EFFECT OF FLUORIDE ON GROWTH AND DEVELOPMENT OF TOTIPOTENT CELLS IN PLANARIA

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Table of Contents

Acknowledgements……...…4

Abstract……5

Introduction……..6

Calculations……..…...10

Protocol Listings……...……12

Materials and Methods……...21

Results……....25

Discussion…….....43

Future Endeavors……...46

Resources……...50

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Abstract

Planaria are free living flatworms that are known for their regenerative elasticity and ability to quickly regenerate body fragments. This is made possible by the totipotent neoblasts distributed throughout their body which is highly undifferentiated, holding the potential to divide into any type of cell in the Planarian body proper. This rapid regenerative property is what makes Planaria a great model system. Since they live in a vast array of environments, it is possible that they are exposed to various amounts of fluoride. Fluoride has been shown to have health benefits by strengthening bone and enamel. To further investigate the effects of fluoride, an experiment was designed to analyze the effect of fluoride on the growth of totipotent cells in Planaria. Planaria were cut midway through the body along the transverse plane, and each head and tail was placed in a solution of NaF ranging in concentrations of 0 parts per million, 1ppm, 25ppm, 50ppm, 75ppm, or 100ppm. Five segments of Planaria were placed in each concentration. Each day, for five days, a Planarian sample from each concentration was immersed in a preservation fluid and underwent histological tissue preparation to be observed under a microscope. The resulting pattern of mitotic activity suggests that fluoride may be beneficial to cell proliferation in concentrations between 25ppm-50ppm; however, further investigation must be done in order fill the gaps in data and verify patterns as being an effect of fluoride and not random chance.

Introduction

Planaria

Planaria are flatworm in the phylum Platyhelminthes. Although they have all three germ layers (endoderm, mesoderm, ectoderm), they are acoelomate organisms. They are able to move via cilia on their ventral side. This allows them to glide over their surface over the mucus they produce (Gavlik, Szymczak 2003).Planaria are free living organisms that typically lives in a marine or fresh water environment. Their diet typically consists of plants; however, there are plenty of foods Planaria will eat. They are flexible and can adapt to their environment, displaying a non – temperamental life style. Its body consists of a very simple nervous system running along the length of the organism and gastrointestinal material filling the inside. The nervous system consists of a central neural ganglia with two nerve cords running down from the ganglia, parallel through the length of the Planarian. The organism has a blind gut that begins and ends with the mid ventral pharynx. The pharynx is a unique feature of Planaria. It retracts and distends out from the body to pierce and obtain food. It is also used to expel digestive wastes. Planaria lack a circulatory system. This is compensated for by their dorsoventrally flattened body, allowing a high surface area to volume ratio to diffuse gases efficiently. The highly branched digestive system aids this exchange of gas by having a high surface area to volume ratio (Newmark and Alvarado 2001).

The most defining feature of Planaria is their ability to regenerate. A single Planarian can be cut into many different fragments, and each fragment will grow into a new individual. If a Planarian is cut in half along the transverse plane, the head will grow a new tail, and the tail will grow a new head. If the Planarian is cut in half through the mid sagittal plane, each half of the head will grow into a new head. In this way, a Planarian may fragment itself as a means of

asexual reproduction. Planaria are also capable of undergoing sexual reproduction. In this case, they are hermaphroditic and cross fertilize one another. They often fluctuate between asexual and sexual reproduction depending on the environment (Hoshi *et. al*. 2003).

The means in which Planaria are able to regenerate an entire body is due to their stem-cell-like cells called neoblasts. Neoblasts are a specific type of cell that is found in Planaria and are totipotent (Rossi, Salvetti, Bastistoni, Deri, Gremigni 2008). Totipotent is the most undifferentiated level of cell potency. It means that the cell is undifferentiated, and can become any type of cell in the body, extracellular cells, extraembryonic cells, and placental cells; it is not specific to only structures of the body proper. These neoblasts have a high amount of RNA, allowing it to divide on command and frequently. They are the only mitotic cell in the Planaria, so they are spread throughout the body and have an undifferentiated feature. When a Planaria is wounded, the wound site is covered over by a thin layer of epithelium. After that, a blastema forms under the epithelium. A blastema is a mass of cells consisting of two different groups of cells: an outer layer of cells and in inner mass of cells. Both of these cell masses are derived from the neoblasts that spread throughout the Planarian body proper. As the cells in the blastema divide, they differentiate into specialized characteristics to regenerate organs and structures (Newmark and Alvarado 2001).

Fluoride

Elemental fluoride (F^-) has shown proposed benefits to the body over the course of the last few decades. It is believed to be beneficial for bone growth and strengthening the enamel of our teeth. Fluoride is an element that is typically incorporated into the bone in place of an OH^-, making the bone form fluorapatite in place of hydroxyapatite, which is a central component of bone. (Mousny *et. al.* 2008). Hydroxyapatite is also a main mineral found in the enamel, which is

why it is suggested that fluoride has an effect on dental enamel as well as bone. Fluoride is believed to be beneficial because the fluorapatite crystal is more stable than a normal hydroxyapatite crystal in bone. In addition, it decreases the solubility of the crystals in bone, further strengthening bone structure because it remains rigid. Because the incorporation of fluoride changes the structure of bone, it also changes its physical features and reaction to other elements. Fluoride isn't incorporated into the bone right away, but only during mineralization. This acts as a protective effect on the bone, and has been shown to increase resistance to acid dissolution (Grynpas 1990).

The benefits of fluoride are so well known that it has been implemented into our daily lives. Drinking water has fluoride added to it, at roughly 1ppm – 4ppm fluoride. In addition, toothpaste has fluoride in it as well. Brushing twice a day with fluoride along with drinking small amounts of fluoride in our drinking water is thought to be the best way to obtain the correct dosage; it is obtained in small amounts every day (Petersen and Lennon 2004). The World Health Organization has collected reviews on how effective this fluoride exposure was, and it concluded that since these policies have been put into place, the prevalence of dental caries (decay and cavities) has decreased. The correlation between increasing daily doses of fluoride and lower amount of dental caries is sufficient enough to determine that fluoride has health benefits. Researchers believe that fluoride works to strengthen the enamel in multiple ways. They believe it strengthens the enamel's chemical structure, thus being more resistant to acid. This, in turn, makes the enamel protected against the acid produced by plaques of bacteria, and encourages the repair of enamel that has previously been damaged (Jones, Burt, Petersen, Lennon, 2005).

Although fluoride has shown to provide many benefits, the amount of exposure must be monitored. Fluoride is still considered a toxin in large doses, and therefore can cause health

problems if an organism is exposed to too much. For example, if too much is consumed, it could result in dental fluorosis, which is diagnosed with brown or black patches on the teeth. If an extreme amount of fluoride is consumed, this can lead to skeletal fluorosis. Because fluoride strengthen bones, and excessive amount will increase bone density and calcification of the bone and surrounding ligaments, the joints will become less flexible (Suresh, Reddy, Ramesh, Rajeswari, Sunitha, Nagendra 2015).

Studying Effects of Fluoride in Planaria

Most research on the effects of fluoride has been in the realm of studying fluoride effect on osseous structures, such as bone and cartilage. Planaria are organism that don't have such structures, for they are very simple invertebrates. What makes them a good model system is that they regenerate fast and have a long lifespan. In addition, the mitotic activity of their cells, in theory, could easily be observed under a compound microscope due to the fact that their totipotent neoblasts are readily available to divide. Investigating fluoride's effects on mitotic activity of totipotent cells could open up the discussion of fluoride's effects on more than just bone and enamel; it could possibly have other health effects that have yet to be investigated. We designed the experiment to include six different concentrations of fluoride: 0ppm (control), 1ppm (normal drinking water), 25ppm, 50ppm, 75ppm, and 100ppm. I hypothesize that there will be the highest amount of mitotic activity in the Planaria that have been exposed to 1ppm – 25ppm of fluoride. Since 1ppm of fluoride is the standard for drinking water, it should be the most beneficial level for mitotic activity, with some room for adjustment up to 25ppm. I expect to see less mitotic activity in the higher concentrations of fluoride, for it is possible that the fluoride will disrupt the mitotic activity.

Calculations

The first solution to be made was the stock solution of 1000ppm NaF. To determine how much NaF was needed for the solution, the following calculations were performed:

First, the amount of moles in 1 liter of H_2O was calculated:

$$1L\ H_2O \times \frac{1kg\ H_2O}{1L\ H_2O} \times \frac{1000g\ H_2O}{1kg\ H_2O} \times \frac{1\ mol\ H_2O}{18.-148\ g\ H_2O} = 55.5099\ mol\ H_2O$$

Next, using the amount of moles of water in a liter, and given that the count for 1000ppm of Fluoride is needed, the amount of molecules of F^- is calculated:

$$55.5099\ mol\ H_2O \times \frac{6.02x10^{23}\ molecules\ H_2O}{1\ mol\ H_2O} \times \frac{1000\ molecules\ F^-}{10^6\ molecules\ H_2O} =$$

$$3.34x10^{22}\ molecules\ F^-$$

Further conversions give the amount of moles of F^- :

$$3.3417x10^{22}\ molecules\ F^- \times \frac{1\ mol\ F^-}{6.02x10^{23}\ molecules\ F^-} = 0.05551\ mol\ F^-$$

Finally, the grams of F^- needed can be calculated:

$$0.05551 \text{ mol } F^- \quad x \quad \frac{41.99 \text{ g } F^-}{1 \text{ mol } F^-} \quad = \quad 2.331 \text{ g } F^-$$

Therefore, 2.331g F^- must be dissolved in 1 L H_2O to create a 1000ppm NaF stock solution.

To prepare the stock solution, 4.655 g of NaF was measured and dissolved in 2 L of spring water, thus giving us 2.327 g NaF/ L.

Next, the stock solutions for each concentration that is tested is made from an aliquot amount from the 1000ppm NaF stock. The amounts of aliquot 1000ppm NaF stock and spring water for each concentration are listed in Table 1.

Table 1. Stock solutions of variable concentrations of NaF. This table describes the amount of 1000ppm NaF in proportion to the amount of spring water that is mixed to create stock solutions for 0ppm, 1ppm, 25ppm, 50ppm, 75ppm, and 100ppm NaF.

Stock concentration NaF (ppm)	**Aliquot 1000ppm NaF Stock (ml)**	**Aliquot spring water (ml)**
0	0	1000.0
1	1	999.0
25	25.0	975.0
50	50.0	950.0
75	75.0	925.0
100	100.0	900.0

Protocol Listings

Experimental Set-up Protocol:

Materials needed: NaF, spring water, Bouin's solution, small fish bowls, Planaria, scalpel, glass slide, bulb pipet, graduated cylinders, microcuvette.

1) Label 12 small fish bowls with the following labels: 0ppm heads, 0ppm tails, 1ppm heads, 1ppm tails, 25ppm heads, 25ppm tails, 50ppm heads, 50ppm tails, 75ppm heads, 75ppm tails, 100ppm heads, 100ppm tails.
2) Pour 150 ml of each of 0ppm, 1ppm, 25ppm, 50ppm, 75ppm, and 100ppm stock in each respectively labeled bowl. Set aside.
3) With the pipet, transfer a Planaria to a glass slide.
4) With the scalpel cut the Planaria in half along the transverse plane.
5) Place the top half with the head in the bowl labeled "0ppm heads." Place the bottom half with the tail in the bowl labeled "0ppm tails."
6) Repeat steps 3-5 until there are five segments of Planaria in each bowl of each NaF concentration.
7) Take one Planaria from each concentration and head/tail subcategory out of the bowl and transfer each to its own individual microcuvette via the bulb pipet.
8) Fill each microcuvette with Bouin's solution. Close the microcuvette cap and mark it as "Day 0."

9) Repeat steps 7-8 each subsequent day at the same time to collect samples for Days 1, 2, 3, and 4. At the end, there should be Planaria samples from each concentration and orientation from days 0-4 (five microcuvettes total for each respective bowl). This results in 60 microcuvettes all together.

Planaria's regenerative feature is the basic characteristic that will allow us to study how fluoride effects the growth and development of the cells. The totipotent cells that will generate at the spliced ends to regrow the missing half will be stopped in their development in 24 hour intervals. From our previous testing, we observed that it takes the Planaria between four and six days to fully regenerate the body part that is missing; therefore, we took samples every 24 hours for four days. Bouin's solution is a fixative that is often used to preserve tissue. As soon as the Planaria is submerged in Bouin's solution, the cells die and are arrested in whatever phase of mitosis they may be in. Bouin's solution contains various amounts of formaldehyde and picric acid which aid in preservation (Grantham and Walmsley 2015). Samples were left in Bouin's solution until all three trials were completed, and the dehydration procedures started.

Dehydration Protocol:

Materials needed: Bulb pipets, 50% EtOH, 75% EtOH, 95% EtOH, 100% EtOH, xylene, beaker (for waste).

1) Decant Bouin's solution with the bulb pipet from microcuvette, careful to not decant Planaria sample.
2) With the bulb pipet, Add 50% EtOH to the microcuvette until filled.
3) Decant 50% EtOH, and refill the cuvette with more 50% EtOH.
4) Repeat step 3 until 3 total washes of 50% EtOH has been done.
5) Let samples sit in the 50% EtOH solution for 5 minutes.
6) Repeat steps 2-5 with 75% EtOH and 95% EtOH, and 100% EtOH
7) When dehydrated, decant 100%EtOH and fill the microcuvette with Xylene in three washes.

Dehydration of the tissue is an important step in preparing the tissue to be embedded in paraffin. This tissue must be dehydrated because it cannot have any moisture or aqueous solution. Otherwise, the paraffin would not be able to infiltrate the tissue, because it is a hydrophobic compound. The tissue sample must be void of aqueous solutions because of paraffin's property. Alcohol gradients are a very efficient way to dehydrate tissue. Dehydration must occur in gradual steps, which is why it began at 50% EtOH and moved up to 100% EtOH. This is because it ensures the replacement of aqueous solutions in an efficient manner and it does not distort the tissue as much as other methods may do. After dehydration takes place, the alcohol needs to be removed from the sample because alcohol and paraffin do not mix. This next

step is called the “clearing” step. Xylene is the ideal clearing agent because it can be homogenous with both alcohol and paraffin (Wilson). Three washes of each solution must be done in order to ensure that all of the previous solution has been replaced. It is important to note that a large sample should not be left in xylene for more than a week, or else it will become very dry and crack upon slicing. Since the Planaria samples are so small, they can remain in xylene for longer than a week. In addition, disposal of xylene must be handled with care in placed back into its own container; it cannot go down the sink.

Paraffin Embedding Protocol:

Materials needed: Paraffin, bulb pipet, razor, incubator, cryo console, small metal trays, plastic wax molds, forceps, and a pencil.

1) Set paraffin pellets in a plastic cup in the incubator at 70°F degrees. Wait until melted.
2) Decant xylene from microcuvette, careful not to lose Planaria, with the bulb pipet.
3) Pour melted paraffin into microcuvette.
4) Place microcuvettes in incubator for the paraffin to melt again.
5) Decant paraffin.
6) Repeat 3-5 until 3 washes of paraffin have been done.
7) Place Planaria sample on metal tray with the cut side down.
8) Place plastic wax mold on the metal tray. Be sure to write with a pencil on the plastic wax mold which concentration, day, and orientation (head or tail) the sample represents.
9) Fill tray with wax until it reaches the top of the plastic wax mold.
10) Set on the cryo console to cool fast.
11) Once cooled, remove wax block from metal tray.
12) With the razor, cut a trapezoid around the Planaria sample, cutting away the excess paraffin surrounding the trapezoid shape.

The Planaria samples must be embedded in a block of paraffin in order to slice it on the Microtome. The plastic wax molds are designed to fit into a Microtome to be sliced and embedded onto a slide. Cutting the paraffin into a trapezoid shape will allow for a smooth cut.

Slide Preparation Protocol:

Materials needed: Glass slides, glue/water solution, cotton swab, water, bulb pipet, small paint brush, forceps, microtome, and slide heater.

1) With the cotton swab, smear the glue and water mixture onto a glass slide, and place on the hot slide warmer.
2) Place the paraffin block into the Microtome with the shorter side of the trapezoid facing down.
3) Place blade of Microtome close to the edge of the paraffin block.
4) With the microtome set to 20 µm, turn the nob to start slicing. With the small paint brush, keep brushing off the wax sheets produced until you see the sample start to show up on the wax sheet that is cut.
5) Switch the increment to 10 µm and continue to slice the wax into small sheets.
6) Place the wax sheets (with the embedded Planaria sample) onto the prepared slide. Place as many samples on the slide that can fit.
7) Taking the mounted slide off of the slide warmer, run cool water over the slide. Set aside to dry.

H&E Staining Procedure:

Materials needed: Xylene, 100% EtOH, 95% EtOH 75% EtOH, 50% EtOH, water, Hematoxylin solution, eosin solution, 70% EtOH + 1%HCl solution, polystyrene, glass wells, forceps, coverslips.

1) Fill four glass wells each with xylene, 100% EtOH, 95% EtOH, 75% EtOH, and 50% EtOH. Fill one glass well each with hematoxylin and eosin. Fill six glass wells with water.
2) Place the glass slide in the wells in the following order for the following amount of time:
    a. Xylene #1 : 1 min
    b. Xylene #2: 1 min
    c. 100% EtOH #1: 1 min
    d. 100% EtOH #2: 1 min
    e. 95% EtOH #1: 1 min
    f. 95% EtOH #2: 1 min
    g. 75% EtOH #1: 1 min
    h. 75% EtOH #2: 1 min
    i. 50% EtOH #1: 1 min
    j. 50% EtOH #2: 1 min
    k. Water #1: 1 min
    l. Water #2: 1 min
    m. Hematoxylin: 5 min
    n. Water #3: 5 min

o. 70% EtOH + 1%HCl solution: dip (1-2 seconds)
p. Water #4: 5 min
q. Eosin: 10 min
r. Water #5: 1 min
s. Water #6: 1 min
t. 50% EtOH #3: 1 min
u. 50% EtOH #4: 1 min
v. 75% EtOH #3: 1 min
w. 75% EtOH #4: 1 min
x. 95% EtOH #3: 1 min
y. 95% EtOH #4: 1 min
z. 100% EtOH #3: 1 min
aa. 100% EtOH #4: 1 min
bb. Xylene #3: 1 min
cc. Xylene #4: 1 min

3) Lay slide on paper towel facing up to dry.
4) Add drops of polystyrene to the dried slide.
5) Add coverslip at a 45 degree angle.
6) Allow to dry for 24 hours.

H & E is one of the most widely used histological staining procedures that is performed. First, the slide must be exposed to xylene in order to dissolve the wax that was embedded onto the slide. Two washes of each solution is done to ensure proper clearing, hydration, and dehydration. Next, a series of alcohol dilutions are exposed to the slides in order to slowly

introduce water back into the sample, rehydrating it. This needs to be done because the stains that are used are aqueous, and thus an aqueous sample is needed in order to penetrate and stain it. Hematoxylin, the first stain, is used to stain nuclear material a slightly blue color. It is then dipped in a slightly acidic solution in order to get rid of excess staining. Following this comes the Eosin staining, which is used to stain cytoplasmic material and other cell types. It is reddish in color. Both stains have a similar color profile. The Hematoxylin stains the nuclear material a darker, purple/blue color which should be noticeable in a mitotically active cell. Following the Eosin, the slide needs to be dehydrated once more, and cleared with xylene. Polystyrene is used to mount the cover slip onto the slide, thus completing the staining procedure (nationaldiagnostics.com).

Materials and Methods

Selecting a Model System

In our first attempt at designing this experiment, we chose silverside fish as our model system. We chose this at first because they have a vertebrate, and so we would be able to note differences in bone growth by measuring the fish in 24 hour intervals after they are exposed to different concentrations of fluoride. Measuring the fish was difficult, for they didn't stay still in a dish. Despite the difficulties, we were able to measure the fish for the first few days. After two days, all of the fish had died. Not only the experimental fish, but the extra fish that we had in a separate tank of spring water had also died. There are many reasons this could have happened. It could be that the fish are not meant to have a long lifespan, or that the conditions we kept them in were not favorable for their survival. The fish may be too temperamental to take care of under our limited time availability. Rather than ordering another shipment of fish, we decided to try a new model system.

Next, we decided to try using *Daphnia magna*. Daphnia are small crustaceans, and commonly known as water fleas. Its body is covered by a carapace, which is a shell like organ that is made out of mostly chitin and other carbohydrates. Although Daphnia are invertebrates, the effect of fluoride could still be tested by investigating the growth, survival, and reproduction of Daphnia in various fluoride concentrations. When looking at Daphnia under a dissecting microscope, we can see eggs that are stored within the Daphnia. However, we had many complications with Daphnia as well. They seemed to get stuck to the bottom of the fish bowls where the food pellet residue decomposed. We resolved this issue by giving them a diet of only algae, which did not sink to the bottom of the bowl and trap the Daphnia. Handling the Daphnia was clearly damaging the carapace and other organs when observed under the dissecting

microscope. In addition, some eggs would fall out of the carapace, in the cases where it was still intact, making it difficult to accurately count eggs. Overall, maintaining their Daphnia was difficult and we did not have the means to correct this. It was then we decided to try to use Brown Planaria as a model system.

Planaria was observed to be the best model system because they had a longer lifespan than silverback fish and Daphnia. They were also less temperamental and were able to live in various environments and temperatures, so they were easy to maintain. They did not need to be in a very specific temperature, and did not need to be fed often. Their lifespan was long enough to have them survive for the entire trial, and allow us to run multiple trials as well. Planaria's regenerative property made for a good means to study mitotic activity, especially given that they have totipotent cells that have a large capacity for differentiation and mitosis. They regenerated fast and easily because of the large amount of neoblasts spread throughout its body.

Once Planaria were selected as the correct model system, brown Planaria were ordered in bulk amounts through Carolina Biological Supply Company and placed in a large tank of spring water. Spring water was used as the media for them to reside because it had some minerals dissolved into them, making it a livable habitat for Planaria. For nutrients, they were fed egg whites on a weekly basis and kept in a room temperature environment, roughly 21°C.

Experimental Set up

Small glass fishbowls were laid out and labeled as follows: “Heads 0ppm,” “Heads 1ppm,” “Heads 25ppm,” “Heads 50ppm,” “Heads 75ppm,” “Heads 100ppm,” “Tail 0ppm,” “Tail 1ppm,” “Tail 25ppm,” “Tail 50ppm,” “Tail 75ppm,” and “Tail 100ppm.” 0ppm, 1ppm, 25ppm, 50ppm, 75ppm, and 100ppm solutions of NaF were created and 150ml of each concentration solution was poured into its respective bowl. Planaria were individually cut in half along their transverse planes. The half that contained the head was placed in one of the containers marked “Head…” whereas the half that contained the tail was placed in one of the containers marked “Tail…” This procedure was repeated until there were five Planaria samples in each bowl concentration.

Following this, one Planarian sample from each of the 12 bowls were taken out and placed into a microcuvette labeled with its appropriate anatomical structure and concentration. Bouin’s solution, a preservative fluid, is placed in each microcuvette, thus killing the Planarian and arresting its cells in whichever phase it is in. One Planarian sample is removed from the bowl into a Bouin’s solution filled microcuvette at the same time for the next four days. The initial day when the bowls are set up is considered to be day 0 (only a few minutes of exposure to the different concentrations.)

Histological Tissue Preparation

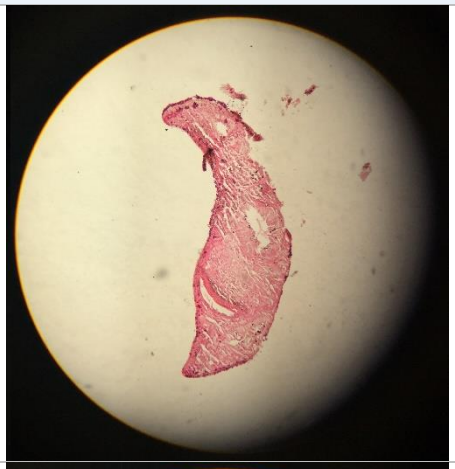
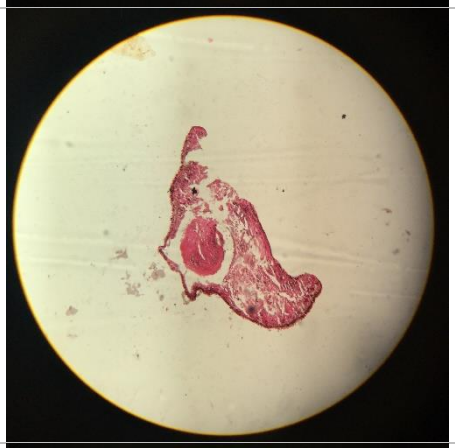
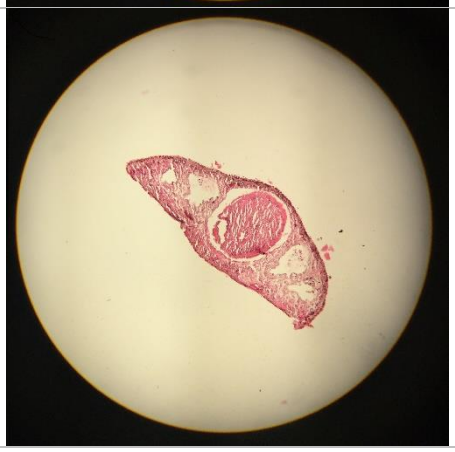
Each Planarian sample is dehydrated through a series of increasing alcohol concentrations from 50% EtOH to 100% EtOH. Dehydration must be done in order to take the water out so paraffin, a hydrophobic substance, is able to penetrate the sample. Xylene is a final wash for Planaria and is used to clear the sample, removing the alcohol and preparing it for paraffin embedding. The sample must stay in xylene for at least 30 minutes to complete clearing, but should not be left in the solution for more than a couple of weeks. Next, the Planaria samples are placed in metal trays with the cut side down, and paraffin is poured over it, forming a block of paraffin which is left out to cool and harden. The paraffin is then trimmed away around the sample, leaving the sample embedded in a smaller trapezoid shape rather than a large square.

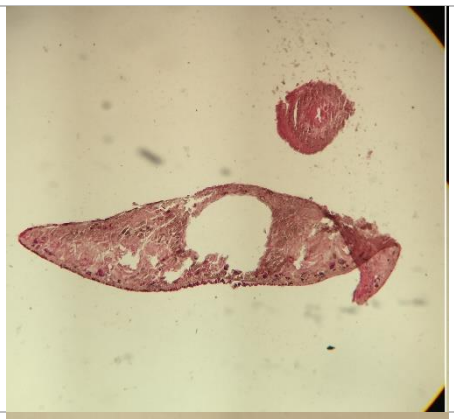
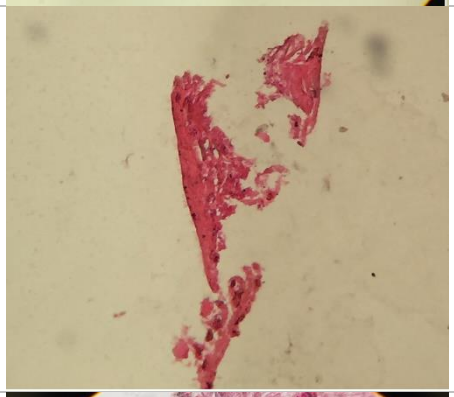
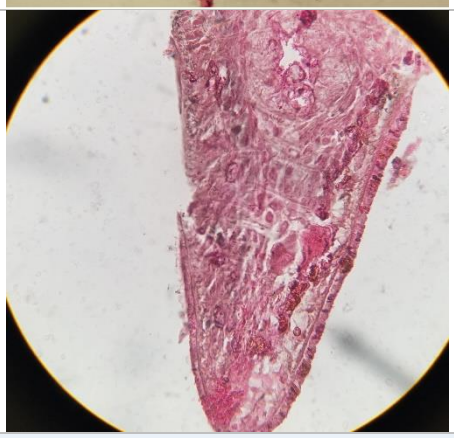
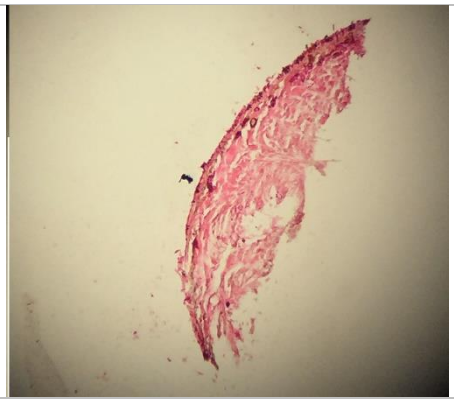
A Microtome is then used to slice the paraffin into 10μm thick slices (roughly the thickness of a red blood cell). As the slices containing the Planarian sample are made, it is placed on a blank slide that has been prepared with a glue and water mix and placed on a slide warmer. The wax will melt immediately upon touching the slide, embedding the sample to the slide. As many slides as possible are placed on each slide. Once complete, the slide is left to cool completely before staining. A standard H & E (Hematoxylin and Eosin) staining procedure was used to stain the sample. The Hematoxylin stains the nuclear material a slightly darker shade than the Eosin, which stains cytoplasmic material. Once dried, each slide is observed under the microscope to observe any trends.

Results

Raw Data

Table 2. Day 0 photographed images of Planaria sections. Pictures of each Planaria sample that could be observed on the initial day of sampling are shown at while looking through a compound microscope on the 10x objective. Descriptions of darkness areas are noted.

Concentration NaF (ppm)	Description	Photograph
HEADS		
0	Darker on lateral edges, dorsal and ventral	
1	Darkness on later edges, dorsal and ventral. No darkness medially.	
25	Dark on entire dorsal side from one end to the other. No darkness on ventral surface	

50	Dark all along ventral surface. Very little darkness on lateral edges slightly dorsal.	
75	Broken sample. Some darkness on later tips on ventral side.	
100	Only half of a sample was visible. Darkness seen on the ventral edge – spotted. No darkness on ventral.	
TAILS		
0	N/A	N/A
1	Partial sample. Darkness on dorsal surface, laterally. Medial dorsal side no darkness.	

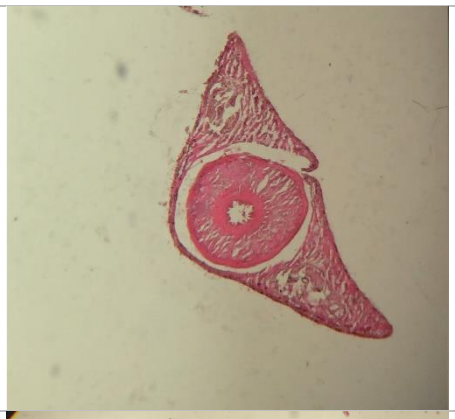
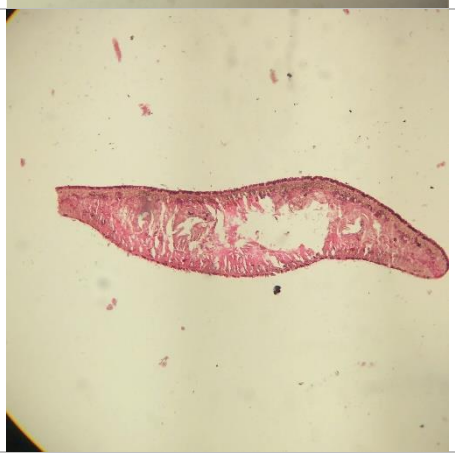
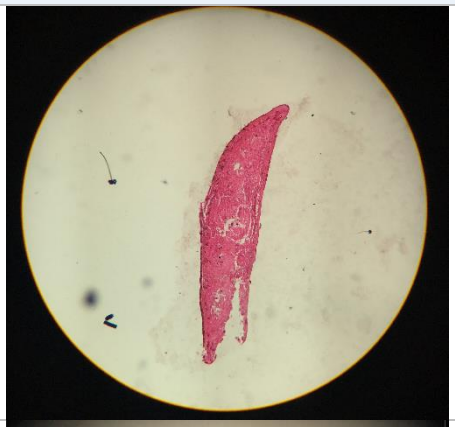
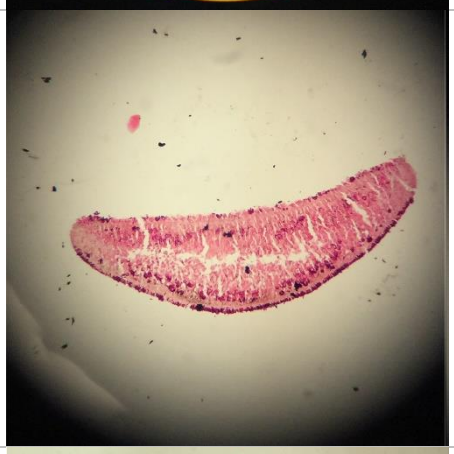
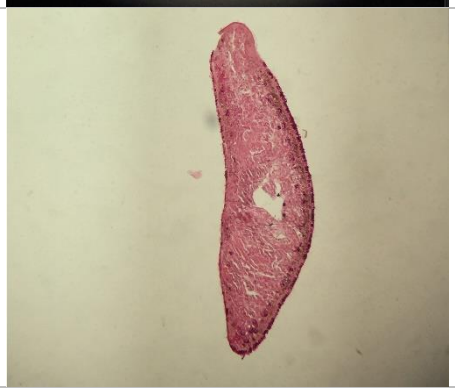
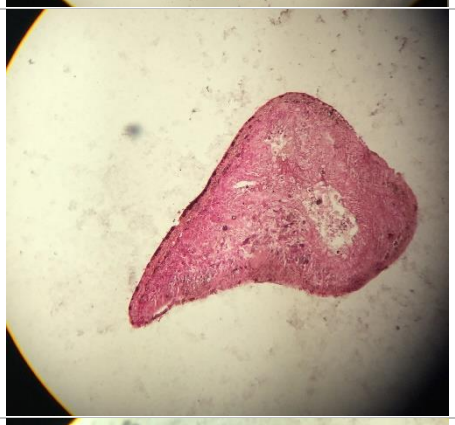
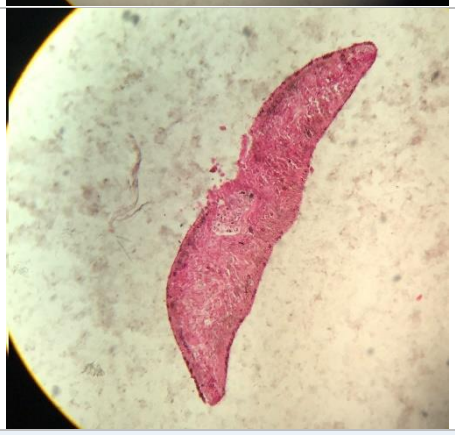
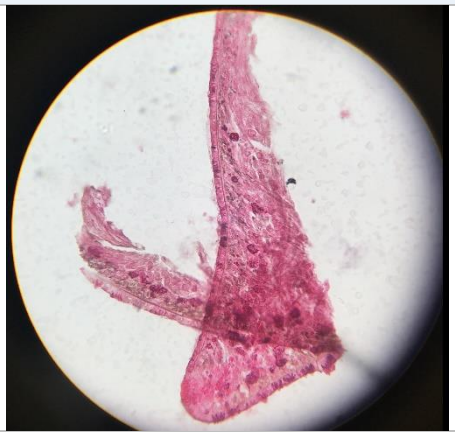
25	Heavy darkness all along dorsal surface. Spotted darkness along ventral surface.	
50	Heavy darkness along dorsal surface, but darkness along ventral surface as well.	
75	N/A	N/A
100	Darkness on ventral side	

Table 3. Day 1 photographed images of Planaria sections. Pictures of each Planaria sample that could be observed on the initial day of sampling are shown at while looking through a compound microscope on the 10x objective. Descriptions of darkness areas are noted.

Concentration NaF (ppm)	Description	Photograph
HEADS		
0	Darkness on dorsal surface only.	
1	Darkness on dorsal of some samples, ventral of others.	
25	Heavier darkness on dorsal, some on ventral.	

50	Broken sample, but can see spotted darkness on dorsal		
75	Darker on ventral		
100	No pattern. Scattered darkness on lateral edges.		
TAILS			
0	Some darkness scattered on lateral edges. Broken sample.		

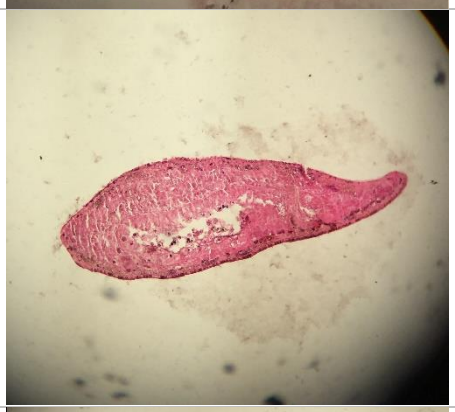
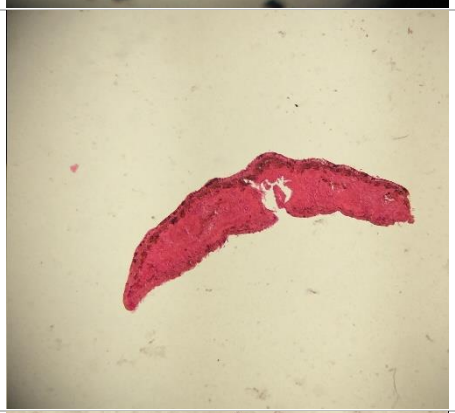
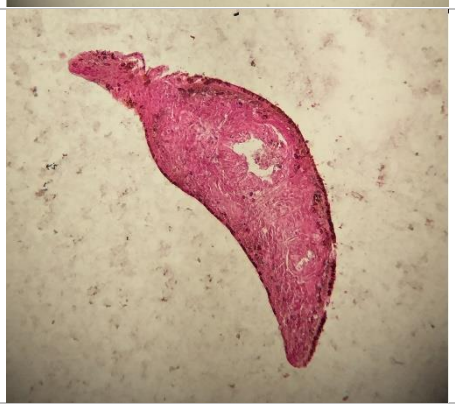
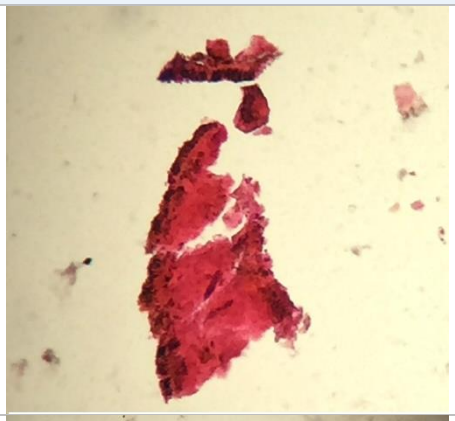
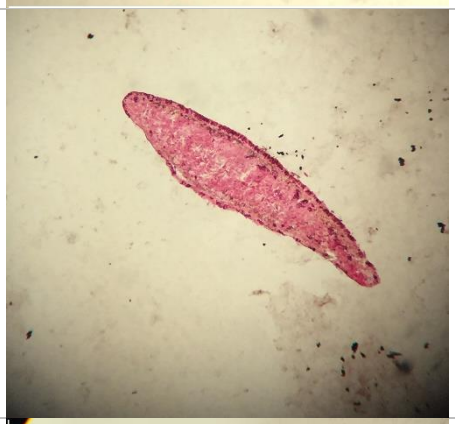
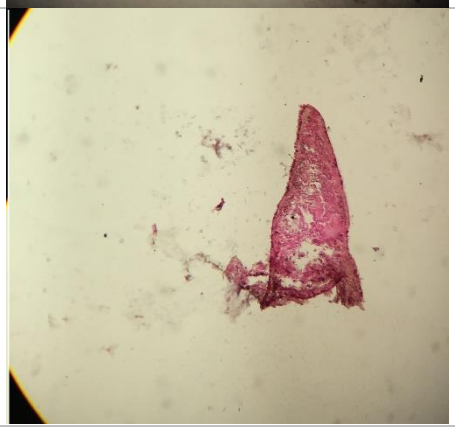
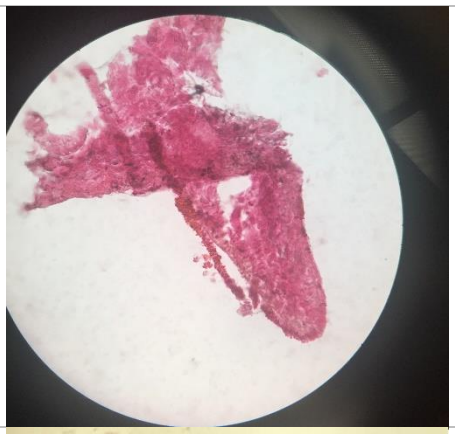
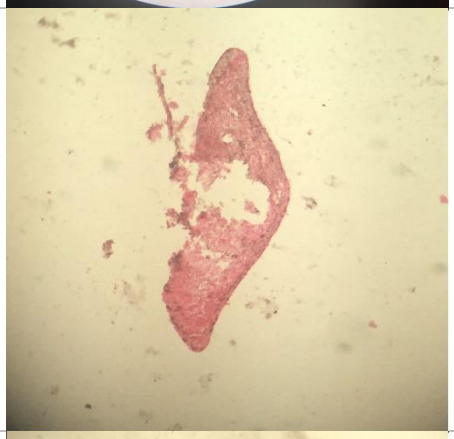
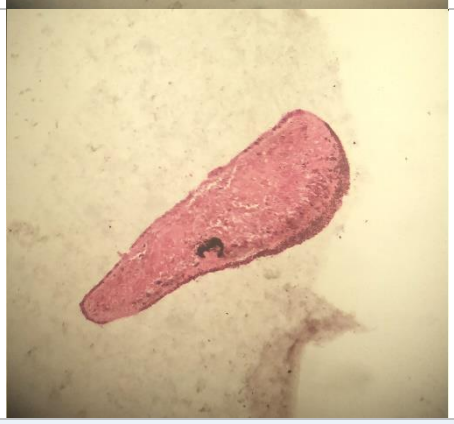
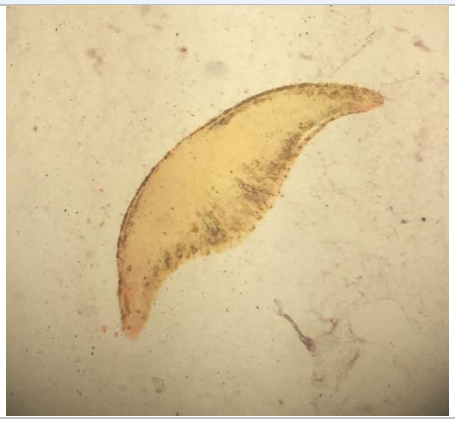
1	Most darkness on dorsal side. Some scattered darkness on ventral side.	
25	Darkness on ventral side.	
50	Darkness on dorsal side.	
75	Most darkness on dorsal side, some on ventral side.	
100	N/A	N/A

Table 4. Day 2 photographed images of Planaria sections. Pictures of each Planaria sample that could be observed on the initial day of sampling are shown at while looking through a compound microscope on the 10x objective. Descriptions of darkness areas are noted.

Concentration NaF (ppm)	Description	Photograph
HEADS		
0	Broken sample. See some darkness on ventral and dorsal.	
1	Darker on ventral side. Some scattered darkness on dorsal side.	
25	Broken sample. Scattered darkness on ventral side.	

50	Heavy darkness on ventral side. Scattered darkness on dorsal side.		
75	No real pattern seen. Scattered darkness; however, not too dark.		
100	Very dark on dorsal side.		
TAILS			
0	Darkness mostly on dorsal. Scattered darkness on ventral.		

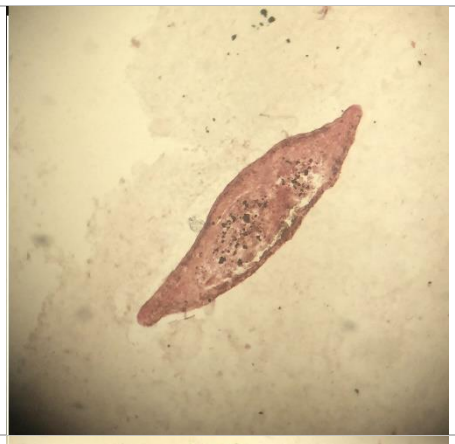
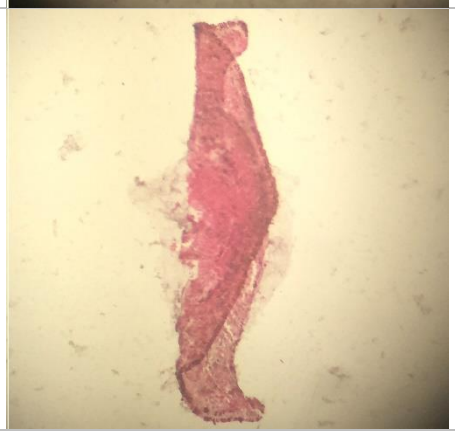
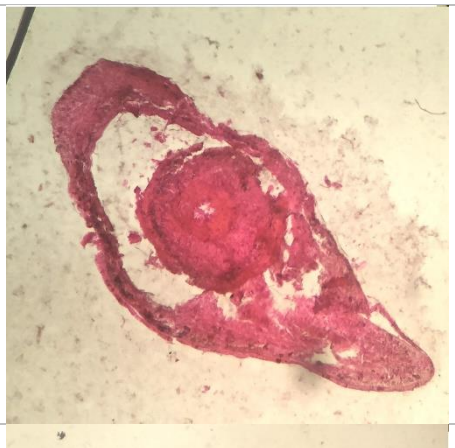
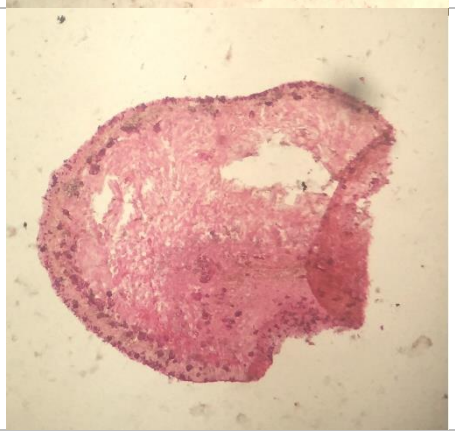
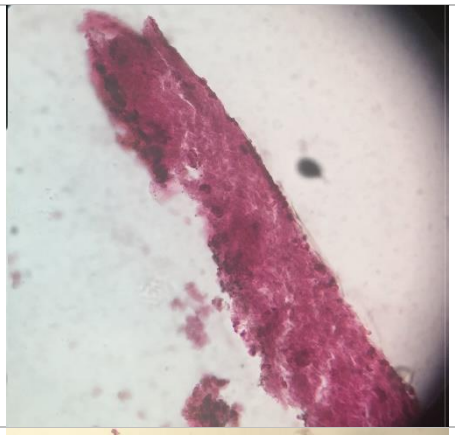
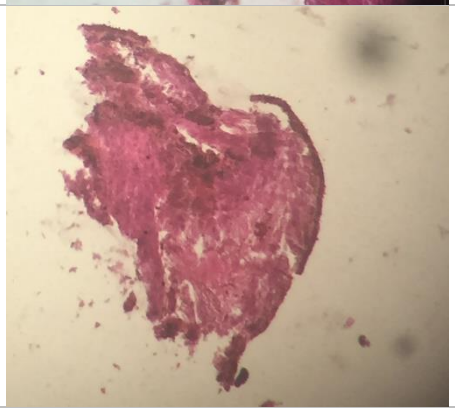
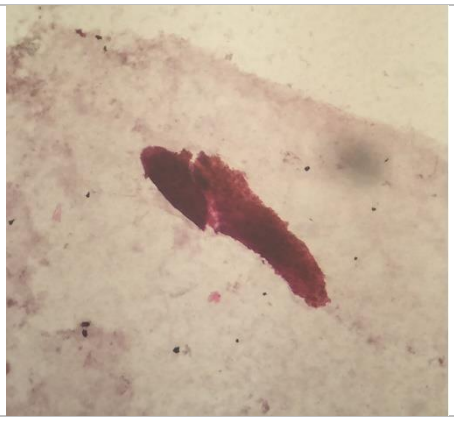
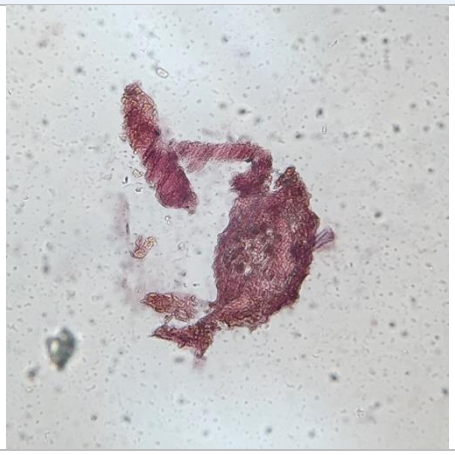
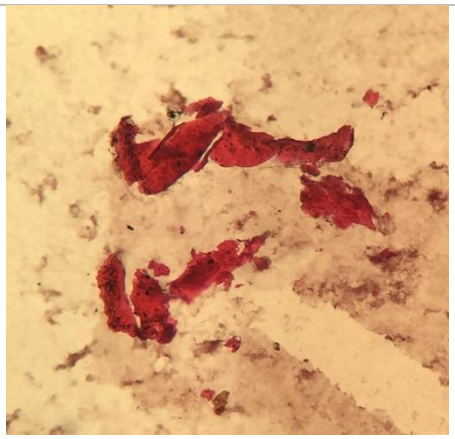
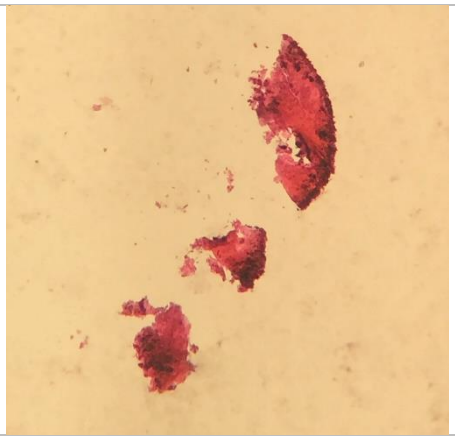
1	Darkness on ventral side.		
25	Darkness on dorsal side.		
50	N/A	N/A	
75	No pattern seen. Very little darkness visible.		
100	Scattered darkness all around the perimeter of the cross section. No pattern.		

Table 5. Day 3 photographed images of Planaria sections. Pictures of each Planaria sample that could be observed on the initial day of sampling are shown at while looking through a compound microscope on the 10x objective. Descriptions of darkness areas are noted.

Concentration NaF (ppm)	Description	Photograph
HEADS		
0	N/A	N/A
1	Broken sample, only see ventral side. Scattered darkness.	
25	Broken sample. Heavy darkness, unable to distinguish whether it is dorsal or ventral.	
50	N/A	N/A
75	Sample indistinguishable.	
100	N/A	N/A

TAILS		
0	Broken sample. No pattern or darkness visible.	
1	N/A	N/A
25	Broken sample. No pattern or darkness distinguishable.	
50	Broken sample. Heavy darkness visible, but unable to distinguish whether it is dorsal or ventra.	

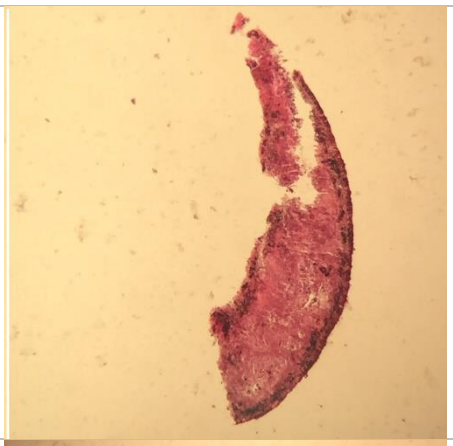
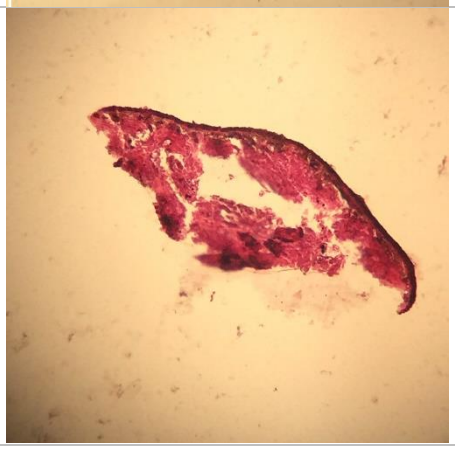
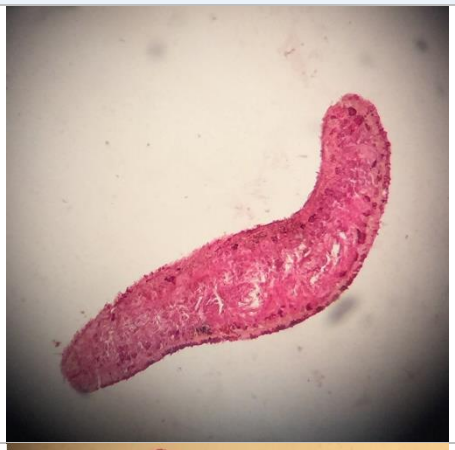
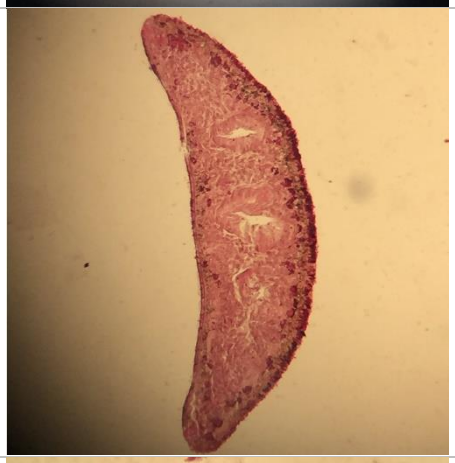
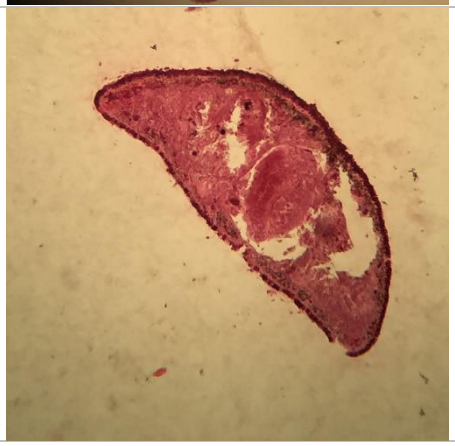
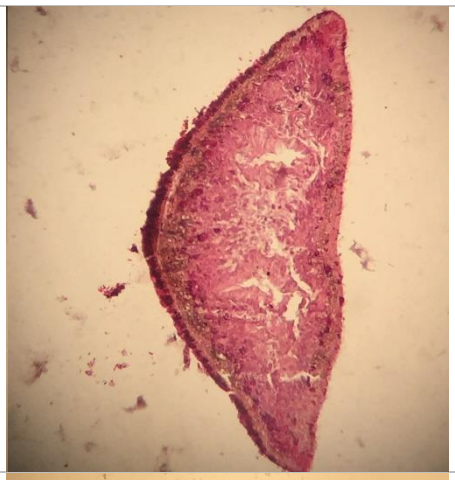
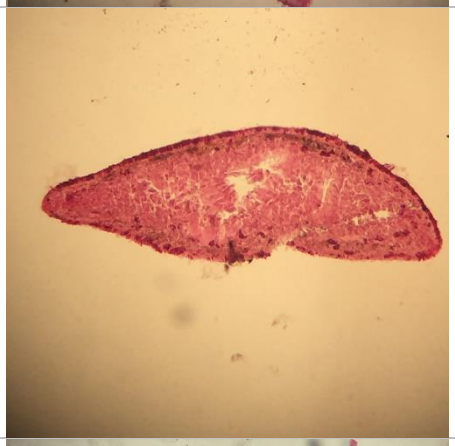
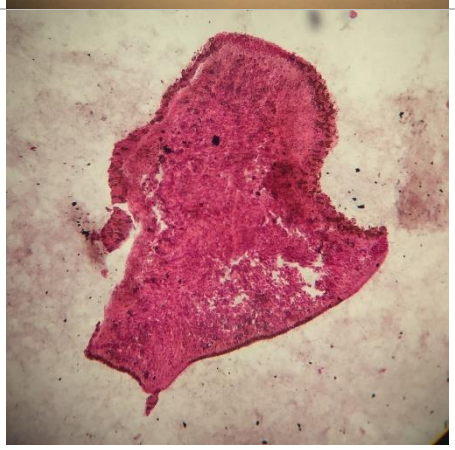
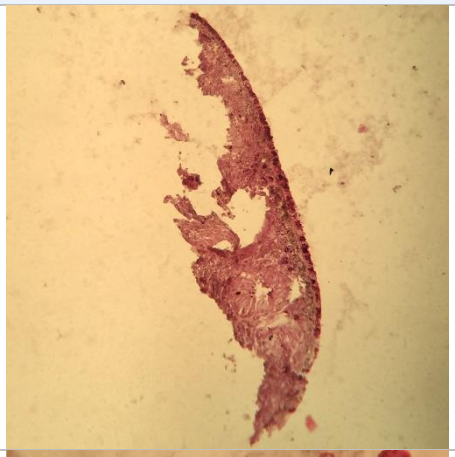
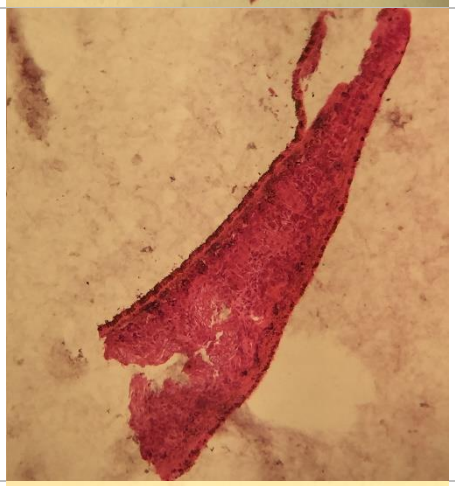
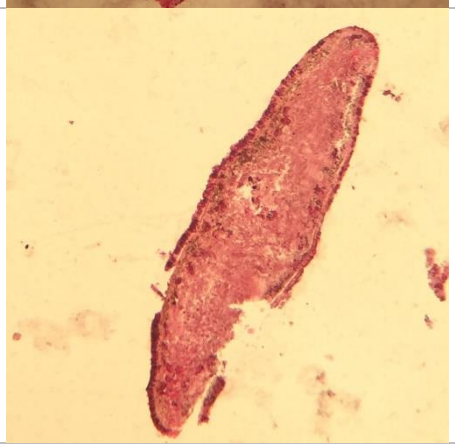
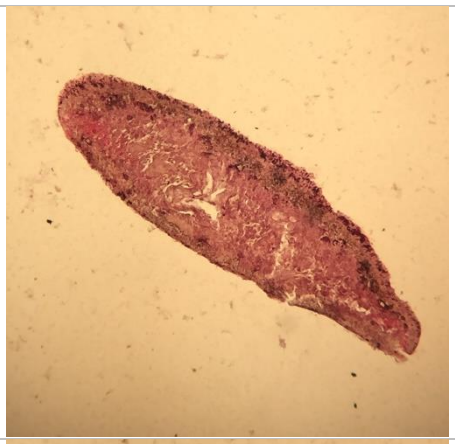
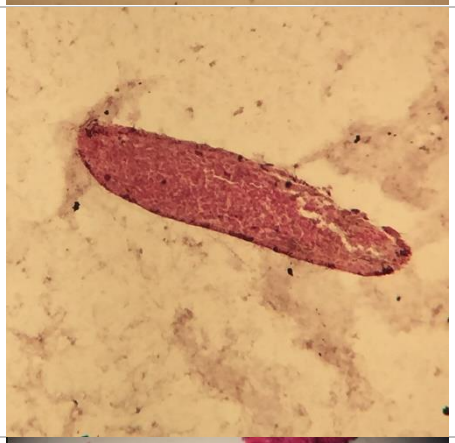
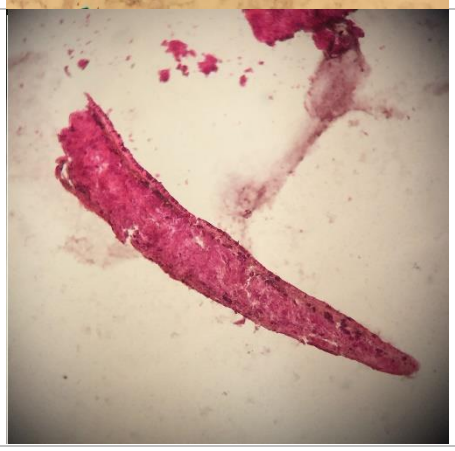
75	Darkness on dorsal side. Scattered darkness on ventral side.		
100	Heavy darkness on dorsal side.		

Table 6. Day 4 photographed images of Planaria sections. Pictures of each Planaria sample that could be observed on the initial day of sampling are shown at while looking through a compound microscope on the 10x objective. Descriptions of darkness areas are noted.

Concentration NaF (ppm)	Description	Photograph
HEADS		
0	Darkness on dorsal side.	
1	Darkness on dorsal side.	
25	Darker on dorsal side. Darkness on ventral side as well.	

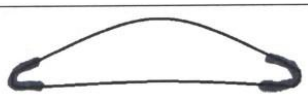
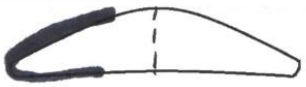
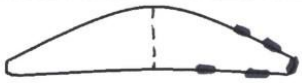
50	Very dark on the dorsal side.		
75	Darkness on dorsal side.		
100	Odd shape, half of the perimeter is dark.		

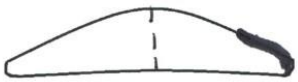
TAILS			
0	Darkness on ventral side		
1	Darkness all along ventral surface. Scattered darkness on dorsal side.		
25	Heavy darkness on dorsal side. Some darkness throughout ventral side.		

50	Scattered darkness on dorsal side.		
75	Some darkness scattered on ventral surface.		
100	Scattered darkness on ventral surface		

Data Analysis

Table 7. Diagrammatic sketch of mitotic activity in Planaria. This table shows diagrams of the Planaria cross section, and shows shading where mitotic activity was observed under the compound microscope.

Concen – tration (ppm)	Day 0	Day 1	Day 2
Heads			
0			
1			
25			
50			
75			
100			
Tails			
0	N/A		
1			
25			
50			N/A
75	N/A		
100		N/A	

Concen – tration (ppm)	Day 3	Day 4	
Heads			
0	N/A		
1			
25			
50	N/A		
75	N/A		
100	N/A		
Tails			
0	N/A		
1	N/A		
25	N/A		
50			
75			
100			

Discussion

When looking for trends in the sketches from *Table 7*, I first looked at in which concentration was there the most mitotic activity. On day 0, the day the experiment was set up, for both the heads and tails sections, the most mitotic activity was seen in concentrations of 25ppm-50ppm. This is somewhat in accordance with my hypothesis that there would be more mitotic activity between 1ppm-25ppm. The results show that the median concentrations of fluoride are resulting in higher mitotic activity. On day 1, for heads, the most mitotic activity was seen at 50ppm, whereas in tails the most mitotic activity was seen in 1ppm and 75ppm. There is a larger range here in day 1 with the distribution of mitotic activity throughout the concentrations. Although there is a broad range, the range is still within the middle three concentrations. For day 2, in heads the most mitotic activity was seen in 50ppm, whereas in tails the most mitotic activity was seen at 25ppm. These results are similar to the pattern that is seen at day 0, where the range of most mitotic activity is in the middle two groups of concentrations.

Day 3 had very few (only two) samples that were available to observe. The rest of the samples were either nonexistent when looking at the slides, or the samples were extremely fragmented and unable to be observed and draw conclusions. Because only two samples were clearly visible from all of the day 3 samples, no conclusions can be drawn from this data. One possible reason for the samples not being displayed on the slide is that the samples lifted off of the slide during the staining procedure. Another reason could be that the Planarian sample was lost somewhere in the dehydration or paraffin embedding procedures. Whatever the cause for this gap in the data, I am unable to include data from day 3 in my evaluation.

Finally, Day 4 showed that for the heads, there was relatively consistent mitotic activity throughout all concentrations. All samples displayed roughly the same amount of mitotic activity

for all of the samples in the heads section. It is possible that at this point, development has progressed far enough where the fluoride has performed the maximum effect it is capable of; now the mitotic activity has normalized throughout all of the concentrations as the totipotent cells become more differentiated. For the tails section in day 4, we again see consistent mitotic activity throughout 0ppm through 25ppm of fluoride.

Although in many of the days we see that there is more mitotic activity in the middle range of concentrations, there is not a consistent concentration in which there is the most mitotic activity; rather, there is only a range (see *Table 8*). This range is roughly between 25ppm-50ppm of fluoride where we see the most mitotic activity in most of the days, whereas in my hypothesis I theorized the most mitotic activity would range between 1ppm-25ppm of fluoride. Despite the slight correlation, I do not believe this correlation is enough to infer causation; that is, I do not believe there is enough evidence here to conclude that fluoride enhances mitotic activity at the optimal level of 25ppm-50ppm. In order to confirm this statement, more trials need to be conducted in order to see if these results can be duplicated, and that it did not just occur by chance. In addition, the fact that day 3 has very little data potentially gives us a false pattern. The additional trials will be able to fill in the gaps that existed in this current trial.

Table 8. Concentration of most mitotic activity in 24 hour intervals. This table shows which concentrations of fluoride displayed the greatest amount of mitotic activity in each of the days the experiment was conducted, running from day 0-4.

	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**
Conc. of most mitotic activity (Heads)	25-50 ppm	50 ppm	50 ppm	N/A	All
Conc. of most mitotic activity (Tails)	25-50 ppm	1 & 75 ppm	25 ppm	N/A	25 ppm

When comparing the total mitotic activity that occurs each day, we can see that the most mitotic activity in all concentrations seems to be on day 1 and day 4. In day 1 and 4, almost all of the samples in every concentration, for both heads and tails subgroups, have roughly half of their epithelium marked as mitotically active. This may occur in day 1 because the Planarian is in the early phase of healing the wound. Within the first 24 hours, the neoblasts proliferate in a great amount to seal the wound and begin repair. This division must occur rapidly to avoid bacteria or other pathogens entering the Planarian body proper. Therefore, it is expected that initially there is a high rate of mitotic activity. At day 4, we notice high mitotic activity as regeneration is almost complete. I believe at this point the cells differentiated by some amount, allowing them to proliferate in their destined cell line and complete the formation of the missing fragment. When looking at the head and tail subgroups, and comparing the same concentrations within (example: Day 0 comparing Heads at 0ppm to Tails at 0ppm, Heads at 1ppm to Tails at 1ppm, etc), there was no correlation observed between the two.

Overall, the only patterns that were seen was that of a similar range of concentrations having the highest amount of mitotic activity throughout the 5 days of collecting samples. There may be a correlation between that mitotic activity and it being an effect of fluoride; however, more trials need to be performed in order to fill the gaps in the current data and confirm that there is a trend. Further investigation is needed.

Future Endeavors

Accounting for Error

When the experiment was first conducted, we had to go through much trial and error to find a favorable model system. Now that the trials and processing has been done, I must go back and refine what caused problems as the experiment was executed. First, this report only represents one trial worth of data. Given that it was just one trial, being a very small sample size, means that it is susceptible to error by random chance. Other trials must be included to confirm the trends that had been observed. Two more trials were performed; however, the amount of time and resources I had did not allow me to process those two trials within the academic year in which all processing was done. More time is needed to complete this processing, which requires a considerable amount of time to complete. In addition, having more assistants to help process the samples would have sped up the process. Given that I did not have any assistants as I processed the samples, little progress was made. I hope that students in the future will continue where I left off, process these other two trials, and compare it to what I have found with the first trial.

It is important to process the other trials because there are many gaps in my current data. In many slides there were no samples on the slide to be observed. One reason for this is from samples being lost somewhere along the process of dehydration or embedding in paraffin. Since the Planarian samples are so small, transferring them to and from different vessels must be done with care and attention. If not, samples will be lost, which is what has occurred in a few groups resulting in gaps of data. Another reason for gaps in the data is from samples of cut Planarian falling off of the slides during the staining procedure. When cutting the paraffin-embedded Planarian samples and placing them on the slide, I had included roughly 15 cuts per slide. In

many of the slides, after I had completed the H & E staining, very few or even no samples remained on the slide. Multiple factors could account for this error. One could be that the slide was not dehydrated enough before beginning the H & E procedure. When the slide is set aside to dry after the wax sheet of the sample is added, it must be completely dehydrated before beginning the H & E staining, or else it may cause the sample to lift off during the procedure. This is one possibility of why the samples came off the slide during the staining procedure. Another possibility is that they samples slid off because the slides were held vertically in the staining solutions, due to the design of the glass wells. In order to correct this, we could attempt to place the slides flat horizontally in the staining solutions. Although this will not allow us to run as many slides in one cycle, it may have a better rate or retaining the samples.

Revisions

In order to enhance the data, much more experimentation needs to be done. Like mentioned previously, all three trials of Planaria need to be processed so they can all be compared for consistency and precision. It will give the data more validity if we are able to reproduce the same results for each concentration and note similar patterns. Since we do not have any other trials to compare this one with, it is difficult to assess if the results are influenced by fluoride or by pure chance. This will also allow us to do statistical analysis between the trials and concentrations. To narrow down the study, samples can be taken on an hourly basis rather than 24 hour periods. Future experimentation would involve taking a sample every four hours for 24 to 48 hours to track the development at closer intervals. This may give us a clue to pinpoint when exactly the regeneration pattern changes between the different concentrations. We performed one trial on an hourly interval. After 72 hours of the Planaria regenerating, we started to take samples every 4 hours for the next 24 hours. We started after 72 hours of regeneration because we

believed the significant transition to fully regenerating the missing body part occurred within the transition between day 3 and 4. Again, although the trial was run, the samples are still submerged in Bouin's solution and are awaiting processing.

A control experiment also needs to be run in order to determine if Fluoride itself is what is effecting the mitotic activity of the neoblasts. NaF was the salt used to introduce different fluoride levels into each solution. As a control experiment, the same procedures must be performed using a neutral salt such as NaCl. The results of this study could possibly tell us if the Fluoride ion itself was making a difference, or if it was a result of a salt. It is possible that by having any salt added to the solutions, whether it is NaF or NaCl, will have an osmotic effect that alters the mitotic activity.

In addition, we must go back to our original experimental set up and evaluate it for accuracy. Mainly, we must make the different concentrations of solution, and test the solutions themselves to measure exactly how much fluoride is in them. We must evaluate if the actual fluoride concentration is equal to what we claim it is. The NaF was mixed with spring water, not distilled water, in order to make solutions. Given that fluoride is often added to drinking water in the United States, it is possible that the actual fluoride level in the solutions is higher than stated (Petersen and Lennon 2004). One such method is to use an ion-selective electrode, which is an electrode that can specifically measure a certain ion (Charles, Battaglia, Chang, Daniel 1980). Fluoride ion selective electrodes exist; however, some believe they may not produce the most reliable results if there are many other negatively charged ions in the solution as well (Guha, Saha 2010). In the case of spring water, there may be a good chance that the fluoride ion selective electrode will work in an efficient manner.

After performing these additional experiments, enhancements can be made to the current protocol. For example, we may wish to switch to a different staining procedure so that we are

more able to see nuclear structures. If we are able to specify between different phases of mitosis, then that gives another parameter in which we can compare the samples. The Giemsa HCl staining method could be a method to stain the nucleus in a different way. The staining procedures explain how it is used to dye nuclei in bacteria (Herr 1979). Further investigation can be done to determine if this staining procedure would be suitable for Planaria.

In the event that there is enough time to look further into the samples, we can count the amount of cells that are mitotically active. This would require a high resolution compound microscope. A microscope that is able to take high quality images would be preferable in order to amplify the image and be able to count the cells. With this set up, we can get a numerical value of mitotic activity, thus obtaining quantitative results rather than qualitative. If we have quantitative results, statistical analysis can be performed in order to determine the significance of the data.

Resources

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