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Effects of Heavy Metal Uptake on Growth, Chlorophyll Content, and Calcium Oxalate Crystals in Lemna minor (Duckweed)

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EFFECTS OF HEAVY METAL UPTAKE ON GROWTH, CHLOROPHYLL CONTENT, AND CALCIUM OXALATE CRYSTALS IN *LEMNA MINOR* (DUCKWEED)

by

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Abstract

Lemna minor L., duckweed, is a common aquatic plant used for the phytoremediation of wastewater. Heavy metal contamination poses a significant issue, and numerous studies have been conducted on the efficiency of *L. minor's* hyperaccumulation ability of these metals. Calcium oxalate crystals are an important aspect of the growth and development of *L. minor*, but how they are influenced by the uptake of heavy metal has not been extensively studied. This study aims to determine the effects that lead, cadmium, and copper have on the formation of calcium oxalate crystals, vegetative growth, and chlorophyll a and b content on *L. minor.* Cultures were grown in a diluted Hoagland's nutrient solution for thirteen days in various concentrations of lead (0, 50, 100, 200 and 300 mg/L), copper (0, 5, 10, and 30 mg/L), and cadmium (0, 5, 20, 35, and 50 mg/L). Samples were collected on days 1, 5, 8, and 13 and analyzed for plantlet number, plantlet fresh weight, frond area, chlorophyll a, b, and ab, and crystal density, size, and number with polarized light microscopy and a spectrophotometer. It was determined that higher concentrations of heavy metals impact the overall growth and photosynthetic capacities of *L. minor*, which are indicators of its toxicity. Additionally, crystal formation was significantly impacted (i.e., crystal size was reduced by 78%, 51%, and 56% for lead, copper, and cadmium) at higher concentrations of heavy metals, indicating calcium sequestering is greatly inhibited and reduced. This contributes to a further understanding of the phytoremediation capabilities of *L. minor* and its tolerance to heavy metal toxicity.

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INTRODUCTION

Lemna minor L. is an aquatic, vascular angiosperm genus in the family Araceae commonly referred to as "duckweed" along with species of the genera *Spirodela, Landoltia, Wolfiella,* and *Wolffia* (Chen, et al., 2020). *L. minor* is a small, floating, vascular aquatic plant that has one of the fastest reproduction rates among plants (Zirschky and Reed, 1988). Asexual reproduction occurs when the daughter frond, a leaf like structure, starts as a bud along the center axis of the mother frond and eventually emerges from the pouch on the side of the mother and is held together by a strip of tissue called the stipule (Cross, 2015). As the daughter frond matures the stipule elongates and when fully matured it breaks off to release a new cluster or colony (Cross, 2015). Each mother frond is able to reproduce at least ten to twenty times in a life cycle (Zirschky and Reed, 1988). Because of this mechanism, *L. minor* can double in frond numbers every four days eventually covering the entire surface area of water creating a bio mat. In addition to fast growth, *L. minor* is able to accumulate high concentrations of various metals and nutrients (Zirschky and Reed, 1988). Because of this, *L. minor* is a desirable species to be used in phytoremediation.

Phytoremediation uses hyper-accumulative plants to remove toxic substances in water and provides a more cost-effective, eco-friendly, and applicable alternative to chemical removal (Yang, et al., 2021). *L. minor* is a beneficial tool because of its fast plant growth and large biomass production, yielding higher phytoextraction results and cleaner waters in significantly less time and waste compared to conventional treatment processes (Bokhari, et al. 2019). At higher concentrations of heavy metals though, select metabolic pathways are heavily impacted, narrowing its range of use (Naumann, et al., 2007).

Many studies focus on the vegetative aspect of the plant and how metals distribute/sequester within plant tissues (Sobrino, et al., 2010; Bokhari, et al., 2019; Chen, et al., 2020). In addition, generalized plant studies reported heavy metals such as lead, strontium, cadmium, and copper are incorporated in the crystals (Franceschi and Nakata, 2005). However, there is a lack of data on the metals' effect on calcium oxalate crystals in *L minor* specifically. Calcium oxalate crystals evolved as a defense mechanism against grazing animals and include different shapes and sizes of crystals called raphides, druses, styloids, prisms, and crystal sands (Cuéllar-Cruz, et al., 2020). Druses and raphide crystals are the most common type found in plants, specifically in *L. minor,* the raphide form (Cuéllar-Cruz, et al., 2020). These crystals form when calcium from the environment is taken up through calcium sequestration, also known as biomineralization, and deposited in specialized crystalline cells called idioblasts (Cuéllar-Cruz, et al., 2020). The synthesis of these crystals starts with ascorbic acid that gives rise to oxalic acid and once sufficient concentrations are met oxalate oxidase is activated (Cuéllar-Cruz, et al., 2020). Oxalate oxidase acts as the regulator of oxalic acid and actively participates in the formation of oxalate crystals (Cuéllar-Cruz, et al., 2020). In addition, proteins called calsequestrin function to regulate the activity of the cytosolic calcium before it is stored in the vacuoles (Cuéllar-Cruz, et al., 2020). A third protein called calreticulin is a high-capacity calcium binding protein that helps buffer calcium as it is mediated by a dihydropyriding type of calcium channel (Franceschi, Nakata, 2005). A simplified model of calcium oxalate formation can be found in Appendix 1 (Franceschi and Nakata, 2005).

Heavy metals include cadmium, chromium, cobalt, copper, mercury, nickel, silver, thallium, zinc, lead, strontium, arsenic (V), and arsenic (III). All these metals are taken up into the vegetative portions of *L. minor* and effect various pathways of the plant (Bokhari, et al.,

2019; Chen, et al., 2020; Kanoun-Boulé 2009; Sobrino, et al., 2010). One study found that the strontium replaced the calcium in calcium oxalate crystals, leading to a decrease in calcium and a change in morphology of the crystal and idioblasts, where the crystals form (Franceschi, Schueren, 1986). There is a lack of research further investigating the issue of heavy metals incorporating into or affecting the morphology of calcium oxalate crystals.

Heavy metals also impact the metabolic pathways of *L. minor*. Morphologically, heavy metals can cause necrosis, colony disintegration, root break up, and physiological inhibitions such as photosynthesis, pigment synthesis and enzyme activity (Banu Doğanlar, 2013). Chlorophyll content is also heavily impacted as seen by a concentration-dependent decrease particularly in chlorophyll a (Hou, et al., 2007). Additionally, colony disintegration is seen when the presence of heavy metals induces the release of daughter fronds from the mother before reaching maturity and may serve as a biomarker for heavy metal toxicity (Ziegler, et al., 2016).

Lead, copper, and cadmium are commonly found in water systems and can enter plants via natural and anthropogenic activities (Winfield, 2022). Lead exposure is associated with a variety of effects in humans ranging from neurodevelopmental effects, mortality, impaired renal function, and impaired fertility (WHO, 2022). Because of such, the guideline value established by the World Health Organization (WHO) states 0.01 mg/l in drinking water (WHO, 2022). However, levels in rivers and lake water bodies across the globe saw a mean level of 0.116 ± 0.025 mg/l of lead in the 2010s, greatly exceeding guideline values (Zhou, et al., 2020).

Copper is an essential plant and human nutrient for growth and development but results in toxicity at higher doses. Long term exposure to copper can include anemia, dementia, jaundice, liver or kidney failure, tremors, convulsions, etc. (WHO, 2022). The WHO guideline value for copper is 2 mg/l however, concentrations in drinking water range from 0.005 to 30 mg/l

(WHO, 2022). Copper is a common heavy metal mined for its use in household plumbing, electrical wiring, household fixtures, and tools. In the 2010s, global concentrations were only 0.120 mg/l of copper contaminated in water bodies (Zhou, et al., 2020).

Cadmium is able to accumulate in the kidneys and has a biological half-life in humans of ten to thirty-five years, however no clear evidence suggests genotoxicity via oral route (Zhou, et al., 2020). Acute cadmium poisoning will occur though if inhaled or ingested causing gastrointestinal related symptoms, bronchitis, chemical pneumonitis, and pulmonary edema (ATSDR, 2008). Long term exposure can cause kidney disease due to accumulation, fragile bones, and possibly cancer (CDC, 2017). The current WHO guideline value is 0.003 mg/L; however, recent global lake concentrations see values of 0.025 mg/L which greatly exceeds the guideline value (WHO, 2022; Zhou, et al., 2020).

These global values only include a select number of lakes and rivers from the main continents and do not take into account the numerous mines around the globe excavating these heavy metals. The mining industry is the primary source of contamination in numerous ecological environments with improper maintenance creating high amounts of waste, sediment run-off, spills, dust, and accidental destruction of the mining site (Karn, et al., 2021). The contamination of those environments may not spill into major rivers or lakes but pollute the soils and streams around it. The use of *L. minor* as a cheaper and cleaner alternative for protecting the environments around mines would be beneficial, but how high of a concentration can *L. minor* survive and how are the metabolic pathways impacted?

The objective of this study was to determine the effects lead, cadmium, and copper, have on the formation of calcium oxalate crystals, overall vegetative growth, and chlorophyll a and b content on *L. minor.*

METHODOLOGY

Plant Material: *L. minor* was purchased from the vendor Aqua Habit (https://www.amazon.com/Duckweed-Lemna-Minor-Plants-Habit/dp/B073XSY7FX/ref=mp_s_a_1_3?keywords=Duckweed&qid=1657763859&sr=8-3&th=1). Cultures were acclimated for 2 weeks in an incubator at 26º C with a 16-hour light timer and a light intensity of 120 µmol/m^2s^2. A diluted Hoagland's E Medium with EDTA-chelated iron (See Appendix) was used as the nutrient solution. Nutrient solutions were replaced once every week for maintenance of stock cultures. After acclimating the cultures, plantlets of *L. minor* were surface sterilized in 0.75% NaClO, vigorously agitated for thirty seconds and thoroughly rinsed with deionized water. The sterilized, or axenic cultures, were then individually placed and sealed into sterile plastic containers with 50 mL of Hoagland's E Medium and allowed to grow for three months until a sufficient amount, minimum of 500 plantlets, was produced for experimentation.

Heavy Metals: Lead stock solutions of 0, 50, 100, 200, and 300 mg L^{-1} of lead nitrate ($Pb(NO_3)_2$); Fisher Scientific) were prepared in Hoagland's E Medium. Concentrations were selected from the known lethal concentration 50 (LC50) referenced in the literature (Verma and Suthar, 2015). Similarly, copper stock solutions of 0, 5, 10, and 30 mg L^{-1} of Cupric Sulphate ($CuSO_4 \bullet 5H_2O$); Fisher Scientific) were prepared in Hoagland's E Medium. Concentrations were selected from the known lethal concentration 50 (LC50) referenced in the literature (WHO, 2022; Hou, et al., 2007). Cadmium stock solutions of 0, 5, 20, 35, and 50 mg $L^{-1}$of Cadmium sulfate hydrate ($CdSO_4 \bullet xH_2O$); Sigma Aldrich) were prepared in Hoagland's E Medium. Concentrations were selected from the known lethal concentration 50 (LC50) referenced in the literature (Verma and Suthar, 2015). All stock solutions were adjusted to a pH of 6 with NaOH.

Ten *L. minor* colonies were placed in plastic containers with 100 mL of stock solutions and containers were covered with plastic wrap. There were three replicates for each heavy metal. Each replicate was randomized and were placed in an incubator at 26º C with a 16-hour light interval and a light intensity of 120 µmol/m^2s^2. Samples were collected on days 1, 5, 8, and 13 for three colonies, plantlets, with two fronds each. One colony was used or the analysis of chlorophyll concentration and the other two were used for viewing crystals in polarized light microscopy.

Chlorophyll a and b Concentration: Clusters with two fronds were selected and blotted on dry tissue paper for collection of fresh weight. The fronds were weighed to determine fresh weight. The fronds were placed in 100 µL of DMSO to extract chlorophyll. Samples were refrigerated for a week at 4° C allowed to come to room temperature before measuring chlorophyll content in the DMSO solution. To determine the chlorophyll concentrations, 2 µL of the DMSO/chlorophyll extract was measured on a Thermo Scientific NanoDrop One^C Spectrophotometer at a wavelength range of 345-700 nm.

Slide Preparation and Polarized Light Microscopy: Two colonies of *L. minor* were soaked in 70% acetone at 60° C for a total of six days to remove chlorophyll. Samples were then dehydrated with an acetone series, and infiltrated with xylene in the refrigerator at 4° C. Under a dissecting microscope, colonies were separated into individual fronds and infiltrated with mounting resin for permanent slides. A Zeiss polarizing microscope attached to a Haier LCD monitor and Canon Vixia HF S21 camera was used to measure crystal density and size and to quantify frond area. Two mature fronds were selected from each slide for a total of six fronds per treatment to determine crystal density (i.e., the number of crystals per frond divided by the frond

area). For crystal size, two mature fronds were selected and five crystals per frond were measured for length and width for a total of 30 crystals per treatment.

Statistical Analysis: Data for growth, fresh weight, frond area, crystal density, crystal size, crystal number, and chlorophyll content were analyzed for separate, individual days and metals with ANOVA in a completely random design with 4 or 5 level of chemical. For data in which the assumptions of ANOVA were not met (normally and independently distribution of means and variances), values were either log or square root transformed and then re-analyzed with ANOVA. Mean differences for levels of chemicals were determined based on Student-Newman-Keuls mean separation tests.

Calculation of Percent Inhibition: The percent inhibition related to the control of the sample.

$$\text{Percent Inhibition (\%)} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

RESULTS

Plantlet number: The presence of lead and cadmium had a greater effect on plantlet number than did copper (Figure 1A-C). For lead, there was a significant difference among treatments at day 5, 8, and 13 ($p<0.01$ for all tests). On day 5, there was a 65% decrease in treatment 300 mg/L compared to the 0 mg/L control (Figure 1A). On day 8 and 13, there was an 82% and 93% decrease, respectively, for the 300 mg/L treatment compared to the control. Similarly, the presence of cadmium effected plantlet number ($p<0.01$ for days 5, 8, and 13). On day 5, there was a 23-24% decrease in plantlet number at concentrations 20, 35, and 50 mg/L (Figure 1C). On day 8, concentrations 20, 35, and 50 mg/L caused a 36-93% reduction in plantlet number compared to the control. Plantlet number on day 13 for concentrations 20 and

35 mg/L was 90 and 96% lower, respectively, then plantlet number for the control (Figure 1C). Cadmium concentration of 50 mg/L killed all plantlets by day 13 (Figure 1C). In contrast to lead and cadmium, growing plantlets in various concentrations of copper had no effect on plantlet number ($p=0.48$-0.94) (Figure 1B).

Plantlet fresh weight: All three heavy metals significantly reduced plantlet fresh weight. As early as day 5, lead effected plantlet fresh weight ($p<0.01$); lead concentrations of 100 and 300 mg/L reduced plantlet weight by 34% and 73%, respectively, relative to the control (Figure 2A). On day 8, concentrations of 50, 100, and 200 mg/L affected plant weight significantly ($p<0.01$) and reduced plantlet weight by 39-40%. The greatest effect for lead on day 8 was seen at 300 mg/L which exhibited a 91% reduction in plantlet weight. Similar results were seen for day 13 in which treatments 50,100, and 300 mg/L reduced plantlet weight by 26, 33, or 69%, respectively, relative to the control.

Cadmium showed a significant reduction in fresh weight consistently from days 5, 8, and 13 ($p < 0.01$ for all test of individual days). Cadmium concentrations of 20, 35, and 50 mg/L were all significantly lower than those for treatments 0 or 5 mg/L (Figure 2C) with a maximum reduction of 84% relative to the control.

The effect of copper was not significant on plantlet fresh weight until day 8 and 13 ($p<0.01$ and $p=0.03$, respectively) (Figure 2B). On days 8 and 13, the concentration 30 mg/L caused 42 and 49% reduction in fresh weight, respectively, compared to that of the control.

Frond Area: The three heavy metals affected frond area differently; the presence of cadmium affected frond area earlier (day 5; $p=0.05$) than did lead (day 8; $p=0.03$) or copper (day 13; $p=0.02$; Figure 3). The greatest reduction in frond area for lead occurred on day 13 for treatment 300 mg/L which caused a 76% reduction in frond area relative to that of the control

(5.76 mm^2 versus 1.29 mm^2 for control and 300 mg/L treatment, respectively; Figure 3A) (Figure 4). In contrast, the copper treatment 300 mg/ml only reduced frond area by 50%. (Figure 3B) Cadmium reduced frond area on day 5 ($p=0.05$), the smallest frond area was 50 mg/L with a 46% reduction, and 5, 20, and 35 mg/L with a minimal reduction, 14%, 19% and 14%, in size (Figure 3C). On day 8 ($p<0.01$), the smallest size was 35 and 50 mg/L with a 76% and 71% reduction, the second smallest was 20 mg/L with a 56% reduction, and the third smallest was 5 mg/L with a 42% reduction. On day 13 ($p<0.01$), the smallest was 20 and 30 mg/L with an 83% and 82% reduction, the second smallest was 5 mg/L with a 59% reduction, and somewhat reduced in size 50 mg/L with a 35% reduction.

Chlorophyll a concentration: Lead, copper, and cadmium affected the concentration of chlorophyll a throughout the thirteen days. For lead, chlorophyll a concentration decreased on day 5 for concentration 300 mg/L by 55% reduction relative to that of the control (Table 1). On day 13, there was a 5, 18, and 44% reduction for treatments 100, 200, and 300 mg/L, respectively (Table 1). Copper affected concentrations of chlorophyll a on day 8 ($p<0.01$) and 13. The highest concentration of copper on either day caused the greatest reduction in chlorophyll a concentration. Oddly, the intermediate levels of copper (5 and 10 mg/L) caused higher chlorophyll levels than did the control. In general, the greatest reduction in chlorophyll a concentration was caused by cadmium. On days 5, 8, and 13, 50 mg/L of cadmium reduced chlorophyll a concentration by 81, 95, and 96%, respectively. Cadmium effected chlorophyll a on day 5 ($p<0.01$), with 5, 20, 35, and 50 mg/L with a 39%, 51%, 54%, and 81% reduction in concentration. On day 13 ($p=0.01$), the lowest concentrations of chlorophyll a were 35 and 50 mg/L with a 77% and 96% reduction, and the second lowest concentrations of 5 and 20 mg/L with a 53% and 51% reduction.

Chlorophyll b concentration: Lead, copper, and cadmium affected the concentration of chlorophyll b throughout the thirteen days. Lead decreased on day 5 ($p=0.01$) for 300 mg/L with a 10% reduction in concentration (Table 2). On day 13 ($p=0.02$), the lowest concentration was 300 mg/L with a 36% reduction, the second lowest was 200 mg/L with a 18% reduction, and the third lowest was 100 mg/L with an 8.6% reduction. The highest concentration was seen in 50 mg/L, and 0 mg/L the second highest. Copper did not affect chlorophyll b until day 8 ($p<0.01$), the lowest concentration was 300 mg/L with a 11% reduction, the second lowest was 0 mg/L, and the highest were 5 and 10 mg/L. On day 13 ($p<0.01$), the lowest concentration was 300 mg/L with a 30% reduction, the second lowest was 0 mg/L, and the highest were 5 and 10 mg/L. Cadmium effected chlorophyll b on day 5 ($p<0.01$) for 5, 20, 35, and 50 mg/L with a 36%, 56%, 63%, and 77% reduction in concentration. On day 13 ($p=0.01$), the lowest concentration of chlorophyll B was 50 mg/L with a 98% reduction, and the second lowest were 5, 20, and 35 mg/L with a 49%, 25%, and 46% reduction.

Chlorophyll ab: Lead, copper, and cadmium affected the concentration of chlorophyll ab throughout the thirteen days. Lead decreased on day 5 ($p<0.01$), for 300 mg/L with a 54% reduction in concentration. On day 13 ($p<0.01$), the lowest concentration was 300 mg/L with a 43% reduction, the second lowest was 200 mg/L with a 18% reduction, and the third lowest was 100 mg/L with a 6% reduction (Table 3). Copper did not affect chlorophyll ab until day 8 ($p<0.01$), the lowest concentration was 30 mg/L with a 17% reduction, and the second lowest was 0 mg/L. On day 13 ($p<0.01$), the lowest concentration was 30 mg/L with a 53% reduction, and the second lowest was 0 mg/L. Cadmium effected chlorophyll ab on day 5 ($p<0.01$), for 5, 20, 35, and 50 mg/L with 38%, 58%, 57%, and 82% reductions in concentrations. On day 13

($p<0.01$), the lowest concentration of chlorophyll ab was 50 mg/L with a 94% reduction in concentration.

Crystal Density: The density of crystals was minimally affected by lead and copper, but cadmium had a significant effect. Lead only affected crystal density on day 13 ($p<0.01$), smaller densities were recorded for 0, 50, 100, and 200 mg/L and the largest density was 300 mg/L (Table 4; Figure 4). Copper did not affect crystal density throughout the experiment. In contrast, cadmium affected crystal density starting at day 5 ($p=0.03$), the largest density was 50 mg/L, and density did not change for concentrations of 5, 20, and 35 mg/L (Table 4). On day 8 ($p=0.02$), the largest crystal density was 35 mg/L, and the second largest was 20 and 50 mg/L. Lastly, on day 13 ($p<0.01$), the largest crystal density was 20 mg/L, and the second largest was 35 mg/L.

Crystal Size: The size of crystals was predominantly affected by adding lead, copper, and cadmium. Lead affected crystal size on day 5 ($p=0.01$), for the smallest crystal size was 300 mg/L with a 55% reduction in size, and concentrations for 50, 100, and 200 mg/L were somewhat smaller than the control with a 31%, 35%, and 35% reduction in size (Table 5; Figure 5). On day 8 ($p < 0.01$), the smallest was 300 mg/L with a 74% reduction, and the second smallest were 50 and 200 mg/L with a 26% and 17% reduction in size. Lastly, on day 13 ($p<0.01$), the smallest crystal size was 300 mg/L with a 78% reduction, and the second smallest was 50 mg/L with a 33% reduction. Copper affected crystal size on day 5 ($p<0.01$) for the smallest were 10 and 30 mg/L with a 36% and 33% reduction in size (Table 5). On day 13, the smallest was 30 mg/L with a 51% reduction, and the second smallest was 10 mg/L with a 30% reduction in size. Cadmium affected crystal size on day 5 ($p=0.02$), the smallest were 5, 20, 35, and 50 mg/Lwith a 33%, 38%, 33% and a 36% reduction in size. On day 8 ($p<0.01$), the smallest were 20, 35, and 50 mg/L with a 32%, 44% and a 56% reduction, and 5 mg/L was somewhat

smaller with a 8% reduction in size. Lastly, on day 13 ($p<0.01$), the smallest was 35 mg/L with a 69% reduction, the second smallest were 20 and 50 mg/L with a 56% and 56% reduction, and the third smallest was 5 mg/L with a 43% reduction in size.

Crystals per Frond: Lead, copper, and cadmium affected the number of crystals per frond of *L. minor*. Lead decreased in crystal number on day 5 ($p=0.0016$) for 300 mg with a 37% reduction (Table 6). On day 8 ($p=0.01$), the lowest amount was 300 mg/L with a 53% reduction, and somewhat lowered for 50 and 200 mg/L with a 25% and 25% reduction. On day 13 ($p<0.01$), the lowest was 300 mg/L with a 50% reduction. Copper decreased crystal number on day 5 ($p<0.01$) the lowest amount was 30 mg/L with a 43% reduction, and the second lowest were 5 and 10 mg/L with a 19% and 27% reduction. Day 8, however, has a significant p-value of $p=0.03$, but the statistical package identified no differing relationships. Lastly, on day 13 ($p<0.01$), the lowest amount was 300 mg/L with a 51% reduction, and the second lowest were 5 and 10 mg/L with a 27% and 35% reduction. Cadmium decreased crystal number on day 5 ($p<0.01$) for concentrations of 5, 20, 35, and 50 mg/L with a 24%, 26%, 31%, and 31% reduction (Table 6). On day 8 ($p<0.01$) the lowest were 5, 20, 35, and 50 mg/L with a 37%, 44%, 51%, and 50% reduction in number. Lastly, on day 13 ($p<0.01$) the lowest were 5, 20, 35, and 50 mg/L with a 36%, 54%, 66% and 46% reduction in crystal number.

DISCUSSION

Lead and cadmium significantly inhibited colony growth (i.e., plantlet number, frond area, and plantlet fresh weight) of *L. minor* at the treatment concentrations during the thirteen-day period. In contrast, the effects of the presence of copper were less. Lead greatly affected plant growth-highest tested concentrations of 300 mg/L but for biomass weight it affected

concentrations 50 mg/L and greater. Sobrino, et al., (2010) suggests the LC_{50} (Lethal Concentration 50) of lead to be 500 $\pm$ 23.4 mg/L. The results reinforced this with the inhibition of higher concentrations and a longer exposure period before the *L. minor* succumbed to toxicity. In addition, one study also found that *L. minor* is an efficient accumulator of lead content that peaked after 6-7 days of exposure before toxicity settled in, and a second study also found that *L. minor* has better growth rates at higher concentrations of lead (Sobrino, et al., 2010; Verma and Suthar, 2015). In contrast one study reported that lead will interfere with mitochondrial activity leading to reduced growth (Kanoun-Boulé, et al., 2009). The results suggest that growth rate was not affected under 300 mg/L, and that all concentrations decreased in weight by day eight. Which would be the period after peak accumulation of lead when toxicity would settle in as noted due to the yellow discoloration of the fronds indicating necrosis. Colony disintegration only occurred in the concentration of 300 mg/L after eight days with the reduction of colony weight and frond area. This suggests that higher concentrations of lead significantly stress mother fronds and force them to release immature daughter fronds sooner. While lead only affected growth factors at higher concentrations, colony weight is affected at all concentrations after a prolonged exposure period.

On the other hand, copper did not affect colony number throughout the experiment, however a reduction in biomass weight occurred eight days after introduction for concentrations of 5 mg/L or greater. Frond area is only inhibited by copper at concentrations of 30 mg/L after extensive exposure. As previously stated, copper is an essential plant nutrient for growth and development and is toxic at high concentrations of 30 ppm or greater (30 mg/L; Banu Doğanlar, 2013). Colony growth was not affected during the thirteen day period since copper is important for that specifically. Literature reports that “excess of copper interferes with respiration,

photosynthesis, pigment synthesis, and enzyme activity," (Kanoun-Boulé, et al., 2009). This would explain the reduction in only colony weight after eight days of exposure as chlorosis was noted at day eight and by day thirteen necrosis was noted for 10 and 30 mg/L further supporting metabolic interference. The inhibition of colony weight and frond area in 30 mg/L after eight days of exposure is exhibited and severely pronounced after thirteen days. Reaffirming *L. minor* can tolerate up to 30 mg/L of copper.

In contrast, cadmium severely and significantly inhibited growth factors involved with *L. minor*. Shortly after exposure, a reduction in number of colonies, biomass weight, and frond area occurred for concentrations of 5 mg/L or greater. Sobrino, et al., (2010) suggests the LC_{50} (Lethal Concentration 50) of cadmium to be 50 ± 31.5 mg/L. For the 5 mg/L treatment, colony number and weight are not inhibited by cadmium which was seen in a previous study. Verma and Suthar (2015) found that *L. minor* exposed to 5 mg/L treatment of cadmium for seven days had a removal efficiency of 74.2% and had a final biomass gain of 17.1%. In comparison the 5 mg/L treatment had a 17.9% final biomass gain suggesting small amounts of cadmium does not severely inhibit growth rate or biomass of *L. minor*. Frond area does decrease for the 5 mg/L treatment, after eight days of exposure and it is not clear as to why since there is no inhibition for growth or weight. In contrast concentrations greater than 20 mg/L of cadmium severely inhibit number, weight, and frond area of *L. minor* colonies. Colony disintegration, the premature release of daughter fronds, occurred as there was significant reduction in weight and frond area suggesting premature release of daughter fronds due to stress from heavy metals. At small concentrations cadmium minimally affects growth factors but as concentration increases, inhibition also significantly increases.

Lead, copper, and cadmium significantly reduce the amount of chlorophyll a, b, and ab in *L. minor*. Lead affected chlorophyll a, b, and ab significantly on day five with 300 mg/L and on day thirteen with concentrations 100 mg/L or greater. It has been reported that lead is involved in the reduction in photosynthesis and will inhibit chlorophyll synthesis (Sobrino, et al., 2015). It is evident this effect occurred as chlorophyll ab within a few days after exposure it decreased for concentrations 300 mg/L or higher, and at concentrations 200 mg/L or higher with prolonged exposure. For chlorophyll a and b within a few days of exposure it decreased for concentrations of 300 mg/L or higher, and at concentrations of 100 mg/L or higher with prolonged exposure.

Copper significantly impacted chlorophyll a, b, and ab in *L. minor*. Previous studies indicate that copper concentrations greater than 5 mg/L reduced total chlorophyll concentrations after four days of exposure (Hou, et al., 2007). However, only the 30 mg/L for chlorophyll a, b, and ab concentrations were reduced after eight days of exposure. Additionally, 5 and 10 mg/L slightly increased and maintained the chlorophyll a, b, and ab concentrations after eight days of exposure while the control began to decline. Studies have reported that slight increases of chlorophyll a and b have occurred but with a copper concentration of 0.05 mg/L but there is a steady decrease in chlorophyll concentration between 5 and 10 mg/L followed by a sharp decline (Hou, et al., 2007). Excess copper does interfere with photosynthesis and pigment synthesis activities, explaining the observation of chlorosis noted on day eight for all concentrations that continued through day thirteen with necrosis present for 10 and 30 mg/L (Kanoun-Boulé, et al., 2009). However, it does not explain why chlorosis was present, but chlorophyll concentration slightly increased for the 5 and 10 mg/L copper concentrations.

Cadmium significantly reduced chlorophyll a, b, and ab in *L. minor*. Cadmium affected a, b, and ab significantly on day five with all concentrations reduced. Comparatively on day eight

there is no significant difference for chlorophyll b and ab, but chlorophyll a was significantly reduced in 50 mg/L and somewhat reduced in the rest. On day 13, chlorophyll ab there is only significant difference in 50 mg/L, whereas chlorophyll a had severe reduction in 35 mg/L or greater and somewhat reduced in the rest, and chlorophyll b had severe reduction in 50 mg/L and somewhat reduced in the rest. Accompanied with the decrease in chlorophyll was the observation of continual chlorosis and eventual necrosis of colonies. The steady decrease in chlorophyll concentration as cadmium concentration increased is supported by the literature with reports of up to a 10 mg/L causing a steady decrease and then a sharp decline as concentration increases (Hou, et al., 2007).

Lead, copper, and cadmium significantly impacted crystal size and number but have varying effects on crystal density. Lead impacted crystal density only on day thirteen with an increase in density in 300 mg/L. This may be impacted though by the frond area size as it was significantly reduced as well. Crystal size was reduced on day five with 300 mg/L the smallest and the rest somewhat reduced. However, on days eight and thirteen 300 mg/L continued to be the smallest but 50 mg/L and 200 mg/l (only day eight) were the second smallest. The number of crystals per frond was similar to crystal density trends as day five with 300 mg/L had the lowest amount of crystals and continued to for days eight and thirteen. The other lead concentrations only had a significant difference in crystal number on day eight with 50 and 200 mg/L slightly lowered. It appears at higher concentrations lead reduces crystal size and number as crystal growth and cell growth are highly coordinated when regarding the idioblast (Franceschi and Nakata, 2005). The colony is not significantly growing either as frond area is decreased and crystal density is increased, suggesting that lead affects *L. minor* metabolically which will eventually and indirectly affect bulk calcium regulation at higher concentrations.

Copper did not affect crystal density and only reduced crystal size and number. Crystal size was reduced on days five through thirteen for concentrations 10 mg/L or greater. Crystal number on days five and thirteen were reduced for concentrations 5 mg/L or greater. It is evident that copper somehow affects bulk calcium regulation. The primary function of idioblasts is to serve as a localized calcium sink and calcium regulation is observed by the disappearance of crystals under conditions of calcium deficiency or developmental maturation (Franceschi and Nakata, 2005). Copper did not inhibit growth, weight, or crystal density of *L. minor,* but the size and amount of crystal were significantly reduced suggesting the activity of calcium channels or pumps are affected in idioblasts, or it is depleting its stores of calcium in an attempt to overcome toxicity and produce mature daughter cells.

Cadmium significantly impacted crystal density, size, and number throughout the experiment. On day five the number and size of crystals were significantly reduced for concentrations of 5 mg/L or greater. In addition, crystal density was larger for 50 mg/L in part due to the reduced frond are size. Days eight through thirteen crystal density increased as frond area decreased, with crystal size and number decreased, for concentrations 20 mg/L or greater. The presence of cadmium proved toxic to *L. minor* which induced colony disintegration as previously stated. It is not clear if cadmium is incorporated in the crystals since neither scanning electron microscopy (SEM) nor energy dispersive X-ray analysis was performed. A previous study with strontium incorporation in calcium oxalate crystals recorded a decrease in density and number of crystals as strontium concentration increased (Franceschi and Schueren, 1986). Suggesting there may be no incorporation of cadmium into the crystals, but there is some effect on calcium regulation exhibited by the decrease in size and number. Since colony disintegration and toxicity occurred it could be suggested that crystals were reduced in number and size in an

attempt to release more clones or metabolic activity was severely inhibited which indirectly affected calcium regulation.

Calcium is an essential plant nutrient required for the growth and development of cells and other metabolic pathways (White and Broadly, 2005). For plants that contain calcium oxalate crystals it serves various functions including, “calcium regulation, plant protection, detoxification, ion balance, [and] tissue support/plant rigidity,” (Franceschi and Nakata, 2005). In general evidence has found that oxalate helps incorporate a variety of heavy metals into the crystals which pose serious health concerns for other plants that are regularly consumed by animals or humans (Franceschi and Nakata, 2005). In addition, the presence of heavy metals in water poses serious toxicity concerns for plant health and studying these effects can help further the understanding the use of detoxification of wastewaters in an organic and chemical free method.

In terms of phytoremediation the significance of this research is to further develop the understanding of the relationship between calcium oxalate crystals and heavy metals. *L. minor* has one of the fastest reproduction rates among plants and require significant calcium and other nutrients to maintain those rates (Zirschky and Reed, 1988). Those traits make *L. minor* a desirable character for the role of phytoremediation as it is an extremely efficient hyperaccumulator of heavy metals (Bokhari, et al., 2019). The difficulties lie in how efficient it could be in aquatic environments with high concentrations of metals and will calcium regulation be impacted before or after toxicity sets in. If the *L. minor* cannot grow well then it will not be an effective tool for phytoremediation.

The collected data from our study suggests *L. minor* is not as severely impacted at lower concentrations of heavy metals with minimal effects on growth, chlorophyll, and crystal

components. For phytoremediation of lead, *L. minor* should be used up to 300 mg/L and replaced weekly to ensure maximal removal due to the dramatic drop seen in weight, chlorophyll, and crystal content. For copper, only 10 mg/L and below should be used and also replaced weekly due to weight and crystal decreases. Lastly, for cadmium a maximum concentration of 5 mg/L for efficiency of removal and replaced weekly due to inhibitions of growth, chlorophyll, and crystal contents at higher concentrations.

Future directions of this research would include further replications of the experiments to confirm the effects seen. Conducting additional tests at concentrations at lower concentrations than suggested, in the previous paragraph, may also be beneficial for determining a more specific range of use. Additionally, outsourcing samples to a lab for dispersive X-ray analysis and SEM would be conclusive if the heavy metals are truly incorporated in the crystals. Lastly, duplication of an experiment with strontium would be beneficial for comparison since its incorporation into the crystal is already known.

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Table 1. Chlorophyll a concentration of *Lemna minor* exposed to lead, coper, and cadmium

	Chl a content (µg/mg)							
	Day 1	Day 5		Day 8		Day 13		
Conc. of lead	Mean	Mean		Mean		Mean		
0 mg/L	1148 ± 451	667 ± 297	a	650 ± 44		606 ± 24	b	
50 mg/L	1652 ± 642	859 ± 48	a	726 ± 98		726 ± 22	a	
100 mg/L	1366 ± 311	877 ± 31	a	316 ± 339		576 ± 33	bc	
200 mg/L	958 ± 191	887 ± 25	a	681 ± 50		499 ± 7	c	
300 mg/L	712 ± 128	300 ± 108	b	472 ± 88		337 ± 87	d	
	p=0.10	p<0.01		p=0.06		p<0.01		
Conc. of copper								
0 mg/L	568 ± 224	569 ± 22		490 ± 51	b	449 ± 43	b	
5 mg/L	748 ± 229	675 ± 35		668 ± 42	a	733 ± 84	a	
10 mg/L	450 ± 249	669 ± 201		716 ± 0	a	702 ± 49	a	
30 mg/L	726 ± 358	564 ± 1		374 ± 36	c	279 ± 46	c	
	p=0.52	p=0.52		p<0.01		p < 0.01		
Conc. of cadmium								
0 mg/L	638 ± 45	792 ± 69	a	570 ± 172	a	603 ± 119	a	
5 mg/L	596 ± 71	485 ± 156	b	449 ± 60	ab	282 ± 10	ab	
20 mg/L	644 ± 105	387 ± 164	b	358 ± 141	ab	296 ± 251	ab	
35 mg/L	665 ± 43	358 ± 238	b	341 ± 294	ab	141 ± 48	b	
50 mg/L	643 ± 130	152 ± 59	b	27 ± 8	b	22 ± 20	b	
	p=0.90	p<0.01		p=0.08		p=0.01		

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

Table 2. Chlorophyll b concentration of *Lemna minor* exposed to lead, copper, and cadmium

	Chl b content (µg/mg)							
	Day 1	Day 5		Day 8		Day 13		
Conc. of lead	Mean	Mean		Mean		Mean		
0 mg/L	291 ± 108	106 ± 97	b	185 ± 16		175 ± 4	a	
50 mg/L	403 ± 164	227 ± 12	a	207 ± 26		189 ± 36	a	
100 mg/L	352 ± 92	226 ± 2	a	99 ± 94		160 ± 17	ab	
200 mg/L	233 ± 46	242 ± 8	a	184 ± 27		144 ± 8	ab	
300 mg/L	173 ± 33	95 ± 40	b	172 ± 22		112 ± 31	b	
	p=0.11	p=0.01		p=0.13		p=0.02		
Conc. of copper								
0 mg/L	159 ± 69	171 ± 8		145 ± 10	b	137 ± 13	b	
5 mg/L	210 ± 74	201 ± 20		231 ± 35	a	201 ± 2	a	
10 mg/L	138 ± 80	195 ± 99		211 ± 18	a	214 ± 12	a	
30 mg/L	218 ± 124	197 ± 96		129 ± 12	b	96 ± 3	c	
	p=0.65	p=0.95		p<0.01		p < 0.01		
Conc. of cadmium								
0 mg/L	208 ± 15	216 ± 14	a	215 ± 2		189 ± 35	a	
5 mg/L	193 ± 30	139 ± 40	b	135 ± 25		96 ± 14	ab	
20 mg/L	219 ± 23	95 ± 39	b	127 ± 66		134 ± 74	ab	
35 mg/L	217 ± 9	80 ± 46	b	162 ± 92		103 ± 36	ab	
50 mg/L	213 ± 38	49 ± 4	b	67 ± 39		33 ± 9	b	
	p=0.72	p<0.01		p=0.11		p=0.01		

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

Table 3. Chlorophyll ab concentration of *Lemna minor* exposed to lead, copper, and cadmium

	Chl ab content (µg/mg)							
	Day 1	Day 5		Day 8		Day 13		
Conc. of lead	Mean	Mean		Mean		Mean		
0 mg/L	1480 ± 575	880 ± 371	a	858 ± 62		803 ± 28	a	
50 mg/L	2113 ± 829	1116 ± 53	a	959 ± 125		864 ± 170	a	
100 mg/L	1767 ± 414	1134 ± 33	a	426 ± 445		757 ± 51	a	
200 mg/L	1225 ± 243	1161 ± 33	a	890 ± 79		660 ± 15	a	
300 mg/L	911 ± 163	405 ± 151	b	662 ± 112		461 ± 122	b	
	p=0.10	p<0.01		p=0.07		p<0.01		
Conc. of copper								
0 mg/L	746 ± 301	759 ± 31		625 ± 61	b	601 ± 57	b	
5 mg/L	984 ± 311	900 ± 52		923 ± 17	a	980 ± 120	a	
10 mg/L	604 ± 337	887 ± 306		912 ± 87	a	941 ± 60	a	
30 mg/L	970 ± 495	727 ± 29		517 ± 47	c	385 ± 51	c	
	p=0.56	p=0.57		p<0.01		p < 0.01		
Conc. of cadmium								
0 mg/L	868 ± 60	1036 ± 85	a	775 ± 231		814 ± 159	a	
5 mg/L	810 ± 103	641 ± 201	b	599 ± 87		389 ± 23	a	
20 mg/L	887 ± 131	436 ± 208	b	497 ± 209		441 ± 333	a	
35 mg/L	906 ± 53	450 ± 292	b	455 ± 425		250 ± 86	a	
50 mg/L	878 ± 171	191 ± 86	b	74 ± 10		46 ± 28	b	
	p=0.87	p<0.01		p=0.12		p<0.01*		

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

Table 4. Crystal Density of *Lemna minor* exposed to lead, copper, and cadmium

	Number of Crystals / mm^2 of frond							
	Day 1		Day 5		Day 8		Day 13	
Conc. of lead	Mean		Mean		Mean		Mean	
0 mg/L	46.9 ± 9.9		23.4 ± 2.1		23.0 ± 0.5		20.2 ± 2.2	b
50 mg/L	41.7 ± 7.7		34.8 ± 2.9		23.4 ± 4.4		17.7 ± 1.5	b
100 mg/L	48.1 ± 4.1		26.5 ± 8.3		23.2 ± 13.0		18.3 ± 2.6	b
200 mg/L	37.0 ± 8.3		27.7 ± 6.9		25.1 ± 6.6		18.2 ± 2.5	b
300 mg/L	46.7 ± 7.4		27.2 ± 3.0		23. 0 ± 5.5		49.3 ± 21.0	a
	p=0.41		p=0.18		p=0.99		p<0.01 *	
Conc. of copper								
0 mg/L	33.4 ± 3.9		22.6 ± 6.3		21.1 ± 4.0		26.9 ± 7.7	
5 mg/L	34.1 ± 10.1		20.6 ± 5.3		23.0 ± 5.0		18.6 ± 2.6	
10 mg/L	30.2 ± 7.9		22.3 ± 5.4		21.4 ± 4.8		16.4 ± 1.2	
30 mg/L	36.3 ± 9.4		14.8 ± 2.0		17.5 ± 3.9		22.1 ± 7.1	
	p=0.83		p=0.27		p=0.51		p=0.17	
Conc. of cadmium								
0 mg/L	19.0 ± 4.8		22.5 ± 4.3	b	15.9 ± 4.4	b	27.7 ± 6.5	b
5 mg/L	24.8 ± 1.5		19.4 ± 2.1	b	20.6 ± 1.1	b	31.4 ± 13.1	b
20 mg/L	20.6 ± 1.4		20.0 ± 2.4	b	24.2 ± 3.8	ab	73.6 ± 16.8	a
35 mg/L	28.8 ± 3.0		17.8 ± 2.0	b	41.7 ± 13.9	a	53.1 ± 17.9	ab
50 mg/L	30.8 ± 17.0		29.8 ± 6.6	a	33.0 ± 8.7	ab	17.8 ± 0.8	b
	p=0.37		p=0.02		p=0.02		p<0.01	

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

Table 5. Crystal Size of *Lemna minor* exposed to lead, copper, and cadmium

	Crystal Size (μm^2)					
	Day 1	Day 5		Day 8		Day 13	
Conc. of lead	Mean	Mean		Mean		Mean	
0 mg/L	723 ± 109	687 ± 68	a	658 ± 63	a	570 ± 102	a
50 mg/L	732 ± 102	477 ± 116	ab	490 ± 30	b	385 ± 54	b
100 mg/L	585 ± 80	449 ± 96	ab	714 ± 80	a	523 ± 2	a
200 mg/L	667 ± 233	447 ± 32	ab	548 ± 66	b	610 ± 54	a
300 mg/L	641 ± 88	309 ± 151	b	170 ± 41	c	126 ± 27	c
	p=0.67	p=0.01		p < 0.01		p < 0.01	
Conc. of copper							
0 mg/L	623 ± 55	625 ± 18	a	670 ± 133		582 ± 118	a
5 mg/L	618 ± 149	630 ± 29	a	517 ± 11		563 ± 97	a
10 mg/L	563 ± 167	401 ± 81	b	610 ± 47		406 ± 36	ab
30 mg/L	688 ± 123	421 ± 58	b	408 ± 112		287 ± 30	b
	p=0.72	p<0.01		p=0.06		p<0.01	
Conc. of cadmium							
0 mg/L	749 ± 235	699 ± 163	a	558 ± 53	a	675 ± 74	a
5 mg/L	685 ± 143	468 ± 3	b	515 ± 86	a	386 ± 58	b
20 mg/L	542 ± 58	437 ± 51	b	379 ± 64	b	299 ± 48	bc
35 mg/L	657 ± 143	467 ± 47	b	312 ± 84	b	212 ± 35	c
50 mg/L	695 ± 46	445 ± 4	b	245 ± 32	b	297 ± 21	bc
	p=0.52	p=0.02		p<0.01		p<0.01	

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

Table 6. Crystal per frond of *Lemna minor* exposed to lead, copper, and cadmium

	Crystals per Frond (number)							
	Day 1	Day 5		Day 8		Day 13		
Conc. of lead	Mean	Mean		Mean		Mean		
0 mg/L	130 + 19	127 + 3	a	146 + 24	a	116 + 8	a	
50 mg/L	117 + 3	135 + 15	a	109 + 27	ab	98 + 6	a	
100 mg/L	123 + 15	119 + 6	a	129 + 15	a	106 + 8	a	
200 mg/L	104 + 56	127 + 21	a	110 + 14	ab	112 + 2	a	
300 mg/L	108 + 24	80 + 3	b	68 + 20	b	58 + 11	b	
	p=0.81	p<0.01		p=0.01		p<0.01		
Conc. of copper								
0 mg/L	110 + 5	124 + 3	a	107 + 17	a	126 + 20	a	
5 mg/L	94 + 28	100 + 9	b	68 + 13	a	92 + 8	b	
10 mg/L	88 + 12	91 + 11	b	102 + 17	a	82 + 8	b	
30 mg/L	87 + 15	71 + 10	c	73 + 13	a	62 + 1	c	
	p=0.40	p<0.01		p=0.03		p<0.01 *		
Conc. of cadmium								
0 mg/L	93 + 20	114 + 3	a	144 + 18	a	109 + 27	a	
5 mg/L	121 + 15	87 + 6	b	91 + 3	b	70 + 12	b	
20 mg/L	101 + 19	84 + 10	b	81 + 5	b	50 + 4	b	
35 mg/L	133 + 10	79 + 10	b	70 + 11	b	37 $\pm$ 11	b	
50 mg/L	118 + 30	79 + 15	b	72 + 19	b	59 $\pm$ 17	b	
	p=0.19	p<0.01		p<0.01		p<0.01		

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

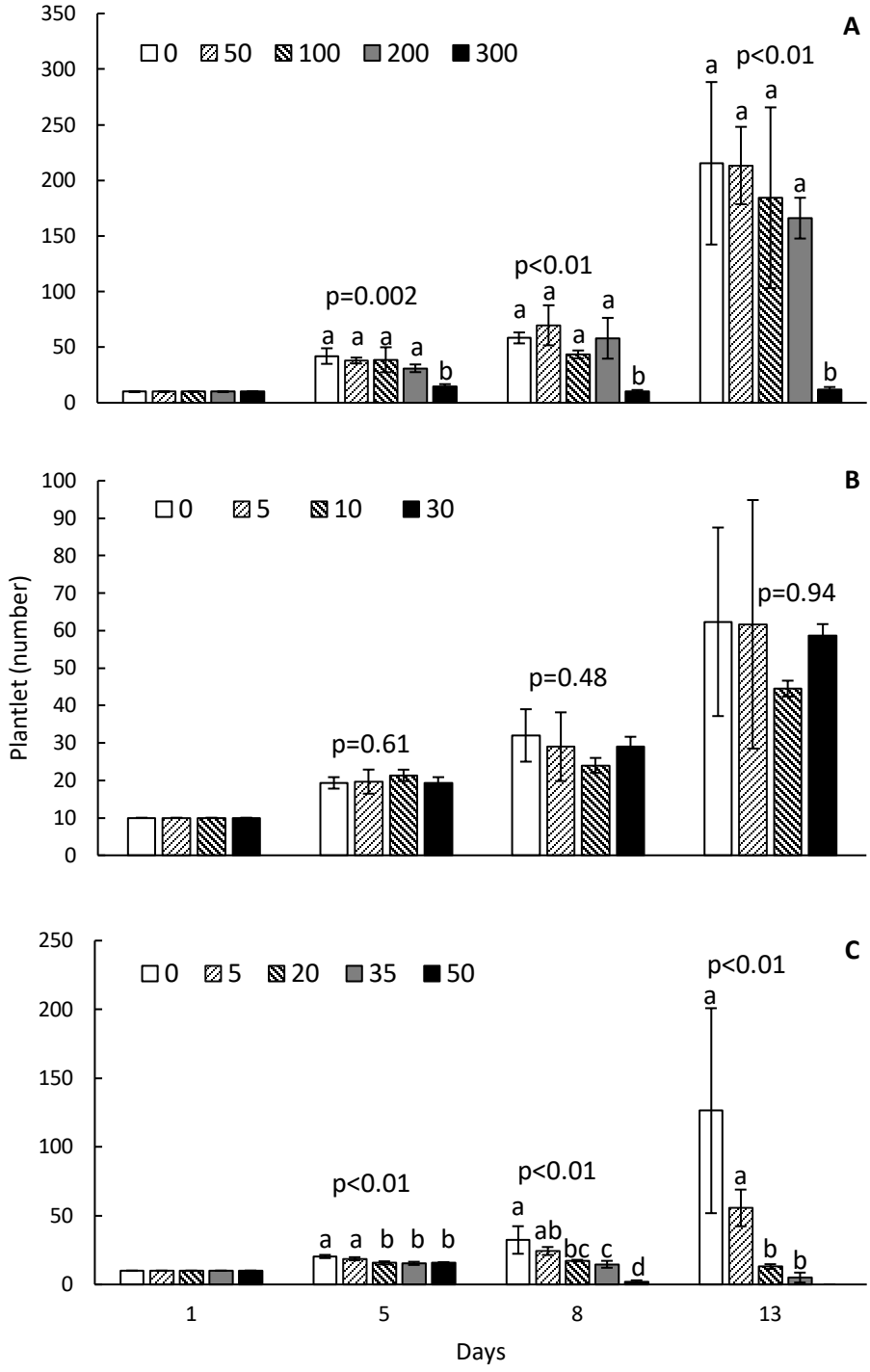


Figure 1. Effect of (A) lead, (B) copper, and (C) cadmium on plantlet number, of *Lemna minor*

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

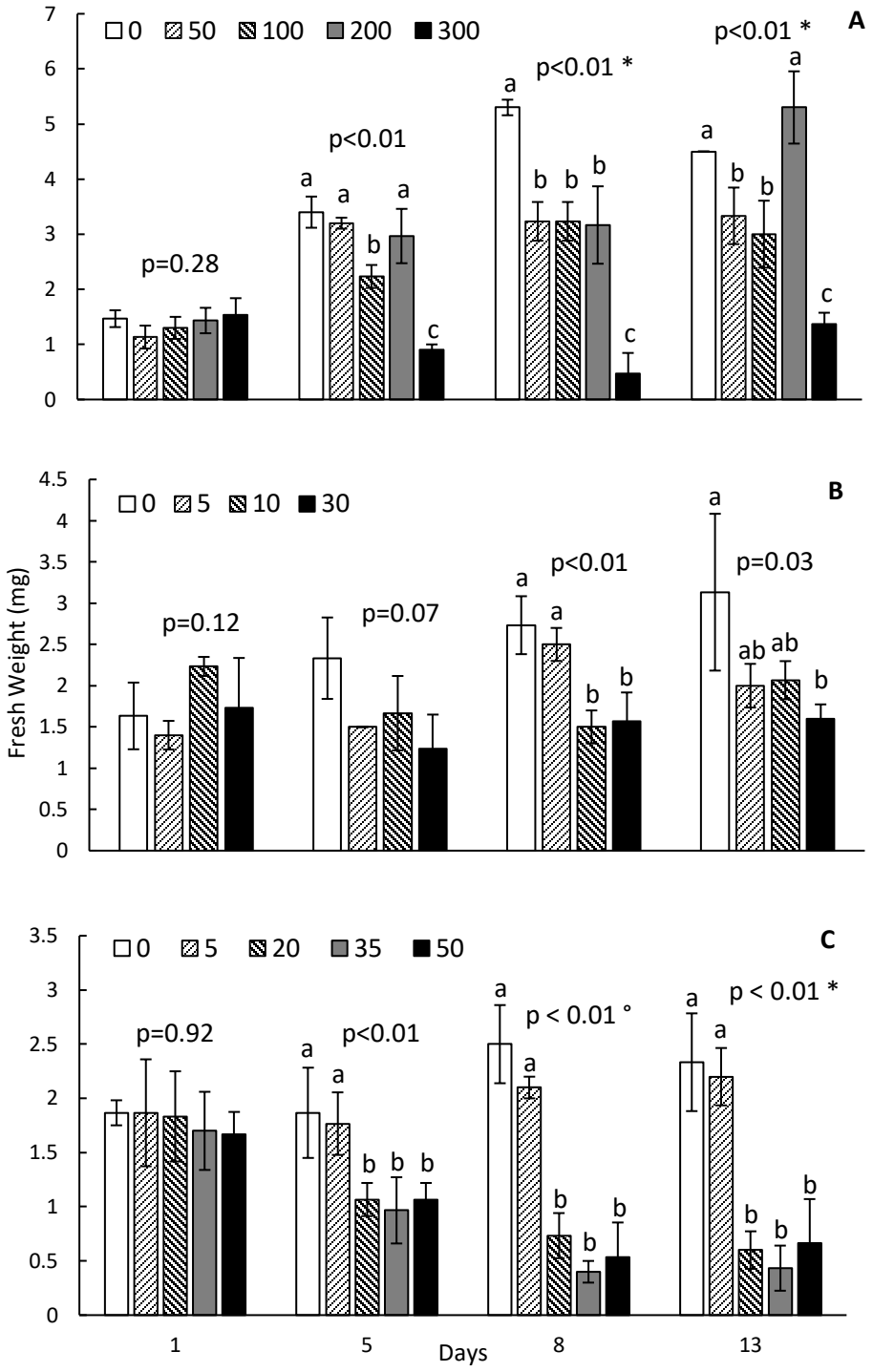


Figure 2. Effect of (A) lead, (B) copper, and (C) cadmium on fresh weight (mg) of *Lemna minor*

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

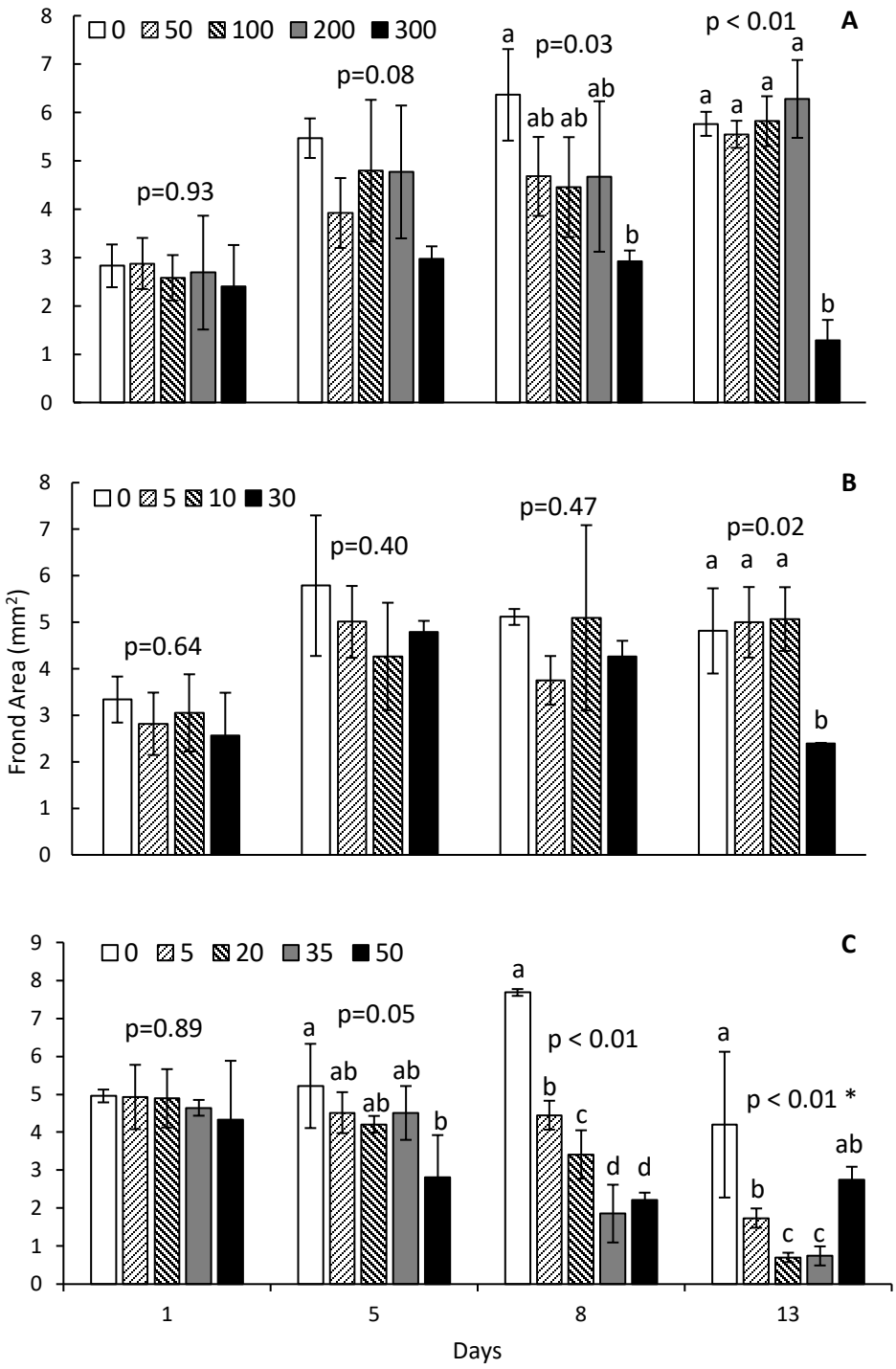


Figure 3. Effect of (A) lead, (B) copper, and (C) cadmium on frond area (mm^2) of *Lemna minor*

* Signifies log transformation for analysis

° Signifies a square root transformation for analysis

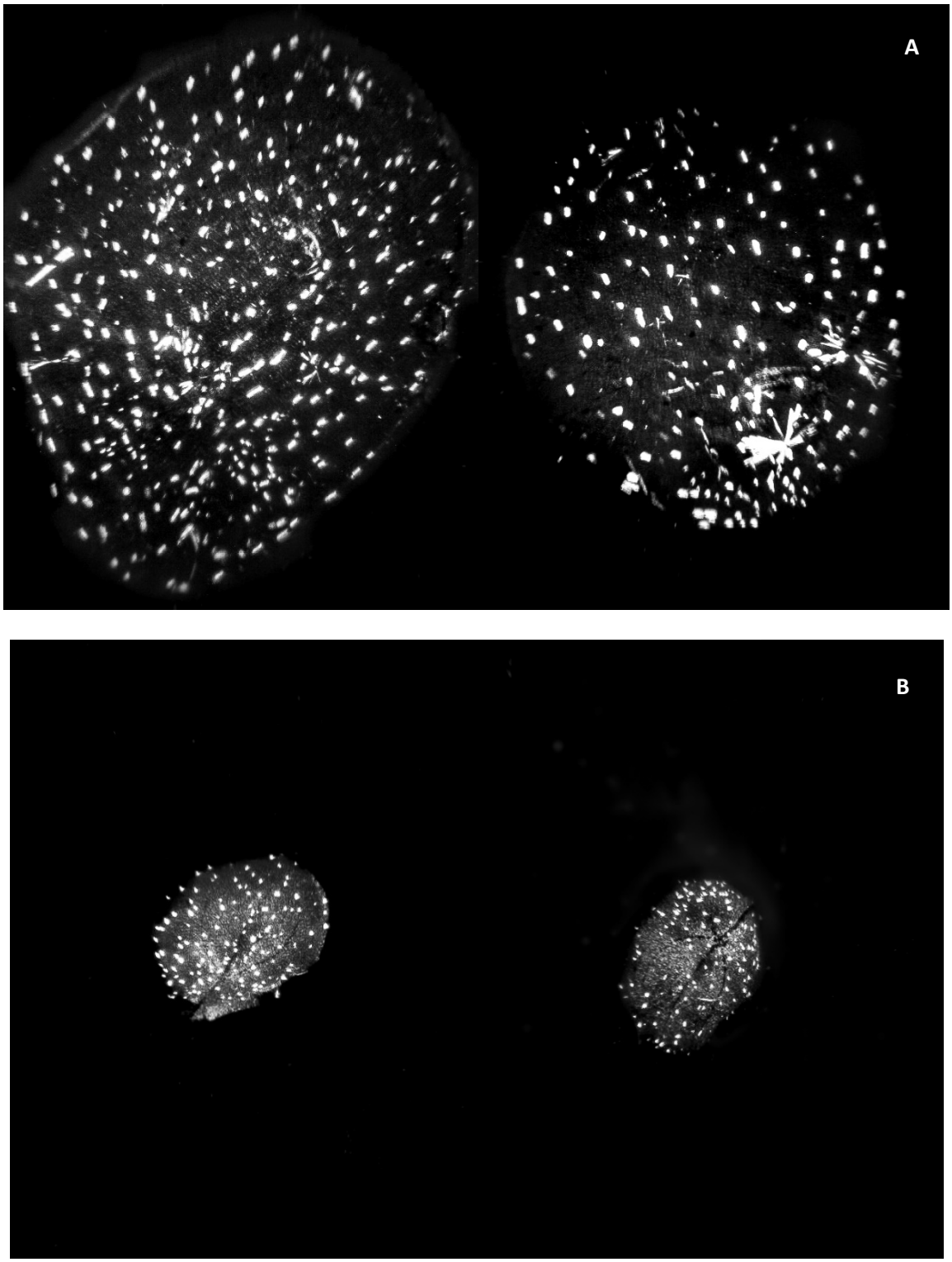


Figure 4. Effect of lead on crystal density and frond area. (A) 0 mg/L treatment, and (B). 300 mg/L treatment on day 13. Magnification 250×

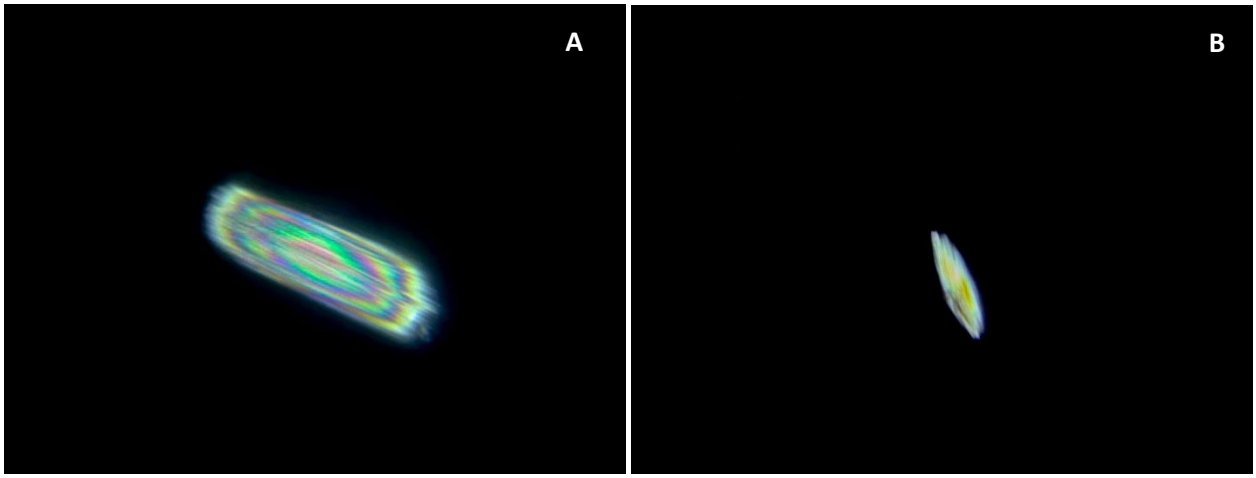
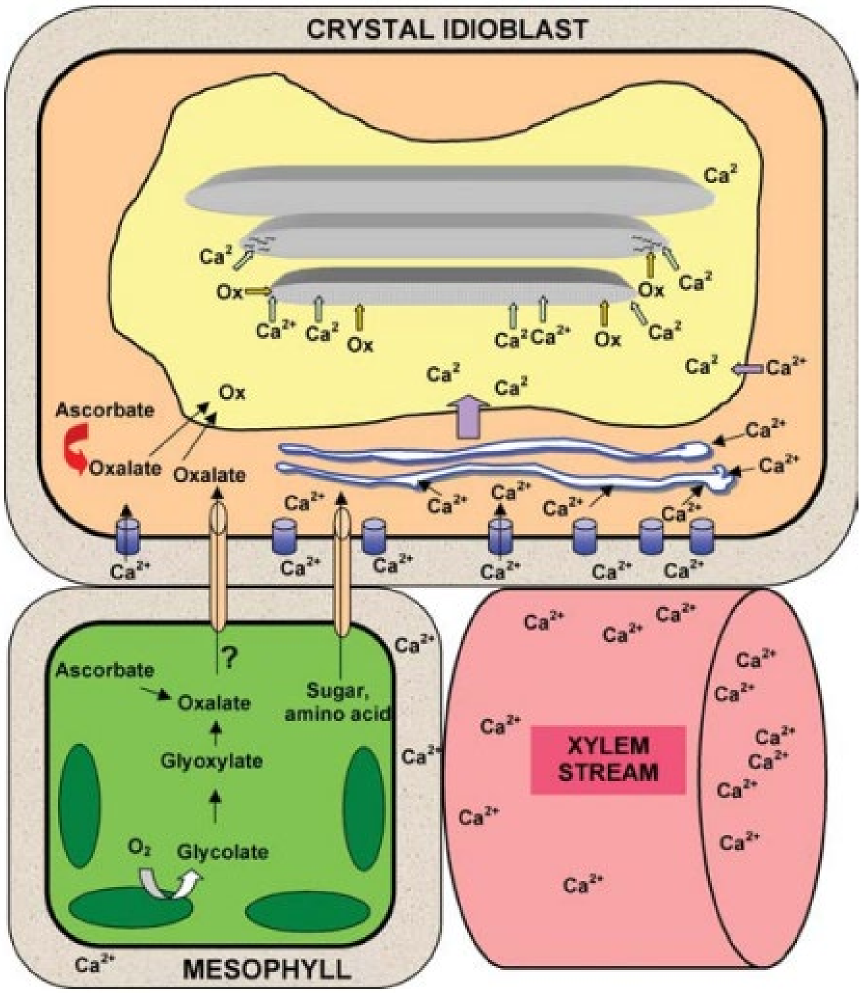


Figure 5. Effect of lead on crystal size. (A) 0 mg/L treatment, and (B). 300 mg/L treatment on day 13. Magnification 630×.

Appendix 1: Simplified Version of Calcium Oxalate Formation. Modified from Franceschi and Nakata (2005). Calcium enters through the plant is distributed among cells while in the xylem stream. In the cell ascorbate is converted to oxalate which with calcium is transferred to the crystal chamber and a mature crystal is formed.



Appendix 2. Media and solutions used in growing *Lemna minor*

Diluted Hoagland's E Medium with EDTA-chelated iron (Cross, 2015)

COMPOSITION	**STOCK SOLUTION**	**Use mL/L**
1. $MgSO_4 \cdot 7H_2O$	24.6 g/100mL	1.0 mL
2. $Ca(NO_3)_2 \cdot 4H_2O$	23.6 g/100mL	2.3 mL
3. KH_2PO_4	13.6 g/100mL	0.5 mL
4. KNO_3	10.1 g/100mL	2.5 mL
5. Micronutrients	Micronutrients Solution (See Appendix 2)	0.5 mL
6. Fe·EDTA	Fe·EDTA Solution (added last, see Appendix 3)	20.0 mL

Preparation of Micronutrients Solution (Cross, 2015)

ADDITION	**STOCK SOLUTION**
1. H_3BO_3	2.86 g/L
2. $MnCl_2 \cdot 4H_2O$	1.82 g/L
3. $ZnSO_4 \cdot 7H_2O$	0.22 g/L
4. $Na_2MoO_4 \cdot 2H_2O$	0.09 g/L
5. $CuSO_4 \cdot 5H_2O$	0.09 g/L

Preparation of Fe·EDTA Solution (Cross, 2015)

ADDITION	**STOCK SOLUTION**
1. $FeCl_3 \cdot 6H_20$	0.121 g/250 mL
2. EDTA	0.375 g/250 mL