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THE EFFECT OF MULTIDRUG RESISTANCE TRANSPORTER ACTIVITY ON MERCURY BIOACCUMULATION IN *STRONGYLOCENTRUTUS PURPURATUS* PLUTEUS LARVAE FROM CONSUMING CONTAMINATED *ISOCHRYSIS GALBANA*

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Abstract

Mercury (Hg) contamination via prey can be significantly affected by bioacculumation of mercury through trophic levels. However, an understanding of how mercury bioaccumulates through trophic levels is not fully complete. This study expands on knowledge of multidrug resistance (MDR) transporters on the uptake of mercury through trophic levels and investigates whether MDR activity in algal cells may alter Hg accumulation. Four-arm pluteus stage larvae of *Strongylocentrotus purpuratus* were exposed to the MDR inhibitor Reversin 205 and then fed inorganic mercury-contaminated algae. Results show that at the four-arm pluteus stage, *S. purpuratus* is more resistant to mercury contamination. Reversin 205 exposure resulted in increased survival of plutei, with no significant difference between Reversin Only and Mercury plus Reversin treatements, although overall health was reduced as compared to controls.

Introduction

THE SERIOUSNESS of mercury poisoning from contaminated marine food sources was first documented in May 1956 in Minamata City, Japan, where victims of what was known as "Minamata disease" suffered from sensory disturbances, ataxia, and dysarthia (Harada, 1995). Fetuses poisoned by mercury from consumption of contaminated food by mothers were observed to have extensive lesions of the brain (Harada, 1995). In Japan, the source of mercury contamination came from a local chemical plant that drained waste directly into the Minamata River that fed into the Shiranui Sea (Harada, 1995). In the United States, mercury contamination has historically come from mining (Alpers et al., 2005). In gold mining, mercury was used to enhance recovery in all types of mining, and much was drained into the environment as waste (Alpers et al., 2005). Today, the largest sources of mercury contamination come from power plants, which currently contribute almost half of all contamination (NRDC, 2013). Other major sources include coal powered boilers and heaters, steel production, and incinerators (NRDC, 2013). It is important to note that over the past two decades, mercury contamination has decreased by 65 percent (NRDC, 2013). However, consumption of mercury-contaminated food is still a critical area of concern of public health worldwide.

The primary exposure of humans to mercury occurs through the consumption of food contaminated with methyl mercury. Mercury released into the environment as inorganic mercury ($HgCl_2$), is often converted to methyl mercury in the environment, and is biomagnified through the food chain (Handy and Noyes, 1975; King et al., 2000). In aquatic organisms, contamination often occurs through sorption and uptake by primary producers (Hill et al., 1996). This causes bioaccumulation, or biomagnification of mercury, so that concentration of mercury

with primary producer cells are thousands to hundreds of thousands of times greater than the concentration of mercury within the water column (Davies, 1976; Hill et al., 1996). With each increase in trophic level, from primary producer to secondary or tertiary consumers, the accumulation of mercury increases (Atwell et al., 1998; Bosnjak et al., 2009). Thus, at higher trophic levels, the concentration of mercury within the tissues of organisms can exceed safe levels and lead to severe health defects. Additionally, bioaccumulation of mercury through trophic levels is affected by the composition of organisms within a trophic food web. For example, the Gulf of the Farallones is represented largely by marine birds, which accumulate mercury at a different rate than invertebrates. As a result, this system has a higher power of biomagnification for mercury than most other systems (Atwell et al., 1998). Time of exposure is also important. Longer-lived organisms not only tend to have higher trophic level positions, but also accumulate larger amount of mercury throughout their lifetime (Atwell et al., 1998). Differences in bioaccumulation are also influenced by the species of mercury, such as inorganic or organic mercury (CH_3Hg). Typically, the species of mercury found in the water column is inorganic mercury. Between primary producer and primary consumers, inorganic mercury is transferred relatively efficiently (Hill et al., 1996). However, at higher trophic levels, organic mercury is even more efficiently transferred between trophic levels than inorganic mercury and is found in higher proportions in the cells of a wide range of marine organisms, from diatoms to piscovorous fish (Bloom, 1992; Mason et al., 1996; Atwell et al., 1998).

In recent literature, the difference in accumulation seen between inorganic and organic mercury has been linked to the effects of efflux activity from multidrug resistant (MDR) transporters. Accumulation of mercury has been studied in the sea urchin *Strongylocentrotus purpuratis* embryo (Bosnjak et al., 2009). These studies have shown than inhibition of MDR transporters, specifically multidrug resistance protein (MRP), lowers the lethal concentration of inorganic mercury, but not organic mercury, which shows that the difference in accumulation rates seen between organic and inorganic mercury is due to removal of inorganic mercury from embryos via MDR transporters (Bosnak et al., 2009). However, the effect of MDR transporters has not been studied in *S. purpuratus* beyond the embryonic stage to the pluteus stage, nor has its presence in algae been studied. At the four-arm pluteus stage (day 5-14 after fertilization), *S. purpuratus* begin feeding (Smith et al., 2008), which makes them in ideal species to study the effects of MDR on the uptake of mercury through trophic cascades. Furthermore, the position of sea urchin larvae within the water column can be used as an indicator of health (Yaroslavtseva and Sergeyeva 2007). The vertical distribution of larvae and their movement in between layers of water is important, natural phenomenon of pelagic invertebrate larvae (Yaroslavtseva and Sergeyeva 2007). In early pluteus stages, normal behavior involves an even distribution from the surface to the bottom.

In this study, the effect of MDR transporters on the uptake of inorganic mercury from a fed source, rather than from the water column, in *S. purpuratus* at the four-arm pluteus stage was tested. This was done by inhibiting MDR activity of *S. purpuratus* with the MDR inhibitor Reversin 205. This inhibitor is general MDR inhibitor that blocks MRP and P-glycoprotein (PGP) activity. Though only MRP has been shown to influence accumulation of inorganic mercury, other MDR transporters may be important at larval stages. *S. purpuratus* were fed either contaminated or non-contaminated *Isochrysis galbana.* This species of algae was chosen

because it has been shown to bioaccumulate inorganic mercury to 1000 mg Hg/kg Dry Weight of *I. galbana* when the concentration of inorganic mercury within the water column is 15 µg/l (Davies, 1974). Therefore, by using *I. galbana*, I was able to estimate the amount of mercury that each larval treatment was exposed to without analysis instrumentation. Additionally, I conducted pilot studies looking for the possibility of MDR activity within *I. galbana* and *Dunaliella tertiolecta* by exposing samples to different MDR inhibitors and analyzing their effect on the uptake of fluorescent dyes. These are appropriate species to use in determining the presence of MDR activity in algae, because they have no cell wall (Zhu and Lee, 1997).

2. Materials and Methods

2.1 Reagents

Calcein-AM (CAM), MK 571, PSC 833, Reversin 205, Rhodopsin were prepared as a 0.5 mM, 10 mM, 3 mM, 3mM, 5 mM stock solutions, respectively. Dimethyl sulfoxide (DMSO) was used as a solvent for CAM, MK 571, PSC 833, Reversin 205, and Rhodamine 123. Fluorescent dye and MDR inhibitors were kept at -20 C. Inorganic mercury ($HgCl_2$) was prepared as 100 mg/l solution using deionized distilled water (DDH_2O). Inorganic mercury stock solution was kept in fume hood at room temperature. Protoslo ® protozoa quieting solution was used to slow the movement of specimens for viewing. All reagents were purchased from Sigma (St. Louis, MO), except for MK 571, PSC 833, CAM, and Protoslo ®, which were purchased from Caymen (Anne Arbor, MI), AdooQ (Irvine, CA), Invitrogen (Grand Island, NY), and Carolina (Burlington, NC).

2.2 Animals and Algae

Ten *S. purpuratus* adults were collected by the Animal Resource Group, at the Bodega Marine Laboratory, Bodega Bay, California. Sizes of the individuals ranged from small to large. Spawned individuals were divided by sex and held in recovery tanks post-spawning at the Bodega Marine Laboratory. Larvae where kept at concentrations suggested by Keahy (1986). *I. galbana* and *D. tertiolecta* samples for algae multidrug resistance experiments were provided by the Animal Resource Group. *I. galbana* for larvae feeding experiments was gifted from Jill Bible at the Bodega Bay Marine Laboratory.

2.3 Multidrug Resistance in Algae

Four pilot studies were performed to determine if multidrug resistant transporter activity within in the membranes of algae might affect the uptake of mercury within cells. Samples were viewed under an Olympus EX 50 WI bright field fluorescent microscope with 20x water lens. Exposure times were 50 and 500 ms for untreated and fluorescent dye treated samples, respectively. Relative fluorescent units (RFU) were measured and analyzed using Metamorph software. CAM treated samples were viewed using Alexa 488 excitation/emission wavelength 488/519 nm and Rhodamine 123 treated samples were viewed using TRITC with excitation/emission wavelengths 554/576 nm.

2.3.1 Algae Pilot 1

In Pilot Experiment 1, *I. galbana* and *D. tertiolecta* were viewed without fluorescent dyes under bright field fluorescent microscopy to verify background fluorescence and optimum exposure times for analysis. Autoexposure was used to determine optimum exposure time for viewing samples without fluorescent dyes.

2.3.2 Algae Pilot 2

Untreated samples of algae were compared to fluorescent dye treated samples to determine the effect of background fluorescence on analysis. *I. galbana* and *D. tertiolecta* were each

treated with either CAM only or CAM plus MK 571. "No Treatment" controls were neither treated with fluorescent dyes nor MDR inhibitors. The final concentration of CAM and MK were 0.5 µM and 10 µM, respectively. Samples were incubated for an hour and a half at 16 °C. Automatic exposure was used to determine exposure times for viewing *I. galbana* and *D. tertiolecta* under Alexa 488.

2.3.3 Algae Pilot 3

To test if there was MDR transporter activity other than MRP activity, samples of *I. galbana* and *D. tertiolecta* were treated with MK 571, PSC 833, or Reversin 205. "No Treatment" controls were neither treated with fluorescent dyes nor MDR inhibitors. The final concentration of CMA was 0.5 µM in all treated samples. The final concentration of MK 571, PSC 833, and Reversin 205 were 10 µM, 3 µM, and 3 µM, respectively. Samples were incubated for an hour at a half at 16 °C. Samples were then centrifuged at 5000 rpm for five minutes and rinsed. After final centrifuging, samples were brought back up to 1 ml. *D. tertiolecta* were fixed for viewing using one drop of 4% paraformalydehyde. *I. galbana* cells died from centrifuging, but were not ruptured, and thus were not fixed.

2.3.4 Algae Pilot 4

This pilot experiment tested if the background fluorescence of both algae species interfered with the fluorescents of an alternative dye. "No Treatment" controls were neither treated with fluorescent dyes nor MDR inhibitors. The final concentration of both CAM and Rhodamine 123 treated samples was 5 µM. The final concentration of MK 571 treated samples was 10 µM. DMSO samples were treated with 2 µl of DMSO. Samples were incubated for an hour at a half at 16 °C. *I. galbana* samples were centrifuged at 2500 rpms for five minutes and rinsed with FSW, twice. *D. tertiolecta* samples were centrifuged at 5000 rpm for five minutes and rinsed with FSW, twice. After the final centrifuging, *I. galbana* and *D. tertiolecta* samples were brought up to final volume of 0.5 ml. Four drops of protozoa quieting solution Protoslo®, from Carolina®, was added to *D. tertiolecta* samples to stop cells from swimming. *I. galbana* died during centrifuging, therefore were treated with Protoslo®.

2.4 Larval Feeding of Mercury Exposed Algae

2.4.1 Sea Urchin Spawning

Individuals were injected with approximately 0.5 ml of KCl to induce contraction of the gonads and release gametes. Individuals were then inverted over 30 ml beakers filled to the brim with 0.45 micron filtered sea water (FSW) to collect gametes. Eight females and one male were spawned. One individual did not spawn and was returned to the containment tank. Eggs were rinsed twice with FSW by allowing eggs to settle, decanting excess fluid off and adding FSW back. The eggs from one female were chosen after testing for fertilization. Sperm from male was not diluted for fertilization, and one drop of sperm was added to 250 ml of eggs and FSW in a 600 ml beaker. Fertilized eggs were allowed to settle and were rinsed twice. Zygotes were divided equally among four large glass bowls filled between half and three-quarters with FSW. Fertilized eggs were held in incubator at ~16 °C for 24 hours, then swimming larvae were divided into two clean bowls with FSW. Debris and dead larvae were removed approximately every three days and FSW was added to maintain water levels. Larvae were checked for feeding stage seven days after fertilization by feeding a sample *I. galbana* and viewing under a microscope.

2.4.2 Preparation of I. galbana cultures

One liter of *I. galbana* was kept in natural sunlight with air bars. For experiment 1, 36 hours before each feeding, two 20 ml samples

of *I. galbana* were taken in 70 ml canted neck polystyrene non-treated flasks. One sample was treated with inorganic mercury, so that the final concentration was 15 µg/l. Samples were laid flat on their widest side, ~4 inches underneath an aquarium grow light for 36 hours.

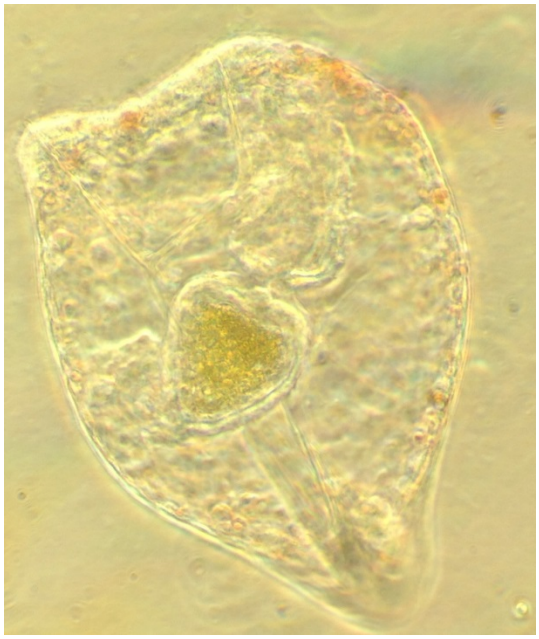

Image 1: *Strongylocentrotus purpuratus* pluteus (7 days from fertilization) with gut filled with *Isochrysis galbana.* 25 May 2013.

2.4.3 Preparation of algae for feeding

Before each feeding, algae were centrifuged in 50 ml plastic centrifuge tubes at 200 RCFs for 5 minutes. To rinse the algae, I removed the supernatant, added FSW until the volume was brought back to 10 ml, and re-centrifuged each sample for 5 minutes at 200 RCFs. Each algae sample was rinsed twice before every feeding. After the last centrifuging, FSW was added to bring the final volume to 5 ml. If the cell density was to high to count via disposable hemocytometer, samples were diluted to 10 ml by adding FSW.

2.4.4 Feeding experiment 1

Four larvae treatments were prepared using 15 ml glass test tubes. These treatments were: control (C), Reversin control (R), mercury only (M), and mercury plus Reversin (MR). At 0 hours, sixty, 7-day old larvae were added into 10 ml of FSW in each test tube. Reversin 205 was added to R and MR treatment yield a 3 µM solution. M and MR treatments were fed mercury treated algae. Each larvae treatment was fed 10,000 cells of algae per ml at 0 hours, 24 hours, and 48 hours. Addition of Reversin 205 and algae feeding did not alter the final volume of each treatment by a significant amount. The experiment lasted 72 hours and every twelve hours the position of larvae within their respective test tubes was observed, recorded, and photographed using a digital Canon T3i with a 28-50 mm lens.

2.4.5 Feeding experiment 2

Four larvae treatments were prepared: control (C), reversin control (R), mercury only (M), and mercury plus Reversin (MR). Larvae in this experiment were 11 days old on Day 0. Thirty, larvae were added into 10 ml of FSW in each well of two six-well plates. Reversin 205 was added to R and MR treatments to a yield a 3 µM solution. C and R treatments were fed non-contaminated algae, while M and MR treatments were fed contaminated mercury. Each larvae treatment was feed 10,000 cells of algae per ml at 0 hours and 5,000 cells of algae per ml, subsequently, at 24 hours and 48 hours. Excess algal cells were removed at 24 hours, before feeding, without changing the end volume by a significant amount, to ensure larvae were not feeding on expired algae. Reversin 205 treatments and algae feeding did not alter the final volume of each treatment by a significant amount. Samples were viewed under dark-field microscopy every 12 hours. Larval position in the water column was recorded as bottom, middle, and surface. Mortality was recorded. Behaviors such as lethargy or activeness were noted.

2.5 Analysis of Mercury Within Algae and Larvae Treatments

2.5.1 Algal Survivability

Before algae were treated with inorganic mercury and before algae were centrifuged, both No Mercury and Mercury treated algae cell density was calculated to estimate the mortality between each treatment. Algae in both treatments settled to the bottom of their 70 ml canted neck polystyrene non-treated flasks after 36 hours. Algae were not re-suspended before counting cell densities via hemocytometer, so that dead cells were not calculated.

2.5.2 Calculation of Percent Dry Weight of I. galbana

To calculate the percent dry matter, an unmeasured volume of *I. galbana* was filtered using nitrocellulose filter paper and a vacuum pump, until the filter become clogged. This was to maximize the amount of dry *I. galbana* in order to ensure a significant amount could be measured. The sample was weighed before and after being heated in an oven at ~60 C with a vacuum pump until final weight became constant. This process was repeated once. To measure the weight of one cell of *I. galbanai*, ~150 ml of distilled and deionized water was filter through nitrocellulose paper with a vacuum pump and weighed wet. Next, 50 ml of *I. galbana* with a cell density of 4,600,000 cell/ml was filtered the wet nitrocellulose filter paper and weighed. The net weight of the algae was calculated and used to determine the approximate weight of one cell of *I. galbana.* The percent dry matter, weight of one cell of *I. galbana*, and the total number of cells fed to each treatment was used to estimate the amount of mercury in each larval feeding treatment.

3. Results

3.1 Multidrug Resistance in Algae

3.1.1 Pilot 1

This pilot experiment determined that *I. galbana* and *D. tertiolecta* can be viewed under the bright-field fluorescent microscopy using Alexa 488 with excitation/emission wavelength of 488/519 nm and that both exhibit background fluorescence. Exposure time for viewing *I. galbana* and *D. tertiolecta* under Alexa 488 was optimal at 50 ms.

3.1.2 Pilot 2

This pilot experiment showed that the background fluorescence *I. galbana* is significantly lower than Calcein-AM treated *I. galbana*, although very slightly, and the addition of MK 571 did not effect the luminescence significantly. Non-treated *D. tertiolecta* had significantly lower luminescence than Calcein-AM treated *D. tertiolecta*, Calcein-AM treated *D. tertiolecta* showed significantly lower luminescence than Calcein-AM and MK 571 treated *D. tertiolecta* (Figure 1). Optimal exposure time for viewing with fluorescent dye was 500 ms.

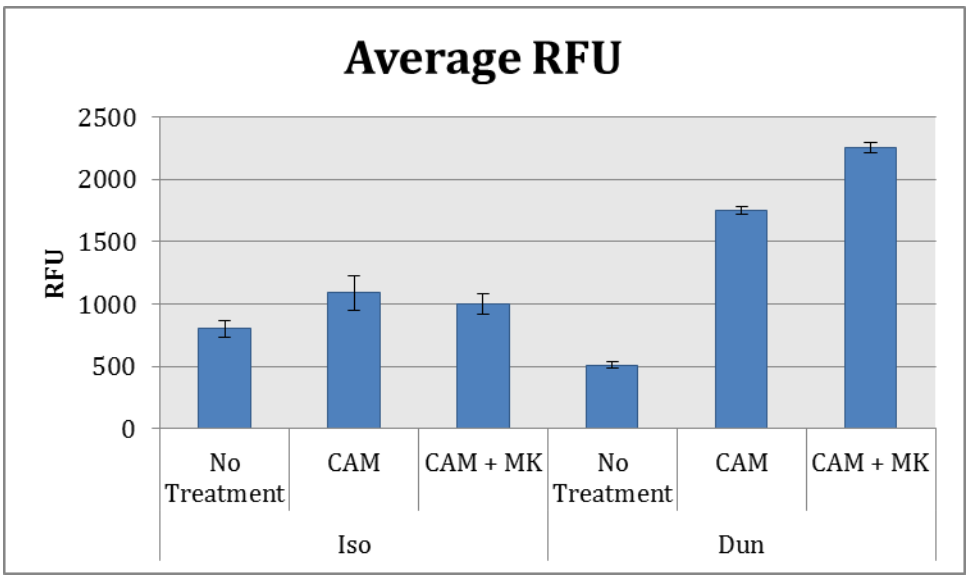

Figure 1: Average relative fluorescent units (RFU) measured using Metamorph software and Olympus EX 50 WI fluorescent microscope with 20x water objective lens under Alexa 488. ISO refers to *Isochrysis galbana* and DUN refers to *Dunaliella tertiolecta*

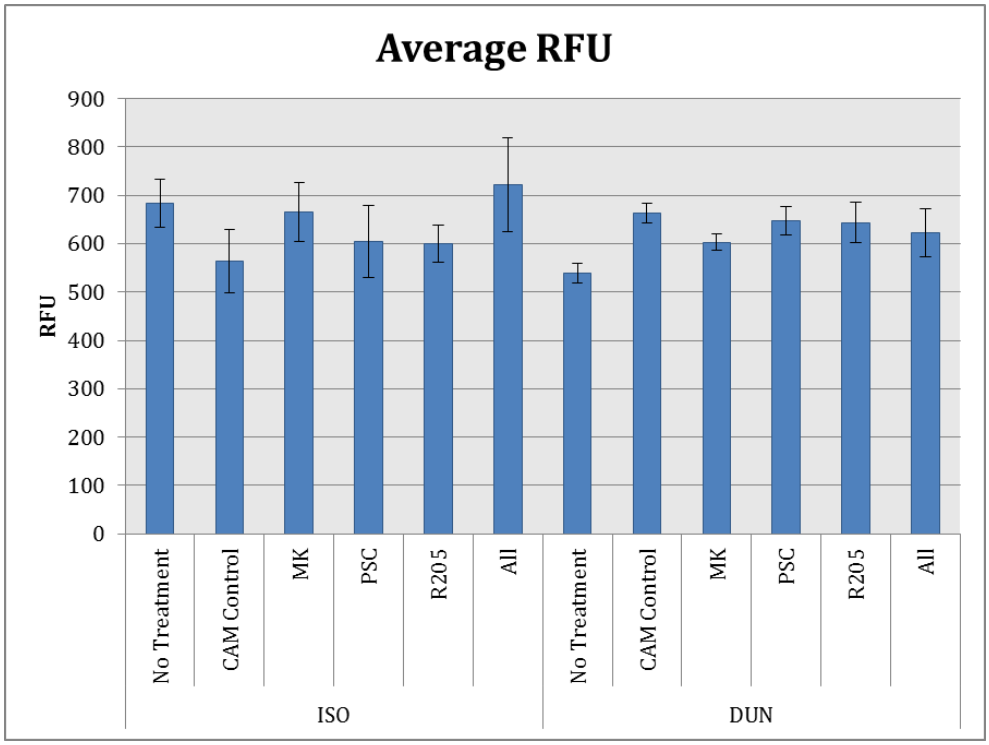

Figure 2: Average luminescence measured in relative fluorescent units using Metamorph software and Olympus EX 50 WI fluorescent microscope with 20x water objective lens under Alexa 488. ISO refers to *Isochrysis galbana* and DUN refers to *Dunaliella tertiolecta*

3.1.3 Pilot 3

The differences in relative fluorescent units between each of the *I. galbana* treatments were non-significant from the non-treated control and CAM control (Figure 2). All *D. tertiolecta* treatments had significantly higher luminosity than the non-treated control, but not the CAM control (Figure 2). Additionally, there was no significant difference between each *D. tertiolecta* treatment (Figure 2).

3.1.4 Pilot 4

Rhodamine 123 was included in this experiment as it is a well-known MDR transport substrate (Lee et al., 1994), but it does not require esterase activity to become fluorescent the way CAM does. DMSO did not affect the survivorship of either *I. galbana* or *D. tertiolecta* (Figure 3a). There were no significant differences between No Treatment, CAM, or CAM plus MK 571 in *I. galbana* (Figure 3a). *I. galbana* treated with Rhodamine 123 measured significantly lower RFUs than No Treatment and Rhodamine 123 plus MK (Figure 3b). Rhodamine 123 plus MK 571 did not significantly differ from No Treatment control (Figure 3b). *D. tertiolecta* treated with CAM was significantly higher than CAM plus MK 571 and No Treatment control (Figure 3a). Rhodamine 123-treated *D. tertiolecta* was significantly higher than No Treatment TRITC control. Rhodamine 123 plus MK 571 was significantly higher than Rhodamine 123 (Figure 3b).

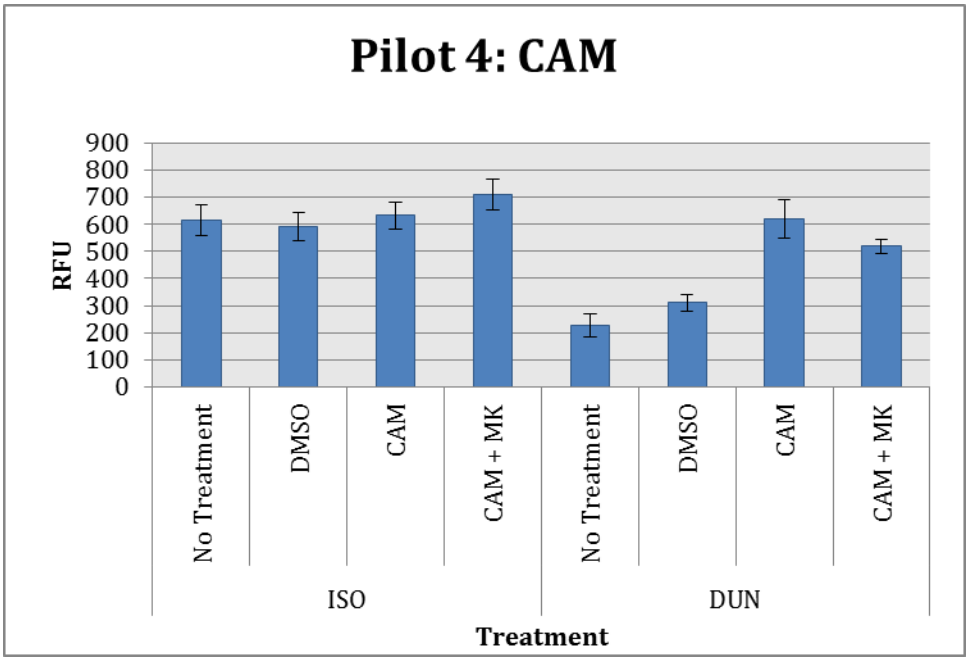

Figure 3a: Average luminescence measured in relative fluorescent units using Metamorph software and Olympus EX 50 WI fluorescent microscope with 20x water objective lens from with excitation/emission wavelength of 488/519 nm. ISO refers to *Isochrysis galbana* and DUN refers to *Dunaliella tertiolecta*

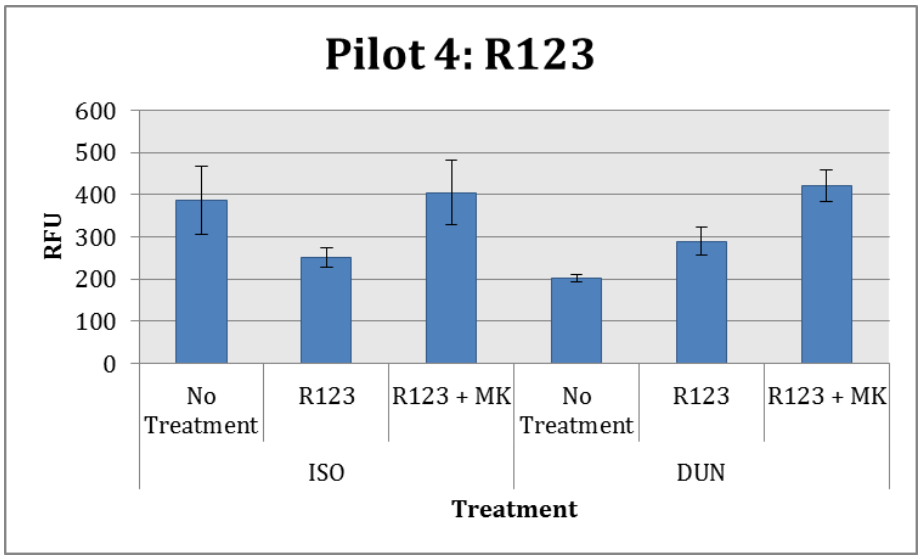

Figure 3b: Average luminescence measured in relative fluorescent units using Metamorph software and Olympus EX 50 WI fluorescent microscope with 20x water objective lens, with excitation/emission wavelength of 554/576 nm. ISO refers to Isochrysis galbana and DUN refers to Dunaliella tertiolecta

3.2 Analysis of Mercury Within Algae and Larvae Treatments

3.2.1 Algae Viability

Data from 0-hour feeding could not be used to determine algal mortality due to technician error. 24-hour and 48-hour feeding samples were used to determine survivability. Cell density was averaged each day per treated to yield the average cell density before treatment and average cell density after 36 hours (Figure 4). Algal cell density in both treatments significantly fell below initial cell densities, though there was no significant difference between the cell density No Mercury and Mercury treatments after 36 hours (Figure 4).

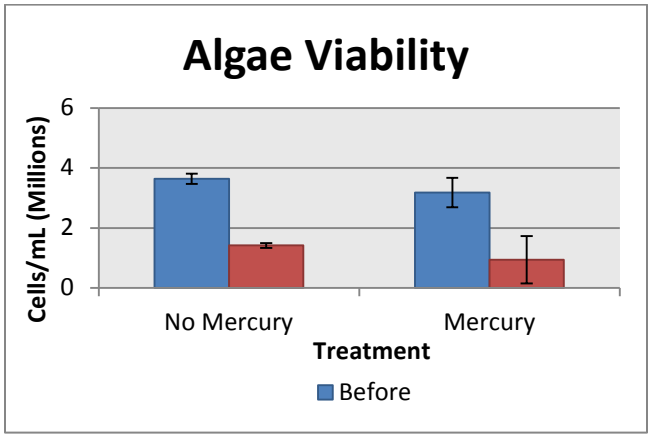

Figure 4: Average cell density of *I. galbana* treatments calculated from 24-hour and 48-hour feeding. Before (blue) indicates the cell density of algae before 36-hour exposure. After (red) indicates the cell density of algae after 36-hour exposure. Error bars were calculated from standard deviation.

3.2.2 Percent Dry Weight of Algae

The percent dry weight was calculated using the following formula:

$$\% DM = \left(\frac{Dry\ Weight}{Wet\ Weight}\right) \times 100$$

This method of calculated dry weight may have error to due volatiles and materials that decompose at high temperatures. The calculated percent dry matter for samples 1 and 2 was 6.25% and 6.44%, respectively. The average percent dry matter was 6.35%. The wet weight of one cell of *I. galbana* was calculated by dividing the net weight of the filtered algae by the total number of cell in 50 ml of water. The weight of one cell of *I. galbana* was 97.8 pg.

The calculated wet weight of one cell of *I. galbana* allowed the estimation of the amount of mercury that each treatment was during the feeding experiment fed to on each day. The calculated weight wet was ~9.78 pg/cell. For 0-hour feeding, approximately 100,000 cells were fed to each treatment, with a wet weight of approximately 9.78 mg. Using the percent dry matter, this was ~0.00062 kg dry weight of *I. galbana*. Assuming the Davies (1974) ratio of milligrams mercury per kg dry weight of *I. galbana*, there were 0.62 mg Hg in each 10 ml treatment. Therefore, the final concentration in each treatment was 62 mg Hg/l. For 24-hour and 48-hour feedings, the final concentration in each treatment was 31 mg Hg/l. Due to the size of the larvae, overfeeding would not have been issue, as larvae would have eaten until satiation and then stopped.

3.3 Larval Feeding of Mercury Exposed Algae

3.3.1 Experiment 1

The proportion of larvae at the bottom, middle, and surface was calculated for each treatment replicate using the number of individuals found at each position divided by the total number of individuals counted. The average proportion of larvae at each position was calculated across each replicate in each treatment. Standard deviations were calculated to compare significant difference between treatments. The average proportion was converted to a percentage and graphed against time. Figure 5 represents the average percent of larvae at each position for Control and Mercury treatments. There was no significant different between in position between these two treatments at every time point. Figure 6 shows the average percent of larvae at each position for Reversin Only and Mercury plus Reversin Treatments. There was no significant difference between these two treatments. Throughout the 72-hour experiment, the majority (60-90%) of larvae in the Control and Mercury Only treatments were positioned in the middle of the water column (Figure 5). In the Reversin Only and Mercury plus Reversin treatments, the majority of the larvae were spread evenly between the bottom and the middle, but with time the majority of the larvae occupied the bottom of the water column (Figure 6).

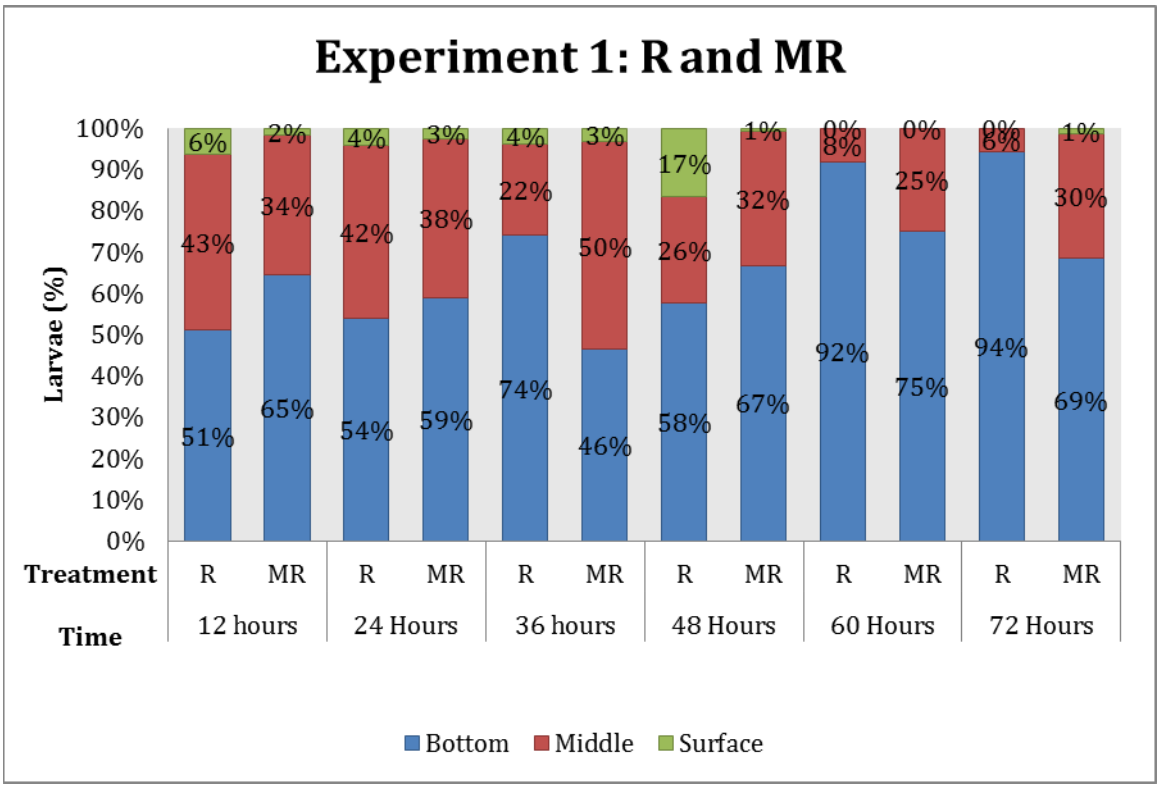

Figure 6: Average percent of larvae in Reversin Only (R) and mercury plus Reversin (MR) treatments according to position in water column. Blue represents bottom, red represents middle, and green represents surface.

3.3.2 Experiment 2

3.3.2.1 Mortality

The decomposition rate of the larvae made quantifying the dead larvae directly difficult. Instead mortality was quantified indirectly by subtracting the total number of individuals observed at every 12-hour check from the number of individuals at the start of experiment (ie 30). These numbers were averaged across replicates in each treatment to yield the average number of missing individuals per treatment. Overall, the average number of missing individuals in Reversin-absent treatments (ie Control and Mercury Only) increased as time progressed with no significant difference (Figure 7). In Reversin-present treatments (ie Reversin Only and Mercury plus Reversin), however, remained relatively constant, following the similar trend lines with no significant difference (Figure 7). As time progressed, differences between Reversin-absent and Reversin-present increased from non-significant to significant (Figure 7).

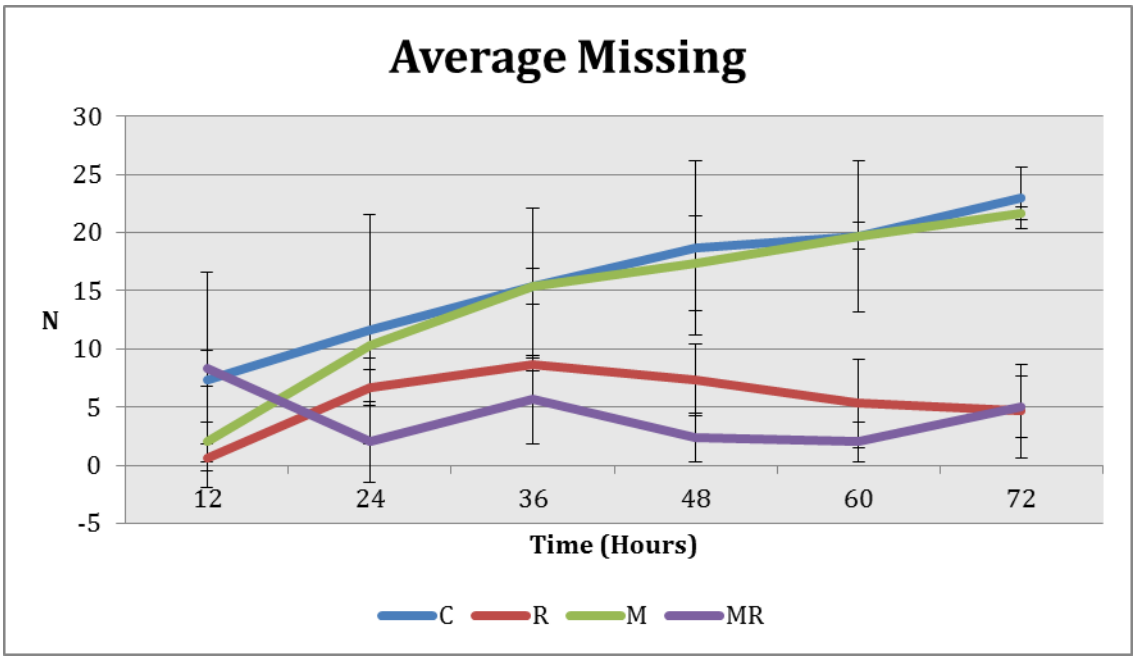

Figure 7: Average number (N) of missing individual larvae from treatments through time. Error bars calculated from standard deviation. Blue is the Control (C), red is the Reversin 205 (R) treatment, green is the Mercury Only (M) treatment, and purple is the Mercury plus Reversin 205 treatment.

3.3.2.2 Sublethal effects

In the Control and Mercury Only treatments, larvae remained in the through the water column (ie the middle) through the majority of the experiment, with exceptions at 12 hours and 72 hours (Figure 8). The average proportion of individuals at the bottom or surface decreased slightly through time, except at 72 hours, where the proportion of individuals at the bottom increased (Figure 8). In the Reversin Only and Mercury plus Reversin Treatments the average proportion of individuals at the bottom increased with time, while the average proportion of individuals in the middle decreased with time (Figure 9). In the Reversin Only treatment the average proportion of individuals at the surface decreased with time, while in the Mercury plus Reversin treatment the average proportion of individuals at the surface remained constant (Figure 9).

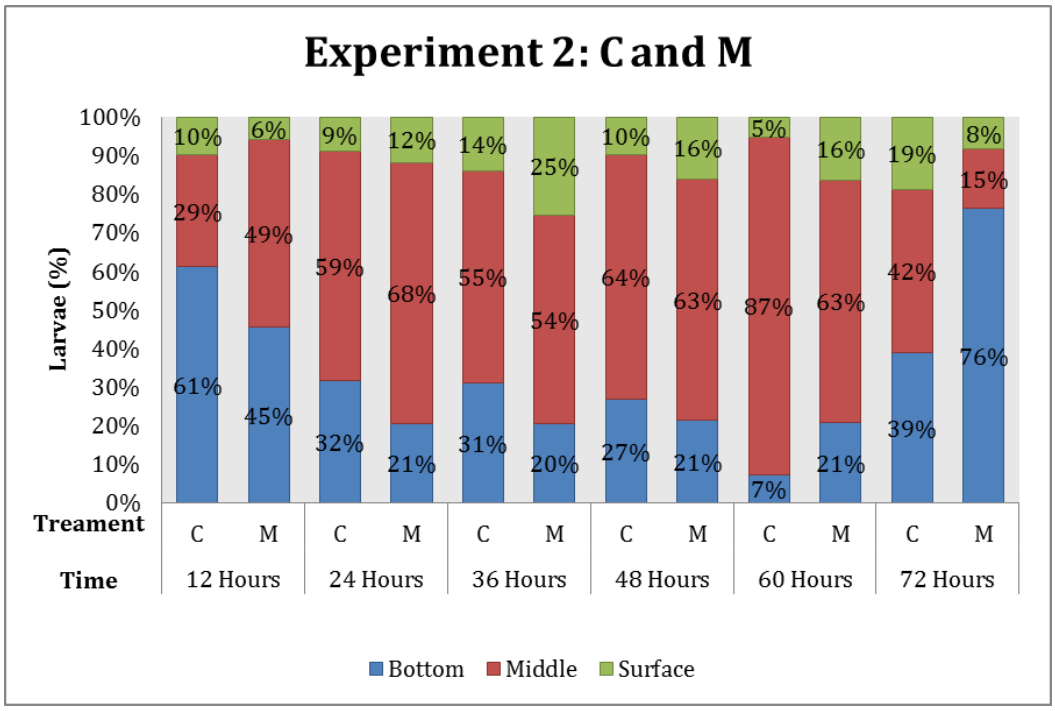

Figure 8: Average percent of larvae in control (C) and mercury only (M) treatments according to position in water column. Blue represents bottom, red represents middle, and green represents surface.

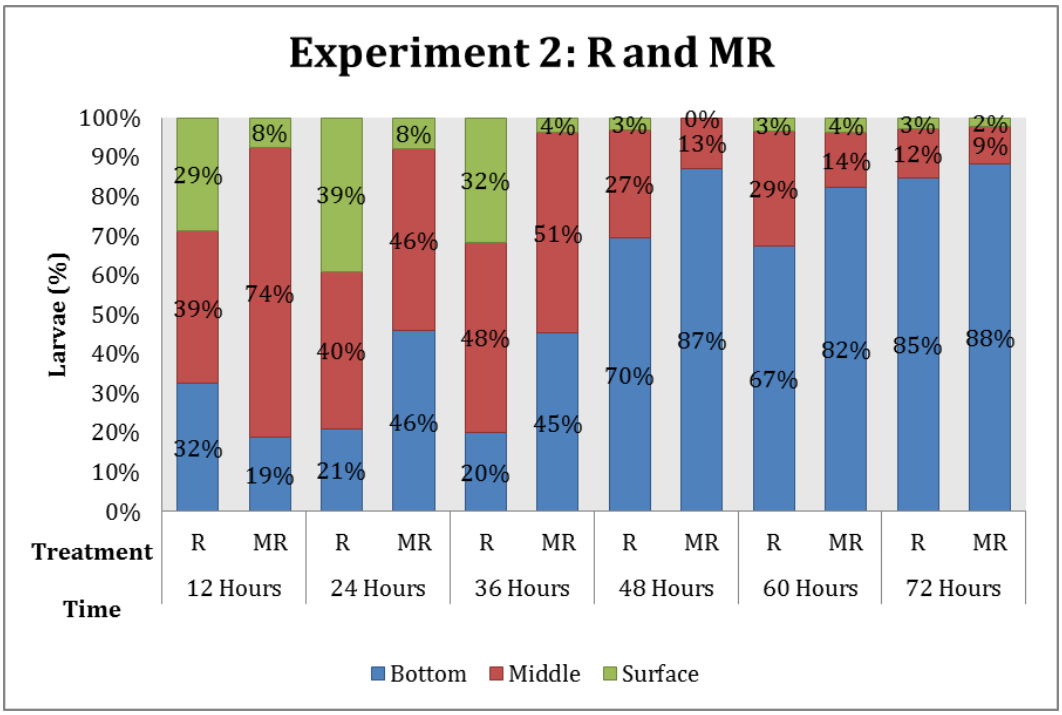

Figure 9: Average percent of larvae in Reversin Only (R) and mercury plus Reversin (MR) treatments according to position in water column. Blue represents bottom, red represents middle, and green represents surface.

4. Discussion

4.1 Multidrug Resistance of Algae

The results of exposure of algae to MDR inhibitors yielded non-significant results for *I. galbana*. The average RFU values of *I. galbana* exposed to MDR inhibitors MK 571, PSC 833 or Reversin 205 were not significantly differently from fluorescent dye controls or untreated controls. *I. galbana* and *D. tertiolecta* do not have cell walls (Zhu and Lee, 1997), thus the fluorescent dye should have been able to diffuse through the cell membrane. However, both species are autofluorescent (Olson et al., 1989; Peterson, 1989) and interference from this natural fluorescence may have made collecting significant data unobtainable in this study. This does not necessarily rule out the possibility of MDR activity within algae, but further studies are needed to determine the optimal wavelength to observe the species under.

4.2 Analysis of Mercury Within Algae and Larvae Treatments

The differences in survivability of *I. galbana* suggests that the mercury has no effect on the mortality of this species of algae. However, past literature has shown that *I. galbana* is sensitive to inorganic mercury 1.0 µg/l (Eisler, 2007). This means that either there was an error in the making of the stock solution, or that there was a sampling error in calculating the survivability that lead to a greater standard deviation and thus a non-significant difference in survivability between treatments. When examining the data from 24-hour and 48-hour feeding after the 36 hour treatment, algal cell density was 1,120,000 cell/ml higher in 48-hour than 24-hour feeding. This difference in cell density could be due to a difference in the location of cell settlement between these to samples or to accidental re-suspension during the treatment. Therefore, there is enough evidence to indirectly say that algal samples were exposed to mercury.

4.3 Larval Feeding of Mercury Exposed Algae

4.3.1 Sublethal Effects

At early pluteus stages, healthy sea urchin larvae are seen at the surface of the water column, and at later stages spread evening throughout the water column from bottom to surface (Yaroslavtseva and Sergeyeva, 2007). The results of Experiment 1 and Experiment 2 both showed that the Reversin 205 treated larvae were unhealthy in comparison to larvae that were not treated. However, the results in either experiment were unable to show that mercury affected the health of larvae. Had mercury affected the health, a greater percentage of larvae would have been found at the bottom. Furthermore, there was no significant difference between Reversin Only and Mercury plus Reversin treatments. This provides further evidence that when MDR transporter activity is blocked, the concentration of mercury within the larvae was not increased to levels that would have lead to death in embryos of this same species (Bosnjak et al., 2009).

4.3.2 Mortality

The concentration of mercury in each of my mercury contaminated larval feeding treatments was well above the concentration at which embryos of *S. purpuratus* begin to die. Previous literature has shown that *S. purpuratus* embryos begin experiencing acute toxic effect at 500 nM, resulting in death (Bosnjak, 2009; Loh, 2009). This molarity translates to 135 µg Hg/l. The larvae in my experiment were exposed to approximately 31 mg Hg/l, which is more than 1,000 times higher. However, analysis of mortality of larvae in the feeding experiment showed that the

Control and Mercury Only treatments did not significantly differ. This could mean that at later stages of development, *S. purpuratus* is more resistant to lethal effects of mercury and that the mortality seen in the Control and Mercury only treatments was due natural mortality of larvae. Furthermore there was no significant difference between Reversin and Mercury plus Reversin treatments, suggesting that even with an MDR inhibitor, the concentration of mercury within the larvae was not increased to lethal levels. Although differences in toxicity of mercury have not been studied in *S. purpuratus* between embryonic and pluteus stages, the effect of mercury on larvae and adult stages of oysters, shrimp, crab, lobsters had been compared (Conner, 1972). Connor (1972) found that larvae were between 14 to 1,000 times more susceptible than adults of the same species. Furthermore, larvae naturally experience very high mortality rates. Therefore, the high mortality seen in the Control and Mercury Only treatments may be due to natural effects.

Interestingly, Reversin Only and Mercury plus Reversin treatments had significantly less mortality than the Control and Mercury Only treatments. Previous literature has shown that adding and MDR inhibitor that blocks MRP activity increases the EC 50 of inorganic mercury in *S. purpuratus* embryos, due to the accumulation of inorganic mercury (Bosnjak et al., 2009). It was therefore expected that larvae would also show higher mortality in the Mercury plus Reversin Treatment than in the Mercury Only and Reversin Only treatments. However, the results of my experiment indicate that Reversin can actually maintain the sea urchins, so that they do not experience significant mortality. This may be due to some advantageous consequence of blocking MDR activity. MDR transporters are non-specific, thus may transport any substrate that binds out of the cell, regardless of whether it is toxic or not. Therefore, by blocking the MDR activity at the four-arm pluteus, some unknown substrate, possibly an essential amino acid, that positively effects the survival of the larvae may have been allowed to sequester within the larvae. Further replication of this experiment and research of the pluteus stages of *S. purpuratus* are needed to very the cause of the results seen in this experiment.

4.4 Conclusion

While there is a lack of evidence at this time that MDR activity may be present in algal cells to influence the uptake of mercury, the results of this experiment show that the pluteus stages of *S. purpuratus* show interesting and unexpected effects from MDR inhibition and mercury toxicity. While there is evidence that the health of Reversin treated larvae was antagonistically affected due to their position in the water column, mortality showed that overall the viability of the larvae was greater. Further replication of this experiment using more advanced methods of mercury analysis and variation of mercury concentrations is needed to determine the concentration at which effects of mercury on the four-arm pluteus stage can be observed. By understanding how mercury affects the pluteus stage of *S. purpuratus*, the effect of MDR inhibition on mercury uptake at this stage, and whether it continues to act positively on survival as mercury concentration within algal cells increases, can be determined.

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