 days	2.5 days	3 days	3.5 days	4 days	4.5 days	5 days	5.5 days	6 days	6.5 days	7 days
0	0	0	0	0	0	0	1	1	1	2	3	4	5	5
5	0	0	0	0	0	0	1	1	1	2	2	4	5	5
10	0	0	0	0	0	1	1	1	2	2	2	4	4	6
50	0	0	0	0	0	0	1	1	2	2	4	4	5	5
100	0	0	0	0	0	1	1	2	2	3	4	4	6	6
500	0	0	0	0	1	1	1	2	2	4	4	6	7	7

Dose of dimethoate versus number of dead *C. elegans* over 7 days at 12 hour increments														
Dose (ppm)	.5 day	1 day	1.5 days	2 days	2.5 days	3 days	3.5 days	4 days	4.5 days	5 days	5.5 days	6 days	6.5 days	7 days
0	0	0	0	0	0	0	1	1	1	3	3	3	4	6
5	0	0	0	0	0	0	1	1	1	2	2	4	4	5
10	0	0	0	0	0	0	1	1	2	2	2	4	4	5
50	0	0	0	0	0	1	1	1	1	3	4	5	5	6
100	0	0	0	0	0	1	1	3	3	3	4	5	6	6
500	0	0	0	0	0	2	2	2	2	4	4	5	7	7