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

Introduction

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.[1] 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.[2] CPS can contaminate the air and water, and sticks very strongly to soil where it has a half life of several months.[3] 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.[3] 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.[4]

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.[3] Planarians, 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.[5]

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.[6] 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.[7]

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.[8] CPS exposure results in a form of competitive inhibition by producing a negative charge at the enzyme's active site.[8] This reaction creates a strong bond between the compound and the enzyme, impairing the enzyme's function.[8] 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.[8]

CPS has a similar inhibitory effect in non-insect organisms, and can have toxic effects in both vertebrates and invertebrates.[3] 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.[9] 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).[10] 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.

Materials

The following materials will be used: petri dishes, 100% ethanol, 100 mg chlorpyrifos, standard pipetting equipment, D. tigrina, abcam acetylcholinesterase activity kit, ELISA Kit and antibodies, spring water, microwell plates, and a plate reader.

Methods

Planarian Culture

Each assay used freshwater brown planaria (*Dugesia tigrina*). The planaria were stored in Poland Springs 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 are 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 (abcam ab138871). The kit uses the absorption intensity of Ellman's reagent (DTNB) to measure the total thiocholine produced, as thiocholine amount is proportional to AChE activity. 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 using an ELISA kit 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.[11] The antibody will then be added to the plate.[11] 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.[11] A standard curve will be generated from the data with concentrations on the x axis and absorbance on the Y axis.[11] This process will be repeated for planaria from each exposure group.

Preliminary Data

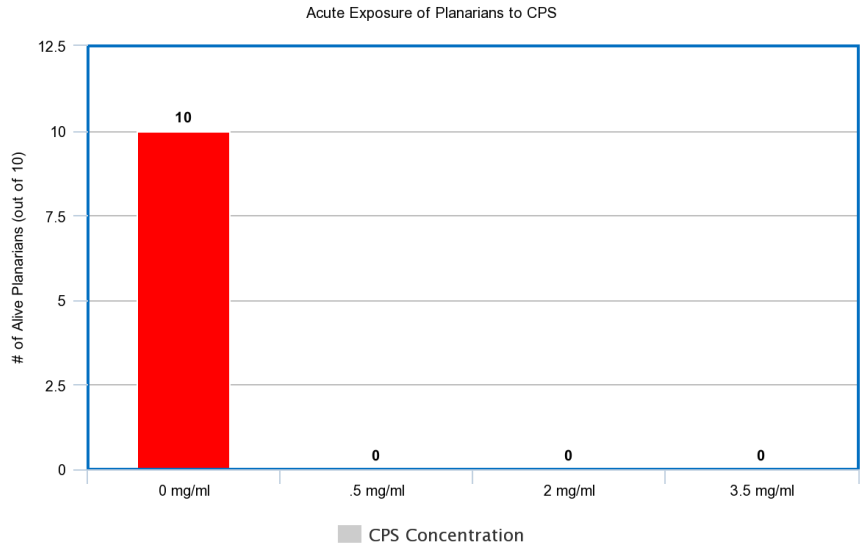
The following is preliminary LC_{50} data, accounting for the smaller sample sizes and lack of repeated trials:

Note that the first batch of CPS used during testing is no longer available, thus all future experiments will use the second batch of CPS received.

Acute Exposures

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}.

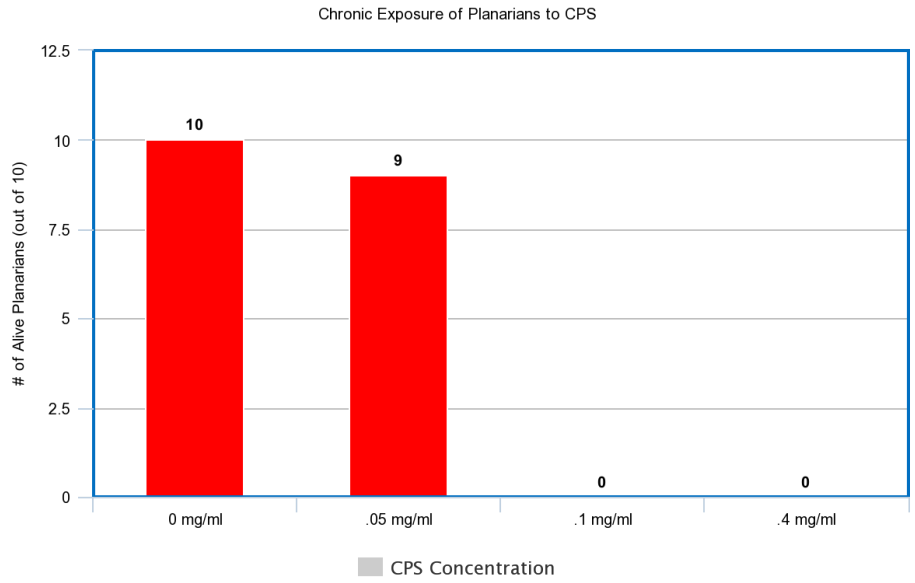
Concentration	0 mg/ml	.00075 mg/ml	.0015 mg/ml	.0030 mg/ml	.1 mg/ml	.5 mg/ml	2 mg/ml	3.5 mg/ml	5 mg/ml
# worms alive : # worms dead (1st batch of CPS)	6:0	6:0	6:0	6:0	6:0	6:0	N/A	N/A	N/A
# worms alive : # worms dead (2st batch of CPS)	10:0	N/A	N/A	N/A	N/A	0:10	0:10	0:10	0:10



Chronic Exposures

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 chronic LC_{50}.

Concentration	0 mg/ml	.00015 mg/ml	.00025 mg/ml	.00035 mg/ml	.00045 mg/ml	.045 mg/ml	.05 mg/ml	.1 mg/ml	.4 mg/ml	.7 mg/ml	1 mg/ml	2 mg/ml
# worms alive : # worms dead	6:0	6:0	6:0	6:0	6:0	6:0	N/A	N/A	N/A	N/A	0:6	0:6
# worms alive : # worms dead (2st order of CPS)	10:0	N/A	N/A	N/A	N/A	N/A	9:1	0:10	0:10	0:10	0:10	N/A



- Both graphs were made using data from the 2nd batch of CPS (all future trials will be conducted using the 2nd batch).

Research Questions and Objectives

2.1 Research Questions

- Does dose-dependent acute/chronic exposure to the pesticide chlorpyrifos have an effect on acetylcholinesterase activity and protein levels in Planarian worms?

2.2 Specific Aims

- Determine the acute and chronic LC_{50} values of CPS for D. tigrina
- Determine the Lowest Observed Effect concentration

- Quantitatively evaluate the dose-dependent inhibitory effect of CPS on AChE using the AChE activity kit
- Investigate the effect that CPS inhibition of AChE has on planarian AChE levels using ELISA assays

2.3 Hypothesis

- If planarian worms are exposed to various concentrations of chlorpyrifos, we expect acetylcholinesterase activity to decrease and protein levels to increase in the worms, as the observed method of action in insects and vertebrates is acetylcholinesterase inhibition.

Timeline

- November
    - Chronic and acute LC50 assays
- December
    - Dose dependent AChE activity assay (chronic and acute)
    - Data analysis
- January
    - Dose dependent ELISA assay on either chronic or acute exposure
- February
    - Data analysis
    - Working on NYCSEF presentation
- March
    - NYCSEF presentation

Hazardous chemicals, activities and devices

- This team will utilize chlorpyrifos at a final concentration of 0.5mg/ml. <u>All stock and working dilutions</u> of chlorpyrifos and will be prepared by the adult P.I. (Ileana Rios) for student use.
- Due to stringent safety protocols, safety training, and direct supervision, the risks to the students are minimal and may involve accidental spills.
- Skin contact and eye exposure are entirely minimal due to personal protective equipment. In addition, there is a shower and eye wash station in the biology lab. First, the BSL-1 prep room which houses the CO2 incubator, cell media reagents, autoclave, and biohazardous waste is secure with a combination door lock. A few instructors in the science department and maintenance are familiar with the key code; the prep room is always closed and locked unless the adult P.I. is present in the room. Students will wear personal protective equipment consisting of lab coats, nitrile disposable gloves, goggles, and facemasks from VWR. All activities and protocols with BSL-1 entities are carried

out in the safety hood in a BSL-1 prep room and under direct supervision by the adult P.I. All working dilutions of chlorpyrifos and will be prepared by the adult P.I. for student use.

- All worm media waste is disinfected with 10% bleach and autoclaved for 20 minutes at 212oF prior to disposable in a red biohazard bag which is picked up for incineration by Sharps Compliance, Inc. All chlorpyrifos-treated liquid waste is collected in amber chemical waste bottles and collected by PEGEX Hazardous Waste Removal (Account NumberA-96207).

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