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Cadmium and Salinity Stressor Antagonism on Vallisneria neotropicalis

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CADMIUM AND SALINITY STRESSOR ANTAGONISM ON *VALLISNERIA NEOTROPICALIS*

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by
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LIST OF ABBREVIATIONS

Fv/Fm = Ratio of variable fluorescence to maximum fluorescence

ppm = Parts per million

ppb = Parts per billion

PSU = Practical salinity units

A = Leaf length

A_o = Initial leaf length

G = Growth coefficient

t = Elapsed time

ABSTRACT

Christopher P. Mikolaitis, M. S., University of South Alabama, July 2023. Cadmium and Salinity Stressor Antagonism on *Vallisneria neotropicalis*. Chair of Committee: John C. Lehrter, Ph.D.

Submerged macrophytes form the foundation of freshwater ecosystems. These organisms are sessile and are very susceptible to shifts in their environment. Heavy metals are of particular concern as they can be sequestered indefinitely in sediments and are readily taken up by rooted vegetation. In the presence of saltwater intrusions, these metals can interact with salt ions potentially changing their availability to submerged vegetation. In this study a freshwater macrophyte, *Vallisneria neotropicalis,* was used as a test species for interactive effects between Cd, a non-essential heavy metal, and salt stress. The metrics used to establish the individual as well as the combined stressor effects were clonal reproduction rate, leaf growth rate, and total Cd body burden after exposure to three levels of salinity (0, 5, and 10 PSU) and three levels of sediment Cd (0, 10, and 40 ppm). In the presence of low salinity (5 PSU), ramet production was impeded less by Cd than it was in the presence of freshwater. There was also a significant decrease in leaf length in these treatments, indicating that the ramet production was balanced by a change in biomass allocation. However, the plant's Cd body burden did not vary as a function of salinity. The trend of Cd accumulation irrespective of salinity suggests that the plants' rate of Cd export could be a function of heavy metal-induced leaf senescence and shedding. We emphasize the need for further research to understand heavy metal export strategies across a salinity gradient in freshwater macrophytes.

INTRODUCTION

Heavy metal contamination is a persistent problem in coastal areas waters. While industrial regulation has grown progressively through the years, legacy inputs still remain as heavy metals precipitate or are scavenged out of the water column and become deposited in the sediment (Atkinson et al. 2007). In the sediments, the metals are primarily bound to organic compounds or are found in insoluble precipitates (Calmano et al. 1993; Javed et al. 2019). These different states are of varying toxicity levels as their bioavailability is heavily state-dependent. Precipitates are insoluble and demonstrate poor bioavailability, thus they are not considered when looking at sediment metal toxicity (Javed et al. 2019). Cadmium is no exception as its high affinity for sulfide (CdS) produces low solubility CdS, which may render Cd as a nontoxic precipitate within the sediment (Di Toro et al. 1996). Cd state is also affected by other environmental conditions. In particular, increasing salinity can lead to an increase in negatively charged chloride ions, which bind to Cd forming mobile $CdCl_2$ complexes and increasing Cd bioavailability and thus toxicity (Xu et al. 2017).

In Mobile Bay, Alabama, where heavy metal contamination is well documented near the ports and industrial areas (Weston Solutions Inc. 2015), the freshwater and oligohaline regions in the upper bay are habitats for submerged macrophytes, such as *Vallisneria neotropicalis*, which form meadows that have immense ecological value. These meadows provide habitats for economically important species such as white shrimp and blue crabs (Heck et al. 2001, Rozas & Minello 2006), can ameliorate nutrient loading (Wigand et al. 1997), and control algal growth (Wigand et al. 2000). Given the abundance and importance of *V. neotropicalis* in the upper Mobile Bay region, this macrophyte was selected as a test species to investigate the impact of heavy metals on aquatic flora.

V. neotropicalis is a strong bioaccumulator of metals, to the point of being considered for bioremediation efforts for contaminated sediments (Javed et al. 2019; Sinha et al. 1994). While submerged macrophytes are capable of metal uptake from the water column, prior studies have demonstrated that root uptake is typically their primary mechanism of metal accumulation (Javed et al. 2019; Wang et al. 2009). Cd is present in the sediment of the upper Mobile Bay (Weston Solutions Inc. 2015) and is taken up by flora and fauna. Cd is ubiquitously toxic and with differing mechanisms of stress as a function of dosage and exposure time. In acute exposures, Cd can reduce antioxidant defenses and enhance iron driven Fenton reactions resulting in the production of reactive oxygen species (Liu et al. 2009). In more chronic exposures, the long-term induction of Cd tolerance compounds can reduce growth through shifts in energy and nutrient allocation, and has carcinogenic effects through decreased sensitivity of apoptotic pathways (Bielmyer-Fraser et al. 2022, Liu et al. 2009, Wang et al. 2008). Notable toxicity mechanisms in *Vallisneria* include: reduction of chlorophyll biosynthesis (Wang et al. 2010), decrease in cell division and subsequent increase in chromosomal aberration frequency (Mukherjee et al. 1990), and decreased antioxidant response (Huang et al. 2020). In this study, the focus was on the generalized cumulative stress on the plant and thus integrated measures of plant health such as growth and clonal reproduction were tracked to account for Cd-induced stress.

V. neotropicalis is also sensitive to changing salinity and thrives at lower salinity of 0-5 PSU (Tootoonchi et al. 2019). *Vallisneria* is a freshwater macrophyte and as such it is also subject to osmotic and ionic stress from increases in salinity in addition to the potential increase in Cd stress (Tootoonchi et al. 2020). Osmotic stress is the short-term stress induced by the change in osmotic gradient across the cell membrane resulting in shifts away from homeostatic turgor pressures (Touchette 2007). This short-term stress is then mediated by the production of osmoprotectants, which can be an energetically costly process (Al-Abbawy et al. 2020, Santini et al. 2022). However, saltwater ions such as Na^+ are also accrued as a function of the hypersaline concentration gradient. While the resultant ionic imbalance may be partially buffered via Hofmeister-relevant osmoticums (Yancey et al. 1982), at high concentrations the ionic imbalance can induce stress via interference with chlorophyll biosynthesis (Petjukevics & Skute 2022) and reactive oxygen species production (Santini et al 2022) Cd stress may increase with increasing salinity due to increasing mobilization of Cd in the sediment due to ionic strength, as discussed above. *Vallisneria neotropicalis* can grow in salinities up to 12 PSU but becomes stressed above 5 PSU (Tootoonchi et al. 2019). The geochemical interaction between a salinity influx and sediment Cd could result in a shift in stressor strength on the macrophytes. Because CdS partitioning leads to reduced bioavailability of Cd in the presence of salinity, this stress would be less than additive (antagonistic) or, in the case of increasing chlorinity mobilizing Cd, the stress could be more than additive (synergistic). Thus, in this study, the Cd and salinity and their interactive impacts on plant growth and clonal reproduction were evaluated.

OBJECTIVES

The purpose of this study was to explore and quantify the impacts of multiple stressors, Cd and salinity, on *V. neotropicalis*. As such, the hypotheses put forth in this study are as follows:

H1 - There will be a decrease in *V. neotropicalis* growth rate as salinity increases.

H2 - There will be a decrease in *V. neotropicalis* growth rate as sediment cadmium concentration increases.

H3 - Where Cd and salinity increase co-locally the *Vallisneria* will demonstrate a greater reduction in growth rate than the stressors would create additively.

H4 - Cd accumulation in plant tissue will be greater in treatments in the presence of salinity.

METHODOLOGY

1.1 Materials

All sediments used in this study were collected from the oligohaline, tidal river region of Mobile Bay. The bulk of the sediment was collected from the intertidal zone along the Apalachee river (Figure 1). Additional sediments used for sediment analysis were sourced from the intertidal zone near the mouth of the Tensaw River and from a submerged macrophyte meadow in Bay John. *Vallisneria neotropicalis* plants were gathered directly east of this site on the Blakely River. Cadmium used for stressor treatments was cadmium chloride hemipentahydrate 99+% (ACROS Organics, Belgium). The synthetic sea salt used was Crystal Sea Marine Salt Mix (Marine Enterprises International, Baltimore).

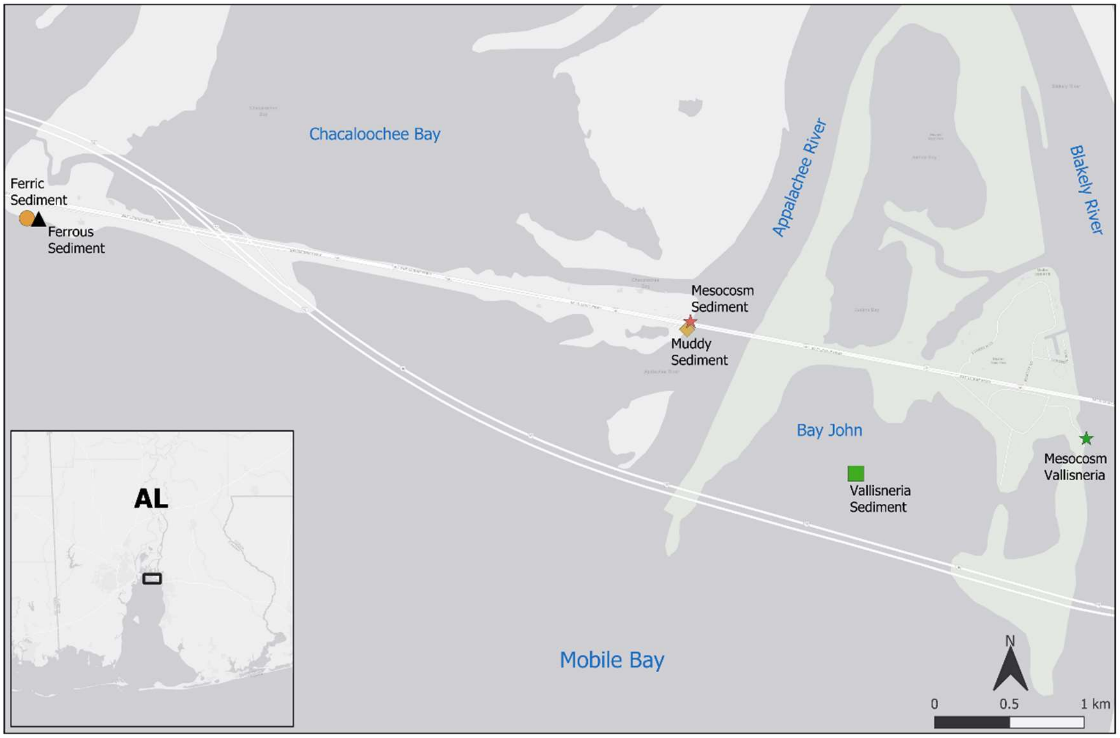

Figure 1. Sediment and Vallisneria collection locations. The Ferric and Ferrous sediments were intertidal iron-rich coarse and fine-grain sands, respectively. The Mud and Vallisneria groups refer to intertidal silty mud and mud obtained from a subtidal Vallisneria meadow, respectively. The sediments used in the factorial experiment and Vallisneria are marked using stars.

1.2 Sediment analysis

Given the potential for organic matter to complex with Cd ions (Sauve et al. 2000), the degree of attenuation of free Cd within the sediment was quantified. Half a gallon of sediment was collected by hand from four different sites near *Vallisneria* communities ranging from the intertidal to subtidal zones. One-hundred-gram fractions of these sediments were then dehydrated overnight using a drying oven at 65 °C and homogenized with a mortar and pestle before being prepared for inductively-coupled plasma mass spectrometry (ICP-MS). The sediment samples were divided into five treatments: a control where nothing was added to the sediment, and four treatments wherein solutions of different mobile (uncomplexed) Cd concentrations (5, 10, 20, 40 ppm) were added to the sediments and allowed to rest prior to drying. The sediment Cd was extracted with a 1M ammonium nitrate solution (Hahn et al. 2019) on a shaker table, followed by vacuum filtration of the extracted sample through a 47 mm GF/F filter (nominal pore size of 0.7 microns; Whatman plc, Maidstone). The extracted sample was then preserved using 5% nitric acid. The preserved samples were then analyzed for their Cd concentration using an Agilent 7700 ICP-MS. The instrument was calibrated using a six-point curve with concentrations ranging from 0 ppm to 80 ppm. Every eight samples a drift check was performed using the 0 ppm standard and a 40 ppm standard. All standards contained an internal 2 ppb indium standard as an additional quality control.

There was no replication of the treatments as this experiment was not meant to provide any statistical inferences about sediment properties, instead its purpose was to determine what sites would have the least in situ Cd contamination as well as provide insight into the necessary starting amount of Cd to be added in the pilot and mesocosm experiments with *Vallisneria neotropicalis* plants discussed below.

1.3 Pilot Study

The primary question to be addressed here was: How will *Vallisneria* growth respond to varying levels of salinity and Cd by themselves? The purpose of this experiment was to address the stressor concentration levels that would demonstrate significantly different and measurable changes in the shoots without risk of mortality. A total of seven experimental units were employed, three to estimate the Cd concentrations that induce change in growth, three to measure the salinity concentrations the plants can tolerate, and a control to understand the baseline stress induced by the replanting process. The salinities used were 5 PSU, 10 PSU, 15 PSU, and the cadmium levels employed were 5 ppm, 10 ppm, and 40 ppm. Each experimental unit consisted of 10 cm of intertidal sandy mud within a five-gallon bucket and a single mature *V. neotropicalis* shoot with a bubbler and some *Neritina* snails to control algal buildup. All the units were contained within an outdoor greenhouse to buffer for temperature changes and minimize salinity variance from precipitation and evaporation. The mesocosm units were also periodically topped off with fresh water to account for any evaporative loss. The replanting of shoots into the five-gallon buckets occurred after the plant's photosystem II activity was equilibrated as determined by Fv/Fm comparisons via PAM fluorometry (Petjukevičs & Skute 2022). The Cd was introduced to the sediment prior to planting to allow time for complexation with compounds in the sediment. The runtime of this pilot was 33 days starting mid-December after the two-week acclimatization period. Plant growth was measured via changes in leaf length as well as the number of ramets produced.

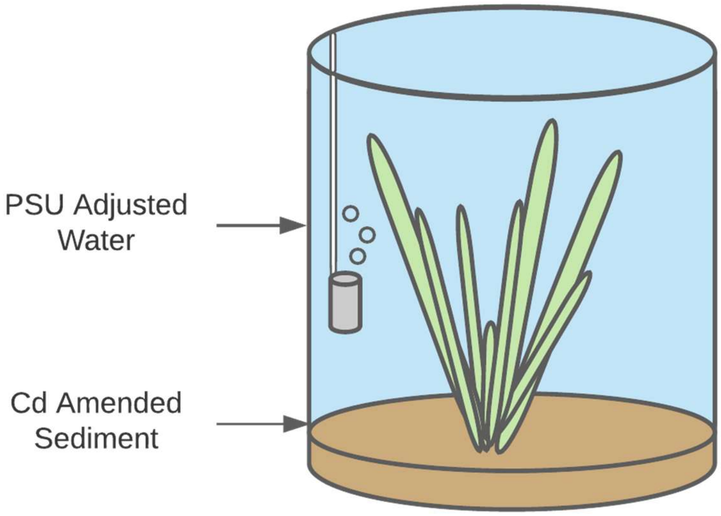

Figure 2. Diagram of pilot mesocosm units. A mature *Vallisneria neotropicalis* shoot in a clean 5-gallon polyethylene bucket with an aerator to disrupt algal buildup. Experimental treatments included manipulating water column salinity and sediment Cd concentration.

1.4 Mesocosm Experimental Design

The experimental design for observing multiple stressor effects of Cd and salinity consisted of nine treatments with three levels of salinity and three levels of (Figure 3). The stressor concentrations were 0, 10 and 40 ppm Cd in the sediment, and salinity of 0, 5 and 10. Each treatment was prepared in triplicate for a total of 27 experimental units where each of the units was the same physical design as the pilot, with a single plant and 10 cm of its original sediment within a five-gallon bucket containing an aerator and *Neritina* snails to control algal growth. Different from the pilot study, the plants used in this experiment were juveniles as opposed to mature plants. These juvenile plants were ramets clipped from mature plants grown in the same acclimation units as those used in the pilot. All experimental units were contained in an outdoor greenhouse to control temperature and minimize salinity variance from precipitation and evaporation. The mesocosm units were also periodically topped off with fresh water to account for any evaporative loss. This refilling procedure was confirmed with a handheld salinity probe to not alter the salinity treatments. Similar to the pilot, the introduction of the plants to the treatment mesocosms began once the plants had acclimated according to the Fv/Fm measured by PAM fluorometry (Petjukevičs & Skute 2022). After the two-week acclimatization period, the run time of the mesocosm experiment was 12 weeks starting in March to mimic the length of a growing season.

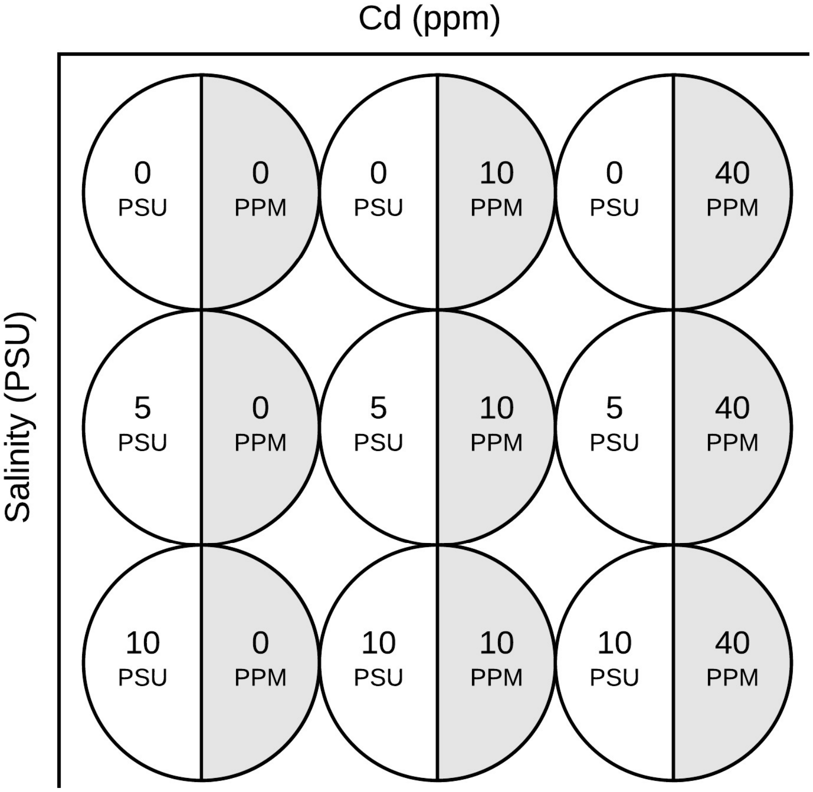

Figure 3. Matrix for all stressor/control combinations in the multiple stressor experiment. The Cd concentration is the amended sediment Cd concentration in units of parts per million (ppm). Salinity treatments are shown with salinity concentrations in practical salinity units (PSU).

1.5 Sampling Procedure

Growth measurements were taken twice a week for each experimental unit. Leaves were marked via needle puncture and then measured from rhizome to tip. Plants were then carefully removed from the sediment and examined for new leaf and ramet development. Afterward, the plants were replanted in the same spot in the sediment and observed to ensure they would not float free. The total examination time did not exceed five minutes for each plant, and the plants were periodically rewet in their mesocosms to minimize any stress caused by the measuring process.

Cd accumulation by the *Vallisneria* was measured at the end of the study. Whole plants were desiccated in a drying oven at 60 °C until a constant weight was reached. The plants were then homogenized in mortar and pestle after freezing with liquid nitrogen. The resultant powder was then pre-digested overnight in a mixture of 8mL of 65% nitric acid and 2mL of 30% hydrogen peroxide in a sealed Teflon vessel. The digestion was then completed via microwave digestion (MARS6, CEM Microwave Technology Ltd., USA) at 200 °C with a 15-minute ramp time and a 15-minute hold time. The digested samples were decanted into nitric acid-washed polypropylene conical tubes for dry-down. Following the dry-down, the remaining volumes were reconstituted to 50mL with 2% nitric acid. The preserved solutions were then analyzed on the Agilent 7700 ICP-MS. The instrument was calibrated using an eight-point curve with standard concentrations ranging from 0 ppm to 250 ppm Cd made from a 1000 ppm stock standard. This broad range was employed since unlike the sediment extraction, there was no estimate for the plants' Cd accumulation prior to analysis, and high levels of bioaccumulation which *Vallisneria* has demonstrated in prior studies (Sinha et al. 1994, Wang et al. 2007, Wang et al. 2010). After each set of treatment replicates were run, a 50 and 100 ppb check standard were inserted into the queue analyzed for instrument drift.

1.6 Statistical Analysis

Statistical analyses were performed using Matlab R2021a. Growth parameters in each treatment were compared using a two-way ANOVA (Matlab: anovan) and post hoc tests were performed using Tukey's honest significant difference test (Matlab: multcompare 'hsd'). As the objective of these experiments was to explore potential interactions that have not been previously documented, all statistical tests were evaluated at an alpha of 0.10. This increased the sensitivity of the tests to detect treatment differences but also increased the chance of a false positive from 5% to 10%. For this study, the potential detection of treatment differences was deemed more important than a false positive.

Leaf length was used as a measure of growth by approximating the growth coefficient as it appears in the function, $A = A_o e^{Gt}$ where G is the growth coefficient, t is the time elapsed since the first measure of a given leaf, A_o is the first measure of a given leaf, and A is the leaf length at a given time. This equation was linearized and then fit to each leaf's dataset (Matlab: fitlm). For number of leaves and number of shoots, only the numbers at the end of the experiments were used to evaluate differences among treatments.

RESULTS

2.1 Sediment Analysis

Tidal river intertidal sediments were found to contain bioavailable Cd in the range of tens to hundreds ppb. This was an order of magnitude lower than the maximum level found in a previous study from this region (Weston Solutions Inc. 2015) and was two to three orders of magnitude lower than what was used for the stressor experiment. In treatments with added Cd, the portion of bioavailable Cd as determined by ammonium nitrate extraction was found to increase relatively linearly as a function of the sediment type. For the ferric and ferrous sand, the trend deviated from linearity at the 10 ppm addition (Figure 4). The *Vallisneria* meadow sediments were fairly linear until the 20 ppm addition, at which point the bioavailable Cd spikes. The muddy sediment exhibited a linear attenuation of Cd throughout the treatments (R-squared = 0.983). Given this linearity, the muddy sediment was found to be the optimal choice for the stressor experiments, as the bioavailable Cd would have the least variance. It should be noted that the 40 ppm amended sediments were excluded from the dataset. The extractions at the 40 ppm concentration yielded almost identical results to that of the 20 ppm addition, perhaps indicating that the 1M concentration of ammonium nitrate used to extract the Cd from the sediment was insufficient to exchange with the 40 ppm Cd amendment.

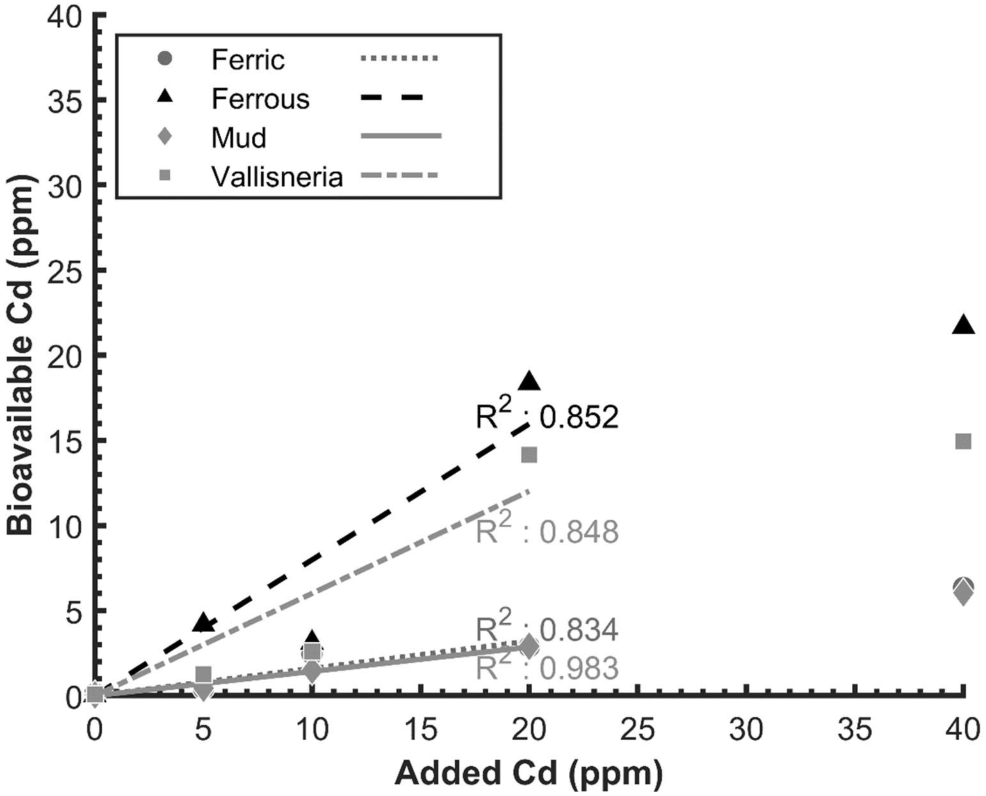

Figure 4. Bioavailable Cd for intertidal and subtidal sediment in the lower Mobile Delta. The Ferric and Ferrous sediments were intertidal iron-rich coarse and fine-grain sands, respectively. The Mud and Vallisneria groups refer to intertidal silty mud and mud obtained from a subtidal *Vallisneria* meadow, respectively. Trendlines only extend to 20 ppm as the extraction methods suitability was questionable for 40 ppm range.

2.2 Pilot

Over the course of the 33-day pilot experiment, no treatments demonstrated any appreciable change in growth. All plants followed similar growth patterns as the control, primarily maintaining their initial biomass. There was no change in leaf length measures (only the oldest leaf was measured in this pilot) with the exception of progressive leaf degradation due to the senescence of the oldest leaf. The primary findings were that multiple leaf measures were necessary to account for leaf shedding and that mature plants are able to withstand individual stressors over the course of a month.

2.3 Multiple Stressor Experiment

There was a significant difference in leaf length and number of shoots among the different treatments. However, there was no difference in the number of leaves per shoot. The number of new ramets generated significantly declined across all cadmium treatments ($p = 0.0001$). A similar trend was observed with respect to increasing salinity ($p = 0.0005$), with the exception that the Cd 0 / PSU 5 treatment was not significantly different from the control. There was also a significant interaction between the Cd and salinity stressors ($p = 0.0037$). Treatments with low salinity and Cd, regardless of Cd concentration, did not statistically differ in the number of ramets from the low salinity no cadmium treatment (Figure 5). All other treatments failed to develop ramets.

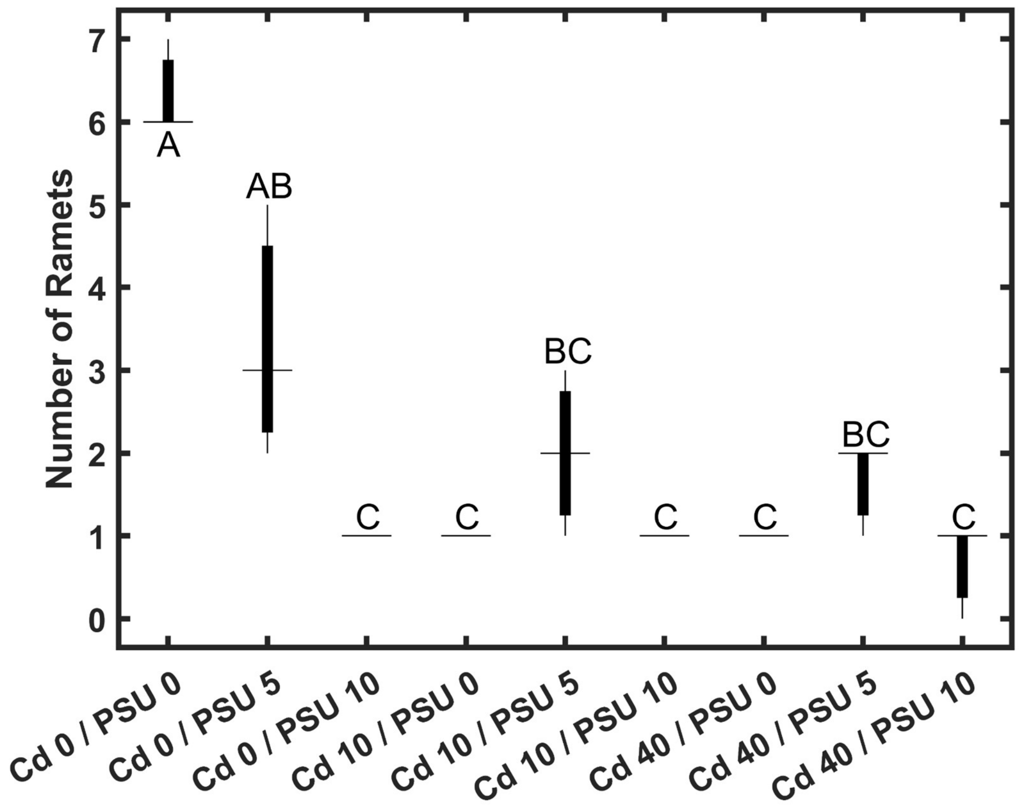

Figure 5. End number of ramets in the multiple stressor experiment. Categories on the x-axis represent the stressor treatment combinations of cadmium (Cd) and salinity (PSU). Letter annotations correspond to statistically significant ($p < 0.10$) differences.

The final (maximum) leaf length was significantly different in the cadmium treatments ($p = 0.0029$) with smaller leaf lengths in higher Cd treatments and demonstrated a suggested interactive effect with salinity ($p = 0.0862$). Leaf length was highest in the control (mean: 3.794 cm) but was significantly lower in the low salinity treatments in which cadmium was present (mean: 3.1 cm) (Figure 6). The Cd 40 / PSU 10 treatment also significantly differed from the control (mean: 2.540 cm).

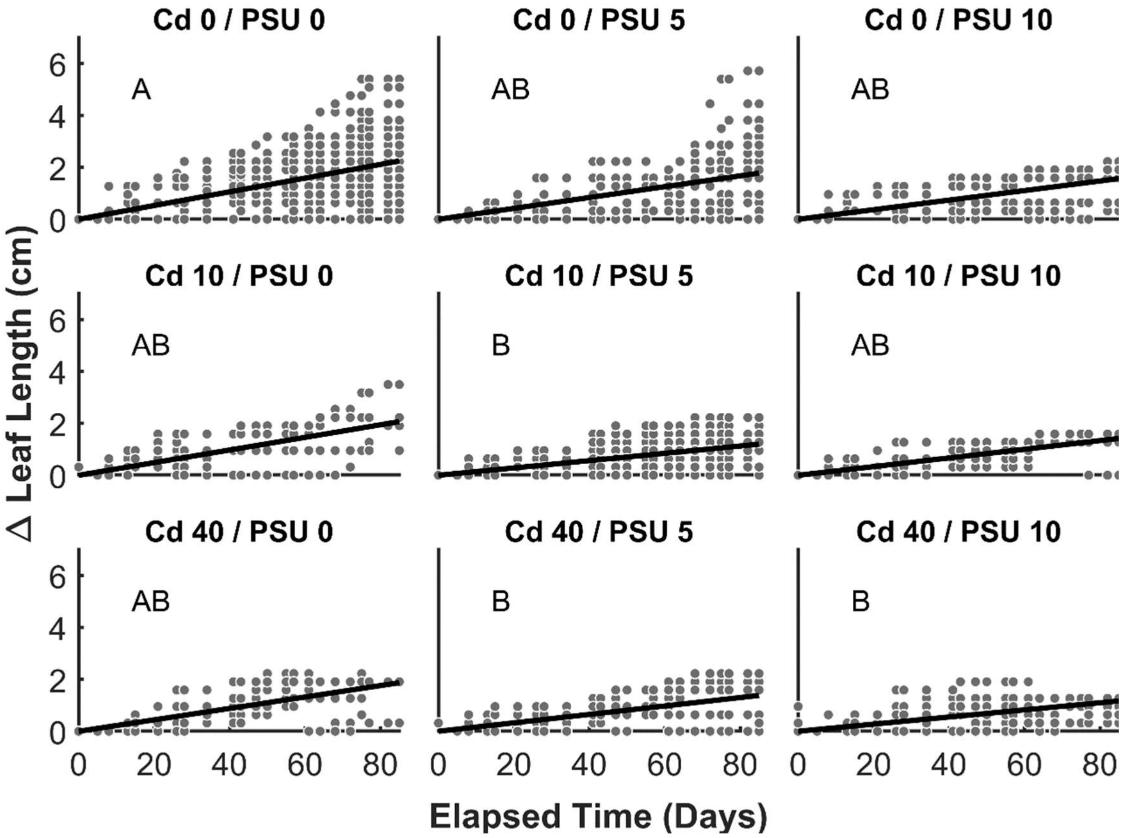

Figure 6. Change in leaf length over time for each experimental treatment after pooling ramets, replicates, and differing leaf ages. The red line is a fitted linear regression to quantify the rate of change and aid in visualizing the difference. Letter annotations correspond to statistically significant ($p < 0.10$) differences in final leaf length.

Leaf growth significantly differed as a function of the Cd treatments ($p = 0.0037$, range: [-0.0063, 0.2110]), and had a significant interaction with the salinity treatments ($p = 0.071$, range: [-0.0063, 0.1352] with salinity, range: [0.0, 0.0407] without salinity). However, there was no statistically significant difference in leaf growth rates between the salinity treatments. As with the number of ramets, the control and the Cd 0 / PSU 5 treatment were not statistically different. However, unlike with the end number of ramets, there was no difference between the low salinity no Cd treatment and the rest of the stressor treatments (Figure 7).

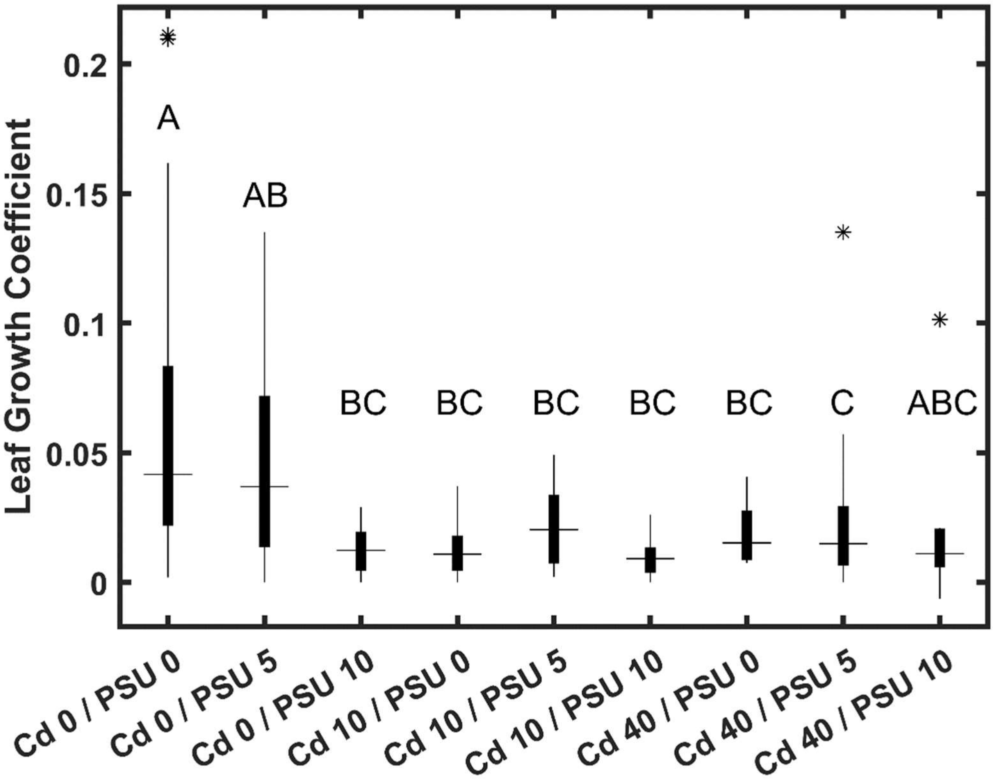

Figure 7. Leaf growth coefficients for each experimental treatment after pooling ramets, replicates, and differing leaf ages. Letter annotations correspond to statistically significant ($p < 0.10$) differences.

2.4 Cadmium Accumulation

The hypothesis (H4) that cadmium accumulation in the plants would increase as a function of salinity was not born out. Cd accumulation also did not differ significantly between the 10 ppm sediment treatment and the 40 ppm sediment treatment, although the mean Cd in the plants was higher in the Cd 40 treatment (Figure 8). This trend was consistent across all isotopes of Cd. Cd-111 is used here since it demonstrates the most precise measurements in the check and spike quality controls.

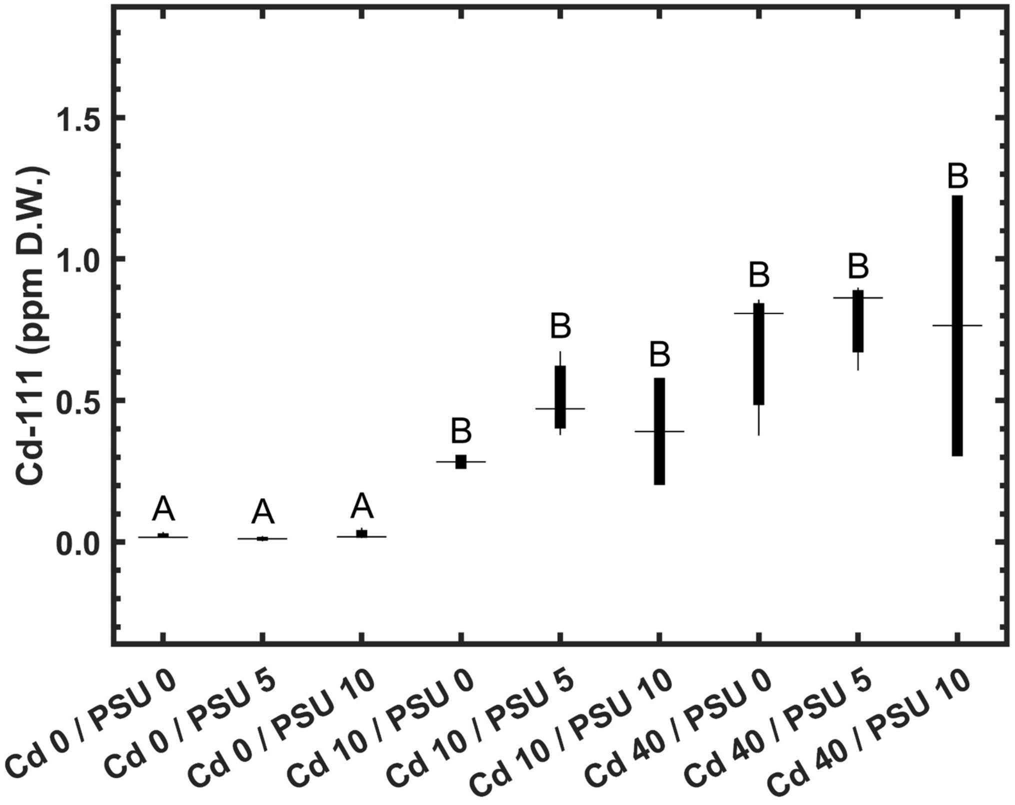

Figure 8. Cadmium accumulation in desiccated whole plant samples (units of parts per million dry weight). Letter annotations correspond to statistically significant ($p < 0.10$) differences.

DISCUSSION

3.1 Sediment Cadmium Retention

Cd attenuation varied greatly between sediment types. These differences were in part a function of the ammonium nitrate extraction method. The silty mud type sediment was ultimately chosen for the experiments since it had the highest linearity of exchangeable to amended Cd, and it was an easily accessible site. A caveat to these results is that our method only measures the readily exchangeable metals from the sediment (Hahn et al. 2019). However, the bioavailable portion is potentially a broader portion than just exchangeable metals, and can include Mn-oxide bound metals, Fe-oxide bound metals, metal-sulfides, and metals complexed with organics such as humic acids (Calmano et al. 1993, Rule & Alden 1990). Assessing the Cd concentrations in these various fractions would require a cascade of different extraction methods. This was deemed unnecessary as the purpose of this experiment was to establish what sediments would have comparatively negligible in-situ Cd so as to not confound the control, as well as to determine the degree of attenuation for Cd capable of mobilization by increasing chlorinity (Jiann & Ho 2014).

3.2 Plant Development

Plant growth was significantly different in multiple aspects between the different stressor combinations. In the single stressor experiments, ramet development only occurred in the low salinity (5 PSU) treatment. In this aspect, H1 and H2 were supported as ramet development was inhibited in the single stressor treatments. However, in multiple stressor treatments where Cd was present along with low salinity levels, the plants' ramet development progressed irrespective of sediment Cd concentration. This new shoot production did not yield less healthy ramets. The new shoots exhibited similar growth rates to the parent plant, and in some cases would even continue leaf and further ramet production as the parent plant decayed. This increased growth in low salinity multi-stressor treatments contradicted our hypothesis (H3). One possible reason for the apparent antagonism as opposed to the hypothesized synergism, is due to increasing concentrations of sulfate ions with increasing salinity. Sulfate can be reduced by the sediment microbial community to sulfide, an ion that forms stable CdS precipitates (Calmano et al. 1993). The introduction of sulfate in the salinity treatments could induce changes in gene expression resulting in a shift towards sulfate reduction as the dominant metabolic pathway (Edmonds et al. 2009). Sulfate reducing bacteria occupy similar niches as the methanogenic microbes in the oligohaline region of estuaries and can outcompete methanogens when sulfate is not limiting (Andreote et al. 2012, Ikenata et al. 2009). Sulfide production via sulfate reducing bacteria is such an efficient mechanism of Cd mitigation that it is being investigated as a potential treatment for acid mining runoff (Zhang & Wang 2016, Zhang et al. 2018). In the case of the pre-existing microbial community being adapted to using multiple metabolic pathways as a function of seasonal salt regime shifts, this induction of sulfate reducing pathways would explain the apparent stressor antagonism. This would hold especially true in the case of poor oxygenation at the root-sediment interface, where a lack of oxic mineral exchange sites would limit the amount of bioavailable Cd (Jiann & Ho 2014, St-Cyr & Campbell 2000). In prior studies of similar duration, the primary growth parameter affected by Cd toxicity was root development (Qian et al. 2019). The *Vallisneria neotropicalis* collected for use in this study had incredibly diminished roots even before transplanting into the stressor treatments, to the point of being non-existent for many individuals. However, only the plants that were cultured in the Mobile Delta sediments were used in order to simulate in situ conditions as closely as possible. This trend held true for the plants in the multi-stressor experiment as well. On a few occasions, a plant floated free from the sediment and began to develop roots. Further research would be required to verify the impacts to root morphology in these multi-stressor environments.

There were significant differences in the leaf length maxima as well as the leaf growth rates. The leaf length was found to be significantly smaller in the saline low Cd treatments. In these treatments, there was also notable ramet development and a decreasing trend in leaf growth rates with increasing Cd. These observations would point to stressor-induced changes in biomass allocation. It should be noted that there was also a significant leaf length decrease in the high salinity/high Cd treatment, but the leaf growth rate was not significantly different. This can be attributed to a short-lived ramet that had developed in a single replicate. The rapid leaf growth of the new ramet provided the two outlier points visible in Figure 7 Cd 40 / PSU 10.

One metric not captured in this sampling scheme was the shedding and replacement of leaves. Partitioning metals into different tissues is a well-documented metal tolerance strategy in plants (Andresen et al. 2013, Lin et al. 2023, Yang & Ye 2009). The general paradigms for submerged monocots are first partitioning in roots, followed by epidermal tissue in leaves, then finally veins and mesophyll. It has also been observed that older leaves hold higher metal concentrations than younger leaves (Andersen et al. 2013). These older leaves can act as sinks for toxic metals as they periodically senesce and are shed by the plants. In this study there was no significant difference in the number of leaves per ramet among the various stressor treatments. There were anecdotal observations for leaf shedding however, the mean residence time of each leaf was not tracked. Further research is needed to ascertain the importance of leaf cycling on cadmium export from the plants.

3.3 Cadmium Accumulation

Given the linearity ($R^2 = 0.983$) of the bioavailable Cd to added Cd for the mesocosm sediment, it was expected that the *Vallisneria* Cd concentration would increase in a linear fashion with added Cd (H4). However, the bioaccumulation of Cd by the juvenile plants did not significantly increase along with amended Cd nor did it demonstrate any interactive effect with the salinity treatments. This weak coupling between the plant's Cd content and the sediment Cd amendments could be a function of cadmium's uptake routes. Prior studies suggest that root uptake is the dominant Cd uptake pathway for *Vallisneria* (Jackson 1998, Lin et al. 2023, Wang et al. 2010). However, the highly diminished roots and lack of significant difference in plant Cd for the Cd 10 and 40 treatments point towards foliar uptake being a major uptake pathway. Heavy metal uptake through leaf sorption has been observed in previous studies using *Vallisneria* (St-Cyr & Campbell 2000, Wang et al. 2008) as well as across other genera (Shahid et al. 2017). Diffusion out of the sediment pore waters as well as disturbance to the sediment during sampling could contribute to the water Cd concentration. Having an appreciable concentration in the water for exchange with the shoots could account for the weaker than expected relationship between body burden and sediment concentration. Unfortunately, our sampling regime did not capture water column Cd as it was not predicted to be an important uptake pathway. However, foliar uptake does not explain the lack of interactive effects (Cd-Salinity antagonism) observed in plant development. One possible explanation is that the apparent lack of antagonism between the Cd and salinity is being masked by leaf senescence. *Vallisneria* has historically been observed to have very high rates of leaf turnover (Hauxwell et al. 2007) which can serve to export Cd out of the plant (Lewis & Devereux 2009). In addition to unstressed patterns of leaf senescence, Cd uptake can induce production of ethylene, which in addition to reactive oxygen species, can initiate a signaling cascade that ultimately ends in leaf senescence (Balestrasse et al. 2004). The shedding of leaves in turn decreases the Cd body burden on the plant. Further research is needed to test this hypothesis, as our sampling scheme did not capture leaf cycling, just the overall number of leaves.

CONCLUSIONS

The multiple stressor effects of Cd and salinity on developing *Vallisneria neotropicalis* plants appear to be antagonistic, resulting in an overall decreased effect on leaf development and clonal reproduction than Cd would have in the absence of higher salinity. This antagonism is not evidenced in the Cd body burden of the plants, suggesting that there is a continual removal of Cd through leaf senescence. Overall, saltwater intrusion-induced metal fluxes do not appear to pose a significant threat to *Vallisneria* meadows in the lower Mobile Delta. Further research is needed to establish how leaf senescence and production serve to buffer heavy metal body burdens.

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