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# Production of Reactive Oxygen Species in H.exemplaris from Cadmium and Copper Exposure

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## Abstract

Cadmium and Copper are both heavy metals of concern and toxic environmental contaminants. Due to copper's excellent electrical conductivity, its most common use is in electrical equipment. Cadmium on the other hand, is not essential, although it is used majorly in batteries. Contamination with cadmium and copper metals stems mainly from anthropogenic activities but other natural sources also exist.

Tardigrades are microscopic aquatic animals renowned for their tolerance towards extreme environmental conditions, this study is to investigate their tolerance towards heavy and how to ameliorate these effects with an antioxidant (Selenium). Antioxidants are substances that may protect the cells against the effects of free radicals, they are intimately involved in the prevention of cellular damage: the common pathway for cancer, aging, and some diseases. We hypothesized that H. exemplaris has a low tolerance to a high concentration of toxicants and if Reactive Oxygen Species is released, some enzymatic pathways will be altered, but a certain concentration of Selenium will abate this effect, confirming the antioxidative capacity of Selenium.

As was observed, cadmium and copper solutions increased Tardigrade sensitivity to oxidative stress as well as significantly reduced metabolism was observed. The produced reactive oxygen species was confirmed with fluorescence microscopy.

Treating the exposed tardigrades with 0.5ppm of selenium using sodium selenite counteracted the cadmium and copper induced reactive oxygen species produced. The antioxidative enzymes produced were quantified using various assay methods. In conclusion, from an environmental perspective, selenium is a relevant antioxidant using the appropriate dosage.

**Keywords:** Cadmium, Copper, Reactive Oxygen Species, Antioxidant, Enzyme Assay

## Introduction

Industrial revolution which took place between the 18th and 19th century was responsible for the majority of the current environmental contamination and ecological disruption of the aquatic ecosystem. As a result of anthropogenic disturbances and urbanization, there are huge discharge of contaminants like, pesticides, pharmaceutical waste, detergents, sewage and heavy metals.

Heavy metals are non-photodegradable and non-biodegradable, these characteristics of heavy metal makes their toxicity highly persistent consequently having an easy environmental bioaccumulation1,2. It is important to effectively monitor the accumulation of heavy metals in aquatic ecosystem3 because there is a tendency for the transference and biomagnification of heavy metal through food chains, which would consequently be a threat to public health4. The presence of heavy metals such as chromium, mercury, lead and copper, even in trace concentrations, in soil, water and atmosphere can cause serious health implications. Heavy metals are naturally emitted into the environment through, volcanic eruptions, earthquake and other natural hazards but the quantity of heavy metals released through mobile and stationary sources exceeds the natural rate of emission5. In recent times, most industrial activities make use of metals, for machines, utensils, walls and a variety of other uses. Cadmium and copper are two of the more commonly used heavy metals6. ATSDR 20127 labelled cadmium (Cd) as a heavy metal of very great concern due to its different known effects.

Cd is highly water soluble; this characteristic increases its bioaccumulation in aquatic organisms8 consequently, posing threats to their existence8,9. In the studies carried out by Wang et al10; Mao et al11 and Liu et al4 on crustaceans and aquatic species, it was discovered that Cd reduces growth and survival. It furthermore results to a decline in population growth.

Copper is known to be an essential micronutrient to marine organisms as well as a toxicant when exceeding a critical concentration12. The toxicity of Copper is directly related to the copper concentration that is bioavailable13, rather than the concentration of dissolved copper in solution13. Chronic

exposure to Cu can lead to adverse effects on survival, growth, reproduction as well as alterations of brain function, enzyme activity, blood chemistry, and metabolism. The World Health Organization (WHO) has set 3ppb as the maximum limit allowable for Cadmium in freshwater while the maximum allowable limit for Copper is 2ppm14. Approaches to minimize the natural and anthropogenic exposures to these heavy metals are important since they are non-biodegradable or photodegradable. Some of these approaches include herbal absorption of heavy metal deposit to neutralize the soil. This method was confirmed by Singh et al15 in their study explaining how the use of herbal medicine has become the major form of treatment in developing countries in recent years. Substitution of elements involved in industrial or mining activities can also reduce exposure to these metals. In jewelry making, for example, pure silver can replace the use of silver-cadmium, zinc, nickel, tin can also replace cadmium in plating16. According to literature, these metals form a serious hazard to the public health and a threat to most life forms. Studies have shown Copper to inhibit osmoregulatory enzymes and inducers of oxidative stress, while Cadmium inhibit cell proliferation due to its carcinogenicity. Human genotoxicity and carcinogenicity due to exposure to insecticide represent a prominent public health hazard.

Tardigrades also known as Water bears were first discovered in 1773. Phylum Tardigrada are minute invertebrates belonging to the supertype Articulata. They can be found all over the Earth and can inhabit remarkably diverse environments (soil, water bodies, mountain tops). Water bears are small, cylindrical invertebrates, up to 2.1 mm in length, the tardigrade body is covered with a flexible cuticle, which is smooth or covered with gibbosities, spines or plates. They are divided into five segments, with the first segment being the head and the next four each have one pair of unsegmented legs ending in claws. Their internal structure includes a complete digestive system that feeds on algae, bacteria, fungal cells and other tardigrades; a well-developed nervous system consisting of rings around mouth (brain) and an abdominal chain with segmental ganglia. They also have various sensory organs like papilla, chemoreceptors and eyes. The reproduction can be dioecious and bisexual; fertilization can be external or internal. The eggs are covered with an additional shell, the smooth or ornamented chorion, and are laid directly into the environment. Terrestrial species can be found in mosses, lichens and soil where they are threatened by drying. In this situation, terrestrial species need a thin water film around their bodies in order to stay active. Formation of tun help to control the drying up of the internal and external organs17.

Selenium is an antioxidant that prevents the formation and/or removes oxidative stress. It plays an important role in reducing the damage caused by reactive oxygen speccies (ROS) to the cell18. Selenium exist as organic and inorganic forms. The organic form of selenium is highly bioavailable and improves growth and antioxidative capacity19,20. Environmental Protection Agency (EPA) has set a maximum contaminant level (MCL) at 0.05 mgL-1 or 50 ppb for environmental selenium. This value is set based on the best available science to prevent potential health problems. Some states, however, may set more stringent drinking water MCL for selenium than EPA21.

Considering what is known about selenium, we postulated that when Reactive Oxygen Species (ROS) is released, some enzymatic pathways will be altered, but a certain concentration of Selenium will abate this effect, confirming the antioxidative capacity of Selenium. Thus, the objectives of this study were to evaluate the toxic effect of cadmium and copper in the formation of free radicals, cytotoxic stress and perform enzymatic assay to determine the effect of the exposure on Superoxide Dismutase (SOD), catalase (CAT) and acetylcholine (Ach).

## Materials and Methods

One hundred grams of Copper (II) chloride hydrate, puratronic, 99.999% and 100g Cadmium chloride hydrate, 99.99% was purchased from VWR International (PA USA).

Image-IT™ LIVE Green Reactive Oxygen Species Detection Kit, for microscopy was purchased from Thermofisher, Life Sciences. 10mM Stock solution of dye was prepared by adding 50µl of DMSO to one vial of carboxy-H2DCFDA (275µg), the solution was vortexed until the powder was completely dissolved.

### Tardigrade culture

We selected adult Tardigrades for further testing, and they were cultured in deionized water to simulate their natural environment. Following the culturing, we incubated the plates at room temperature.

## Heavy Metal Preparation

### Copper

Stock solution (2ppm) was prepared with 2mg of Copper (II) chloride hydrate in 1000ml of deionized water and serial dilution was carried out to get the other concentrations; 1ppm, 0.8ppm, 0.6ppm, 0.4ppm, 0.2ppm, 0.1ppm and 0.05ppm

### Cadmium

Stock solution (1000ppb) was prepared with 1mg of Cadmium chloride hydrate in 1000ml of deionized water and serial dilution was carried out to get the other concentrations; 500ppb, 250ppb, 100ppb, 50ppb, 5ppb, 2.5ppb, 2.0ppb, 1.5ppb, 1.0ppb, 0.5ppb, 0.1ppb, 0.05ppb and 0.025ppb

### Toxicity Testing

Prior to cadmium and copper exposure, single tardigrades were collected from the stock culture under microscope22 using an ordinary laboratory pipette with plastic tip. We transferred the tardigrades to glass petri dish containing deionized water.

We then assigned tardigrades into groups of ca. 50 in each plate and exposed to 2 ml of a given cadmium solution. The cadmium concentration in these solutions ranged from ~0.025ppb to 500ppb (500ppb, 250ppb, 100ppb, 50ppb, 5ppb,

2.5ppb, 2.0ppb, 1.5ppb, 1.0ppb, 0.5ppb, 0.1ppb, 0.05ppb and 0.025ppb) for periods of 2, 4, 6 and 24h and 2ml of copper solution was exposed to the experimental animal at various concentrations ranging between 0.05 ppm and 2ppm (0.05, 0.1, 0.2, 0.4, and 0.6ppm) at 2, 4, 6 and 24h.

Based on preliminary experiments, reactions that occur between 5 to 6 hours remain fixed, therefore 6 hours seemed sufficient to allow toxic effects of the cadmium and copper to occur.

## ROS Assay

Immediately following the exposure, 5 µl of the dye was added to the plate and incubated for 30 minutes at 220C. After incubation, we transferred the tardigrades with a Pasteur pipette into 2-ml glass centrifuge tubes. They were washed by allowing the tardigrades to gravity settle into a pellet, removing the supernatant, adding 2.0 ml of deionized water, gently mixing the tardigrades by creating bubbles with a Pasteur pipette and repeating the process for a total of three washes. We then transferred 2ml of the washed tardigrades (~45-50 tardigrades) to a glass petri dish.

Images were acquired using a Nikon Ti2 Eclipse inverted microscope with 4x objective lens and DS-Qi1Mc camera. Figures 1a-e are the fluorescence image of copper-exposed organisms, while figure 1f is the control plate. Figures 2a-f are the fluorescence image of cadmium-exposed organisms, while figure 2g is the control plate. Image capture was automated in Nikon NIS-Elements AR software to capture images of the tardigrades. The microscope software stores images in Nikon nd2 format. To begin analysis, we converted the file format to TIFF images with the filenames identifying the concentration and duration of exposure23.

Interestingly, the fluorescent image seemed to be observed in almost all the concentrations, though the brightness is greatly reduced (Figure 3 a-b) and this can be explained by the research conducted by Mittler24, which stated that ROS are essential for promoting normal cellular processes, as opposed to having a toxic effect on life.

Hydrogen peroxide 3% was introduced to the Tardigrade as a positive control and a very bright fluorescence was observed after 2h. (Figure 4).

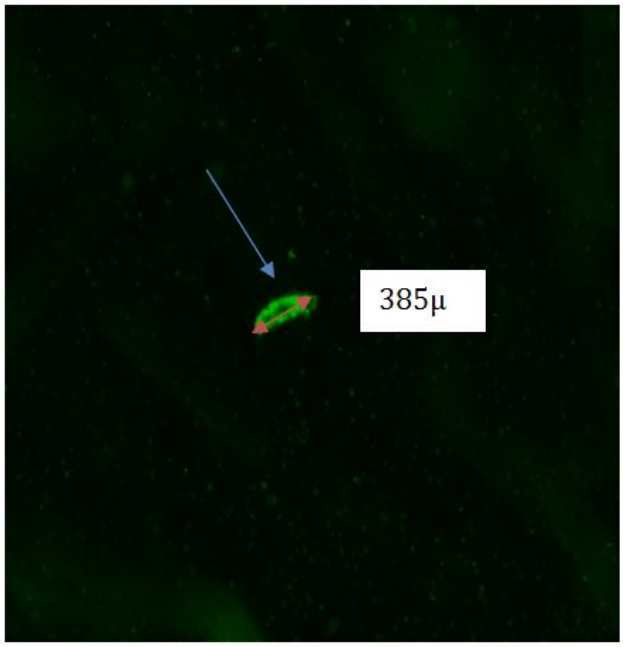



**Fig 1a:** Fluorescence at 0.4ppm of Cu after 4hr Mg(4X).

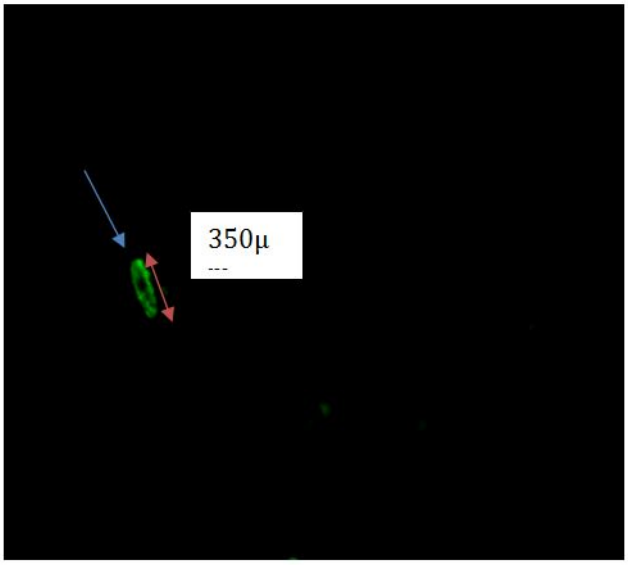



**Fig 1b:** Fluorescence at 0.4ppm of Cu after 6hr Mg (4X).

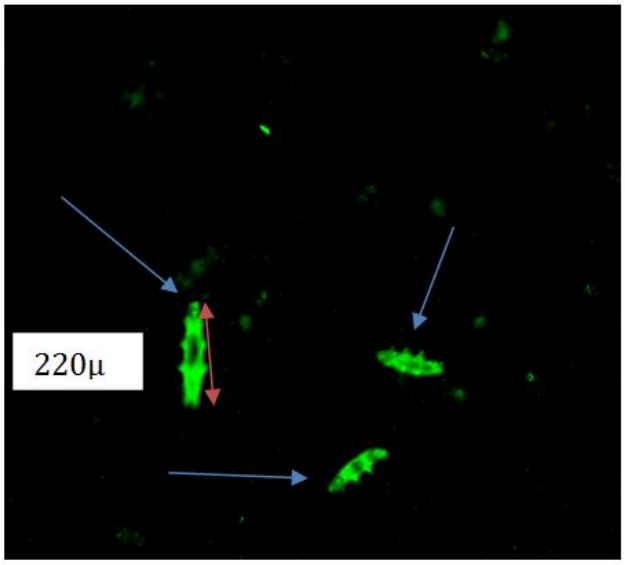



**Fig 1c:** Fluorescence at 0.4ppm of Cu after 24hr Mg(40X).

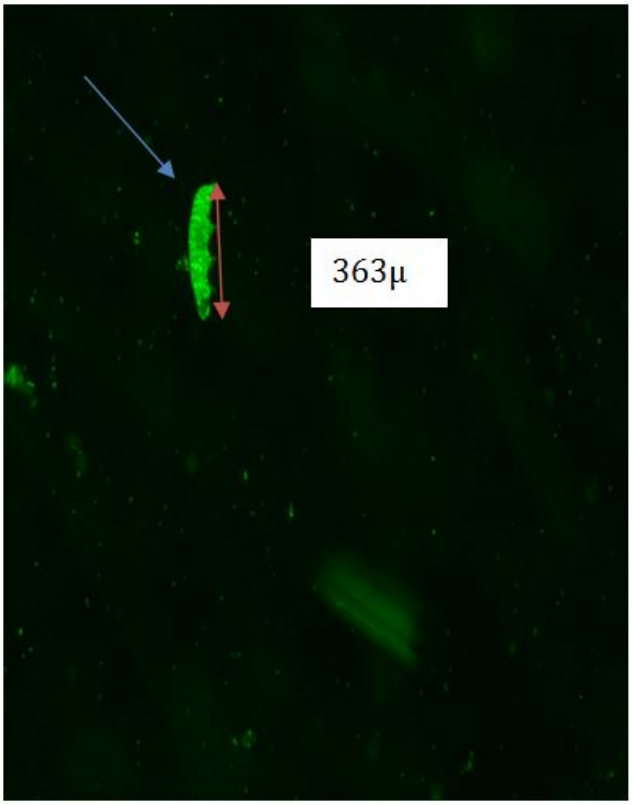



**Fig 1d:** Fluorescence at 0.6ppm of Cu after 4hr Mg(4X).

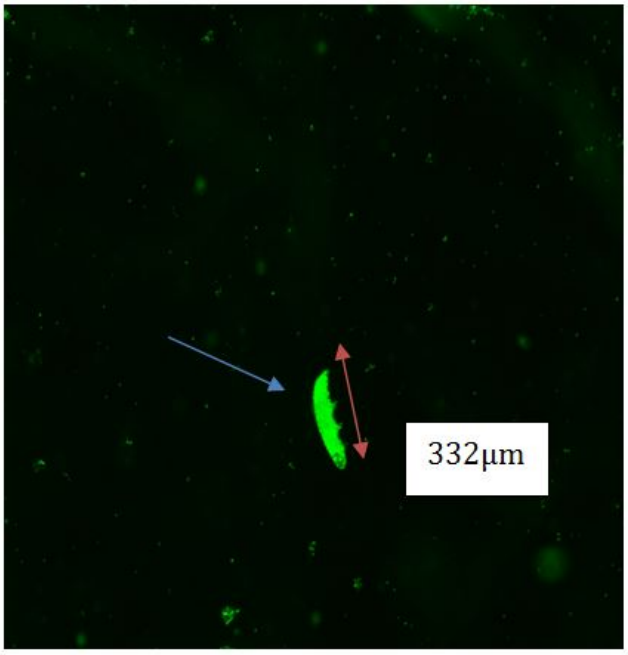



**Fig 1e:** Fluorescence at 0.6ppm of Cu after 6hr Mg(4X).

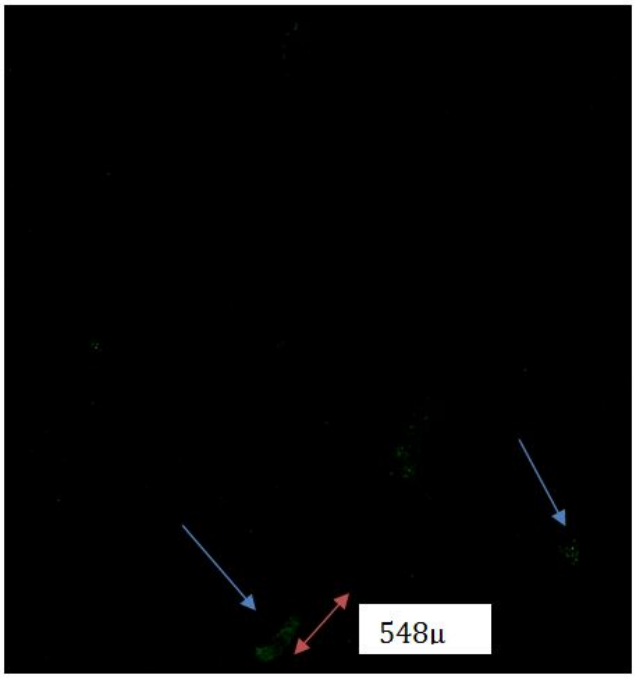



**Fig 1f:** Copper control, No Fluorescence Mg(4X).

Figure 1: Exposed to Copper concentrations, the metabolic activities of tardigrades were observed, with activities decreasing, over time, the morphology was also noticed to be affected as the body size reduced as the exposure time increased. (A) Fluorescence was produced after 4hr of exposure to 0.4ppm. (B) The fluorescence increased after 6hr and (C) highest after 24hr when the metabolic activity was down to about 20%. (D) Exposure to 0.6ppm for 4h with a high fluorescence when compared with the control (F), interestingly, the fluorescence kept increasing and at (E) 6hr, the metabolic activity was down to 20%.

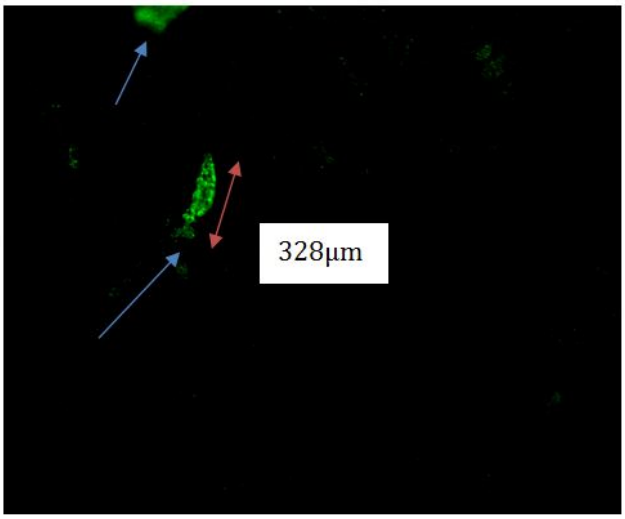



**Fig 2a:** Fluorescence at 0.5ppb of Cd after 6hr Mg(4X).

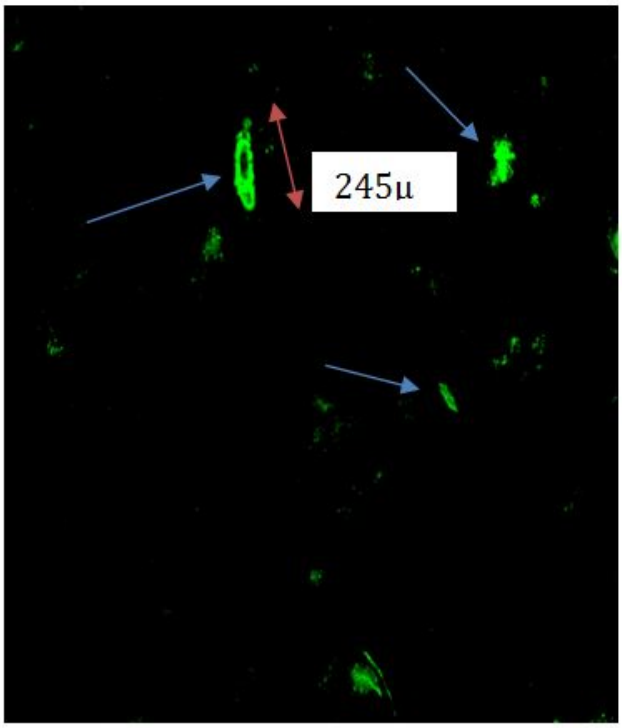



**Fig 2b:** Fluorescence at 0.5ppb of Cd after 24hr Mg(40X).

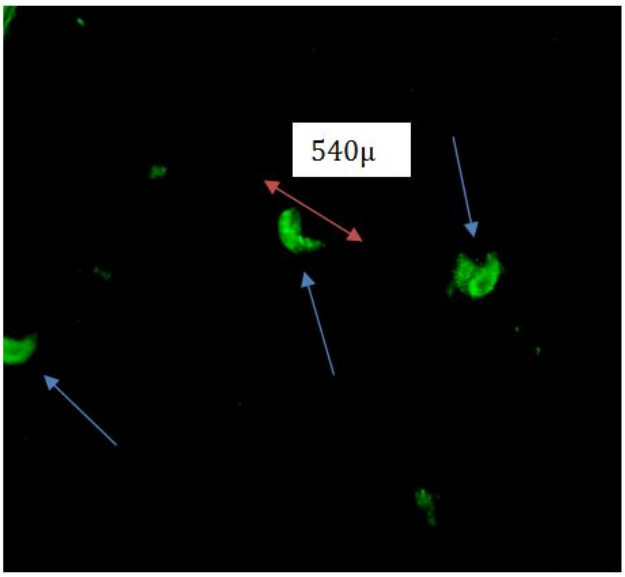



**Fig 2c:** Fluorescence at 1.0ppb of Cd after 2hr Mg(4X).

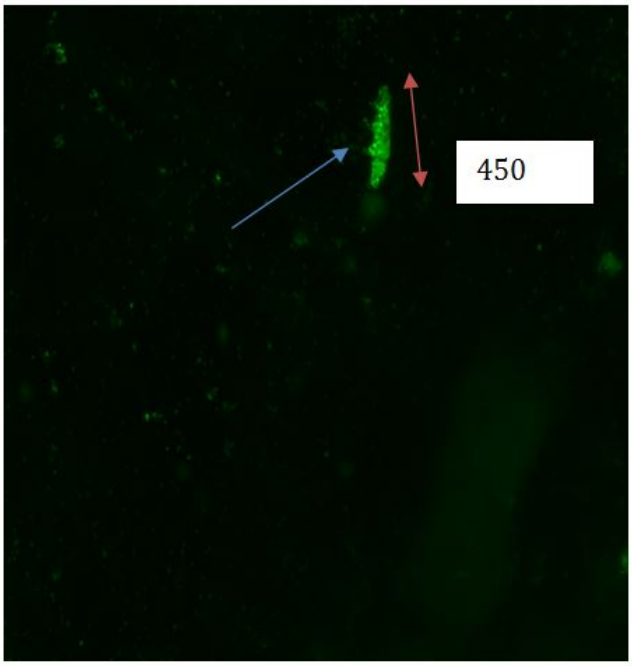



**Fig 2d:** Fluorescence at 1.0ppb of Cd after 4hr Mg(4X).

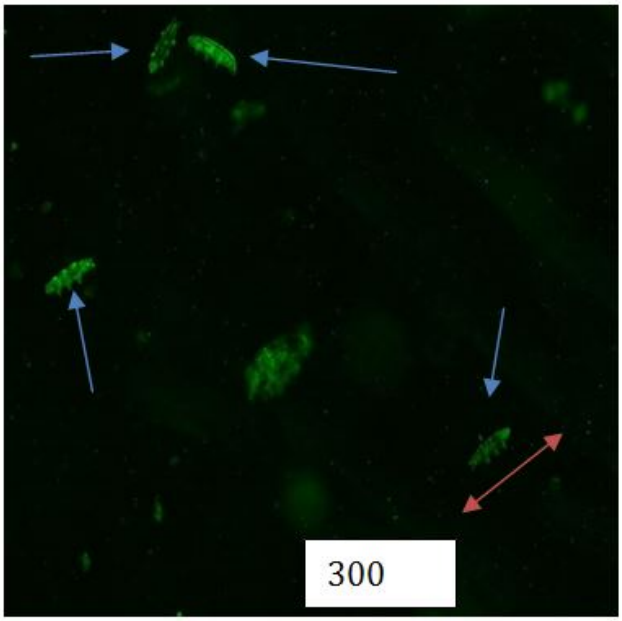



**Fig 2e:** Fluorescence at 1.0ppb of Cd after 6hr Mg(4X)s.

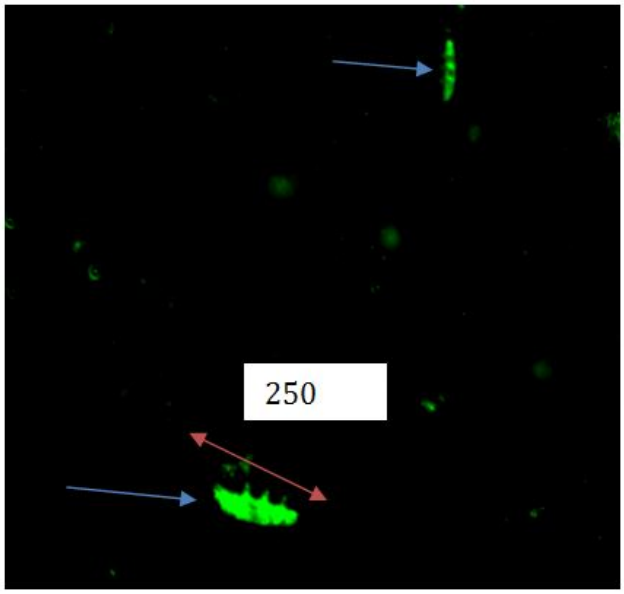



**Fig 2f:** Fluorescence at 1.0ppb of Cd after 24hr Mg(40X).

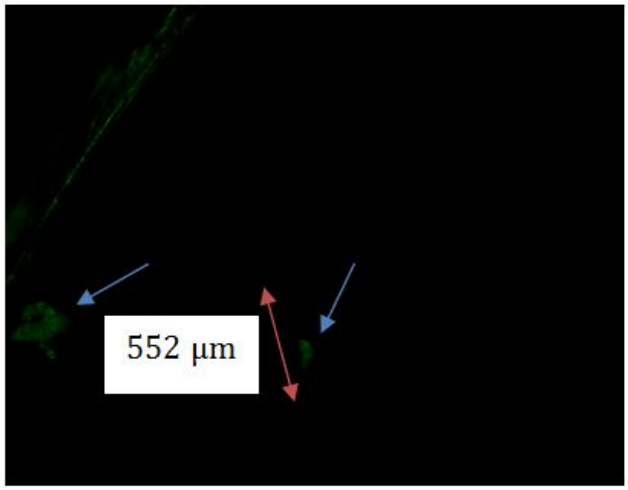



**Fig 2g:** Cadmium control, No Fluorescence Mg(4X).

Figure 2: After exposure to increasing Cadmium concentrations, we observed a gradual decline in the metabolic activities of tardigrades as the time of exposure increased. Morphologically, it was observed that the body size gradually reduced as the concentration and duration of exposure increased. (A) Fluorescence was produced after 6hr of exposure to 0.5ppb. (B) The fluorescence increased after 24hr and (C) after 2hr of exposure to 1.0ppb, the metabolic activity was relatively good but after (D) Exposure to 1.0ppb for 4hr the fluorescence increased and highest at (E) 1.0ppb after 6hr and metabolic activity down at (F) 1.0ppb after 24hr when compared with the control (G).

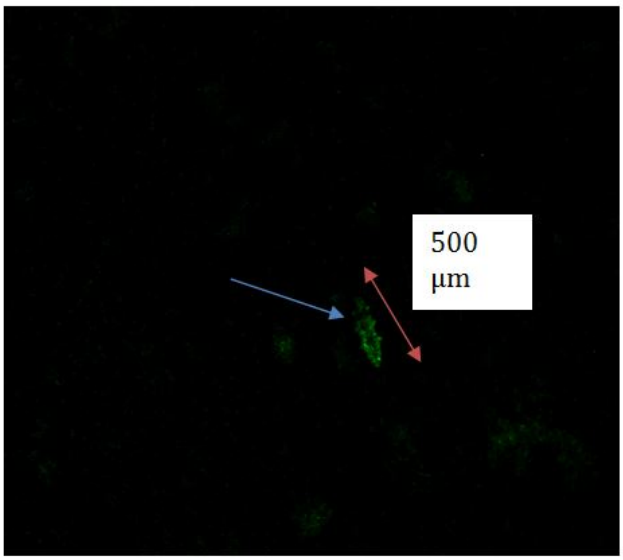



**Fig 3a:** Fluorescence at 0.025ppb of Cd after 24hr Mg(40X).

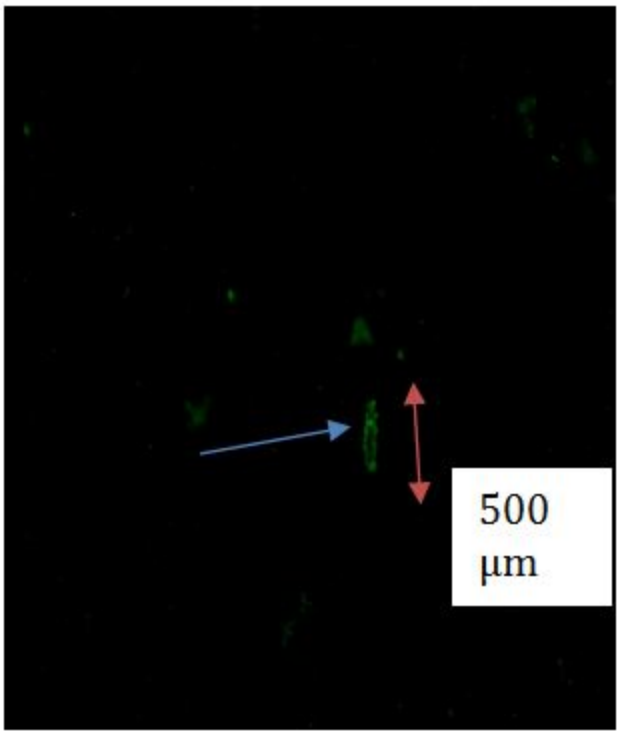


**Fig 3b:** Fluorescence at 0.05ppb of Cd after 24hr Mg(4X).

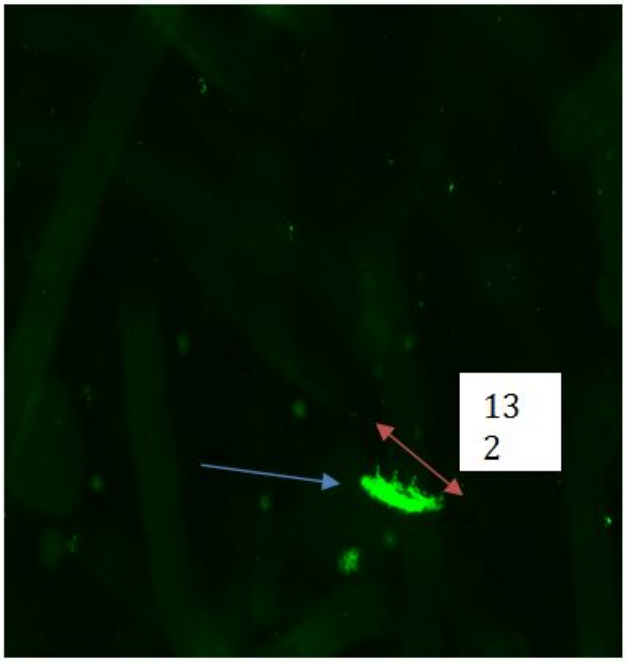


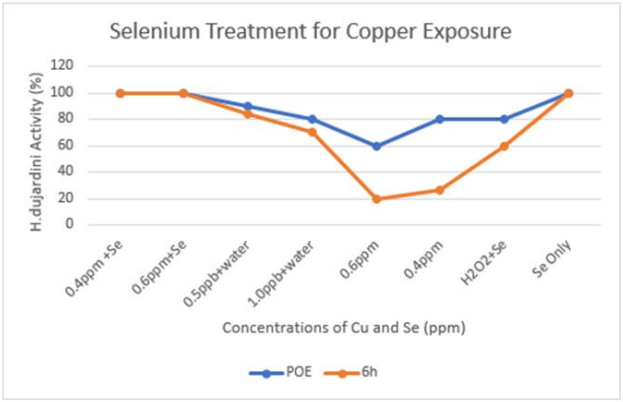

**Fig 4:** Fluorescence at 0.05ppb of H2O2 as positive control after 2hr Mg(4X).

Figures 3 and 4: Figure: These confirmed that ROS was being produced in the Tardigrades even at minute concentrations, however, the stressed condition that led to the production of these ROS is not enough to alter the metabolic activity (A) and the morphology also remained the same (B) with the control Tardigrade.

## Treatment

Prior to exposure of H. exemplaris to both Cd and Cu solutions, we added 1ml of 1ppb Se solution to the H. exemplaris culture plates. Interestingly, there was no toxic effect from the point of exposure through the duration of exposure (graph 1-2, chart 1-2, Fig 5a-d) To confirm the actual concentrations that we treated, exposure to the cadmium concentrations (0.5ppb and 1.0ppb), copper concentrations (0.4ppm and 0.6ppm), Hydrogen peroxide solution (H2O2) was done again and the same result was reported. There was not much difference in the activity of H. exemplaris at the POE and after 6h of exposure, when water was used as a treatment. It is suggested that water is not an effective treatment of production of ROS, but it most likely diluted the concentration of the cadmium and copper in the H. exemplaris culture plate, resulting in a decreased toxic effect on the H. exemplaris.

We added 1ml of 1ppb Se solution to the H. exemplaris as a positive control (Fig. 20), This result confirmed the antioxidative capacity of Se.




**Graph 1:** Selenium Treatment for Copper Exposure.

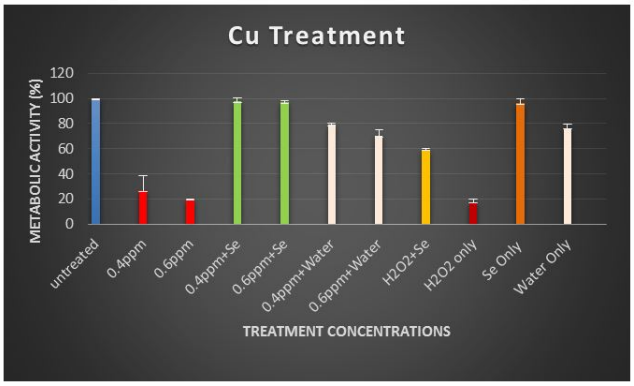



**Chart 1:** Selenium Treatment for Copper Exposure.

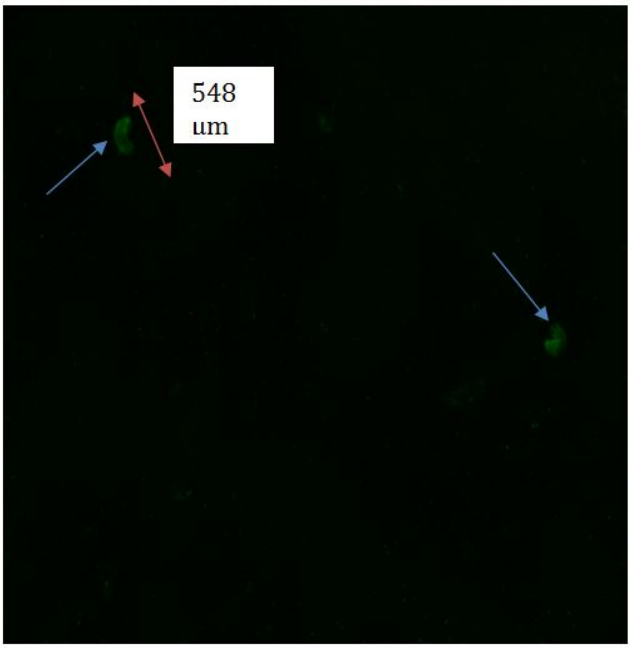



**Fig 5a:** Se +0.4ppm Cu after 6hr Mg(X4).

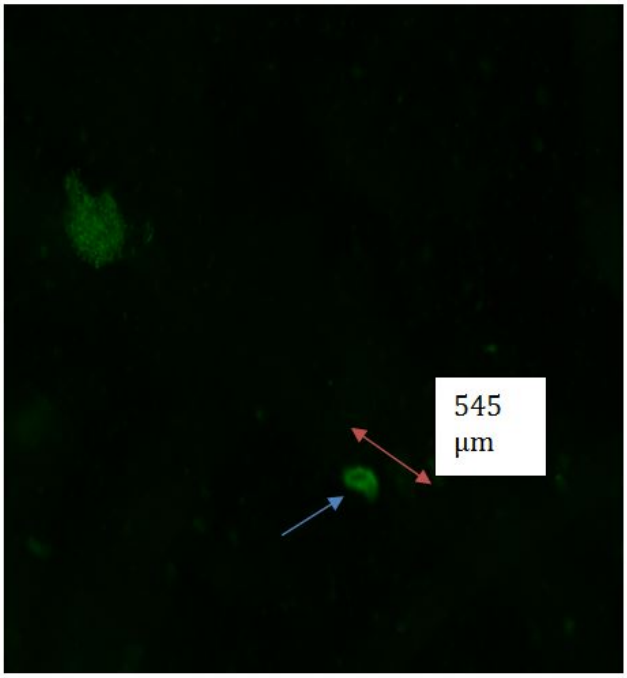
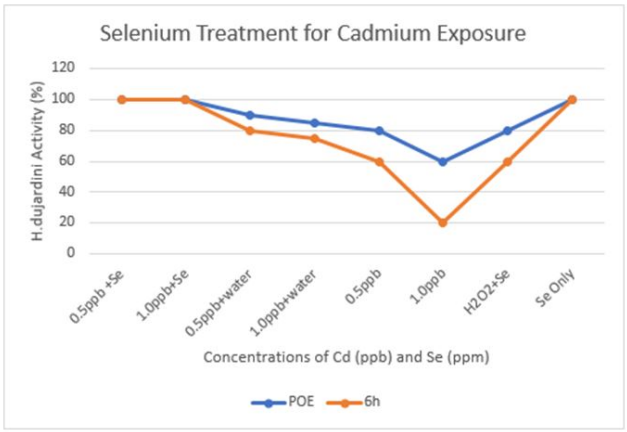



**Fig 5b:** Se+0.6ppm Cu after 6h.

Figure 5: Figure: Treatment with 1ml of 1.0ppm of Se showed that the metabolic activity peaked back to about 99% for the Tardigrade that was exposed to 0.4ppm copper (A) and the body size was relatively the same as the control Tardigrade for the tardigrade that was exposed to 0.6ppm.




**Graph 2:** Selenium Treatment for Cadmium Exposure.

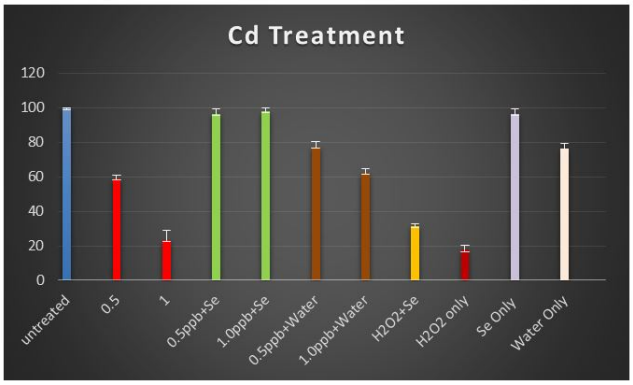



**Chart 2:** Selenium Treatment for Cadmium Exposure.


**Fig 5c:** Se+ 0.5ppb Cd after 6hr Mg(4X).


**Fig 5d:** Se+ 1.0ppb Cd after 6h Mg(4X).

## Quantification

### SOD Assay

The SOD activity assay was designed to quantitatively measure SOD activity in the treated tardigrades. The assay was carried out using the Superoxide Dismutase (SOD) Colorimetric Activity Kit with manufacturer's instruction for cells. Using a 96 well plate, the standards, experimental samples and treatments were plated. The plate was incubated for 20 minutes in room temperature and after gently mixing, the reaction mixture is monitored spectrophotometrically at 450 nm every 10minute for 6hrs using a microplate reader25. The value of absorbance reduced with increase in enzyme production, this result confirmed that the Se-treated organisms produced more antioxidative enzyme (SOD), hence the low absorbance reading (Chart 3-6 ang Graph 3).

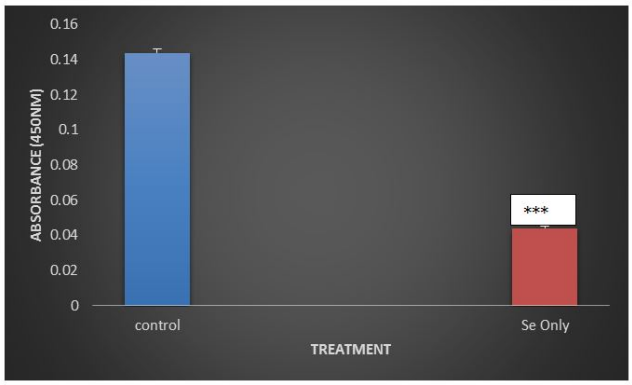



**Chart 3:** control against Se (positive control).

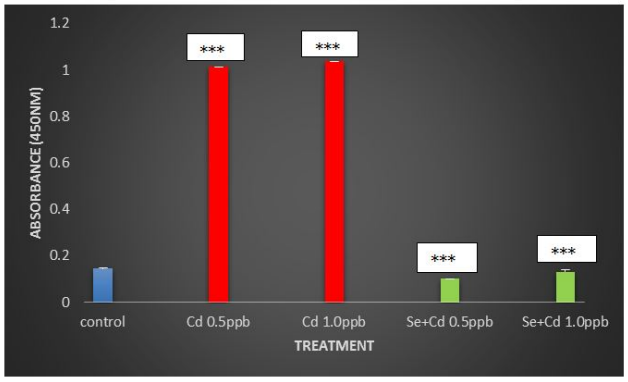



**Chart 4:** Control against Cd treatment.

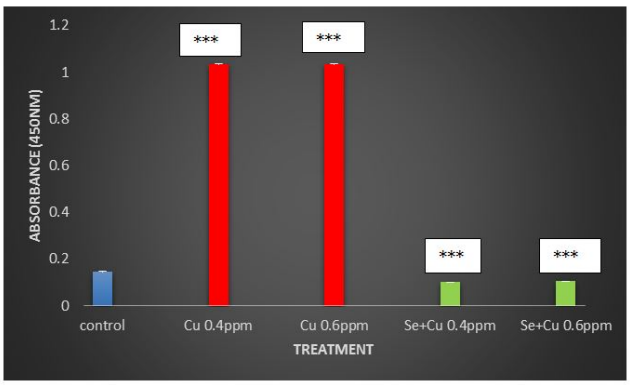



**Chart 5:** Se against Cu treatment.

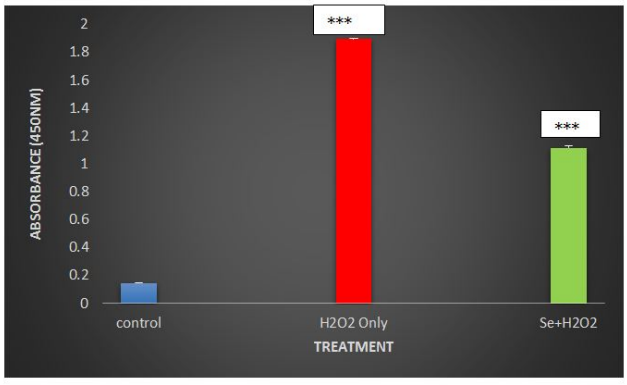
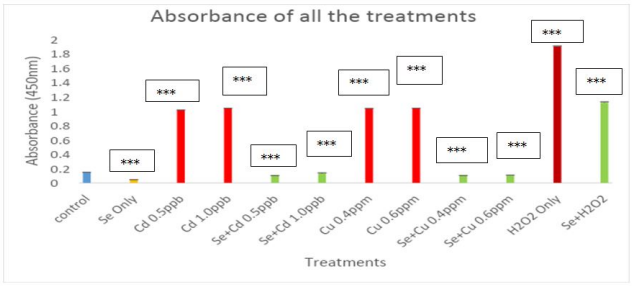



**Chart 6:** Control against Hydrogen Peroxide.




**Graph 3:** Comparison of SOD Absorbance.

## Catalase Assay

Catalase is an enzymatic antioxidant that decomposes H2O2 produced as a result of oxidative stress to give H2O and O2. This result is in agreement with Wahid et al26 who explained that catalase decomposes hydrogen peroxide and protects the cell tissues from hydroxyl free radicals, hence a decrease in catalase activity result in reduced metabolism in tardigrades due to the absorption of superoxide and hydrogen peroxide free radicals.

The assay was carried out using the Catalase (CAT) Assay Kit with manufacturer's instruction for cells. Using a 96 well plate, the standards, experimental samples and treatments were plated.

The plate was incubated for 30 minutes in room temperature and a ter gently mixing, the reaction mixture is monitored spectrophotometrically at 530-560 nm every 10minute for 6hrs using a microplate reader25. The value of absorbance reduced with increase in enzyme production, this result con irmed that the Se-treated organisms produced more antioxidative enzyme (CAT), hence the low absorbance reading (Chart 7-11 and graph 4-5).

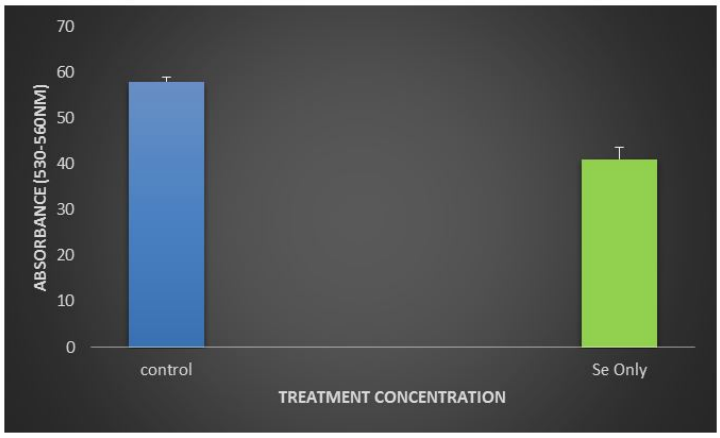



**Chart 7:** Control against Se.

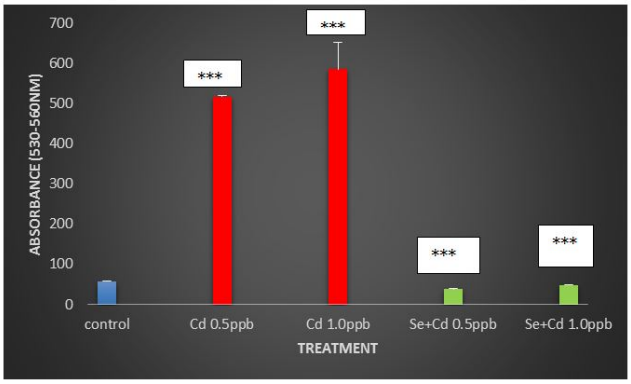



**Chart 8:** Control against Cd Treatment.

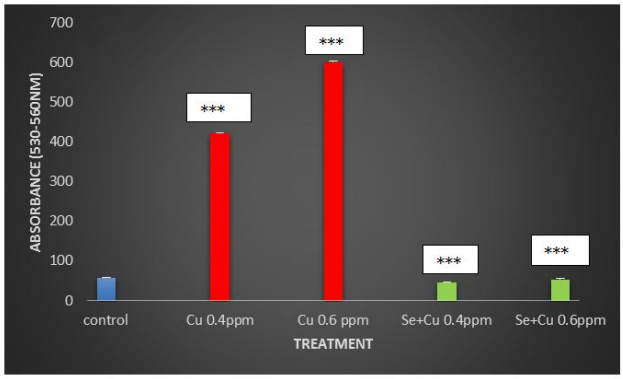



**Chart 9:** Control against Cu Treatment.

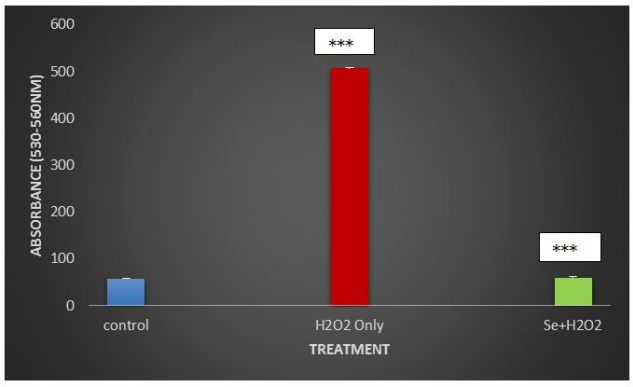



**Chart 10:** Control against Hydrogen Peroxide (Positive control).

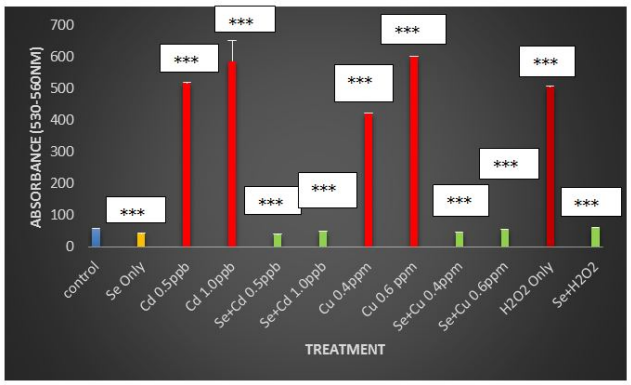
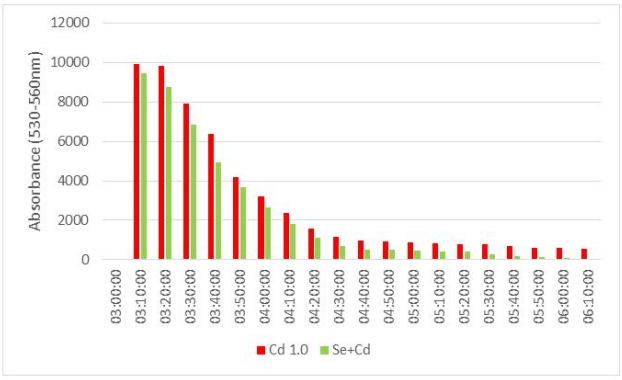
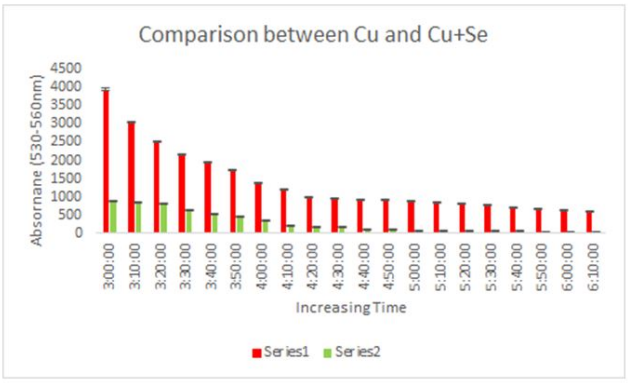



**Chart 11:** Absorbance of all the treatments.




**Graph 4:** Absorbance of Cd treatment every 10 minutes.




**Graph 5:** Absorbance of Cu treatment every 10 minutes.

## Acetylcholine Assay

Acetylcholine is a chemical that sends signal for muscle activation, Cd and Cu has the ability to cause harm by inactivating tardigrade muscles through their influence on the neuromuscular junction, hence the slowed movement in the exposed tardigrades can be traced to inadequate acetylcholine.

Acetylcholine Assay kit was used to detect the Acetylcholine (in a fluorescence microplate reader.

Using a 96 well plate, the standards, experimental samples and treatments were plated. The plate was incubated for 30 minutes in room temperature and a ter gently mixing, the reaction mixture is monitored spectrophotometrically at 530-56025.

The fluorescence reading increased with an increase in enzyme production, this result con irmed that the Se-treated organisms produced more antioxidative enzyme (ACh), hence the high fluorescence reading (Chart 12-14 and

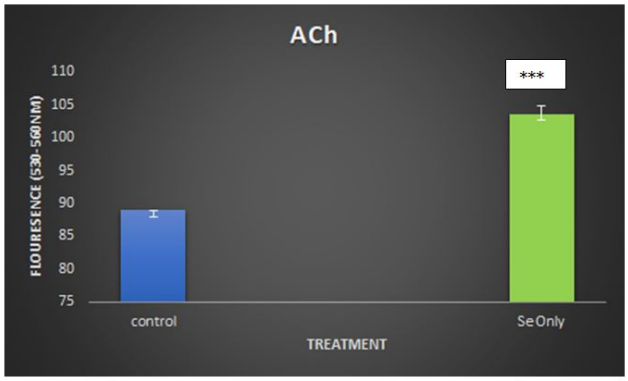



**Chart 12:** Control against Se.

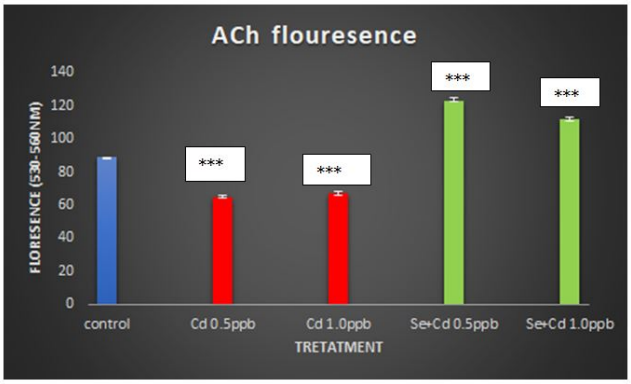



**Chart 13:** Control against Cd Treatment.

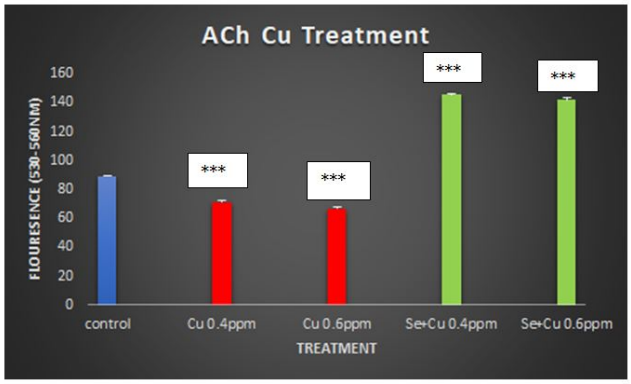
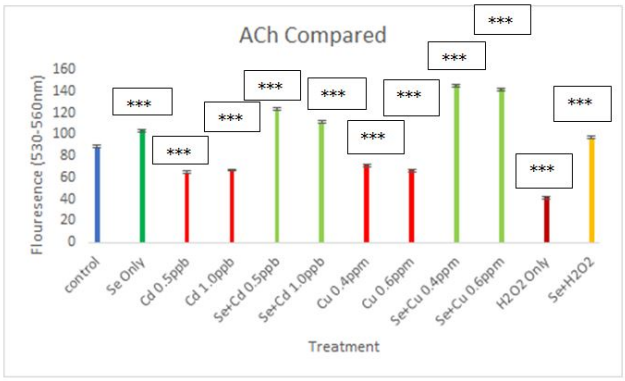



**Chart 14:** Control against Cu Treatment.




**Graph 6:** Comparison of ACh fluorescence.

**Statistical analysis of concentrations compared to the control. ***P<0.001**

## Conclusion

The heavy metals do have a high impact on the morphology and metabolic activities of H. exemplaris as they reduced in body size and metabolic activities dropped to 20% over the period of exposure to cadmium and copper.

Comparing the three enzymatic antioxidant, it was observed that catalase production was the most altered because functionally catalase prevents the effect of free radicals to the cell by transforming harmful superoxide radicals into hydrogen peroxide which later breaks down into water and oxygen, however, after the cadmium and copper exposure, there was a rapid oxidation of the fluorogenic probe producing fluorescence and more absorbance value when the enzyme was quantified.

Summarily, our findings suggest that exposure of Tardigrades to cadmium and copper results in a toxic effect that induces the release of antioxidative enzymes, however, continuous exposure overtime, reduces the production of the antioxidative enzyme resulting in harmful conditions in tardigrades. Treatment with Se, which is environmentally available, upregulates the antioxidative enzymes.

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