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Characterization of heavy metal bioremediation pathways in local moss species

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Characterization of heavy metal bioremediation pathways in local moss species

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DEDICATION

This thesis is dedicated to my family,

both those who are scientifically inclined and those who are not.

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Abstract

Heavy metals are found in natural water sources and are toxic to plants and animals. Metal removal remains a goal of environmental protection; one promising approach is to use plants that have been genetically modified to better remove metals from water. A known metabolic pathway to neutralize metals uses the Phytochelatin Synthase (PCS) enzyme to catalyze the production of phytochelatins. Our research characterizes the potential of Indiana moss species for heavy metal bioremediation as foundational research for future genetic modification. Mosses grow well in water and absorb metals through their entire surface area, and using locally collected moss species will ensure that any future genetically modified organisms developed for bioremediation are well adapted to the local environment. To determine factors involved in heavy metal response and phenotypic variance, we measured survival and heavy metal uptake of three local moss species after growth in various doses of copper, cobalt, and cadmium sulfates. A three-way ANOVA showed no significant effect of metal identity, metal dose, or moss species on chlorophyll levels, indicating no survival advantage for any moss in any metal or any dose. A separate three-way ANOVA showed significant effects of metal identity, metal dose, and moss species on metal absorbance rate, indicating the need to select a specific moss to best absorb a particular metal at a particular dose. Future work will probe for and sequence *PCS* genes in several local moss species. This study of moss phenotypic and genetic response to heavy metals is a prerequisite to the development of a moss genetically modified for bioremediation.

Introduction

Heavy metals, such as copper, cadmium, cobalt, and lead, are found as dissolved ions in natural water sources and are toxic to plants and animals, including humans (Morais *et al*., 2012). Of modern social and political relevance, heavy metal contamination in the water supply has been a crisis in many areas, including recently in Flint, Michigan (CNN, 2019); as such, heavy metal removal remains a common goal of environmental protection. One promising approach to large-scale water purification is bioremediation, the application of biology for environmental benefit. Several organisms, including *Arabidopsis thaliana* (Chaney *et al*., 1997), *Schizosaccharomyces pombe* (Ha *et al*., 1999), microalgae (Perales-Vela *et* al., 2005), model bacteria species (Mullen *et al*., 1989), and rhizospheric bacteria (Macek *et al*., 2007) have been studied for bioremediation. After characterizing a common pathway used by these species, researchers have been able to genetically modify them to improve their potential for bioremediation; however, limitations still exist.

Common setbacks to bioremediation through current methods include low uptake of metals and other toxins through roots, low overall biomass, and low organism survival when introduced to a new environment (Chaney *et al*., 1997; Meagher, 2000; Macek *et al*., 2007). Bryophytes (small nonvascular plants, including mosses) grow well in water and absorb nutrients (and metals) through their entire surface area, making them attractive candidates for water bioremediation. Contrary to previous scientific belief, a recent study showed that mosses utilize the common phytochelatin plant pathway for

heavy metal metabolism, demonstrating the potential for genetic modification using existing knowledge (Petraglia *et al*., 2014).

A prerequisite to the development of a genetically modified organism is the characterization of the genetic pathway being manipulated. The phytochelatin pathway for heavy metal metabolism is well-studied and highly conserved across plants and animals (Cobbett and Meagher, 2002; Beck *et al*., 2003; Petraglia *et al*., 2014; Filiz *et al*., 2019). In this pathway, the dissolved metals are transported into plant tissue through roots and surface contact. From there, the Phytochelatin Synthase (PCS) enzyme, activated by the presence of the metals, catalyzes the production of phytochelatins (metal chelating peptides) from Glutathione (GSH). GSH is a tripeptide (Glu-Cys-Gly) common in plants and animals, and PCS cleaves the C-terminal Gly, conjugating the remaining Glu-Cys dipeptide to other Glu-Cys dipeptides to create a chain between 4 and 22 amino acids long. The resulting peptide, a phytochelatin, bonds with the dissolved metal ions, forming interactions between the divalent ion and the nucleophilic sulfurs of the Cys residues. In this conjugated form, the metal is no longer reactive or harmful to the plant or to humans. The complex is then transported into vacuoles by ABC (ATP binding cassette) transporters for storage. The cleavage of GSH by PCS is the rate-limiting step of this process, making PCS an appropriate target for genetic manipulation. While the *PCS* gene is constitutively expressed, with the PCS enzyme activated by the presence of heavy metals, common genetic manipulation of this pathway involves increasing expression of the *PCS* gene to facilitate a more sensitive and robust response to heavy metals (Dixit *et al*., 2015).

Based on knowledge of this common pathway, this study characterizes the potential of local Indiana moss species for heavy metal bioremediation as foundational research for future genetic modification. Using locally collected moss species will ensure that any future genetically modified organisms developed for bioremediation are well adapted for the local environment. To determine factors involved in heavy metal response and phenotypic variance, we measured survival and heavy metal uptake of three local moss species when grown in various doses of copper, cobalt, and cadmium sulfates. This study of moss phenotypic and genetic response to heavy metals is a prerequisite to the development of a moss genetically modified for bioremediation.

Materials and Methods

Moss collection

Local moss species were collected near the campus of Butler University in Indianapolis and were washed with water and isolated with tweezers before use. The species collected likely included *Hypnum* spp., *Amblystegium* spp., and/or *Mnium* spp. Identification of moss species relies largely on analysis of mating structures present only for a short time in the spring, so the actual identification of each moss species could not be accurately determined.

Growth in heavy metal

A standardized volume of moss was grown in 10 mL of DI water containing copper sulfate, cobalt sulfate, or cadmium sulfate at doses of 0, 1,000, 5,000, or 20,000 ppb (w/v). Samples were wrapped to prevent evaporation and grown under growth lamps at room temperature for 8 days, at which time the moss and the water were collected. Each combination of moss, metal, and dose was assayed in triplicate, and a control of 1,000 ppb metal with no moss was covered and stored under the same conditions as the other samples for the 8 days with each batch.

Chlorophyll Assay

Moss samples were collected both fresh from the environment and after growth in heavy metal as described above. Portions of each sample were randomly selected and dried with drying paper. To measure chlorophyll in each sample, 0.015g dry moss was ground

by hand in 1.5 mL microcentrifuge tubes with microcentrifuge tube pestles for 2 minutes with 0.5 mL 80% acetone. Samples were centrifuged at 16.3 x 1000 RCF for 5 minutes, then diluted with 4.5 mL 80% acetone. Absorbance was read at 645 nm and 663 nm, and chlorophyll per gram of moss was calculated with the following formula (from Frank *et al*., 2005):

Chlorophyll/gram = [A663 (0.00802) + A645 (0.202)] * 1.5 / mass (g)

Results were normalized to the 0 ppb response for each moss and metal combination and to chlorophyll content of freshly-collected moss for each moss species. Results were then analyzed in a three-way ANOVA.

Heavy metal concentration assay

Free divalent ion concentration remaining in solution was measured using SenSafe Water Metals Check Kit (part number 480309). Due to the range and sensitivity of the strips, samples were diluted to a readable concentration and measured to the nearest 100 ppb, and actual sample concentrations were back-calculated. Results were normalized to the 0 ppb response for each moss and metal combination and analyzed in a three-way ANOVA. Control samples (1000 ppb metal with no moss stored under the same conditions as the other samples for 8 days) were measured with each batch. All controls were measured to contain at least 1000 ppb metal after the 8 days, and some higher measurements indicated some evaporation occurred. Cadmium concentration could not be measured due to limitations of the test strips.

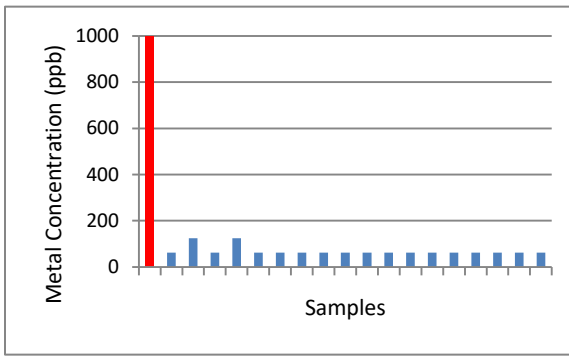
Results

This study generated prerequisite information for the genetic modification of local species for bioremediation. To be useful for genetic modification, a moss must survive well in relevant doses of the desired metal and uptake and metabolize the metal at a practical rate. To determine heavy metal response and phenotypic variance, we measured survival (through a chlorophyll assay) and heavy metal uptake (through a metal uptake assay) of three local moss species when grown in various doses of copper, cobalt, and cadmium sulfates. This work was performed not to determine which particular species of moss was best at absorbing/surviving in which metal at which dose, but rather to see whether the moss species, metal, or dose are factors that influence absorbance/tolerance of heavy metal so that these factors may be considered in the development of a GMO moss for bioremediation.

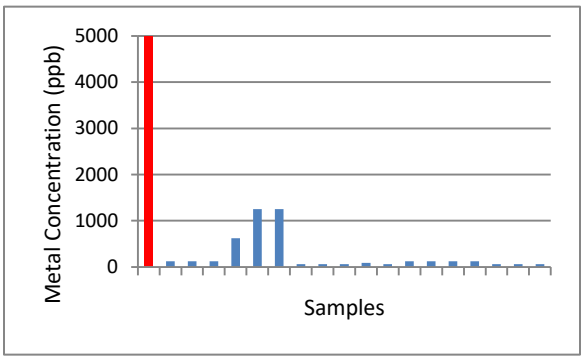
Preliminary analysis of metal absorbance data indicated that mosses absorbed an average of 93.82% of the metals added to each sample (Figure 1). Due to limitations in the metal concentration test strips used, concentration of Cadmium could not be measured, so data was obtained only from samples with Cobalt and Copper. A three-way ANOVA showed significant effects of metal identity ($F = 18.63$, $df = 1$, $p = 1.18 \times 10^{-4}$), metal dose ($F = 79.64$, $df = 2$, $p = 6.05 \times 10^{-14}$), and moss species ($F = 215.71$, $df = 2$, $p = 9.1 \times 10^{-21}$) on metal absorbance rate, indicating the need to select a specific moss to best absorb a particular metal at a particular dose (Figure 2).

Visual analysis of moss after growth in the metals showed no clear difference between metals, mosses, and doses (Figure 3). A three-way ANOVA also showed no significant effect of metal identity ($F = 0.30$, $df = 2$, $p = 0.744$), metal dose ($F = 2.89$, $df = 2$, $p = 0.064$), or moss species ($F = 0.81$, $df = 2$, $p = 0.450$) on chlorophyll levels, indicating no survival advantage for any moss in any metal or any dose (Figure 4). A regression analysis showed no correlation between chlorophyll levels and metal uptake ($r = 0.026$), further supporting that differences in metal absorbance are not caused by differences in moss survival and viability.

a. 1,000 ppb



b. 5,000 ppb



c. 20,000 ppb

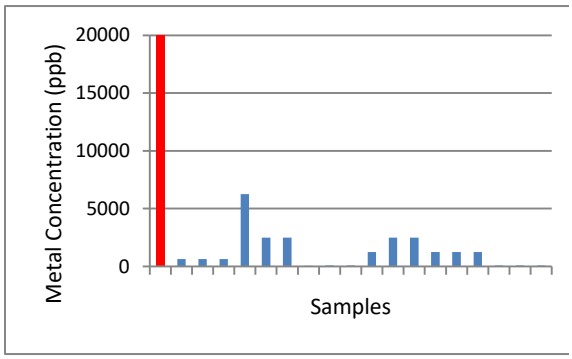


Figure 1. Comparison of metal concentration before and after moss growth. Three moss species were grown in $CuSO_4$ or $CoSO_4$ at doses of 1,000 ppb, 5,000 ppb, or 20,000 ppb, and the final metal concentrations were measured after 8 days. The initial metal concentration (red) and final metal concentrations (blue) are shown for each starting concentration regardless of moss type or metal: 1,000 ppb in a), 5,000 ppb in b), and 20,000 ppb in c). Replicates (N=3) are all shown as independent bars.

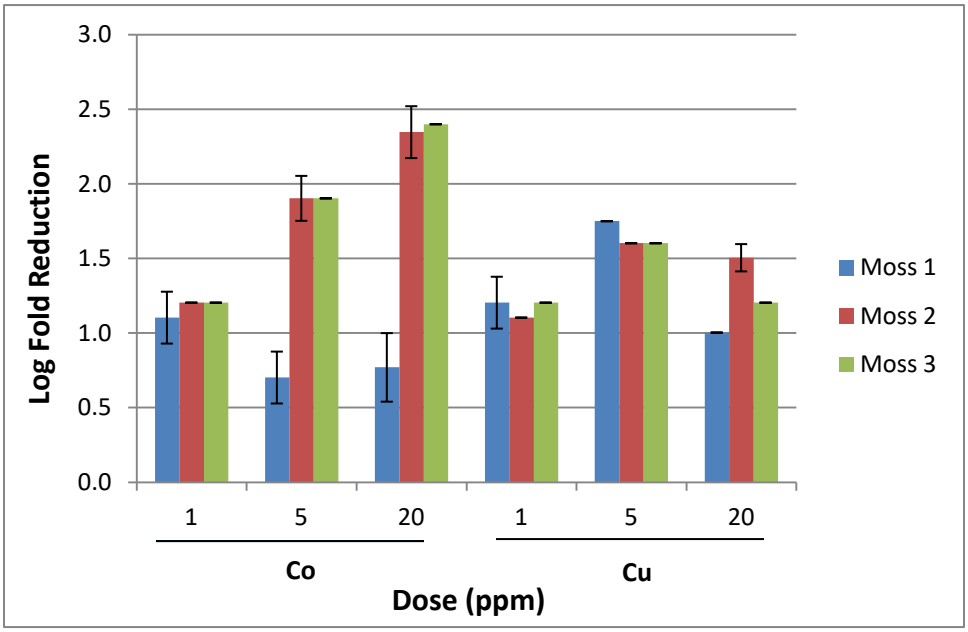


Figure 2. Relative log fold metal reduction. Three mosses were grown in $CuSO_4$ or $CoSO_4$ at doses of 1,000 ppb, 5,000 ppb, or 20,000 ppb, and final metal levels were measured after 8 days**.** The graph shows the average log fold reduction, and error bars show standard deviation (N=3). A three-way ANOVA showed significant effects of metal identity ($p=1.18 \times 10^{-4}$), metal dose ($p=6.05 \times 10^{-14}$), and moss species ($p=9.1 \times 10^{-21}$) on metal absorbance rate.

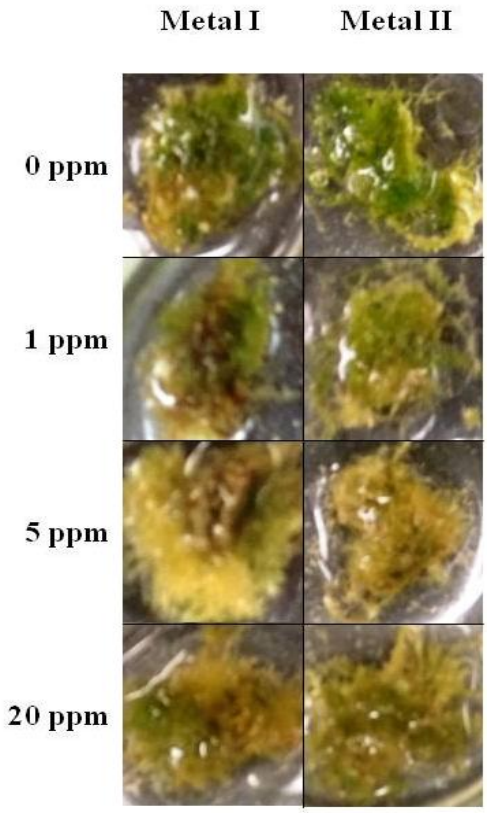


Figure 3. Representative images of dose and metal response. Moss was grown in $CuSO_4$, $CoSO_4$, or $CdSO_4$ at doses of 1,000 ppb, 5,000 ppb, or 20,000 ppb, and images were collected after 8 days**.** The figure shows the same moss species grown in two different metals.

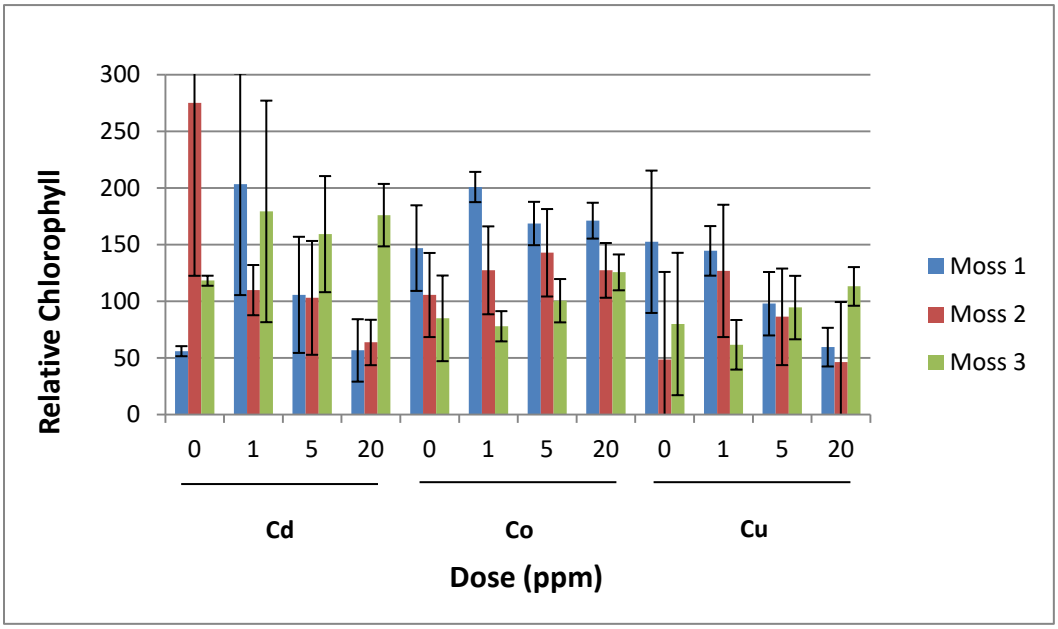


Figure 4. Relative chlorophyll by dose and moss. Three mosses were grown in $CuSO_4$, $CoSO_4$, or $CdSO_4$ at doses of 1,000 ppb, 5,000 ppb, or 20,000 ppb, and final chlorophyll levels were measured after 8 days**.** The graph shows the average log chlorophyll (in AU/mg) normalized to the 0 ppb dose and day 0 chlorophyll levels for each moss, and error bars show standard deviation (N=3). To maintain the scale of the graph, the entirety of the upper error bar for Moss 2 grown in 0 ppb $CdSO_4$ is not shown. A three-way ANOVA showed no significant effect of metal identity (p=0.744), metal dose (p=0.064), or moss species (p=0.450) on chlorophyll levels.

Discussion and Conclusion

Most current efforts for bioremediation use vascular plants, and the use of Bryophytes for heavy metal bioremediation has not yet been well studied. In this study, we show that local moss species are suitable candidates for genetic modification for bioremediation based on their ability to survive in and absorb heavy metals at significant rates. We demonstrate that moss species, metal identity, and metal dose do not have a significant effect on the short term survival of the moss but all affect how well the moss absorbs/metabolizes metal from its environment. This work provides necessary background information for future genetic modification of local moss species.

The doses of heavy metals employed in this study are higher than maximum concentrations currently allowed by federal regulations, but consistent with concentrations measured during water contamination crises. To regulate heavy metals and other water impurities, the EPA, supported by the 1974 Safe Drinking Water Act (Overview of the Safe Drinking Water Act), puts forth water quality requirements; based on these standards, the maximum water level before nationally-enforceable corrective action is required is 15 ppb for lead, 1,300 ppb for copper, and 5 ppb for cadmium (Lead and Copper Rule; National Primary Drinking Water Regulations). Cobalt is not currently regulated but was on the 2016 Contaminant Candidate List 5, suggesting public and governmental interest in adding it to future water quality legislation. In contrast to these standards, home water samples from Flint, Michigan during the 2014 lead water crisis contained as high as 13,200 ppb lead (CNN, 2019), leading to a national crisis. The

doses used in this study (1,000, 5,000, and 20,000 ppb) are well above the maximum national standards for action but are consistent with and or above the range of crisis levels, indicating the potential for mosses to be used for bioremediation in extreme circumstances. Future work could characterize response to lower doses of metals. In addition, we could probe for potential *PCS* genes (from the well-studied phytochelatin pathway) homologous to the *A. thaliana PCS* gene in several local moss species with a Southern Blot analysis, followed by a DNA pulldown assay using the same probe to isolate the PCS gene for sequencing and future modification.

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