# Modified acetylcholinesterase activity and protein modulation as a result of chlorpyrifos exposure in *D. tigrina*: Preliminary Results

By Cindy Hou and Nicholas Madoff

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**Abstract**

Chlorpyrifos (CPS) is an organophosphate that since its registration in 1965, has both agricultural and nonagricultural uses, being sprayed on crops, animals, golf courses, and buildings to kill insects. Six million pounds of CPS was used across 10 million acres of land, making it the most commonly used insecticide in the United States, and it can contaminate the air, water, and soil. This study aims to first determine if various doses of the pesticide chlorpyrifos (CPS) are toxic to planaria through $LC_{50}$ assays. This study will also examine if CPS has a similar inhibitory effect on AChE in planaria as it does in other organisms by quantifying acetylcholinesterase activity levels using a colorimetric assay kit and if planaria exposed to CPS will have altered AChE protein levels by running a quantitative ELISA. We have identified that CPS exposure is toxic to planaria when acutely exposed to concentrations between .1 mg/ml and .5 mg/ml for 20 minutes and between .05 mg/ml and .1 mg/ml when chronically exposed for 72 hours. We expect that worms exposed to various concentrations of CPS will have decreased AChE activity levels, more notably at higher concentrations. In addition, exposed planaria will increase their acetylcholinesterase protein levels in order to counteract lowered activity due to AChE inhibition.

**Rationale**

Introduced in 1965, Chlorpyrifos (CPS) is an organophosphate that has a variety of agricultural and nonagricultural uses. CPS is sprayed on crops, animals, golf courses, and buildings in order to kill insects (Cornell PMEP 1993). Six million pounds of CPS have been used across 10 million acres of land between 2009 and 2013, making it one of the most commonly used insecticides in the United States (Vogel 2016). CPS can contaminate the air and water, and sticks very strongly to soil where it has a half life of several months. CPS can drift into waterways from spray applications, and although CPS has a low solubility in water due to its non-polar nature and is unlikely to be washed into bodies of water on its own, CPS contaminates ponds and rivers when treated soil ends up in runoff (Christensen et al 2009). Due to the common usage of CPS and its prevalence in soil, it is likely that a runoff event can have a particularly damaging effect on aquatic ecosystems (Moore et al 2002).

CPS exposure occurs through ingestion, inhalation, and dermal means and has been shown to have toxic effects on a variety of organisms including mammals, fish, and nematodes (Christensen et al 2009). Planaria, however, represent a unique approach to measuring the effects

of CPS in aquatic environments. Due to their inherent sensitivity to environmental toxicants and alterations, planaria can be employed as sentinel organisms to provide advanced warning of how a pollutant could affect other aquatic invertebrates or even humans (Knakievicz 2014).

In the U.S., CPS has been banned in Hawaii, while California has ruled to ban the sale of CPS by 2020. However, CPS is currently not banned or restricted in the rest of the US due to a 2017 decision by the EPA, despite extensive clinical research outlining its toxic effects (Beech 2017). However, CPS is more tightly regulated internationally and is either banned or restricted to specific use in nations like the U.K., South Africa, and Singapore (Schlueter 2017)

When insects are exposed to CPS, a CPS metabolite called Chlorpyrifos-oxon irreversibly inhibits the acetylcholinesterase enzyme, which is responsible for the breakdown of the neurotransmitter acetylcholine. CPS exposure results in a form of competitive inhibition by producing a negative charge at the enzyme's active site. This reaction creates a strong bond between the compound and the enzyme, impairing the enzyme's function. In healthy organisms, acetylcholine is critical to the function of the central nervous system, and AChE is responsible for ensuring the neurotransmitter is present at appropriate levels. However, overaccumulation of acetylcholine due to AChE inhibition causes an overstimulation of neuron cells that eventually results in neurotoxicity and death (Purves et al 2011).

CPS has a similar inhibitory effect in non-insect organisms, and can have toxic effects in both vertebrates and invertebrates (Christensen et al 2009). In sublethal doses, CPS and its resulting AChE inhibition often result in reduced heart rate, greater sweating and secretion of bodily fluids, and muscle spasms (Elersek 2011). While this interaction has been investigated in insects, mammals, and fish, the effects of CPS have not been extensively researched in aquatic invertebrates such as planaria.

Further investigation of CPS and its effects is critical, as the U.S. government is still unwilling to acknowledge the compound's toxicity, while research with the compound has only been conducted using a limited group of model organisms. This study aims to first determine if various doses of CPS have a similar inhibitory effect on AChE in planaria as they do in other organisms. In addition, this study also will examine if CPS exposure alters AChE protein levels in planaria, as a further extension of our investigation of CPS's inhibitory function. As demonstrated in a paper published in 2008, it is possible to measure protein levels in planaria using ELISAs (Enzyme-Linked Immunosorbent Assay) (Fukushima et al 2008). We hope to characterize the potential upstream effects of CPS exposure on AChE activity and levels in planaria. Through better characterizing the pesticide's toxic nature, we hope to provide a more conclusive answer to the debate on whether CPS poses a threat to ecosystems and humans.

**Research questions, hypothesis, and goal**

We want to investigate how dose-dependent acute and/or chronic exposure to the pesticide chlorpyrifos affects acetylcholinesterase activity and acetylcholinesterase levels in Planarian worms. Our project centers around planaria due to the organism's novel nature and the lack of extensive characterization of CPS's effects in planaria or any other aquatic invertebrate. Through quantifying the potential toxicity of CPS in planaria, our projects hopes to enable broader predictions of how other aquatic invertebrates will respond to CPS exposure. Based on the research that CPS inhibits acetylcholinesterase activity in other organisms, we hypothesize that worms exposed to various concentrations will also have decreased AChE activity levels. We chose to conduct a dose dependent study because we wanted to investigate the most dramatic effects of CPS in planaria and at what minimum dose these complications can occur. We also wanted to explore the sub-lethal symptoms of CPS exposure, and how they vary at different doses.

In addition, we wanted to look into how planaria respond to the effects of CPS, and if they have existing proteomic responses to mitigate the toxicant's influence. We hypothesize that planaria exposed to CPS will increase their acetylcholinesterase levels in order to counteract lowered activity due to AChE inhibition. Due to the observed similarities between planarian and human nervous systems, it is likely that planarian response to CPS is applicable to humans as the toxicant has a similar method of action in both organisms.

**Methodology, Data Analysis, and Discussion**

*Planarian Culture*

Each assay used freshwater brown planaria (*Dugesia tigrina*). The planaria were stored in spring water when not used for the experiments, and their water was changed twice a week. The planaria were fed a diet of hard-boiled egg yolk once a week. Planaria used in experiments were starved for at least a week prior.

*$LC_{50}$ Assay*

In order to find the concentration of CPS in water that kills 50% of the adult planaria population ($LC_{50}$) during acute exposure, planaria will be exposed for 20 minutes to different concentrations of CPS dissolved in spring water. For each concentration, there will be ten planaria exposed. The exposed worms will be removed, washed, and placed into clean water post exposure. After 72 hours, the planaria will be harvested for the final lethality level at the tested concentration to empirically determine the acute $LC_{50}$. This entire process will be repeated again, this time exposing the planaria for 48 hours and harvesting them immediately to find the chronic $LC_{50}$ value.

*Acetylcholinesterase Activity Assay*

After determining the acute and chronic $LC_{50}$ values, the planaria will be subjected to acute (20 minute) and chronic (48 hour) exposures of CPS at five different doses, ranging from the acute or chronic $LC_{50}$'s to a toxicant free bath. Immediately after exposure, the changes in AChE activity in the living planaria will be determined using by following the procedures

outlined for the Colorimetric Acetylcholinesterase Activity kit (ab138871). Once these assays have been completed, the AChE activity will be compared between acute and chronic exposure to find which leads to a more dramatic difference in activity between the respective experimental groups and the control group.

*ELISA*

A direct ELISA will also be performed on all dosage exposure groups in order to measure the AChE protein levels in either the chronic or acute exposure planaria, depending on which resulted in a bigger change in AChE activity. After coating the antigen-containing planarian tissue sample to the microplate, a blocking buffer will be added to block remaining protein-binding sites in the well. The antibody will then be added to the plate. We will then add the substrate solution and wait for color development, at which point we will use the plate reader to determine the absorbance. A standard curve will be generated from the data with concentrations on the x axis and absorbance on the Y axis.

This process will be repeated for planaria from each exposure group.

The following is preliminary $LC_{50}$ data:

In the acute exposures, six planaria were exposed to six different concentrations of CPS between 0 mg/ml and .5 mg/ml that were made with the first batch of CPS. In all of the trials, the six planaria remained alive. However, using our new batch of CPS, ten planaria were exposed to four concentrations between .5 mg/ml and 5 mg/ml. In all four of these trials, 100% of planaria died. A control group containing ten planaria was also exposed to a 0 mg/ml concentration, all of which survived. As a result, for future experiments using the new batch of CPS, planaria will be exposed to concentrations between 0 and .5 mg/ml to determine the $LC_{50}$.

In the chronic exposures, researchers employed two different batches of CPS, testing planaria in groups of six with the first batch and in groups of 10 for the second. For the first batch, planaria were exposed to CPS at concentrations of 0 mg/ml, .00015 mg/ml, .00025 mg/ml, .00035 mg/ml, .00045 mg/ml, .045 mg/ml, 1 mg/ml, and 2 mg/ml. In these trials, all six planaria lived, except for in the 1 mg/ml and 2 mg/ml trials in which all six planaria died. For the second batch, planaria were exposed to CPS at concentrations of 0 mg/ml, .05 mg/ml, .1 mg/ml, .4 mg/ml, .7 mg/ml, and 1 mg/ml. In these trials, all ten planaria died, except for those in the 0 mg/ml (control) trial in which all planaria survived, and the .05 mg/ml trial in which 9 planaria survived and one died. The planaria will thus be exposed to concentrations between .05 mg/ml and .1 mg/ml to find the $LC_{50}$.

**Literature review:**


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