

THE EFFECTS OF ANTHROPOGENIC COPPER ON THE NATIVE MARINE MUSSEL *MYTILUS CALIFORNIANUS* IN SPUD POINT MARINA, BODEGA BAY, CALIFORNIA

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Abstract

Established marinas act as gateways for human impact on the natural environment. One of the major ways this occurs is through the leaching of biocides from antifouling paints applied to marine vessels. These paints often contain copper, which can have an effect on non-target organisms, especially those in fouling communities within marinas. In this study, I hypothesized that the level of copper in Spud Point Marina in Bodega Bay, CA., would affect the local population of the native mussel *Mytilus californianus* at the larval stage. I tested this by measuring the level of copper in the marina using diffusive gradients in thin films (DGTs), and exposing larvae to different copper concentrations in the laboratory. I found that while increasing copper does decrease normal larval development, increased exposure time has the same effect on development. These factors interact with each other to produce a combined influence on larval development.

I. Introduction

SINCE THE BAN of tributyltin (TBT) as an antifouling compound in 2003, other compounds have been used more prevalently as biocides in antifouling paints, specifically those containing heavy metals like zinc, nickel, cobalt and copper (Bao et al., 2008; Mayer-Pinto et al., 2010; Valkirs et al., 2003). Out of these metals, copper is used most often in antifouling paints applied to recreational vessels and therefore can accumulate within areas surrounding marinas and harbors that have restricted water circulation and are frequented by recreational boats (Schiff et al., 2007; Schiff et al., 2004). Copper in solution can create reactive oxygen species (ROS) and can be detrimental to organisms when ingested or absorbed, damaging DNA and other macromolecules in cells (Keyhani et al., 2006). This effect makes copper ideal in its use as a biocide for recreational vessels, but the leaching of copper into the surrounding environment can affect non-target organisms. This is the same reason that TBT antifouling paints were banned and raises the question of whether copper is really a better alternative as an antifouling biocide (Valkirs et al., 2003).

The presence and leaching of cupric ions into seawater occurs at different rates depending on the number of copper sources. These can be recreational boats, urban runoff, or industrial waste (Piola et al., 2009). Copper presence is also dependent on the amount of local water movement, making copper density higher in areas with less water circulation and tidal flushing (Pineda et al., 2012). As marinas and harbors are subject to the constant presence of boats, potential pollution caused by human interactions, and minimal water movement because of their sheltered locations, it is expected that there would be higher copper concentrations within marinas and harbors, and lower copper concentrations in less sheltered areas.

These concentrated levels of copper pose greater risks to non-target organisms within marinas and harbors, effects that can be observed through the impacts copper has on the make-up and overall health of fouling communities. At some level of stress, all fouling organisms are going to be affected, but stressors in their environment, especially those that are anthropogenic in nature, may have greater

negative impacts on organisms in early-life stages than adult organisms (Pineda et al., 2012). Because of this, non-target larvae are at risk from copper leaching off boats into marinas and harbors.

To investigate how mussel larvae are affected by elevated copper levels, I collected adult mussels from Spud Point Marina in Bodega Bay, Ca., and induced them to spawn in the laboratory. I then exposed their larvae to different levels of copper in the laboratory to observe effects that this trace metal has on development. To apply these data to actual field results, I measured the level of copper in the water column in Spud Point marina in order to correlate with laboratory larval results.

I hypothesized that developmental abnormalities in mussel larvae would rise with increasing copper concentrations in the

laboratory. I compared these results to copper-induced abnormalities in the field through comparison of the measured copper concentrations in water. My results indicated the impacts that copper is likely having on mussel species in the field. To supplement this assumption, I surveyed adult mussel populations of *Mytilus californianus* and *Mytilus galloprovincialis* on two docks in Spud Point Marina, one of which was analyzed for copper presence.

2. Materials and Methods

2.1 Field Copper Testing

Diffusive gradients in thin films (DGTs) were used to measure levels of copper in Spud Point Marina in Bodega Bay, California (Figure 1, Figure 2).



Figure 1. A map of Bodega Bay Harbor, California. The red arrow indicates the location of Spud Point marina within the harbor (Grewell, 2008). DGTs were placed in six different slips along dock B, with two at each relative dock region: near, mid, and far from the dock origin (Figure 3). Fishing line and zip ties were used to attach DGTs directly to mussel aggregations already present on dock B. After exposure to the marina water for approximately six days, DGT sampling units were collected, rinsed in distilled water and immersed in 1 ml 1M HNO₃ for 24 hours. They were then delivered to the UC Davis Interdisciplinary Center of Plasma Mass Spectrometry for analysis.

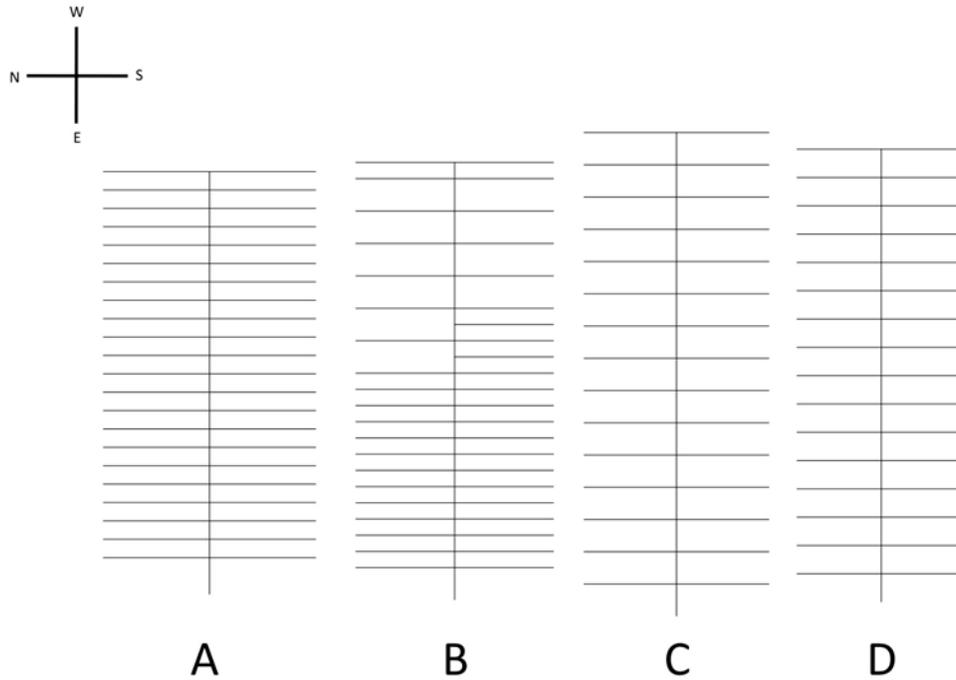


Figure 2: A map of the docks in Spud Point Marina with a compass to orient the docks within the harbor. The slips on the docks are numbered so that odd numbered slips are on the north side of the docks and even numbered slips are on the south side of the docks.

2.2 Field Mussel Surveys and Mussel Collection

Before collecting mussels, both docks B and C were surveyed for the presence of the two species of mussels: *Mytilus californianus* and *Mytilus galloprovincialis*. Four quadrat samples were collected in each slip surveyed, with the first sample always nearest to the slip origin and the fourth always furthest away from the slip origin. Ten slips on both docks were surveyed,

five on both the north and south sides of the docks (Figure 3).

After the mussel populations for both *M. californianus* and *M. galloprovincialis* were surveyed, ten individuals of each species were collected for spawning in the laboratory. The individuals collected ranged in size from four to ten inches in length.

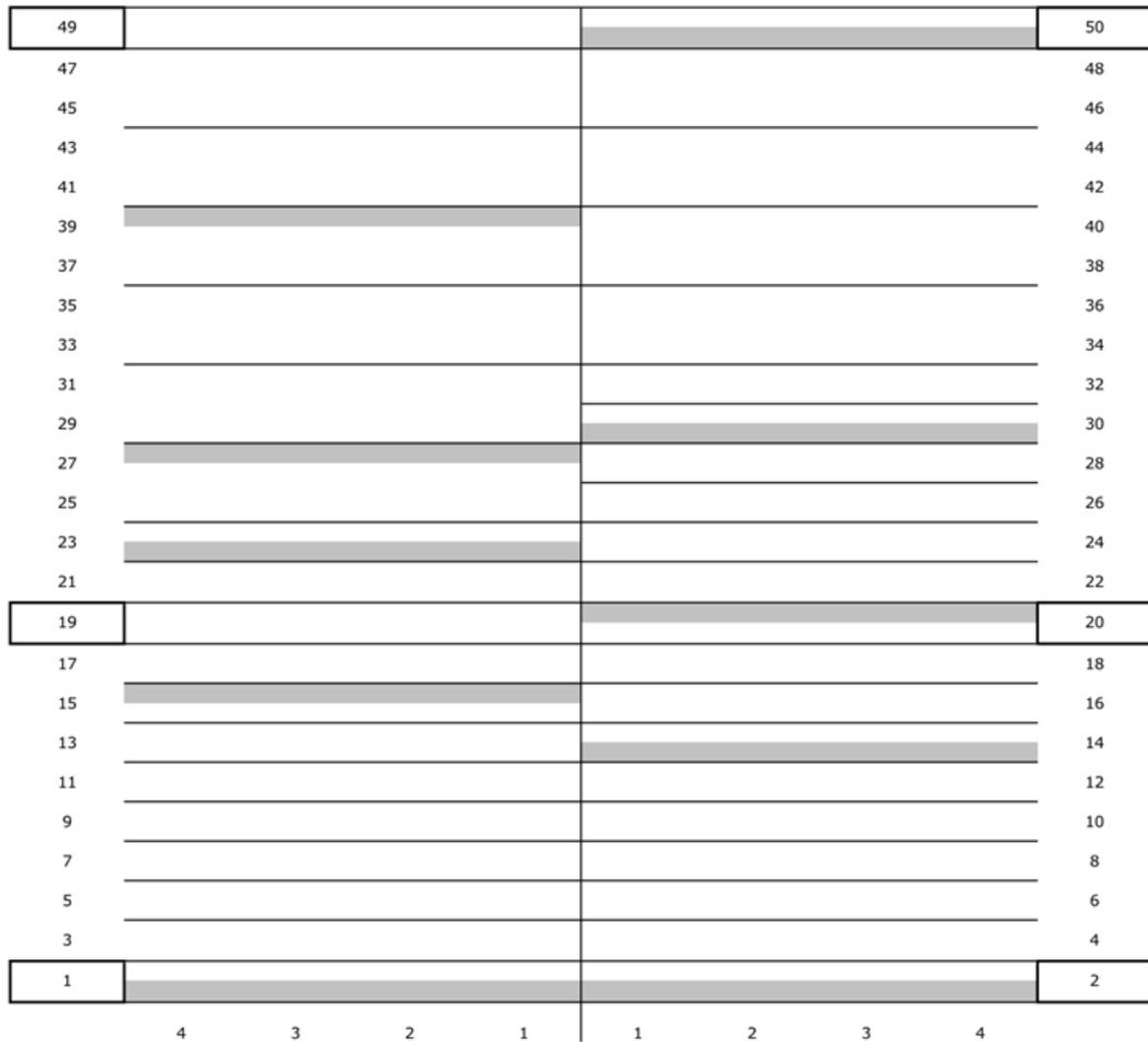


Figure 3: A diagram of dock B in Spud Point Marina, Bodega Bay, California. Odd numbered slips are on the north-facing side of the dock and even numbered slips are on the south facing side of the dock. Shaded areas represent the areas that I surveyed for *M. californianus* and *M. galloprovincialis* using four quadrat replicates in each area (replicate number areas are noted at the bottom near the dock origin). Slip numbers outlined in a thick border represent slips in which I deployed DGTs to measure copper concentrations. Many slips further from the dock origin are double-wide slips to accommodate larger vessels; this is represented by their relative width.

2.3 Preparation of Copper Solutions

To prepare different concentrations of copper solution for use in larval exposure trials, two stock solutions of copper chloride (CuCl_2) measuring $1 \times 10^6 \mu\text{g/L}$ and $1 \times 10^3 \mu\text{g/L}$ were made by adding solid CuCl_2 to distilled water. Volumes of either of these stock solutions were then transferred to six containers each with 75 mL of

filtered seawater to create copper concentrations of $1 \mu\text{g/L}$, $5 \mu\text{g/L}$, $10 \mu\text{g/L}$, $25 \mu\text{g/L}$, $50 \mu\text{g/L}$, and $100 \mu\text{g/L}$. These solutions were stored at room temperature until 9 mL of each solution were transferred into 12-well polystyrene plates for the laboratory copper tests.

2.4 Laboratory Copper Tests

After collecting the mussels, they were placed in a tank with constantly moving water and allowed to acclimate for two days before spawning procedures began. To induce spawning, mussels were immersed in 0.45 μm filtered seawater containing between 2 and 4 million cells per ml *Isochrysis galbana* algae to allow mussels to feed to excess. Mussels were allowed to clear the water of algae for about two hours before they were agitated to induce spawning. Individual mussels were placed in a plastic tub with a lid and shaken violently for one minute. They were then returned to the Pyrex dishes with clear filtered seawater for up to six hours to allow for spawning. This technique resulted in the successful spawning of multiple females and one male of *M. californianus* and only one male of *M. galloprovincialis*. This process was repeated for two more days without successful spawning of both a male and female individual of *M. galloprovincialis*, preventing copper larval exposures of this species.

After the successful spawning of *M. californianus*, male and female gametes were put on ice in separate beakers filled with 0.45 μm filtered seawater. They were then mixed together in a larger container. Fertilization took place for thirty minutes before embryos were transferred to prepared copper solutions, after which, 9 ml of each copper solution and a control composed of 9 ml of filtered seawater were then transferred to labeled 12-well plates with 12 ml wells, and 1 ml of 0.45 μm filtered seawater containing mussel larvae was added to each well. Three replicates of these solutions were created for a total of 21 samples. After transfer, the plates were placed in an incubator set to 15°C. At 2, 12, 24 and 48 hours after initial exposure, samples of each copper treatment were transferred to 24-well plates and fixed with 4% paraformaldehyde for later observation. These time points were used to observe larvae at different developmental

stages: fertilized embryo, trochophore larva, veliger larva, and the D-hinged shell stage.

3. Results

3.1 Field Copper Assessments

The UC Davis Interdisciplinary Center of Plasma Mass Spectrometry processed the DGTs deployed in Spud Point Marina and returned copper measurements in parts per billion (ppb) for the entire time period that the instruments were deployed for both stable isotopes of copper, ^{63}Cu and ^{65}Cu . These values were used to calculate the amount of copper entering the DGT every second in $\mu\text{g/L}$, accounting for time using the following equations:

$$M = \frac{Ce(V_{\text{HNO}_3} + V_{\text{gel}})}{fe} \quad C_{\text{DGT}} = \frac{M\Delta g}{(DtA)}$$

In the first equation, M is the mass of the accumulated copper, Ce is the concentration of metals in the 1M HNO_3 (measured in $\mu\text{g/L}$), V_{HNO_3} is the volume of HNO_3 added to the resin gel, V_{gel} is the volume of the resin gel, and fe is the elution factor for each metal, typically measuring 0.8. In the second equation, C_{DGT} is the concentration of metal measured by the DGT, Δg is the thickness of the diffusive gel plus the thickness of the filter membrane, D is the diffusion coefficient of metal in the gel based on the temperature of the water tested, t is deployment time in seconds, and A is the exposure area of the DGT (Practical Guide for Using DGT in Waters).

The copper present in each DGT was calculated using these equations for both stable isotopes (Table 1). While the raw data shown in the table shows the amount of each isotope of copper accumulated in the DGT over the course of six days, the calculated value of C_{DGT} shows the rate of copper accumulating within the DGT for every second.

Sample Location	Sample Number	Copper 63			Copper 65		
		Raw ($\mu\text{g/L}$)	M ($\mu\text{g/L}$)	C_{DGT} ($\mu\text{g/L}$)	Raw ($\mu\text{g/L}$)	M ($\mu\text{g/L}$)	C_{DGT} ($\mu\text{g/L}$)
Slip 1	2	36.87	0.05299	0.00111	37.02	0.05321	0.00112
Slip 2	3	31.31	0.04500	0.00094	31.70	0.04557	0.00096
Slip 19	4	42.41	0.06096	0.00128	42.24	0.06072	0.00127
Slip 20	5	58.55	0.08417	0.00177	58.60	0.08424	0.00177
Slip 49	6	66.50	0.09559	0.00201	66.50	0.09559	0.00201
Slip 50	7	28.85	0.04147	0.00087	28.40	0.04083	0.00086

Table 1: Analysis of DGT copper concentrations. The Raw category represents the amount of copper ($\mu\text{g/L}$) accumulated in the DGT over the course of six days while deployed in Spud Point Marina analyzed by the UC Davis Interdisciplinary Center of Plasma Mass Spectrometry. The M value is the mass of copper accumulated in the DGT and the C_{DGT} value is the amount of copper absorbed by the DGT during every second of deployment.

3.2 Field Mussel Surveys

The field data collected show that there are more *M. californianus* present on the docks both in slips further from the dock and slip origins. Because copper in the water column was only tested for on dock B, the survey results are limited to reflect the populations of *M. californianus* in this specific area. To analyze how *M. californianus* presence differed from the origin of the dock to points further out in the marina, quadrat results were collected at different slip

ranges and the numbers of *M. californianus* present in each slip range were totaled. Each slip range was assigned a number to represent their distance from the dock origin, with 1 being the closest to and 5 being the furthest from the origin. When these data were graphed, it showed that *M. californianus* increased with increased distance from the dock origin (Figure 4). It is also shown that along dock B, there were more mussels in north-facing slips than in south-facing slips.

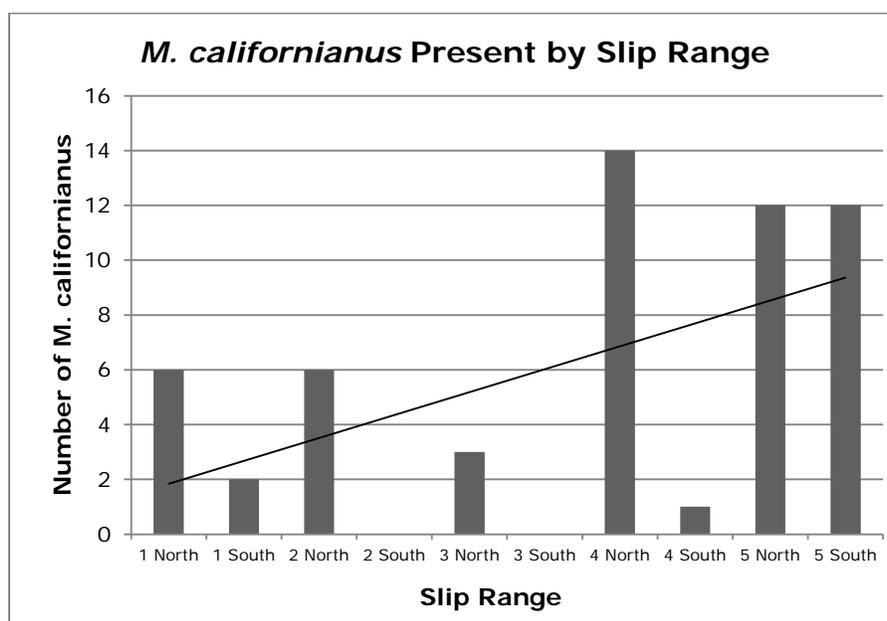


Figure 4: The number of *M. californianus* present on dock B by slip range. Slip ranges represent relative distance from the dock origin with 1 being the closest to and 5 being the furthest from the dock origin. Slips surveyed are distinguished as either north or south-facing. North-facing slips generally have higher populations of *M. californianus* than south-facing slips.

To analyze the distribution of *M. californianus* from slip origins to endpoints, the number of mussels present at different distances from the slip origins were compared. When the docks were surveyed, four quadrats were measured on the side of each slip at regular intervals. To compare these,

the number of mussels present in each replicate range were graphed, with 1 being closest and 4 being furthest from the slip origin (Figure 5). This analysis showed that mussel presence increased with increased distance from the slip origin.

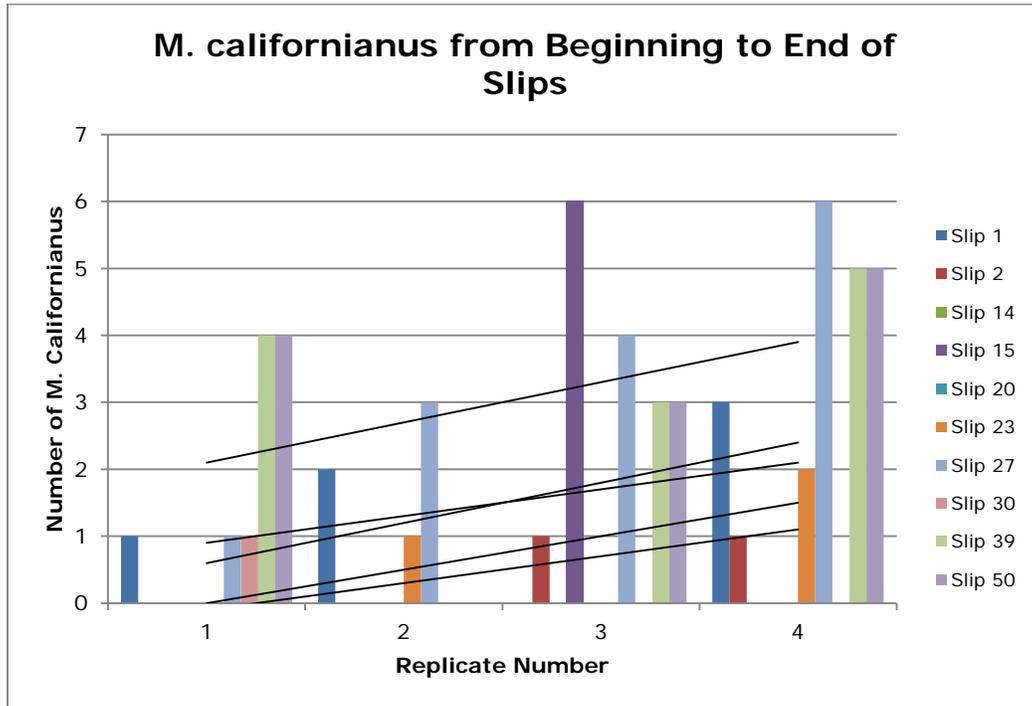


Figure 5: The number of *M. californianus* in replicate numbers. Replicate numbers represent the distance from the slip origin, with 1 being the closest to and 4 being the furthest from the slip origin. These ranges are described visually in Figure 1.

3.3 Laboratory Copper Tests

In observations of the copper-exposed *M. californianus* larvae, the occurrence of larvae developing normally and abnormally for each copper concentration at 2, 12, 24, and 48 hours after initial copper exposure was recorded. Larvae were determined to be developing

normally or abnormally based on their appearance. They were compared at different stages to photos of *M. californianus* larvae included in a report by Breese et al. (1963) to establish the appearance of normal larval development.

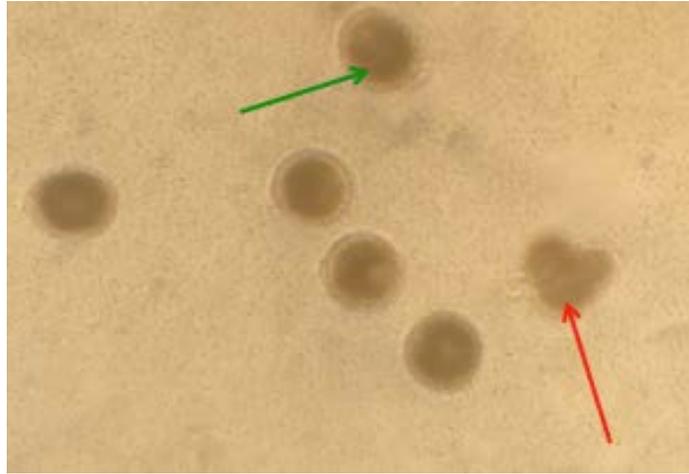


Figure 6: An example of normally and abnormally developing *M. californianus* larvae at two hours after fertilization taken from my laboratory copper tests. The green arrow shows a normally developing fertilized embryo and the red arrow shows an abnormally developing embryo with signs of cellular blebbing.

Abnormal larvae showed signs of cellular blebbing or necrotic deformation (Figure 6), while normal larvae shared most characteristics with the Breese larvae with no apparent cellular damage (Figure 7). At later time points,

development appeared to slow, especially in treatments with higher concentrations of copper where almost none of the larvae reached the D-hinged shell stage at the 48-hour time point.

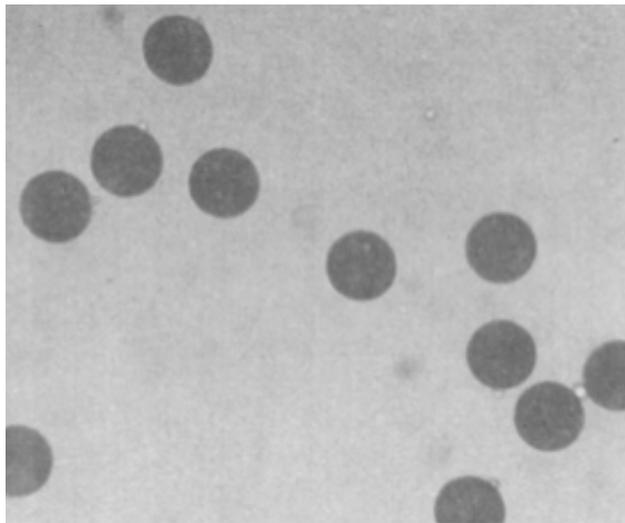


Figure 7: A historical example of normally developing *M. californianus* embryos from Breese et al. (1963).

As expected, the percent of normal larvae decreased with increasing copper concentration (Figure 8). This result was especially apparent in the higher concentrations of 50 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ known to be developmentally harmful to

invertebrate species (Pineda et al., 2012). At lower levels of copper (1 to 25 $\mu\text{g/L}$), higher percentages of normal larvae were observed when compared to the control treatment at the first three time points.

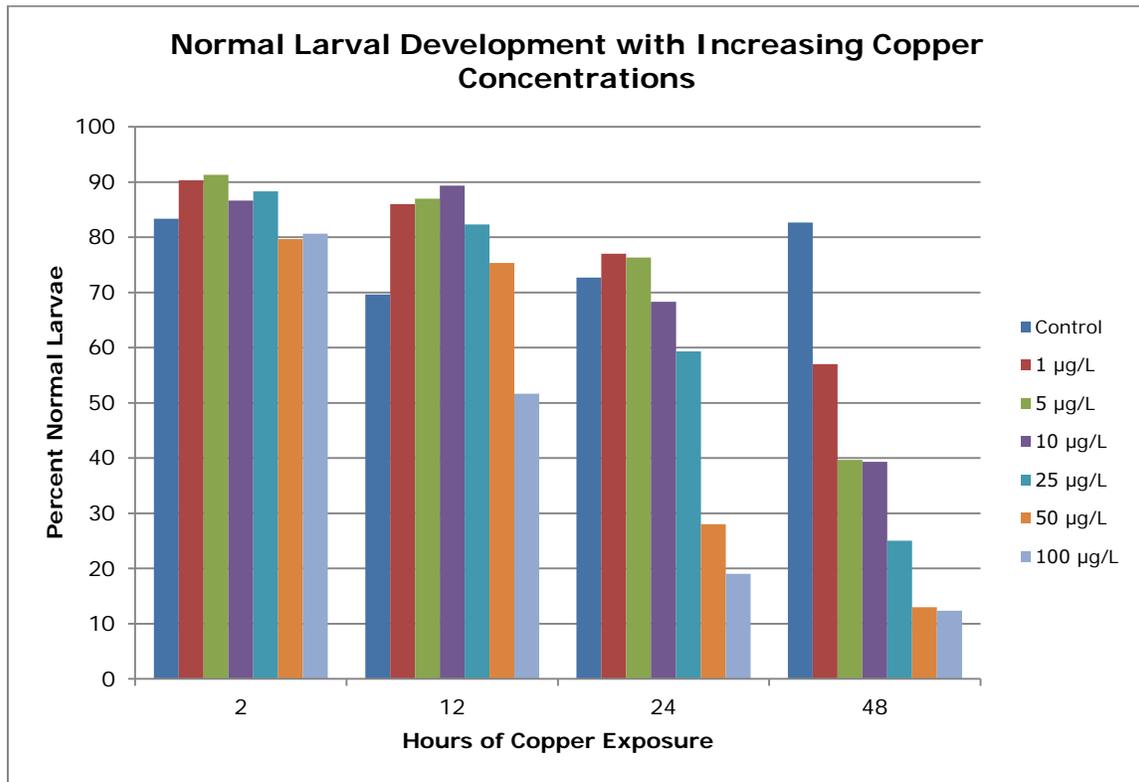


Figure 8: Percent of normal development over time with different concentrations of copper exposure. While higher concentrations of copper reduce normal larval development at almost all time points, lower concentrations also reduce normal development with increased time of exposure.

Unexpectedly, the amount of time that larvae were exposed to copper at any concentration had a significant effect on the percent of normal larvae present. At later time points, higher concentrations of copper affected development more than at earlier time points. The rate at

which the copper had effects on development generally increased with increasing copper concentrations, which were analyzed through finding the slope of the best-fit lines for each concentration over time (Figure 9, Table 2).

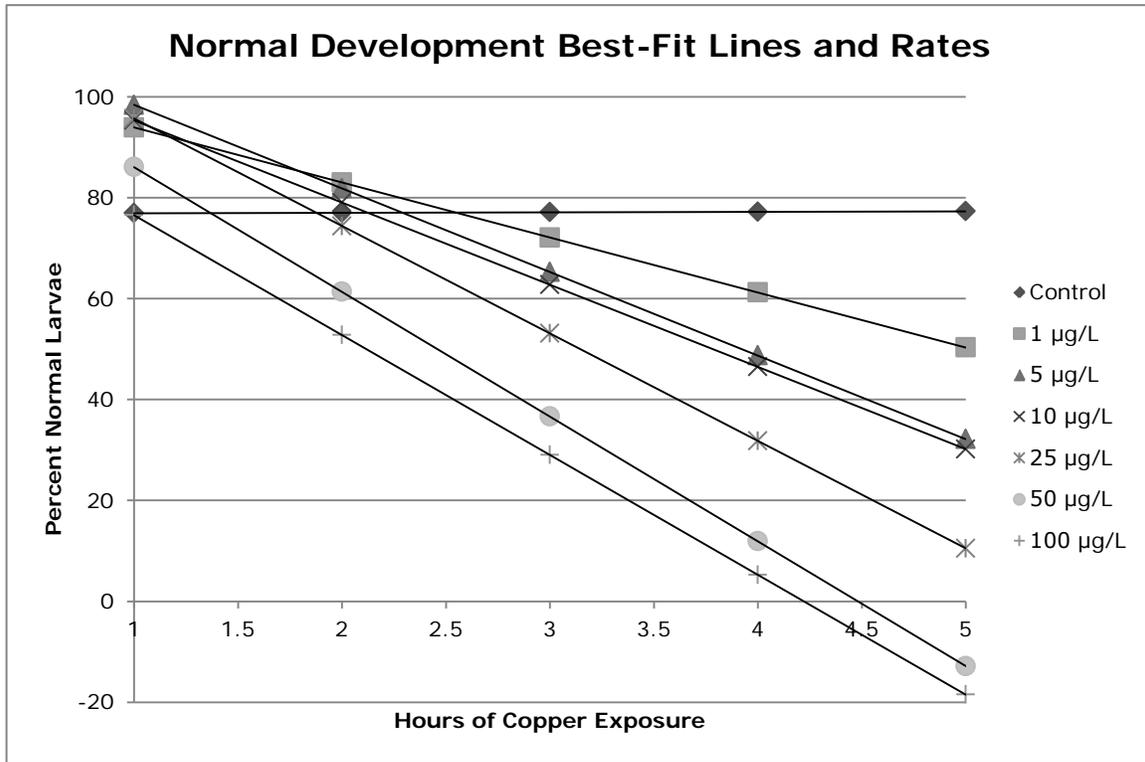


Figure 9: Best-fit lines from the data in Figure 6. In treatments with higher copper concentrations, the rate at which normal larval development decreases shows signs of increasing. This shows a connection between copper concentration and the length of time larvae are exposed to any concentration of copper.

Concentration	Best-Fit Line Slope
Control	0.1
1 µg/L	-10.9
5 µg/L	-16.567
10 µg/L	-16.3
25 µg/L	-21.3
50 µg/L	-24.733
100 µg/L	-23.767

Table 2: The slopes of the best-fit lines from Figure 7. These slopes represent the rates of decrease of normal larval development and show a steady decrease in normal development with increased copper concentration and increased exposure time.

3.4 Laboratory Copper Tests Data Analysis

To determine the significance of copper concentration and time of exposure on abnormal development of *M. californianus*, a two-way analysis of variance (ANOVA) was used with JMP software. From this, it was determined that both copper concentration and time of exposure had significant effects on larval development in *M. californianus*, with $P < 0.001$. From this analysis, it was also determined that both of these factors impacted each other, making them positively correlated with $P < 0.001$. This statistical analysis also showed that the power of performance was sufficient for this analysis.

4. Discussion

The survey of the docks at Spud Point Marina showed that *M. californianus* generally occur in greater numbers at points farthest away from both dock and slip origins. This suggests that these mussels thrive best in areas with high levels of water movement with little shelter from the abiotic stressors in the marina. There were also more mussels in north-facing slips than south-facing slips, where the north facing slips were more exposed to the mouth of the marina, allowing for more water movement from surface winds. These data, however, do not correlate with the data on copper concentration determined from DGTs that were deployed and analyzed. It is likely that these mussels thrive in areas with higher water movement because of their stability in high movement environments and their ability to outcompete other species because of this toughness. A study by Johnson et al. (2006) found that when compared to *M. galloprovincialis*, *M. californianus* are able to withstand harsher physical conditions and stay attached to substrate better than other mussel species. The pattern that was observed is most likely caused by physical factors other than effects of copper toxicity on larval survival and development, although more research could be done to investigate whether or not there is a connection between copper concentration and *M. californianus* adult distribution.

In the placement of the DGTs, there was no correlation between cardinal direction of the slips or distance from the dock origin. It is possible that the concentration of copper found in each slip could depend on the activity of boats within the slips, making a trend impossible to determine without information on boat use in individual slips. With the limited number of DGTs deployed, it is also possible that a trend based on oceanographic factors could exist, but more data points would be needed for this to be confirmed. With information on boat activity and further replication of DGT deployment, it could be possible to determine a pattern of copper concentration on this dock and others in Spud Point marina.

In measuring the levels of copper present in Spud Point Marina, it was determined that because copper levels are at such low levels instantaneously, it is unlikely that the copper in the marina could harm any organism exposed to the surrounding water for a short amount of time. However, it seems that copper would be able to build up over a short amount of time and because of this, could pose a threat to fouling community organisms. Before calculating instantaneous copper amounts, the raw data showing the amount of copper absorbed by the DGTs over the course of six days ranges between 28.40 µg/L and 66.50 µg/L (Table 1). These levels of copper pose serious health risks to invertebrates, and if a fouling organism is unable to eliminate or process copper quickly, it could build up within tissues much like it did in the DGTs deployed in the marina (Pineda et al., 2012).

In the analysis of the laboratory copper test results, it was interesting that development slowed with increased concentration and time exposed to copper. The time points to observe the *M. californianus* larvae were based on information provided by Breese, et al. (1963) and an instructional handout by Cherr, et al. (1996) on the methods for spawning a related species, *Mytilus edulis* in the laboratory. By the 48-hour time point, only a few larvae had reached the shell stage in the control treatments, and very few had in all of the copper-treated groups combined. A definition for normal development was then determined to be relative to whatever stage the larvae had reached at each time point, with the majority of individuals at the trochophore and veliger stage at the 24 and 48-hour stages, respectively. This result could be due to an inaccurate estimate of the time needed to reach certain developmental stages. In order to better observe the effects of copper on specific stages of larval development, more information is needed describing the times at which these developmental stages occur.

M. californianus and other fouling organisms could be at risk to copper toxicity because of the amount of time they are exposed to copper at any

level. In the laboratory copper tests, it was determined that in addition to the concentration of copper used in each exposure, the amount of time an organism is exposed to copper significantly affects the development of mussel larvae, even at low concentrations of copper. Over the course of 48 hours, larvae exposed to copper at a concentration of 1 µg/L had over 30% decreased normal development while treatments exposed to higher copper concentrations decreased in even higher proportions (Figure 6). Although the rate at which copper leaches into seawater is relatively low, 8.2 µg/cm-day for recreational vessels, it can take up to 3 months for these release rates to decrease, allowing copper to build up in the environment surrounding recreational boating areas for a great amount of time (Valkirs et al., 2003). Future research could focus on the effects of time of exposure and concentration on copper toxicity both individually and as a combined factor. Further investigation of how copper builds up within the tissues of invertebrates would also be useful in analyzing the physiological limits of copper intake as they relate to exposure time.

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