A Dissertation

entitled

An Ecologically Engineered System for Remediation of Arsenic-Contaminated Water:

Selecting Plant Species for Northwest Ohio

by

Jordan R. Rofkar

Submitted to the Graduate Faculty as partial fulfillment of the requirements for

the Doctor of Philosophy Degree in Biology (Ecology track)

Dr. Daryl F. Dwyer, Committee Chair

Dr. Defne Apul, Committee Member

Dr. Jonathan M. Frantz, Committee Member

Dr. Alison L. Spongberg, Committee Member

Dr. Michael N. Weintraub, Committee Member

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

May 2010
An Abstract of

An Ecologically Engineered System for Remediation of Arsenic-Contaminated Water: Selecting Plant Species for Northwest Ohio

by

Jordan R. Rofkar

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biology (Ecology track) The University of Toledo

May 2010

Arsenic has become a contaminant of significant concern around the world due to its prevalence and toxicity. The industrial and agricultural history of northwest Ohio created a legacy of arsenic contamination in the area, leaving current and future landowners with the task of remediation. Engineered wetlands could be part of an inexpensive and aesthetically-pleasing treatment strategy for removing arsenic from contaminated water because they rely on naturally-occurring physical, chemical, and biological processes. The role that wetland plants can play in arsenic remediation in temperate climates is not well understood. We do know that in order to maximize the removal of arsenic using wetlands, these plant species need to accumulate arsenic, survive prolonged arsenic stress, function during the course of a growing season, and to survive secondary contaminants that co-occur with arsenic.
During the course of this dissertation I hypothesized that native wetland plants could be used for arsenic phytoremediation and that using mixtures of plant species would maximize arsenic removal in the temperate climate of northwest Ohio. To test this hypothesis I performed four laboratory experiments using native plant species that accumulated arsenic in preliminary tests and have different growing seasons.

(i) *Carex stricta, Pycnanthemum virginianum,* and *Spartina pectinata* tolerated an environmentally relevant concentration (1.5 mg As L\(^{-1}\)) and continued removing arsenic when irrigated with arsenic-laden solutions for seven weeks. Their tolerance toward arsenic was evaluated in terms of contaminant uptake, growth, and chlorophyll content.

(ii) When exposed to arsenic in conditions representative of spring and summer in northwest Ohio, the warm-season species (*S. pectinata*) performed best in summer conditions, while the cool-season species (*C. stricta*) exhibited consistent uptake in both sets of conditions. A variety of warm- and cool-season species could be used to maximize the period of arsenic uptake during a growing season.

(iii) The age of *C. stricta* and *S. pectinata* did not effect uptake by roots, but older plants transferred a greater portion of arsenic to leaves and stems than younger plants. Arsenic extraction could be maximized by allowing plants to accumulate arsenic throughout the growing season, and harvesting aboveground portions in the fall.
The effects of arsenic, copper, and silicon varied in terms of uptake and toxicity in *Azolla caroliniana* and *Lemna minor*. Again, a mixture of plant species could be used to maximize the removal of each contaminant.

The results of this research indicate that native plants could be used in wetlands engineered for arsenic remediation, and that a community of plants could maximize uptake throughout the growing season. These findings will be used during the construction of microcosm and pilot-scale systems at a local, industrial site.
To

Katie

for all of your love, support, patience, and inspiration.
Acknowledgements

Dr. Daryl F. Dwyer – I’d like to express my sincere appreciation for your friendship, patience, and encouragement. I will carry your advice throughout my career.

Drs. Defne Apul, Jonathan M. Frantz, Alison L. Spongberg, and Michael N. Weintraub –

Thank you for your advice and suggestions throughout my time in this program.

Kris Barnswell – I’m glad I was able to complete this process alongside a great friend who inspired and pushed me throughout.

Deanna Bobak – Thank you for your friendship and assistance with lab work, without which, this research could not have been finished.

Doug Sturtz – Thank you for analyzing countless samples on the ICP-OES.

Mom, Dad, Kalena, Kelsey, Ryan, Robin, Dan, and Kelly – You’ve always been supportive and I cannot thank you enough. I’m incredibly fortunate to have such an amazing family.

Mackenzie – You’ve made the past couple of years wonderful. You’ve inspired me throughout this process, and I know you’ll do great things in the years to come.

Katie – You’ve been inspirational, patient, extremely supportive, and an amazing mother to Mackenzie. You’re always interested and involved in my research while still progressing through your own successful career. I am incredibly thankful and proud of you.
Table of Contents

Abstract iii

Acknowledgements vii

List of Tables xii

List of Figures xiii

Chapter One

Engineered wetlands might be a solution for the worldwide arsenic problem 1

1.1. Arsenic around the world 1

1.2. Wetlands for remediation of arsenic-contaminated water 4

1.3. Goals and Objectives 5

References 8

Chapter Two

Using native plant species to maximize arsenic removal in constructed wetlands 11

2.1. Aqueous chemistry of inorganic arsenic 11

2.2. Remediation methods for arsenic-contaminated water 12

2.3. Constructed wetlands for remediation of arsenic-contaminated water 13

2.4. Significance of native macrophytes in engineered wetlands 14

2.5. Mixtures of plant species could maximize arsenic uptake 15

References 21

Chapter Three

viii
Growth, nutrient status, and chlorophyll content of three wetland plant species after prolonged irrigation with arsenic-laden solutions

Abstract

3.1. Introduction

3.2. Materials and Methods

3.2.1. Plants and Growth Conditions

3.2.2. Chlorophyll Content of Leaf Tissues

3.2.3. Leaf Area and Biomass

3.2.4. Arsenic and Nutrients in Plant Tissues

3.2.5. Statistical Analyses

3.3. Results and Discussion

3.3.1. Tolerance Index, Leaf Area, and Biomass

3.3.2. Arsenic in Plant Tissues

3.3.3. Nutrients in Plant Tissues

3.3.4. Chlorophyll Content of Leaf Tissues

3.4. Conclusions

Acknowledgements

References

Chapter Four

Effects of light regime, temperature, and plant age on uptake of arsenic by Spartina pectinata and Carex stricta

Abstract

4.1. Introduction
4.2.  Materials & Methods  

4.2.1. Plants and Growth Conditions  

4.2.2. Variations in uptake kinetics under different temperature and light regimes  

4.2.3. Effects of plant age on arsenic uptake and translocation  

4.2.4. Chemical analyses  

4.2.5. Statistical Analyses  

4.3. Results & Discussion  

4.3.1. Variations in uptake kinetics under different temperature and light regimes  

4.3.2. Effects of plant age and growth rate on arsenic uptake and translocation  

4.4. Conclusions  

References  

Chapter Five  

Uptake and toxicity of arsenic, copper, and silicon in Azolla caroliniana and Lemna minor  

Abstract  

5.1. Introduction  

5.2. Materials and Methods  

5.2.1. Plant material and growth conditions  

5.2.2. Experimental design  

5.2.3. Arsenic, copper, and silicon in plant tissues  

5.2.4. Total chlorophyll and anthocyanins  

5.2.5. Statistical analyses  

5.3. Results and Discussion  

References  

x
5.3.1. Accumulation of arsenic, copper, and silicon 75
5.3.2. Effects of arsenic, copper, and silicon on plant growth 78
5.3.3. Effects of arsenic, copper, and silicon on chlorophyll and anthocyanin content 82

5.5. Conclusions 84

Acknowledgements 85

References 86

Chapter Six

Designing a Wetland for Northwest Ohio 92
List of Tables

Table 1-1. Arsenic concentrations and potentially exposed populations. 3
Table 1-2. Number of Superfund sites with arsenic as a contaminant of concern. 4
Table 2-1. Summaries of technologies for remediation of arsenic. 13
Table 2-2. Concentrations of arsenic in wetland plant species. 17
Table 3-1. Concentrations of arsenic and nutrients in shoots of Carex stricta, Pycnanthemum virginianum, Spartina pectinata, and Pteris vittata. 39
Table 3-2. Concentrations of arsenic and nutrients in roots of Carex stricta, Pycnanthemum virginianum, Spartina pectinata, and Pteris vittata. 39
Table 4-1. Settings used in growth chambers to simulate spring and summer. 51
Table 4-2. Fresh biomass, relative growth rates, and translocation factors of Carex stricta and Spartina pectinata. 53
Table 4-3. Concentrations of arsenic and kinetic parameters for roots of Carex stricta and Spartina pectinata. 55
Table 5-1. Concentrations of compounds used to produce treatment solutions. 73
Table 5-2. Concentrations of arsenic, copper, and silicon in Azolla caroliniana and Lemna minor. 78
List of Figures

Figure 1-1. Diagram of constructed wetlands for treatment of arsenic. 7

Figure 3-1. Tolerance index values for C. stricta, P. virginianum, S. pectinata, and P. vittata. 34

Figure 3-2. Leaf area (A), root biomass (B), and shoot biomass (C) of C. stricta, P. virginianum, S. pectinata, and P. vittata. 35

Figure 3-3. Concentrations of chlorophyll $a+b$ in leaves of C. stricta, P. virginianum, S. pectinata, and P. vittata. 40

Figure 4-1. Non-linear regression of uptake rates by C. stricta and S. pectinata in spring and summer conditions. 57

Figure 4-2. Concentrations of arsenic in shoots of C. stricta and S. pectinata. 59

Figure 4-3. Concentrations of arsenic in roots of C. stricta and S. pectinata. 60

Figure 4-4. Translocation factors of young and old C. stricta and S. pectinata at different growth rates. 62

Figure 5-1. Dry biomass of Azolla caroliniana (a) and Lemna minor (b) treated with combinations of arsenic, copper, and silicon. 80

Figure 5-2. Relative growth rates of A. caroliniana (a) and L. minor (b) treated with combinations of arsenic, copper, and silicon. 81
Figure 5-3. Total chlorophyll \((a+b)\) of *Azolla caroliniana* (a) and *Lemna minor* (b) treated with combinations of arsenic, copper, and silicon.

Figure 5-4. Anthocyanin content of *A. caroliniana* treated with combinations of arsenic, copper, and silicon.
Chapter One

Engineered wetlands might be a solution for the worldwide arsenic problem

1.1. Arsenic around the world

Arsenic naturally enters groundwater in areas where dissolution and mobilization of arsenic-enriched minerals are enhanced by weathering, biological, and volcanic activity (Cullen & Reimer, 1989). Anthropogenic sources include burning of fossil fuels, mining and smelting processes, and agricultural practices that utilize arsenic-based pesticides. Accumulation of arsenic in the environment can lead to human exposure, particularly in areas where groundwater is used for drinking, bathing, and irrigation of crops. The World Health Organization has recommended a limit of 10 µg As L⁻¹ in drinking water, but chronic exposure, even to low levels of arsenic (i.e. less than 50 µg L⁻¹), can cause skin lesions, hyperkeratosis, skin cancer, and liver disease (Karim, 2000).

Arsenic contamination has become a major problem around the world (Table 1-1). The most widely studied incidences of arsenic-contaminated groundwater occur in southeast Asia. Since the late 20th century, people in Bangladesh have been using shallow tube wells to extract drinking water that is free of harmful bacteria (Nickson, et al. 2000). In many areas of the country, however, water from these wells is naturally contaminated by arsenic that is derived from dissolution of iron oxyhydroxides in
subsurface minerals (Nickson, et al. 1998; Nickson, et al. 2000). It is estimated that 30 million people in Bangladesh risk exposure, either by drinking or irrigating crops with groundwater that contains up to 2500 µg As L\(^{-1}\) (Nordstrom 2002).

The United States also has regions where arsenic occurs naturally in groundwater – portions of Alaska, the desert southwest, the Midwest, and hot springs in the central U.S. (Table 1-1). Human activities, such as pesticide use, mining, and burning fossil fuels, also release significant quantities of arsenic which endanger human and ecosystem health (Cullen & Reimer 1989). In the United States, arsenic is the contaminant of greatest concern on the CERCLA Priority List of Hazardous Substances (U.S. Department of Health and Human Services, 2007). A legacy of arsenic waste exists in the form of landfills and sludge reservoirs across the U.S. Over 700 Superfund sites list arsenic as a contaminant of concern in groundwater, surface water, leachate, or liquid waste (Table 1-2; CERCLIS Database 2010). There is an inherent risk of extensive environmental contamination at sites where arsenic is either stored or produced. In 2008, a coal ash spill in Tennessee released 4.1 million m\(^3\) of coal ash (75 mg As kg\(^{-1}\)) into the Emory River and its tributaries (Ruhl, et al. 2009). Downstream waters contained elevated levels of arsenic (0.1 – 95.2 µg L\(^{-1}\)), trace metals, and radioactivity. The abundance and toxicity of arsenic necessitate the development of inexpensive, effective remediation strategies for arsenic-contaminated water.
Table 1-1. Arsenic concentrations and potentially exposed populations around the world.

<table>
<thead>
<tr>
<th>Country/Region</th>
<th>Exposed Population #</th>
<th>Concentration range (µg L⁻¹)</th>
<th>Source(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>30,000,000</td>
<td>&lt; 1 to 2500</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>West Bengal, India</td>
<td>5,000,000</td>
<td>&lt; 10 to &gt; 1000</td>
<td>GW</td>
<td>Chowdhury, et al. 2000</td>
</tr>
<tr>
<td>Vietnam</td>
<td>1,000,000</td>
<td>1 to 3050</td>
<td>GW</td>
<td>Berg, et al. 2001</td>
</tr>
<tr>
<td>Thailand</td>
<td>15,000</td>
<td>1 to &gt; 5000</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Taiwan</td>
<td>200,000</td>
<td>10 to 1820</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Inner Mongolia</td>
<td>600,000</td>
<td>&lt; 1 to 2400</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Argentina</td>
<td>2,000,000</td>
<td>&lt; 1 to 9900</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Chile</td>
<td>400,000</td>
<td>100 to 1000</td>
<td>GW, SW</td>
<td>Karcher, et al. 1999</td>
</tr>
<tr>
<td>Bolivia</td>
<td>50,000</td>
<td>N/A</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Brazil</td>
<td>N/A</td>
<td>0.4 to 350</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Mexico</td>
<td>400,000</td>
<td>8 to 620</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Germany</td>
<td>N/A</td>
<td>&lt; 10 to 150</td>
<td>GW</td>
<td>Heinrichs &amp; Ulduft 1999</td>
</tr>
<tr>
<td>Hungary, Romania</td>
<td>400,000</td>
<td>&lt; 2 to 176</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Spain</td>
<td>50,000</td>
<td>&lt; 1 to 100</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>N/A</td>
<td>&lt; 1 to 80</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Ghana</td>
<td>100,000</td>
<td>&lt; 1 to 175</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>Canada</td>
<td>N/A</td>
<td>&lt; 1 to &gt; 100,000</td>
<td>GW</td>
<td>Nordstrom 2002</td>
</tr>
<tr>
<td>United States</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arizona</td>
<td>N/A</td>
<td>10 to 210</td>
<td>GW</td>
<td>Foust, et al. 2004</td>
</tr>
<tr>
<td>Yellowstone</td>
<td>N/A</td>
<td>160 to 3600</td>
<td>HS</td>
<td>Stauffner &amp; Thompson 1984</td>
</tr>
<tr>
<td>Nevada</td>
<td>N/A</td>
<td>&lt; 2600</td>
<td>GW</td>
<td>Welch &amp; Lico 1998</td>
</tr>
<tr>
<td>South Dakota</td>
<td>N/A</td>
<td>&lt; 2000</td>
<td>GW</td>
<td>Smedley 2002</td>
</tr>
<tr>
<td>Alaska</td>
<td>N/A</td>
<td>&lt; 10,000</td>
<td>GW</td>
<td>Smedley 2002</td>
</tr>
<tr>
<td>Tennessee</td>
<td>N/A</td>
<td>0.1 – 95.2</td>
<td>SW</td>
<td>Ruhl, et al. 2009</td>
</tr>
<tr>
<td>Ohio</td>
<td>N/A</td>
<td>≤ 1500</td>
<td>L</td>
<td>Personal Communication</td>
</tr>
</tbody>
</table>

GW = ground water; SW = surface water; HS = hot springs; L = leachate
Table 1-2. Number of Superfund sites with arsenic as a contaminant of concern.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Number of Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groundwater</td>
<td>541</td>
</tr>
<tr>
<td>Surface water</td>
<td>126</td>
</tr>
<tr>
<td>Leachate</td>
<td>38</td>
</tr>
<tr>
<td>Liquid waste</td>
<td>16</td>
</tr>
</tbody>
</table>


1.2. Wetlands for remediation of arsenic-contaminated water

Humans have been benefiting from the ability of wetlands to clean water for many years. Ecologically engineered wetlands, that rely on natural processes, could be designed as part of a treatment strategy for removing arsenic from contaminated water (Ye et al., 2003). These systems have a number of benefits beyond contaminant removal:

i) Treatment wetlands are essentially fuelled by the sun and require little maintenance.

ii) Ecologically engineered wetlands may be used to restore vanishing wetland habitat, thus maintaining or increasing biodiversity, a critical component of ecosystem stability (Naeem & Li, 1997).

iii) Wetlands can be integrated into an environment using existing soils, vegetation, topography, and climate (Van der Ryn & Cowan, 1996).
We have compiled a team of environmental engineers, geologists, and ecologists to collaborate on the design and construction of wetlands, that incorporate the aforementioned qualities, to remove arsenic from contaminated wastewater. Our focus is on an industrial site in northwest Ohio that produces wastewater with arsenic and silicon as the primary contaminants (1500 µg As L\(^{-1}\); unknown level of silicon). The system will be designed to address this local problem, but we are hopeful that the concepts used in this design will be applicable to other circumstances (e.g. remediating groundwater for drinking or wastewater at other industrial sites). The conceptual model for the engineered wetlands includes primary treatment in a sedimentation basin, followed by secondary treatment in a horizontal flow subsurface wetland, and finally, polishing in a shallow, free water surface wetland (Figure 1-1). As a group, we are studying numerous physical, chemical, and biological processes that can contribute to arsenic removal in this system of wetlands, including (i) adsorption to soil, (ii) chemical transformations due to oxidation and reduction, (iii) chemical transformations mediated by microorganisms, (iv) formation of insoluble arsenic compounds, (v) and uptake and accumulation by plants. The focus of this dissertation is on selection of plant species for accumulation of arsenic in treatment wetlands.

1.3. Goals and Objectives

The goals of this dissertation were (i) to identify whether native wetland plant species could be used to remove arsenic from contaminated water, and (ii) to determine whether a monoculture or a diverse group of species would be better suited to accomplish
this task in the climatic conditions of northwest Ohio. To meet these goals, I established the following objectives:

i) Identify native plant species that are potentially useful for incorporation into an ecologically engineered wetland system for removing arsenic from water.

ii) Determine the effects of long-term arsenic exposure on arsenic uptake, nutrient status, and chlorophyll content in *Spartina pectinata*, *Carex stricta*, and *Pycnanthemum virginianum*.

iii) Determine the rate of arsenic uptake by a warm-season species (*S. pectinata*) and a cool-season species (*C. stricta*) at different temperature and light regimes.

iv) Determine the effects of plant age on uptake of arsenic by *S. pectinata* and *C. stricta*.

v) Determine the effects of copper and silicon on arsenic uptake and growth in wetland plant species (*Lemna minor* and *Azolla caroliniana*).

These objectives were evaluated during the course of three laboratory experiments that are presented hereafter (Chapters 3 – 5). Each of those chapters has been submitted for publication in a refereed, scientific journal.
Figure 1-1. Schematic diagram of a constructed wetland for treatment of arsenic-contaminated water. Primary treatment occurs in a settling basin to remove particulate silicon. Secondary treatment occurs as water flows through a horizontal subsurface flow wetland and arsenic is removed by adsorption to soil, precipitation of insoluble compounds, and uptake by plants. The water is polished by floating macrophytes in shallow free water surface wetlands.
References


Chapter Two

Using native plant species to maximize arsenic removal in constructed wetlands

2.1. Aqueous chemistry of inorganic arsenic

Arsenic is a common contaminant in the environment and exists in four oxidation states (-3, 0, +3, and +5). The most common inorganic forms in water are oxyanions of arsenate [As(V)] and arsenite [As(III)]. Speciation of arsenic in water is primarily determined by pH and reduction/oxidation potential (Smedley & Kinniburgh, 2002). Arsenite is the predominant form of arsenic in anaerobic, aqueous environments, whereas arsenate is more common in aerobic environments (Smedley & Kinniburgh, 2002). Oxidation to arsenate is slow in the presence of pure oxygen, but is more rapid with addition of other oxidants (e.g. iron or aluminum; Welch and Stollenwerk 2003). Arsenate tends to be less mobile than arsenite because it readily adsorbs to the surfaces of common minerals (e.g. ferrihydrite and alumina). Arsenite adsorbs less strongly and, therefore, tends to be more bioavailable than arsenate, one of the factors making arsenite the more toxic inorganic arsenic species.
2.2. Remediation methods for arsenic-contaminated water

The most commonly used methods for remediation of arsenic-contaminated water can be divided into two groups: physico-chemical and biological remediation (Table 2-1; USEPA 2002). Selecting an appropriate remediation strategy depends on a number of factors, including the level of arsenic contamination, the goal for final concentration in treated water, time allotted for remediation, and funding availability. Most commonly, physical or chemical processes are used because they can be engineered to treat any level of arsenic contamination and meet standards for water quality (USEPA 2002). The disadvantages of these systems are their relatively high costs of installation and maintenance, the need for specialized training for maintenance personnel, and disposal of toxic wastes generated during treatment (Mohan & Pittman 2007).

With the spread of arsenic contamination around the world, alternative technologies are being studied to improve the efficiency and cost-effectiveness of arsenic remediation. The use of biological systems to remove contaminants (i.e. bioremediation) is a modern technology that relies on microorganisms and/or plants and could be used to remove or otherwise reduce the harmful impacts of arsenic in the environment. Historically, bioremediation has been overlooked as a feasible remediation strategy for arsenic because it tends to require more time and planning than physico-chemical techniques. In recent years, however, phytoremediation (i.e. using plants) has gained widespread attention and support because it is inexpensive, low-maintenance, and could facilitate habitat restoration (Cunningham and Berti 1993).
Table 2-1. Summaries of technologies for remediation of arsenic.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Physico-chemical</strong></td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>Contaminated water is passed through a column packed with adsorbent material. When adsorption</td>
</tr>
<tr>
<td></td>
<td>sites are saturated with arsenic, the column must be regenerated or replaced.</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Ions held on the surface of a solid are exchanged for arsenic ions of similar charge.</td>
</tr>
<tr>
<td>Precipitation</td>
<td>Dissolved arsenic is transformed into an insoluble solid that is removed from the liquid phase</td>
</tr>
<tr>
<td></td>
<td>via filtration.</td>
</tr>
<tr>
<td>Membrane filtration</td>
<td>Contaminated water is passed through a semi-permeable membrane which allows some constituents</td>
</tr>
<tr>
<td></td>
<td>to pass through while blocking arsenic.</td>
</tr>
<tr>
<td>Permeable reactive barriers</td>
<td>A reactive medium is installed across the flow-path of an arsenic-contaminated groundwater plume.</td>
</tr>
<tr>
<td><strong>II. Biological</strong></td>
<td></td>
</tr>
<tr>
<td>Biofiltration</td>
<td>Microorganisms create ambient conditions that cause precipitation of arsenic in soil or water.</td>
</tr>
<tr>
<td>Phytoremediation</td>
<td>Plants are used to extract, immobilize, or contain arsenic in soil or water.</td>
</tr>
</tbody>
</table>

2.3. Constructed wetlands for remediation of arsenic-contaminated water

Ecologically engineered wetlands have been successfully used to remove metals, metalloids, and organic contaminants from mine waste, agricultural runoff, and industrial effluent (Williams 2002). Wetland systems are typically less expensive and require less maintenance than traditional remediation technologies because they utilize naturally-occurring physical, chemical, and biological processes to remove contaminants. These processes include precipitation, physical interactions with the substrate, chemical complexation, and uptake by plants. The processes at work in treatment wetlands depend on the types of contaminants, biota, and wetland hydrology:
• Free water surface wetlands treat water as it flows across the surface of a planted bed.

• Horizontal subsurface flow wetlands treat water as it flows below the surface of a planted bed.

• Vertical flow wetlands allow water to flood the planted bed and treat the water as it percolates through the root zone.

Although plants are not the only removal mechanism at work in constructed wetlands, they are the focus of this dissertation.

2.4. Significance of native macrophytes in engineered wetlands

The physical functions of wetland plants make them an important component of engineered wetlands. Plants contribute to contaminant removal by altering hydrology, sequestering particulates, and accumulating pollutants (Kadlec and Wallace, 2008). These processes can be utilized to design wetlands with a number of treatment approaches:

1) Phytoextraction relies on plants to take up and accumulate contaminants.

2) Rhizofiltration is the use of plant roots, submerged in water, to take up and accumulate contaminants.

3) Phytostabilization is the use of plants to retain contaminants within a given area, due to either accumulation or stabilization.
Judicious selection of plant species could maximize the amount of arsenic removed using phytoremediation. In most cases, plants have had minimal impact on arsenic removal in engineered wetlands, accumulating only a small portion of the total arsenic supply (Mays & Edwards, 2001; Ye et al., 2003; examples in Brisson and Chazarenc 2009). Many studies have focused on uptake of arsenic by wetland plants (Table 2-2). Plant species should accumulate arsenic, but also must tolerate prolonged arsenic stress and survive the environmental conditions in the treatment area. Most of the known arsenic-hyperaccumulating plant species are subtropical ferns (Meharg 2002), and likely would not be useful in wetlands in temperate regions. Alternatively, native plants are a logical choice for phytoremediation because they are adapted to the local environment and could foster habitat restoration.

2.5. Mixtures of plant species could maximize arsenic uptake

Mixtures of native plant species could potentially be used to maximize arsenic uptake throughout a growing season in engineered wetlands. Temperate climates have seasonal variations in temperature, light regime, and light intensity which limit the effective growing seasons for most native plants. Among aquatic systems, wetlands generally experience the greatest seasonal variability, in terms of photosynthesis, respiration, and growth (Kalff, 2002). Plants can exhibit seasonal variability in phosphate uptake (Jonasson and Chapin 1991; Tate, et al. 1991) and the same might be expected for arsenate, a chemical analog of phosphate. In tropical or sub-tropical climates, where the growing season lasts most or all of the year, a single plant species might be sufficient for phytoremediation. But in temperate climates, using mixtures
warm- and cool-season species could maximize the length of the “uptake season,” thereby maximizing arsenic removal.

Creating mixtures of plant species is also a possible strategy for phytoremediation of contaminant mixtures that contain arsenic. Most research has focused on identifying plant species that could be used for phytoremediation of arsenic alone (e.g. Ma, et al. 2001). Unfortunately, arsenic rarely exists alone in the environment. Instead, contaminated waters often contain a combination of potentially hazardous chemicals and the interactions between those contaminants, and the effects on phytoremediation, are not well understood. Creating wetlands with a mixture of plant species that vary in their affinity for each contaminant, could maximize the amount of contaminants removed, and ensures that remediation of multiple contaminants occurs simultaneously.
Table 2-2. Concentrations of arsenic in wetland plant species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue concentration (mg As kg(^{-1}))</th>
<th>Treatment</th>
<th>Matrix</th>
<th>Duration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemopsis californica</td>
<td>5 / 3</td>
<td>58 mg kg(^{-1})</td>
<td>Soil</td>
<td>n/a</td>
<td>Flores-Tavizon, et al. 2003</td>
</tr>
<tr>
<td>Atriplex portulacoides</td>
<td>310 (root)</td>
<td>481 mg kg(^{-1})</td>
<td>Soil</td>
<td>n/a</td>
<td>Doyle &amp; Otte 1997</td>
</tr>
<tr>
<td>Carex stricta</td>
<td>501 (root)</td>
<td>1.5 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Carex stricta</td>
<td>2930 / 372</td>
<td>25 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Equisetum hyemale</td>
<td>86.2</td>
<td>100 mg kg(^{-1})</td>
<td>Soil</td>
<td>2 mo</td>
<td>Meharg 2003</td>
</tr>
<tr>
<td>Leersia oryzoides</td>
<td>140 / 140</td>
<td>110 mg kg(^{-1})</td>
<td>Soil</td>
<td>6 wk</td>
<td>Ampiah-Bonney, et al. 2007</td>
</tr>
<tr>
<td>Pteris vittata*</td>
<td>15,861</td>
<td>1500 mg kg(^{-1})</td>
<td>Soil</td>
<td>2 wk</td>
<td>Ma, et al. 2001</td>
</tr>
<tr>
<td>Pteris vittata*</td>
<td>97.6 / 1290</td>
<td>1.5 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Pteris vittata*</td>
<td>949 / 9130</td>
<td>25 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Pycnanthemum virginianum</td>
<td>292 / 31.4</td>
<td>1.5 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Pycnanthemum virginianum</td>
<td>1940 / 1470</td>
<td>25 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Samolus ebracteatus</td>
<td>13 / 8</td>
<td>58 mg kg(^{-1})</td>
<td>Soil</td>
<td>n/a</td>
<td>Flores-Tavizon, et al. 2003</td>
</tr>
<tr>
<td>Spartina pectinata</td>
<td>288 (root)</td>
<td>1.5 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Spartina pectinata</td>
<td>1480 / 50</td>
<td>25 ppm</td>
<td>Soil</td>
<td>52 d</td>
<td>This document</td>
</tr>
<tr>
<td>Spartina townsendii</td>
<td>53 (root)</td>
<td>186 mg kg(^{-1})</td>
<td>Soil</td>
<td>n/a</td>
<td>Doyle &amp; Otte 1997</td>
</tr>
<tr>
<td>Equisetum fluviatile</td>
<td>352 / 34</td>
<td>1793 mg kg(^{-1})</td>
<td>Sediment</td>
<td>n/a</td>
<td>Dushenko, et al. 1995</td>
</tr>
<tr>
<td>Species</td>
<td>Tissue concentration (mg As kg(^{-1}))</td>
<td>Treatment</td>
<td>Matrix</td>
<td>Duration</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------------------</td>
<td>----------------------------</td>
<td>---------------</td>
<td>----------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><em>Myriophyllum exalbescens</em></td>
<td>143</td>
<td>1793 mg kg(^{-1})</td>
<td>Sediment</td>
<td>n/a</td>
<td>Dushenko, et al. 1995</td>
</tr>
<tr>
<td><em>Potamogeton pectinatus</em></td>
<td>592 / 751</td>
<td>1793 mg kg(^{-1})</td>
<td>Sediment</td>
<td>n/a</td>
<td>Dushenko, et al. 1995</td>
</tr>
<tr>
<td><em>Scirpus maritimus</em></td>
<td>570 / 8.1</td>
<td>532 mg kg(^{-1})</td>
<td>Sediment</td>
<td>3 yr</td>
<td>Taggart, et al. 2009</td>
</tr>
<tr>
<td><em>Sparganium sp.</em></td>
<td>133 / 28</td>
<td>1793 mg kg(^{-1})</td>
<td>Sediment</td>
<td>n/a</td>
<td>Dushenko, et al. 1995</td>
</tr>
<tr>
<td><em>Triglochin palustre</em></td>
<td>470 / 40</td>
<td>1793 mg kg(^{-1})</td>
<td>Sediment</td>
<td>n/a</td>
<td>Dushenko, et al. 1995</td>
</tr>
<tr>
<td><em>Typha dominguensis</em></td>
<td>355 / 28.5</td>
<td>532 mg kg(^{-1})</td>
<td>Sediment</td>
<td>3 yr</td>
<td>Taggart, et al. 2009</td>
</tr>
<tr>
<td><em>Typha latifolia</em></td>
<td>232 / 17.2</td>
<td>1793 mg kg(^{-1})</td>
<td>Sediment</td>
<td>n/a</td>
<td>Dushenko, et al. 1995</td>
</tr>
<tr>
<td><em>Ceratophyllum demersum</em></td>
<td>650</td>
<td>70 µg L(^{-1})</td>
<td>Water</td>
<td>n/a</td>
<td>Reay 1972</td>
</tr>
<tr>
<td><em>Elodea canadensis</em></td>
<td>307</td>
<td>70 µg L(^{-1})</td>
<td>Water</td>
<td>n/a</td>
<td>Reay 1972</td>
</tr>
<tr>
<td><em>Iris pseudacorus</em></td>
<td>15 / 1</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td><em>Lagarosiphon major</em></td>
<td>251</td>
<td>70 µg L(^{-1})</td>
<td>Water</td>
<td>n/a</td>
<td>Reay 1972</td>
</tr>
<tr>
<td><em>Nitella hookeri</em></td>
<td>182</td>
<td>70 µg L(^{-1})</td>
<td>Water</td>
<td>n/a</td>
<td>Reay 1972</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>200 / 200</td>
<td>8 mg L(^{-1})</td>
<td>Water</td>
<td>220 d</td>
<td>Abedin, et al. 2002</td>
</tr>
<tr>
<td><em>Phragmites australis</em></td>
<td>30 / 3</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td><em>Polypogon monspeliensis</em></td>
<td>20 / 2</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td><em>Sagittaria latifolia</em></td>
<td>25 / 3</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td><em>Scirpus validus</em></td>
<td>5 / 1</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td><em>Thalia dealbata</em></td>
<td>20 / 0.5</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td>Species</td>
<td>Tissue concentration (mg As kg(^{-1}))</td>
<td>Treatment</td>
<td>Matrix</td>
<td>Duration</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------------------------------</td>
<td>-----------</td>
<td>------------</td>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td><em>Typha latifolia</em></td>
<td>10 / 1</td>
<td>0.5 mg L(^{-1})</td>
<td>Water</td>
<td>54 d</td>
<td>Ye, et al. 2003</td>
</tr>
<tr>
<td><em>Aster novae-angliae</em></td>
<td>387.3</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td><em>Azolla caroliniana</em></td>
<td>284</td>
<td>50 µM</td>
<td>Hydroponics</td>
<td>10 d</td>
<td>Zhang, et al. 2008</td>
</tr>
<tr>
<td><em>Azolla caroliniana</em></td>
<td>2.5</td>
<td>1 µM</td>
<td>Hydroponics</td>
<td>5 d</td>
<td>Zhang, et al. 2009</td>
</tr>
<tr>
<td><em>Azolla caroliniana</em></td>
<td>76.8</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 wk</td>
<td>This document</td>
</tr>
<tr>
<td><em>Azolla filiculoides</em></td>
<td>54</td>
<td>50 µM</td>
<td>Hydroponics</td>
<td>10 d</td>
<td>Zhang, et al. 2008</td>
</tr>
<tr>
<td><em>Azolla filiculoides</em></td>
<td>2.5</td>
<td>1 µM</td>
<td>Hydroponics</td>
<td>5 d</td>
<td>Zhang, et al. 2009</td>
</tr>
<tr>
<td><em>Azolla mexicana</em></td>
<td>75</td>
<td>50 µM</td>
<td>Hydroponics</td>
<td>10 d</td>
<td>Zhang, et al. 2008</td>
</tr>
<tr>
<td><em>Azolla microphylla</em></td>
<td>275</td>
<td>50 µM</td>
<td>Hydroponics</td>
<td>10 d</td>
<td>Zhang, et al. 2008</td>
</tr>
<tr>
<td><em>Azolla pinnate</em></td>
<td>175</td>
<td>50 µM</td>
<td>Hydroponics</td>
<td>10 d</td>
<td>Zhang, et al. 2008</td>
</tr>
<tr>
<td><em>Calamagrostis canadensis</em></td>
<td>429.1</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td><em>Carex stricta</em></td>
<td>571.4</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td><em>Chelone glabra</em></td>
<td>256.9</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td><em>Lemna gibba</em></td>
<td>1022</td>
<td>100 µg L(^{-1})</td>
<td>Hydroponics</td>
<td>21 d</td>
<td>Mkandawire, et al. 2004</td>
</tr>
<tr>
<td><em>Lemna minor</em></td>
<td>12</td>
<td>1 µM</td>
<td>Hydroponics</td>
<td>5 d</td>
<td>Zhang, et al. 2009</td>
</tr>
<tr>
<td><em>Lemna minor</em></td>
<td>140.5</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 wk</td>
<td>This document</td>
</tr>
<tr>
<td><em>Panicum virgatum</em></td>
<td>474.9</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td>Species</td>
<td>Tissue concentration (mg As kg(^{-1}))</td>
<td>Treatment</td>
<td>Matrix</td>
<td>Duration</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------------------------------</td>
<td>-----------</td>
<td>------------</td>
<td>----------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><em>Pteris cretica</em></td>
<td>1000</td>
<td>500 µM</td>
<td>Hydroponics</td>
<td>n/a</td>
<td>Baldwin &amp; Butcher 2007</td>
</tr>
<tr>
<td><em>Pteris vittata</em></td>
<td>1000</td>
<td>500 µM</td>
<td>Hydroponics</td>
<td>n/a</td>
<td>Baldwin &amp; Butcher 2007</td>
</tr>
<tr>
<td><em>Pycnanthemum virginianum</em></td>
<td>149.3 / 7.1</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 wk</td>
<td>This document</td>
</tr>
<tr>
<td><em>Pycnanthemum virginianum</em></td>
<td>353.7</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td><em>Spartina alterniflora</em></td>
<td>500 / 10</td>
<td>2 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>30 d</td>
<td>Carbonell, et al. 1998</td>
</tr>
<tr>
<td><em>Spartina pectinata</em></td>
<td>273.4 / 9.1</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 wk</td>
<td>This document</td>
</tr>
<tr>
<td><em>Spartina pectinata</em></td>
<td>525.8</td>
<td>1.5 mg L(^{-1})</td>
<td>Hydroponics</td>
<td>2 d</td>
<td>This document</td>
</tr>
<tr>
<td><em>Spirodea polyrhiza</em></td>
<td>26.5</td>
<td>4 µM</td>
<td>Hydroponics</td>
<td>6 d</td>
<td>Rahman, et al. 2007</td>
</tr>
<tr>
<td><em>Spirodea polyrhiza</em></td>
<td>12</td>
<td>1 µM</td>
<td>Hydroponics</td>
<td>5 d</td>
<td>Zhang, et al. 2009</td>
</tr>
<tr>
<td><em>Wolfsia globosa</em></td>
<td>25</td>
<td>1 µM</td>
<td>Hydroponics</td>
<td>5 d</td>
<td>Zhang, et al. 2009</td>
</tr>
<tr>
<td><em>Agrostis stolonifera</em></td>
<td>10</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Baroni, et al. 2004</td>
</tr>
<tr>
<td><em>Mentha aquatica</em></td>
<td>540 / 37</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Baroni, et al. 2004</td>
</tr>
</tbody>
</table>

* *P. vittata* and *P. cretica* are not considered wetland species, but they were included in this table, for comparison, as known arsenic hyperaccumulators.
References


Chapter Three

Growth, nutrient status, and chlorophyll content of three wetland plant species after prolonged irrigation with arsenic-laden solutions

(Submitted to Journal of Hazardous Materials)

Abstract

Engineered wetlands can be an integral part of a treatment strategy for remediating arsenic-contaminated wastewater, wherein, arsenic (As) is removed by adsorption to soil particles, chemical transformation, precipitation, or accumulation by plants. It is desirable to optimize the remediation process by choosing plant species that exhibit an ability to take up As throughout the seasonal growing period. This report details experiments that utilize wetland plant species native to Ohio (Carex stricta, Pycnanthemum virginianum, and Spartina pectinata) that exhibit seasonally related maximal growth rates, plus one non-native, fern (Pteris vittata) that hyperaccumulates arsenic and was used to compare arsenic tolerance. All plants were cultured in potting mix and irrigated with control or As-laden nutrient solutions (either 0, 1.5, or 25 mg As L$^{-1}$) for 52 d. Biomass, nutrient content, and chlorophyll
content were compared between plants treated and control plants (n = 5). At the higher concentration of arsenic (25 mg L\(^{-1}\)), plant biomass, leaf area, and total chlorophyll were all decreased from values in control plants. A tolerance index, based on biomass at the end of the experiment, indicated \(C.\) \emph{stricta} (0.99) and \(S.\) \emph{pectinata} (0.84) were more tolerant than the other plant species when irrigated with 1.5 mg As L\(^{-1}\). These plant species can be considered as candidates for engineered wetlands.

**Keywords:** phytoremediation, wetlands, arsenic, tolerance

3.1. Introduction

Current strategies for the remediation of arsenic-contaminated water utilize expensive, labor-intensive, chemical and physical filtration processes [1] that are not viable for regions that lack the necessary resources. Engineered wetlands may serve as an economical alternative, with sequestration of arsenic (As) occurring through a combination of chemical transformations [2] and adsorption to soil [3-5]. Unfortunately, these processes may be limited by the quantities of reactants and adsorption sites, which may limit the effective lifespan of a treatment wetland.

For this reason, uptake of As by plants should be considered as a primary component of the remediation process. This is not a straightforward consideration, because As plays no known role in plant nutrition and for a number of reasons, may negatively affect biological functions of plant tissues. (i) Chemical similarities between As and phosphorus (P) can create competition for molecular transporters that
limits uptake of P and displace P in some biochemical reactions [6]. The addition of
As may alter the uptake and transport of various other essential macro- and
micronutrients, as well [7]. (ii) Arsenic can inhibit photosynthesis by causing
reductions in chlorophyll content, as documented in Zea mays [8] and Oryza sativa
[9]. (iii) Most importantly, exposure to As can inhibit growth in many plant species
[10-12]. Each of these factors might limit phytoremediation by inhibiting growth and
As uptake by plants. This led us to a need to identify plant species that can tolerate
and accumulate As.

We hypothesized that a mixture of plant species would increase the likelihood of
As uptake throughout the growing season. In preliminary experiments, we exposed
ten native wetland plant species, which have been recommended for wetland
restoration in Ohio [13], to As in hydroponic systems for two weeks, and observed
accumulation in each. However, their abilities to tolerate a more extensive period of
As exposure, in terms of growth and As uptake, were not assessed. The objective of
the current study was to utilize representative plant species from the larger group to
evaluate As tolerance in a more applicable experimental system with soil and longer
exposure to As. We selected three plant species (Carex stricta, Pycnanthemum
virginianum, and Spartina pectinata) with different growth forms (i.e. graminoid and
forb) and optimum growing conditions (i.e. cool- and warm-season) to determine
whether they could survive and maintain growth and As uptake during an extended
period of exposure. They were compared to Pteris vittata, a non-indigenous fern,
because it accumulates high levels of As [14] and has a relatively high tolerance
during prolonged exposure [15].
The plants were exposed to As for 52 d at concentrations of 1.5 mg As L\(^{-1}\), to simulate leachate from a specific site in northwest Ohio, and 25 mg As L\(^{-1}\), to simulate waste spills [16]. Accumulation of As in roots and shoots was assayed over time along with measurements of biomass, nutrient content, and chlorophyll content to compare the health of treated versus control plants. Based on these data, we calculated a tolerance index, and predicted that \textit{C. stricta} and \textit{S. pectinata} were most tolerant of As, and thus suitable for \textit{in situ} phytoremediation.

3.2. Materials and Methods

3.2.1. Plants and Growth Conditions

\textit{C. stricta} (commonly known as tussock sedge) is a perennial, C\(_3\) graminoid that forms dense clumps and spreads primarily via rhizomes; \textit{P. virginianum} (commonly known as Virginia mountain mint) is a perennial, C\(_3\) herb that produces multiple dense shoots from a single plant; \textit{S. pectinata} (commonly known as prairie cordgrass) is a perennial, C\(_4\) graminoid that forms dense stands via fast-growing rhizomes.

Seeds (Prairie Moon Nursery, Winona, MN) and \textit{P. vittata} spores were germinated on sand in a greenhouse (15 – 20°C, 12-h photoperiod, 50% relative humidity). Seedlings and young sporophytes were transferred to Oasis Horticubes (Smithers-Oasis, Kent, OH) and cultured for ten weeks with a modified Hoagland’s nutrient solution (1.0 mM Ca(NO\(_3\))\(_2\), 1.5 mM KNO\(_3\), 0.25 mM KH\(_2\)PO\(_4\), 0.5 mM MgSO\(_4\), 0.1 mM EDTA-Fe, 0.5 µM ZnSO\(_4\), 6.0 µM MnCl\(_2\), 50 µM H\(_3\)BO\(_3\), 2.0 µM CuSO\(_4\), and 0.09 µM Na\(_2\)MoO\(_4\); pH 5.5 – 6.0). Seedlings and sporophytes were transferred to 4.5-inch plastic pots filled with potting mix (75 g dry wt.; Sunshine Mix
#1, Sun Gro Horticulture, Vancouver, BC) and grown another two weeks. During this acclimation period, the plants were irrigated with nutrient solution to maintain adequate soil nutrients and moisture. For the next 52 d, plants (five replicates per treatment) were maintained in the pots and irrigated twice per week with nutrient solution (100 mL) amended with As (either 0, 1.5, or 25 mg As L\(^{-1}\) as Na\(_2\)HAsO\(_4\)). The pots were irrigated concurrently so that the total volume added for the duration of the experiment (1500 mL), and timing of irrigation were the same for all replicates.

3.2.2. Chlorophyll Content of Leaf Tissues

At the time of harvest, a fresh sample of leaf tissue (1 g) was collected from the youngest fully unfurled leaf of each plant to determine chlorophyll content. These samples were cut into pieces (approximately 1 mm wide) and placed into vials with 80%-acetone solution (5 mL) and kept in the dark (4°C, 24 h). A subsample (3 mL) of the supernatant was collected for analysis in a spectrophotometer (Aquamate, Thermo Electron Corporation, Waltham, MA). Optical densities were recorded at 646.6 nm and 663.6 nm to determine the concentrations (µg g\(^{-1}\) fresh mass) of chlorophyll \(a\), chlorophyll \(b\), and total chlorophyll \((a + b)\) in each leaf [17].

3.2.3. Leaf Area and Biomass

Following the treatment period (52 d), all plants were harvested, rinsed with tap water and 0.1 M HCl to remove adsorbed As, and separated into roots and shoots. Leaves were removed from stems, laid out on a white background, and photographed with a digital camera. Total leaf area was determined for each plant from digital
photographs using Assess: Image Analysis Software for Plant Disease Quantification (APS Press, St. Paul, MN). Plant tissues were then dried at 60°C for 72 h and weighed to determine dry root and shoot biomass. A tolerance index was calculated for each species as the ratio of mean dry weight in a particular treatment to the mean dry weight in the control [18].

3.2.4. Arsenic and Nutrients in Plant Tissues

Dried samples of roots and shoots (0.1 g) from each replicate were mixed with nitric acid (HNO₃; 5 mL) in Teflon digestion vessels and digested in a microwave (15 min ramp time, 200°C, 20 min hold time; Mars Xpress, CEM, Matthews, NC). After cooling, hydrogen peroxide (H₂O₂; 1.5 mL) was added to each vessel and they were returned to the microwave (15 min ramp time, 200°C, 20 min hold time). Resultant solutions were filtered (8 µm paper) and diluted to 3.5% HNO₃ prior to analysis. Inductively coupled plasma-optical emission spectroscopy (ICP-OES; IRIS Intrepid II, Thermo Electron Corporation, Waltham, MA) was used to quantify total As, macro- (P, K, Ca, Mg, and S), and micronutrients (B, Cu, Mn, and Zn).

The ICP-OES was standardized for arsenic detection as previously described [19]. Briefly, peach leaves (NIST No. 1547, National Institute of Standards and Technology, Gaithersburg, MD) were digested and spiked with known amounts of arsenic to determine the appropriate wavelength (189.042 nm) for arsenic analysis. The limit of detection for arsenic in digested solutions (10 µg As L⁻¹) corresponded to a concentration of 13.7 mg As kg⁻¹ in plant tissues. The appropriate wavelengths for analysis of other elements were determined using peach and spinach leaves (NIST
Nos. 1547 and 1570a, respectively). Quality control measures for analyses by ICP-OES included (1) low and high standards analyzed after every tenth sample, (2) certified reference materials (NIST No. 1547 and 1570a) analyzed after every twentieth sample, (3) a multi-element standard (LPC Standard 1, SPEX Certiprep, Metuchen, NJ) analyzed after every sixtieth sample, and (4) procedural blanks in each analytical batch.

3.2.5. Statistical Analyses

Statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). One-way analysis of variance followed by Tukey’s pair-wise comparison test was used to determine significant differences between treatment means for chlorophyll, leaf area, biomass, and log-transformed nutrient data (p < 0.05). The student’s t-test was used to determine whether tolerance indices were significantly different from 1.0 (p < 0.05).

3.3. Results and Discussion

3.3.1. Tolerance Index, Leaf Area, and Biomass

The relatively high index values for *C. stricta* and *S. pectinata* indicate that these species might survive well in a wetland designed for treatment of As-contaminated water at, or below, 1.5 mg L$^{-1}$. Of the plants irrigated with 1.5 mg As L$^{-1}$, *C. stricta* and *S. pectinata* had the highest tolerance indices (0.99 and 0.84, respectively), while indices were less than 0.5 for all plant species at the 25 mg As L$^{-1}$ treatment level (Figure 3-1). Similar indices have been used to quantify plant responses to many
types of stressors, including heavy metals [18,20,21]. Some wetland plants survive in environments with levels of arsenic comparable to the lower treatment [22]. *P. vittata* is not a wetland species and its low tolerance index might reflect an intolerance to the irrigation regime used in this study, rather than to arsenic itself. *P. vittata* is a hyperaccumulator that benefits from As concentrations up to 100 mg kg$^{-1}$ in soil [11], while growth of many other species is inhibited at As levels below 20 mg kg$^{-1}$ [10]. Saturation of the substrate would be expected in engineered wetlands, and based on our observations, might prevent phytoremediation by *P. vittata* in those circumstances.

Although tolerance indices were highest in *C. stricta* and *S. pectinata*, a low level of arsenic apparently was not detrimental to any of the plant species tested. In actuality, all of the individual plants survived the exposure period. When they were irrigated with 1.5 mg As L$^{-1}$, leaf area, aboveground biomass, root biomass, and root:shoot ratios were comparable to controls (Figure 3-2). These observations indicate that *P. virginianum*, *C. stricta*, and *S. pectinata* meet an important criterion for inclusion in a treatment wetland for As – they can survive prolonged exposure to As.

Reductions in leaf area, aboveground biomass, and root biomass indicated that 25 mg As L$^{-1}$ negatively affected the wetland plant species. In fact, the effects were so great that some of the individual plants died prior to the end of the experiment. It is possible that wetland plants might experience comparable levels of As in cases of localized contamination [23]. If the species studied here were to experience such an
extreme concentration in the environment, they might not survive, making their use impractical for remediation wetlands in those circumstances.

![Figure 3-1](image)

Figure 3-1. Tolerance index calculated as the ratio of total biomass in individual treated plants to total biomass of individual control plants. A value of 1.0 indicates no change in biomass between treated and control plants. Stars (*) indicate mean values that are significantly different from 1.0 ($P < 0.05$). Bars represent mean ± 1 standard error.
(A) Leaf Area (cm²)

- Control
- 1.5 mg As L⁻¹
- 25 mg As L⁻¹

C. stricta, P. virginianum, S. pectinata, P. vittata

(B) Root Biomass (g dw)

- Control
- 1.5 mg As L⁻¹
- 25 mg As L⁻¹

C. stricta, P. virginianum, S. pectinata, P. vittata
Figure 3-2. Leaf area (A), root biomass (B), and shoot biomass (C), of individual plants after 52 d of irrigation with treatment solutions. Bars represent mean ± 1 standard error. Letters indicate significant differences between treatments within a species ($P < 0.05$).

3.3.2. Arsenic in Plant Tissues

Similar to previous experiments with As [10,19], tissue concentrations of each species increased along with treatment level (Tables 3-1 and 3-2). Most of that As was stored in roots, as is typical of non-hyperaccumulators [e.g. 24]. On the other hand, *P. vittata* transferred most of its accumulated As to the fronds, similar to previously reported observations [14]. The translocation factor (TF), a ratio of As concentration in shoots to roots, is often used as a metric for determining the value of a plant species for phytoextraction [25]. By using plants that transfer As to shoots, contaminated tissues can potentially be harvested without removing the entire plant. Of the three wetland species, *P. virginianum* irrigated with 25 mg As L$^{-1}$ had the highest TF (0.76), while overall, the highest TF (13.2) was in *P. vittata* treated with
1.5 mg As L\textsuperscript{-1}. Although they had significantly lower TFs, \textit{C. stricta} and \textit{S. pectinata} might still be useful for phytoextraction because of their growth habits. These graminoids are able to spread under the soil surface and the location of their meristems allow for periodic removal (\textit{i.e.} mowing) of their aboveground tissues without permanently damaging the plants.

### 3.3.3. Nutrients in Plant Tissues

Overall, irrigation with 1.5 mg As L\textsuperscript{-1} had a minimal effect on nutrient content of \textit{C. stricta}, \textit{P. virginianum}, \textit{S. pectinata}, and \textit{P. vittata}. All of the plant species maintained normal levels of each nutrient that was examined [26], although the nutrient content within each species shifted after exposure to As. Similar changes in nutrient content following As exposure have been reported for \textit{Spartina alterniflora}, a wetland grass species [24]. Nutrient composition is an important consideration when choosing plant species for phytoremediation applications because deficiencies could diminish a plant’s ability to grow and survive, and ultimately might reduce the rate of As uptake. In this study, the changes in nutrient content had no apparent effect on growth or tolerance of the wetland species at 1.5 mg As L\textsuperscript{-1}.

Arsenic did not affect P content of any of the plant species except \textit{P. virginianum} treated with 25 mg As L\textsuperscript{-1}. This reduction in P content is likely due to phytotoxicity; some of the \textit{P. virginianum} died while others had low biomass and leaf area relative to controls. Arsenic is chemically analogous to P and may interfere with biochemical functions performed by P in plants (\textit{e.g.} energy transfer and protein metabolism). Reductions in P content might be expected when plants are exposed to As, due to
either competitive uptake or phytotoxicity [27], but some species accumulate higher concentrations of P when exposed to As [11]. The species tested here showed neither increased nor decreased P content, critical for maintaining growth for continued As uptake.

Concentrations of some other nutrients in the wetland species were affected by irrigation with 25 mg As L\(^{-1}\). Calcium (Ca) levels decreased in shoots, but increased in roots of each wetland species. Ca has naturally low mobility in plants, which may have been compounded by decreased growth and transpiration due to arsenic toxicity. When exposed to arsenic, \(P. \text{vittata}\) [11] and \(Spartina \text{alterniflora}\) [24] accumulate potassium (K), possibly to counterbalance accumulation of As anions. In this study, however, K decreased in roots of \(C. \text{stricta}\) but remained constant in tissues of the other species. Magnesium (Mg) is the central atom in chlorophyll molecules, and decreased in shoots of \(C. \text{stricta}\) and \(S. \text{pectinata}\). Accordingly, this corresponded to a decrease in chlorophyll in \(S. \text{pectinata}\), but not \(C. \text{stricta}\) (see Section 3.4.).
Table 3.1. Concentrations of As and nutrients in shoots of *C. stricta*, *P. virginianum*, *S. pectinata*, and *P. vittata* after 52 d of irrigation with treatment solutions. Values represent means ± standard error. Letters indicate statistically significant differences within each species (*P < 0.05*). Values that are not followed by a letter are not significantly different within a species.

<table>
<thead>
<tr>
<th></th>
<th><em>C. stricta</em></th>
<th><em>P. virginianum</em></th>
<th><em>S. pectinata</em></th>
<th><em>P. vittata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1.5 mg As L⁻¹</td>
<td>25 mg As L⁻¹</td>
<td>Control</td>
</tr>
<tr>
<td>As</td>
<td>nd</td>
<td>nd</td>
<td>293 ± 771b</td>
<td>nd</td>
</tr>
<tr>
<td>B</td>
<td>14.6 ± 1.10</td>
<td>12.0 ± 2.02</td>
<td>18.1 ± 2.06</td>
<td>13.2 ± 1.17</td>
</tr>
<tr>
<td>Ca</td>
<td>4140 ± 234a</td>
<td>4020 ± 53.4a</td>
<td>2280 ± 261b</td>
<td>2010 ± 1840</td>
</tr>
<tr>
<td>Cu</td>
<td>12.3 ± 1.51</td>
<td>11.3 ± 1.59</td>
<td>8.11 ± 0.60</td>
<td>6.93 ± 0.603</td>
</tr>
<tr>
<td>Mg</td>
<td>31500 ± 1310ab</td>
<td>35700 ± 1720a</td>
<td>29000 ± 653b</td>
<td>43000 ± 1730</td>
</tr>
<tr>
<td>Mn</td>
<td>3660 ± 194a</td>
<td>3260 ± 68.0ab</td>
<td>2530 ± 245b</td>
<td>4930 ± 512</td>
</tr>
<tr>
<td>Na</td>
<td>182 ± 8.20</td>
<td>149 ± 10.8</td>
<td>nd</td>
<td>199 ± 45.4</td>
</tr>
<tr>
<td>P</td>
<td>991 ± 54.5a</td>
<td>957 ± 86.7a</td>
<td>1350 ± 125b</td>
<td>881 ± 50.4</td>
</tr>
<tr>
<td>Zn</td>
<td>2670 ± 100.0</td>
<td>2860 ± 120.0</td>
<td>2870 ± 183</td>
<td>4730 ± 829</td>
</tr>
</tbody>
</table>

Table 3.2. Concentrations of As and nutrients in roots of *C. stricta*, *P. virginianum*, *S. pectinata*, and *P. vittata* after 52 d of irrigation with treatment solutions. Values represent means ± standard error. Letters indicate statistically significant differences within each species (*P < 0.05*). Values that are not followed by a letter are not significantly different within a species.

<table>
<thead>
<tr>
<th></th>
<th><em>C. stricta</em></th>
<th><em>P. virginianum</em></th>
<th><em>S. pectinata</em></th>
<th><em>P. vittata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1.5 mg As L⁻¹</td>
<td>25 mg As L⁻¹</td>
<td>Control</td>
</tr>
<tr>
<td>As</td>
<td>nd</td>
<td>nd</td>
<td>2930 ± 771b</td>
<td>nd</td>
</tr>
<tr>
<td>B</td>
<td>510 ± 1.84</td>
<td>293 ± 52.3</td>
<td>3.33 ± 3.41</td>
<td>16.2 ± 1.43</td>
</tr>
<tr>
<td>Ca</td>
<td>2460 ± 155.38ab</td>
<td>2010 ± 139a</td>
<td>4280 ± 1100b</td>
<td>3620 ± 167ab</td>
</tr>
<tr>
<td>Cu</td>
<td>12.8 ± 1.92</td>
<td>10.2 ± 1.65</td>
<td>20.9 ± 6.03</td>
<td>10.4 ± 0.74</td>
</tr>
<tr>
<td>Mg</td>
<td>28600 ± 1490a</td>
<td>26500 ± 1250a</td>
<td>10500 ± 1008b</td>
<td>20500 ± 2200</td>
</tr>
<tr>
<td>Mn</td>
<td>3470 ± 161a</td>
<td>2890 ± 243ab</td>
<td>2460 ± 349b</td>
<td>6100 ± 411a</td>
</tr>
<tr>
<td>Na</td>
<td>48.3 ± 5.41</td>
<td>62.2 ± 11.9a</td>
<td>161 ± 38.0b</td>
<td>49.6 ± 4.08</td>
</tr>
<tr>
<td>P</td>
<td>12200 ± 401a</td>
<td>2350 ± 302a</td>
<td>7310 ± 2190b</td>
<td>1370 ± 106a</td>
</tr>
<tr>
<td>Zn</td>
<td>59.2 ± 7.97a</td>
<td>54.1 ± 9.02a</td>
<td>112 ± 17.8b</td>
<td>34.2 ± 1.60</td>
</tr>
</tbody>
</table>
3.3.4. Chlorophyll Content of Leaf Tissues

For the duration of this study, total chlorophyll concentrations were maintained in all plant species irrigated with 1.5 mg As L$^{-1}$ (Figure 3-3), one factor which may have contributed to their continued growth. At the higher As level, however, only *C. stricta* had a chlorophyll level comparable to controls. When exposed to metals, several plant species have exhibited declines in chlorophyll content that can result in decreased photosynthesis and growth [8,9]. A decline in chlorophyll content typically takes time to manifest, and is generally considered an indicator of prolonged plant stress. Metals have been shown to interfere with chlorophyll synthesis by inhibiting enzymes involved in production or by inducing nutrient deficiency [28]. Nutrient levels were normal in all plants in this study, but the higher level of As may have inhibited enzymes critical for chlorophyll synthesis.

![Figure 3-3](image)

**Figure 3-3.** Concentrations of chlorophyll $a + b$ in the youngest unfurled leaves of each species. Bars represent mean ± 1 standard error (n = 6). Letters indicate significant differences between treatments within a species ($P < 0.05$).
3.4. Conclusions

(1) *C. stricta* and *S. pectinata* were the more tolerant of 1.5 mg As L\(^{-1}\) than the other plant species and should be considered for *in situ* As phytoremediation.

(2) The plant species used in this were not tolerant of 25 mg As L\(^{-1}\) and might not be suitable for phytoremediation of waters contaminated with similarly elevated levels of As. Nutrient concentrations were within normal ranges, so factors other than nutrient uptake probably caused low tolerance in plants irrigated with 25 mg As L\(^{-1}\).

(3) The wetland species stored most of the accumulated As in plant roots. These species could be used for phytostabilization (i.e. long-term storage of As) and/or mowed periodically to remove the As that has translocated to aboveground shoots.

(4) Utilizing a diverse group of plant species that tolerate As, like those studied here, might allow a wetland to perform over a greater portion of the growing season than a single species alone.

Acknowledgements

We would like to acknowledge the U.S. Department of Agriculture (Grant No. 2005-38894-02307) for funding; Deanna Bobak for laboratory and administrative assistance; Jonathan Frantz, Defne Apul, Kris Barnswell, Steven Choc, and Matthew Gorr for suggestions during preparation of the manuscript; and Doug Sturtz for operation of the ICP-OES.
References


Chapter Four

Effects of light regime, temperature, and plant age on uptake of arsenic by *Spartina pectinata* and *Carex stricta*

*(Submitted to *International Journal of Phytoremediation*)

Abstract

Wetlands designed for arsenic remediation in temperate climates could be optimized by selecting a group of plant species that together accumulate arsenic throughout the growing season. We report here on efforts to show that a combination of native wetland plant species might perform better than a monoculture by supplementing weaknesses such as plant age and seasonal variability. Two wetland plant species (*Carex stricta* and *Spartina pectinata*) native to Ohio, were used in hydroponic experiments. (i) Arsenic uptake was first assessed for the plants at two ages via exposure to control or arsenic-laden solutions (0 or 1.5 mg As L\(^{-1}\) as Na\(_2\)HAsO\(_4\)) for two weeks. Plant age had no significant effect on arsenic concentrations in root tissue, but translocation factors were greater in older plants of *C. stricta* and *S. pectinata* (0.45 and 0.07, respectively) than in younger plants (0.10 and 0.01, respectively). (ii) Seasonal effects were assessed by
determining uptake kinetics for both species in conditions representative of spring temperatures (15/5°C) and light regimes (1050 µmol m⁻² s⁻¹, 13 h day⁻¹) and summer temperatures (28/17°C) and light regimes (1300 µmol m⁻² s⁻¹, 15 h day⁻¹). Both species had comparable rates of arsenic uptake into root tissue in summer conditions (44.0 and 46.5 mg As kg⁻¹ dry wt. h⁻¹ in C. stricta and S. pectinata, respectively), but C. stricta had a higher maximum net influx rate in spring conditions (24.5 versus 10.4 mg As kg⁻¹ dry wt. h⁻¹). We are currently constructing wetland microcosms at an arsenic-contaminated site that will include a combination of plant species to maximize arsenic removal throughout the growing season.

**Keywords:** seasonal variation, kinetics, wetland plants, phytoremediation

### 4.1. Introduction

Arsenic is a ubiquitous element that naturally occurs in soil, plants, and water. Natural processes and anthropogenic activities have redistributed arsenic in the environment, elevating levels in drinking and surface waters in many areas of the world (Nordstrom 2002; Mandal and Suzuki 2002). The increased risk of human exposure and threats to natural ecosystems necessitate the development of cost-effective technologies for the remediation of arsenic-contaminated water. Engineered wetlands are one possible technology. They rely on naturally-occurring physical, chemical, and biological processes to remove arsenic from contaminated water, meaning they have relatively low energy and maintenance requirements.
Many of the processes that contribute to arsenic removal have been studied in controlled environments. Primary removal mechanisms generally include adsorption to substrate and co-precipitation with iron, aluminum, or manganese oxides (Ye, et al. 2003; Buddhawong, et al. 2005). These processes are limited by the quantity of adsorption sites or reactants that are available within a wetland system. By way of contrast, biological processes, mediated by microorganisms (e.g. chemical transformation) and plants (e.g. uptake), might not be limited like other processes because they rely on living organisms. If the appropriate environmental conditions are maintained, then organisms’ biological processes might be extended indefinitely which by itself helps create a cost-effective process.

One possible biological removal mechanism relies on the ability of plants to take up arsenic, a process referred to as phytoextraction. The goal of our research is to select wetland plant species that will maximize phytoextraction at a site that produces leachate with 1.5 mg As L\(^{-1}\) in the temperate climate of northwest Ohio. Unfortunately, phytoextraction is often considered insignificant in the scope of an entire treatment wetland. For example, *Thalia* sp. can accumulate up to 80 mg As kg\(^{-1}\) when grown in hydroponics (Aksorn and Visoottiviseth 2004). However, their arsenic accumulation combined with other wetland plant species was only 2 – 4% of the total quantity of arsenic removal in wetland microcosms (Ye, et al. 2003). The remaining 96 – 98% was adsorbed to soil or precipitated as insoluble compounds of arsenic. To increase the role of plants in engineered wetlands in temperate climates, we need to understand some of the factors, including plant age and seasonal variations, that influence the ability of a plant to accumulate arsenic.
Temperate climates are marked by seasonal variations which create gradients in temperature, light intensity, and light regime. In these conditions, many native plant species have distinct growing seasons which could prevent them from functioning within the context of a treatment wetland for a portion of the year. The amount of phosphorus accumulated by plants can fluctuate throughout a growing season (Jonasson and Chapin 1991; Tate, et al. 1991). The same might be expected for arsenic, because arsenate and phosphate are taken up by the same mechanisms in plants (Asher and Reay 1979; Meharg and Macnair 1990).

Another likely factor affecting arsenic accumulation is the age of the plants. Generally, young roots grow faster and have higher nutrient uptake rates than older roots (Yanai 1994), but the effects of plant age on arsenic uptake have not been widely researched. In the only previous study, eight week old Pteris vittata took up and translocated arsenic to shoots more rapidly than plants that were 16 months old (Silva Gonzaga, et al. 2007), suggesting that young plants would be most efficient for phytoextraction. P. vittata is not adapted to the environmental conditions found in wetlands in temperate climates. So, we would like to know whether native wetland plant species exhibit similar age-dependent variations in arsenic uptake. It would be prudent to utilize or harvest plants at an age that maximizes arsenic phytoextraction during a growing season.

We report here on the effects of (i) plant age and (ii) light and temperature conditions on uptake of arsenic from hydroponic solutions by a cool-season sedge (Carex stricta) and a warm-season grass (Spartina pectinata). Our findings suggest that (i) older plants transfer greater quantities of arsenic to shoots, so aboveground portions should
only be harvested at the ends of growing seasons; and (ii) a mixture of warm-season and cool-season plant species could be used to maximize arsenic uptake in temperate climates. This information will be used in the design of a pilot-scale treatment wetland at a local, arsenic-contaminated site.

4.2. Materials & Methods

4.2.1. Plants and Growth Conditions

Two wetland plant species (*C. stricta* and *S. pectinata*) were previously shown to accumulate and tolerate arsenic (Rofkar and Dwyer, *in review*) and have been recommended for use in wetland restoration projects in Ohio (OEPA 2007). They were selected for this study as representatives of the diverse community of wetland species native to the climate of northwest Ohio. *C. stricta* (tussock sedge) is a C\textsubscript{3}, cool-season, perennial sedge and *S. pectinata* (prairie cordgrass) is a C\textsubscript{4}, warm-season, perennial grass.

Seeds of *C. stricta* and *S. pectinata* (Prairie Moon Nursery, Winona, MN) were sown in a fibrous medium for hydroponics (Oasis Horticubes, Smithers-Oasis, Kent, OH) and placed in a greenhouse (20 – 25°C, 12-h photoperiod, 30 – 70% humidity). When roots protruded from the bottoms of the cubes (4 – 8 weeks after sowing), seedlings were transferred to plastic containers filled with aerated nutrient solution (4 L; 1.0 mM Ca(NO\textsubscript{3})\textsubscript{2}, 1.5 mM K\textsubscript{2}NO\textsubscript{3}, 0.25 mM KH\textsubscript{2}PO\textsubscript{4}, 0.5 mM MgSO\textsubscript{4}, 0.1 mM EDTA-Fe, 0.5 μM ZnSO\textsubscript{4}, 6.0 μM MnCl\textsubscript{2}, 50 μM H\textsubscript{3}BO\textsubscript{3}, 2.0 μM CuSO\textsubscript{4}, and 0.09 μM Na\textsubscript{2}MoO\textsubscript{4}; pH 5.5 – 6.0) and allowed to acclimate to the hydroponic environment (4 – 6 weeks). The solution was replenished as needed and completely replaced weekly.
4.2.2. Variations in uptake kinetics under different temperature and light regimes

Following the initial acclimation period, plastic containers filled with nutrient solution (4 L) and either *S. pectinata* or *C. stricta* were transferred to a growth chamber with light and temperature conditions that simulated either spring *(i.e. mid-April)* or summer *(i.e. mid-July)* in northwest Ohio (Table 4-1). Other environmental variables *(e.g. precipitation)* were not included in this experiment. Plants were left to acclimate for one week. They were then transferred to individual amber HDPE bottles filled with nutrient solution amended with arsenic (0, 0.375, 0.75, 1.5, 3.75, 7.5, 15, or 75 mg L\(^{-1}\) as Na\(_2\)HAsO\(_4\)). Roots were harvested after 4 h *(this time was selected based on preliminary experiments)*. The harvested tissues were rinsed first with tap water, then HCl *(10%)*, with tap water again, then dried *(55°C; 48 h)*, and weighed. Arsenic content of root tissues was then determined by ICP-OES. Uptake rates were used to calculate kinetic parameters, including the maximum net influx rate *(I\(_{\text{max}}\)*) and the affinity constant *(K\(_{\text{m}}\))*.

Table 4-1. Settings used in growth chambers to simulate spring and summer. Values for light intensity indicate average photosynthetically active radiation at canopy height. Values for light regime indicate the timing of simulated daylight during each 24 h period.

<table>
<thead>
<tr>
<th></th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>15°C</td>
<td>28°C</td>
</tr>
<tr>
<td>Night</td>
<td>5°C</td>
<td>17°C</td>
</tr>
<tr>
<td><strong>Light Intensity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>1050 µmol m(^{-2}) s(^{-1})</td>
<td>1300 µmol m(^{-2}) s(^{-1})</td>
</tr>
<tr>
<td>Night</td>
<td>dark</td>
<td>dark</td>
</tr>
<tr>
<td><strong>Light Regime</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>13 h</td>
<td>15 h</td>
</tr>
<tr>
<td>Night</td>
<td>11 h</td>
<td>9 h</td>
</tr>
</tbody>
</table>
4.2.3. Effects of plant age on arsenic uptake and translocation

Plants of two different ages were used for this experiment. Seeds were germinated and the seedlings were cultured as described above until 12 weeks of age (younger plants). The older plants (approximately 6 months in age) were purchased from a nursery (JFNew, Walkerton, IN), washed to remove adhered soil, and transferred to plastic containers filled with nutrient solution (4 L). The plants were acclimated to the hydroponic environment for two weeks, during which the nutrient solution was replaced every five days. Following acclimation, the plants (n = 4) were weighed to determine fresh biomass. The younger plants had significantly lower fresh biomass than older plants in each species (Table 4-2).

After being weighed, the plants were returned to the containers, and the acclimation solutions were replaced with nutrient solutions amended with arsenic (either 0 or 1.5 mg As L\textsuperscript{-1}). The plants were grown for two weeks with the solutions refreshed every five days. Plant tissues were treated as above to obtain dry weights. Arsenic concentrations in roots and shoots were determined by ICP-OES; relative growth rates (RGRs) were calculated from fresh biomass before and after treatment.
Table 4-2. Fresh biomass, relative growth rates (RGR) and translocation factors of C. stricta and S. pectinata exposed to arsenic at different ages.

<table>
<thead>
<tr>
<th></th>
<th>Initial fresh biomass (g)</th>
<th>RGR (mg g⁻¹ d⁻¹)</th>
<th>TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. stricta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young –As</td>
<td>4.28 ± 1.2 a</td>
<td>55.16 ± 2.3 a</td>
<td>N/A</td>
</tr>
<tr>
<td>Young +As</td>
<td>4.22 ± 0.8 a</td>
<td>34.54 ± 7.0 b</td>
<td>0.10 ± 0.05 a</td>
</tr>
<tr>
<td>Old –As</td>
<td>14.3 ± 2.3 b</td>
<td>17.38 ± 2.9 c</td>
<td>N/A</td>
</tr>
<tr>
<td>Old +As</td>
<td>13.0 ± 1.7 b</td>
<td>8.15 ± 3.2 c</td>
<td>0.45 ± 0.12 b</td>
</tr>
<tr>
<td>S. pectinata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young –As</td>
<td>5.62 ± 2.8 a</td>
<td>45.94 ± 7.1 a</td>
<td>N/A</td>
</tr>
<tr>
<td>Young +As</td>
<td>4.86 ± 1.6 a</td>
<td>41.33 ± 5.4 ab</td>
<td>0.01 ± 0.01 a</td>
</tr>
<tr>
<td>Old –As</td>
<td>26.7 ± 2.4 b</td>
<td>18.44 ± 5.3 bc</td>
<td>N/A</td>
</tr>
<tr>
<td>Old +As</td>
<td>29.7 ± 3.9 b</td>
<td>17.82 ± 3.8 c</td>
<td>0.07 ± 0.02 b</td>
</tr>
</tbody>
</table>

Values represent means ± standard error. Letters indicate statistically significant differences within each species (p < 0.05).

4.2.4. Chemical analyses

Samples of dried plant tissues (0.15 g) were digested in a microwave according to a modification of USEPA Method 3051. Briefly, each sample was combined with HNO₃ (5 mL; Fisher Scientific, Pittsburgh, PA) in a 120-mL Teflon® digestion vessel and heated for 45 min (15 min ramp time, 200°C). Then, H₂O₂ (30%; 1.5 mL) was added to each vessel and reheated (15 min ramp time, 200°C, 45 min hold time). After cooling, deionized water (12 mL; 18 mΩ) was added to each vessel. Resulting solutions were filtered and diluted to 3.5% HNO₃ prior to analysis by ICP-OES. Standardization of the ICP-OES was previously described (Rofkar et al. 2007). The limit of detection was 10 µg As L⁻¹ in the ICP-OES solution matrix, which corresponded to a method detection limit of 13.7 mg As kg⁻¹ in plant tissue. Values below that were set at one-half of the
method detection limit (6.85 mg As kg\(^{-1}\)) for statistical analyses. This transformation has been used previously for statistical analyses that include values below a detection limit (Hornung and Reed 1990).

4.2.5. Statistical Analyses

Uptake of arsenic by plants of different ages were compared using the Student’s t-test (\(p = 0.05\); Prism 5, GraphPad Software, Inc.). Translocation factors were calculated from the ratio of arsenic concentrations in shoots to those in roots. Kinetic parameters (\(I_{\text{max}}\) and \(K_{\text{m}}\)) were calculated using non-linear regression of root concentrations fitted to a model for Michaelis-Menten kinetics (Prism 5, GraphPad Software, Inc.).

4.3. Results & Discussion

4.3.1. Variations in uptake kinetics under different temperature and light regimes

When designing wetlands for arsenic phytoremediation, it is possible to select a variety of plant species that maximize uptake throughout the growing season. Several observations suggested that \textit{C. stricta} and \textit{S. pectinata} would be most effective for uptake of arsenic during summer, although \textit{C. stricta} might better maintain its rate of uptake throughout the growing season. The highest values of \(I_{\text{max}}\) were observed for both species when growing in the summer treatment (Table 4-3; Figure 4-1) and the values were not considerably different between the species. In the spring treatment, however, \(I_{\text{max}}\) values were 1.8 and 4.5 times less than in the summer treatment for \textit{C. stricta} and \textit{S. pectinata}, respectively. There was also no notable change in the affinity (\(K_{\text{m}}\)) of \textit{C. stricta} for arsenic between the spring and summer treatments. In contrast, \textit{S. pectinata} had a much
greater $K_m$ value in the summer treatment (6.3 times the $K_m$ value in the spring treatment), indicating a greater affinity for arsenic in summer.

Table 4-3. Concentrations of arsenic in roots of *C. stricta* and *S. pectinata* after 4 h of exposure to 75 mg As L$^{-1}$, and kinetic parameters ($I_{\text{max}}$ and $K_m$) determined from non-linear regression.

<table>
<thead>
<tr>
<th></th>
<th>Root concentration (mg As kg$^{-1}$ dry wt)</th>
<th>$I_{\text{max}}$ (mg As kg$^{-1}$ dry wt h$^{-1}$)</th>
<th>$K_m$ (mg As L$^{-1}$)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. stricta</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>61.0 ± 3.8</td>
<td>24.5 ± 4.5</td>
<td>74.4 ± 23.8</td>
<td>0.9421</td>
</tr>
<tr>
<td>Summer</td>
<td>109.9 ± 21.4</td>
<td>44.0 ± 11.2</td>
<td>44.3 ± 16.7</td>
<td>0.8779</td>
</tr>
<tr>
<td><em>S. pectinata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>37.5 ± 10.7 a</td>
<td>10.4 ± 2.0</td>
<td>21.3 ± 15.2</td>
<td>0.5225</td>
</tr>
<tr>
<td>Summer</td>
<td>108.1 ± 21.2 b</td>
<td>46.5 ± 14.3</td>
<td>60.0 ± 27.0</td>
<td>0.8574</td>
</tr>
</tbody>
</table>

Values represent means ± standard error. Letters indicate statistically significant differences within each species (p < 0.05); kinetic parameters were not compared statistically.

Seasonal growth patterns and photosynthetic mechanisms might explain some of the effects of seasonality observed in *C. stricta* and *S. pectinata*. *C. stricta* actively grows during the spring and summer months, amid a range of temperature and light conditions. The lengthy growing season of *C. stricta* might be reflected in its ability to maintain uptake through both the spring and summer treatments. In addition, organisms like *C. stricta* that perform C$_3$ photosynthesis typically experience maximum photosynthetic efficiency at temperatures below 20°C (Sage and Kubien, 2007 and references therein). *S. pectinata*, on the other hand, performs C$_4$ photosynthesis, and like
other C₄ species might be most efficient at temperatures above 20°C (Sage and Kubien, 2007 and references therein). Therefore, *S. pectinata* tends to be most active during the summer months in temperate climates, which might explain the higher uptake rate and affinity observed in the summer treatment.

Understanding seasonal variations in arsenic uptake is important for design and implementation of phytoremediation systems, particularly in temperate regions where phytoextraction is minimal during winter months. Each plant species likely has a specific set of conditions – a “sweet spot” – during which they extract arsenic most efficiently. Engineering biologically diverse wetlands might foster overlap of those “sweet spots,” thereby maximizing phytoextraction during the course of a growing season. In the setting of a constructed wetland, plant species could be selected to insure this redundancy within the system. Selecting a diverse group of warm- and cool-season plant species could be one strategy to maximize arsenic uptake throughout the growing season of a temperate climate.
Figure 4-1. Rates of arsenic uptake by (A) *C. stricta* and (B) *S. pectinata* treated with different concentrations of arsenic for 4 h. Data represent means ± standard error (n = 4). Kinetic parameters were calculated from non-linear regression of these data.
4.3.2. Effects of plant age and growth rate on arsenic uptake and translocation

Within each species, RGRs were highest in the younger plants that were not exposed to arsenic, followed by younger plants with arsenic, older plants without arsenic, and older plants with arsenic (Table 4-3). Based on these observations, the two factors controlling growth rates of *C. stricta* and *S. pectinata* during this study were plant age and arsenic availability. Typically, young plants have higher growth rates than older plants of the same species (Poorter 1989 and references therein), so this observation was expected. Interestingly, arsenic inhibited growth of the younger *C. stricta*, but did not affect growth in the older *C. stricta* or either age of *S. pectinata*. This result was unexpected because arsenic inhibits growth of other non-hyperaccumulating species including *Oryza sativa* (Azizur Rahman, et al. 2007), mesquite (Mokgalaka-Matlala, et al. 2008), and spinach and tomato (Hartley and Lepp 2008). In contrast, the hyperaccumulating species *Pteris vittata* (Caille, et al. 2005) tolerates higher levels of arsenic and maintains or even increases growth. Neither *C. stricta* nor *S. pectinata* could be considered a hyperaccumulator, but because they maintained growth, both species apparently have relatively high tolerance for arsenic.

The only significant effect of age on uptake of arsenic was observed in shoots of *S. pectinata* – older shoots contained 10.9 mg As kg\(^{-1}\) while the concentration was below the limit of detection in younger shoots (Figures 4-3). Plant age did not affect concentrations of arsenic in roots of *S. pectinata*, or roots or shoots of *C. stricta* (Figures 4-2 and 4-3). In general, the average rate of uptake per unit of root is highest while plants are young, and decreases with age (Barber 1984), but this pattern was not observed for uptake of arsenic by roots of *C. stricta* and *S. pectinata*. If our observations translate to
the field, then *C. stricta* and *S. pectinata* might accumulate arsenic in root tissues throughout the growing season, regardless of their age.

Figure 4-2. Concentrations of arsenic in shoots of (A) *C. stricta* and (B) *S. pectinata*.

Data represent means ± standard error (n = 3 or 4).
Plant age did, however, affect transfer of arsenic from roots to shoots. Translocation factors (TF; the ratio of arsenic concentrations in shoots to roots), were highest in the older plants of each species (Table 4-3). In addition, we observed a decline in TF as RGR increased for each species (Figure 4-4). This might indicate a greater tendency for *C. stricta* and *S. pectinata* to translocate arsenic as they age and their growth
rate declines. Previously, phytoremediation using young P. vittata was recommended because TFs were generally lower in older plants (Silva Gonzaga, et al. 2007). The most efficient time for harvesting C. stricta or S. pectinata, however, might not be when they’re young. Rather, harvest should occur when plants are older (i.e. at the end of a growing season) and growth rates are low.

Long-term effects of plant age on phytoremediation might be more significant than the results of this short-term study suggest. The primary mechanism for removal of arsenic in wetlands usually is adsorption to substrate (Buddhawong, et al. 2005) and in the long-term would likely be limited by the quantity of available adsorption sites. A community of diverse wetland species could extract arsenic throughout the growing seasons, but the effects of plant age beyond the first year, have not been studied in depth. One focus of future research should be the long-term effects of plant age on arsenic removal in engineered wetlands.
Figure 4-4. Translocation factors (TFs) of young and old *C. stricta* and *S. pectinata* at different relative growth rates (RGRs). Each point represents a single replicate.

### 4.4. Conclusions

i) A mixture of cool- and warm-season plant species could maximize arsenic removal by supplementing uptake during seasonal variations. In this study, we observed that *S. pectinata* had a much greater rate of arsenic uptake in summer conditions than in spring conditions, while *C. stricta* was more consistent across both treatments.

ii) To maximize arsenic removal, it might be best to allow plants to accumulate arsenic for the duration of a growing season, and then harvest older plants that have had time to transfer a significant portion of the accumulated arsenic to shoots. As *S. pectinata* and *C. stricta* aged and their growth rates declined, they began to transfer greater quantities of arsenic to aboveground tissues.
References


Chapter Five

Uptake and toxicity of arsenic, copper, and silicon in *Azolla caroliniana*
and *Lemna minor* 

*(Submitted to Chemosphere)*

Abstract

Arsenic is the contaminant of greatest concern in the U.S., but often exists as part
of a chemical cocktail in polluted waters. Ecologically engineered wetlands are a
potential treatment strategy for remediation and ideally may include plant species that
tolerate these mixtures and remove contaminants. In this study, this strategy was
attempted using two aquatic macrophytes (*Azolla caroliniana* and *Lemna minor*), which
were exposed to mixtures of arsenic (0 or 20 µM As), copper (2 or 78 µM), and silicon (0
or 1.8 mM) in a hydroponic system. Biomass and relative growth rates were determined
by weighing plant tissues before and after a treatment period (14 d). Concentrations of
arsenic, copper, and silicon in plant tissues were determined by inductively coupled
plasma-optical emission spectrometry. Chlorophyll and anthocyanin contents were
determined by colorimetric methods following solvent extraction. The plant species used
in this study accumulated each contaminant, although *L. minor* accumulated higher concentrations than *A. caroliniana* in most treatments. Arsenic negatively affected biomass, RGR, and anthocyanin content in *A. caroliniana*, while copper had a greater negative effect on *L. minor*. Copper apparently inhibited uptake of arsenic by *A. caroliniana*, but facilitated uptake of arsenic by *L. minor*. Silicon negatively affected growth and uptake by *A. caroliniana*, but increased uptake and alleviated toxicity of copper in *L. minor*. Although the effects of the chemical mixtures varied between species, *A. caroliniana* and *L. minor* tolerated and accumulated these contaminants, and could be used for phytoremediation.

**Keywords:** phytoremediation, mixed contaminants, chlorophyll, anthocyanins

### 5.1. Introduction

Inexpensive, environmentally-friendly treatment technologies are needed to remediate multiple contaminants in aquatic systems. Unfortunately, traditional technologies such as filtration and precipitation can be effective, but are often expensive and environmentally disruptive (USEPA 2002). Engineered wetlands might function as economical treatment technologies that can remove arsenic, copper, and silicon via chemical transformations, adsorption to sediment, precipitation, and uptake by plants. Little is known about the role of wetland plants and their responses to mixtures of these contaminants. Prior to field application, it would be prudent to determine the effects of these commonly co-occurring contaminants on plant growth, plant stress, and phytoextraction. Understanding the effects of mixed contaminants on uptake and
tolerance by plants, is a step toward the judicious selection plant species for successful long-term phytoremediation.

Arsenic is the contaminant of greatest concern in the United States (USDHHS 2007), and often exists as part of a mixture of contaminants. These mixtures commonly include copper in wastewater from mining and wood preservation facilities (Bech, et al. 1997; Nico et al. 2006), or silicon in areas where arsenic has been used for manufacturing glass (Atkarskaya & Bykov 2003). Arsenic occurs as a natural constituent of ground and surface water in many regions, but has been redistributed in the environment due to human activities.

In plants, prolonged exposure to arsenic can result in decreased growth rates (e.g. Mokgalaka-Matlala, et al. 2008) and chlorophyll content (Jain & Gadre 1997; Azizur Rahman, et al. 2007). However, copper is an essential micronutrient for plants (Arnon and Stout 1939) and acts as a cofactor for a number of physiological processes including electron transfer during photosynthesis and respiration, and the scavenging of free oxygen radicals (Marschner 1995). Copper is particularly harmful to chloroplasts, the reaction centers for photosynthesis (Abdel-Ghany, et al. 2005; Shikanai, et al. 2003). At elevated levels, copper can become toxic, forming harmful, reactive oxygen species (Sancenon, et al. 2004), and resulting in reduced biomass and altered nutrient content (Li, et al. 2008).

Although silicon is not an essential nutrient, it alleviates symptoms of metal toxicity in some higher plants (Epstein 1994; Pilon-Smits et al., 2009). Increased tolerance to metals can result from reduced bioavailability, detoxification within plant tissues, or stimulation of protective antioxidants (Liang, et al. 2007). For example,
silicon inhibits uptake of arsenic by rice (*Oryza sativa*), a silicon accumulating species (Guo, et al. 2005; Guo, et al. 2007), making it more suitable for human consumption. In terms of phytoremediation, it would be best to select plant species for which silicon promotes the uptake and tolerance of arsenic.

Plant species that accumulate the high concentrations of arsenic, copper, and silicon are known (e.g. Ma, et al. 2001; Meharg 2002; Deng, et al. 2004), but the effect(s) of interactions among these contaminants on plant growth and contaminant uptake are unknown. In this report, the interactive effects of arsenic, copper, and silicon on uptake of each contaminant and toxicity in two aquatic plant species (*Azolla caroliniana* and *Lemna minor*) were studied. The specific objectives of this research were to quantify the effects of arsenic, copper, and silicon on (i) contaminant uptake, (ii) biomass and growth rate, and (iii) chlorophyll and anthocyanin content in *A. caroliniana* and *L. minor* following exposure to these contaminants. These species were chosen because they are recommended for use in wetland restoration in Ohio (OEPA 2007) and were observed to accumulate arsenic in previous laboratory studies (Duncan and Gottgens, *in preparation*).

Because copper and arsenic are both toxic to plants at elevated concentrations, but do not inhibit or increase the rate of contaminant uptake, we hypothesized that negative effects on growth and chlorophyll concentrations would be observed in *A. caroliniana* and *L. minor*. Based on preliminary evidence, we further hypothesized that silicon would cause a decrease in arsenic uptake and positively affect growth and chlorophyll content in these species. Hydroponic experiments were designed to test these hypotheses, in which *A. caroliniana* and *L. minor* were exposed to combinations of arsenic, copper, and silicon. Both species extracted each of the contaminants, and were able to continue
growing regardless of the mixture, making them candidates for phytoremediation of arsenic, copper, and silicon.

5.2. Materials and Methods

5.2.1. Plant material and growth conditions

*A. caroliniana* was purchased from Carolina Biological Supply Company (Burlington, NC) and *L. minor* was collected from a pond in northwest Ohio. The plants were cultured separately in a modified Hoagland’s solution (1.0 mM Ca(NO$_3$)$_2$, 1.5 mM KNO$_3$, 0.25 mM KH$_2$PO$_4$, 0.5 mM MgSO$_4$, 0.1 mM EDTA-Fe, 0.5 µM ZnSO$_4$, 6.0 µM MnCl$_2$, 50 µM H$_3$BO$_3$, 2.0 µM CuSO$_4$, and 0.09 µM Na$_2$MoO$_4$; pH 5.5 – 6.0) in a greenhouse (20 – 30 °C, 12-h photoperiod).

5.2.2. Experimental design

*A. caroliniana* and *L. minor* (2 g fresh mass) were placed into rectangular plastic containers (n = 3) filled with the various treatment solutions (250 mL; Table 5-1), which were comprised of modified Hoagland’s solution with arsenic (0 or 20 µM as Na$_2$HAsO$_4$), copper (2 or 78 µM as CuSO$_4$) and silicon (0 or 1.8 mM as K$_2$SiO$_3$). Potassium sulfate (K$_2$SO$_4$) was added to the solutions that lacked silicon to standardize the concentrations of potassium in all treatments. The solutions were discarded and replaced after 4, 7, and 10 days. Plants were transferred to larger plastic containers and one liter of solution on the 10$^{th}$ day because the biomass had outgrown the smaller containers.
On the 14th day of treatment, all plant biomass was rinsed with tap water, a solution of HCl (10%), and tap water. After removing excess surface moisture by using a salad-spinner, fresh biomass was determined for each replicate. Subsamples (0.1 g) were removed for determination of chlorophyll and anthocyanin contents (see Section 2.4.). The remaining plant biomass was placed in an oven (65°C; 72 h), and dry weights were determined. Relative growth rates (RGR) were calculated from the changes in fresh biomass from the start of treatment (2 g) to the end of the treatment (Equation 1; Chiarello, et al. 1990).

\[ \text{RGR (mg g}^{-1} \text{d}^{-1}) = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}, \]

where \( W_1 \) is initial fresh biomass, \( W_2 \) is fresh biomass at harvest, and \( t_2 - t_1 \) represents the treatment period (14 d).

5.2.3. Arsenic, copper, and silicon in plant tissues

For the determination of arsenic and copper concentrations, dried tissue samples (0.1 g) were mixed with analytical grade HNO\(_3\) (12 N; 5 mL) in Teflon vessels and digested in a microwave (20 min ramp time, 200°C, 20 min hold time; Mars Xpress, CEM, Matthews, NC). After cooling, H\(_2\)O\(_2\) (1.5 mL) was added to the vessels and they were returned to the microwave (20 min ramp time, 200°C, 20 min hold time). For determination of silicon content, dried tissue samples (0.1 g) were mixed with KOH (8.9 M, 3 mL) in Teflon vessels and digested in a microwave (20 min ramp time, 200°C, 20 min hold time; Frantz, et al. 2008). All of the resulting solutions were diluted with
deionized water (12 mL) and filtered (No. 2 Whatman filter paper). The solutions were further diluted to 3.5% HNO₃ or 2% KOH prior to analysis by inductively coupled plasma-optical emission spectroscopy (ICP-OES) as previously described (Rofkar et al., 2007).

Table 5-1. Concentrations of the compounds used to produce treatment solutions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Si</th>
<th>Cu</th>
<th>Cu+Si</th>
<th>As</th>
<th>As+Si</th>
<th>As+Cu</th>
<th>As+Cu+Si</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂H₃AsO₄ (µM)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>CuSO₄ (µM)</td>
<td>2</td>
<td>2</td>
<td>78</td>
<td>78</td>
<td>2</td>
<td>2</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>K₂SiO₄ (mM)</td>
<td>0</td>
<td>1.8</td>
<td>0</td>
<td>1.8</td>
<td>0</td>
<td>1.8</td>
<td>0</td>
<td>1.8</td>
</tr>
<tr>
<td>In mM:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>1.05</td>
<td>0</td>
<td>1.05</td>
<td>0</td>
<td>1.05</td>
<td>0</td>
<td>1.05</td>
<td>0</td>
</tr>
<tr>
<td>In µM:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

5.2.4. Total chlorophyll and anthocyanins

Chlorophyll contents were determined for both plant species following the treatment period. Subsamples (either three fronds of A. caroliniana or 1 g fresh mass of L. minor) from each replicate were ground and incubated in 80% acetone (5 mL) in the dark (24 h; 4°C). Absorbances of the extractants were measured with a spectrophotometer (Aquamate, Thermo Electron Corporation, Waltham, MA) at 646.6 and 663.6 nm. Concentrations (µg g⁻¹ fresh mass) of chlorophyll a, chlorophyll b, and
total chlorophyll \((a + b)\) were determined using published extinction coefficients and equations (Equation 2; Porra, et al. 1989).

\textit{Equation 2.}

\begin{align*}
\text{Chlorophyll a} &= 12.25A_{663.6} - 2.55A_{646.6}, \\
\text{Chlorophyll b} &= 20.31A_{646.6} - 4.91A_{663.6},
\end{align*}

where \(A_{663.6}\) is the absorbance at 663.6 nm and \(A_{646.6}\) is the absorbance at 646.6 nm.

In preliminary experiments, anthocyanins were not present in \textit{L. minor}. Therefore, total anthocyanins were measured only in \textit{A. caroliniana}. Three fronds from each replicate were combined and incubated in 1\% HCl-methanol (5 mL) in the dark (24 h; 4°C). The extracts were filtered (0.45 \(\mu\)m PTFE syringe filters) and the absorbance measured at 530 and 600 nm. Anthocyanin concentrations (OD units g\(^{-1}\) fresh mass) were calculated from the difference between the absorbance measurements at the two wavelengths (Equation 3; Dai, et al. 2006).

\textit{Equation 3.}

\begin{align*}
\text{Anthocyanin content (OD units g\(^{-1}\) fresh mass)} &= (A_{530} - A_{600}) / M_t,
\end{align*}

where \(A_{530}\) is the absorbance at 530 nm, \(A_{600}\) is the absorbance at 600 nm, and \(M_t\) is fresh biomass at harvest.
5.2.5. **Statistical analyses**

Data are presented as means ± standard error (n = 3). One-way analysis of variance (p = 0.05) was used to determine significant differences in biomass, chlorophyll, and anthocyanin content among treatments, within each species.

5.3. **Results and Discussion**

5.3.1. **Accumulation of arsenic, copper, and silicon**

*A. caroliniana* accumulated arsenic from the test solutions, a primary criteria for inclusion in a treatment wetland. After the treatment period, arsenic concentrations in *A. caroliniana* ranged from 33.7 to 76.8 mg As kg\(^{-1}\) in the As+Cu+Si and As treatments, respectively (Table 5-2). These values are between previously reported concentrations of 3 and 284 mg As kg\(^{-1}\), when exposed to 1 and 50 µM As, respectively (Zhang, et al. 2008; Zhang, et al. 2009). Addition of silicon to the treatment solutions resulted in decreased arsenic uptake by *A. caroliniana*, a potentially negative result in terms of phytoextraction. Silicon had previously been shown to decrease uptake of arsenate (As(V)) by *Oryza sativa* (Guo, et al. 2005). In addition, Ma, et al. 2008 reported that silicon and arsenite (As(III)) are taken up by the same transporters in plant roots. In our study, the form of arsenic added to the treatment solutions was arsenate, and we did not analyze treatment solutions for speciation of arsenic. Therefore, there is no way of knowing whether interactions between silicon and arsenite played a role in this result.

Elevated copper in the treatment solutions also caused a decline (not statistically significant) in arsenic uptake by *A. caroliniana*. The decline might be due to an interaction between copper and arsenic or an indirect result of copper toxicity in the
plants. The mechanism of this interaction has not been previously reported, and cannot be determined from this study.

*L. minor* accumulated arsenic to levels 13-fold higher than *A. caroliniana* in some treatments. Arsenic concentrations in *L. minor* ranged from 140.5 to 450.6 mg As kg\(^{-1}\) in the As and As+Cu treatments, respectively (Table 5-2). A related species (*Lemna gibba*) has been shown to accumulate more than 1000 mg As kg\(^{-1}\), a level that would be toxic to most plant species (Mkandawire, et al. 2004). Unlike in *A. caroliniana*, the lowest arsenic concentrations were in *L. minor* without silicon or elevated copper. The addition of copper caused an increase in arsenic uptake to a level almost 2.5-fold greater than arsenic alone. This interaction was opposite of that in *A. caroliniana*. Silicon also caused increases in arsenic uptake in both the As+Si and As+Cu+Si treatments (Table 5-2). This was contrary to silicon’s effect in *A. caroliniana* and *O. sativa* (Guo, et al. 2005), but in agreement with recently published results for *Pteris vittata* (Wang, et al. 2010). It seems likely that the effect of silicon on arsenic uptake is specific to each plant species.

Both species accumulated copper when the concentration in treatment solutions was elevated above controls (Table 5-2). *A. caroliniana* accumulated higher levels of copper from control solutions than *L. minor*, but *L. minor* generally accumulated more copper when exposed to the elevated level copper. Concentrations of copper in *A. caroliniana* ranged from 52.8 to 331.6 mg Cu kg\(^{-1}\) in the As and Cu+Si treatments, respectively (Table 5-2). The highest levels of copper accumulation in *A. caroliniana* occurred in the absence of arsenic. This is further indication of an interaction between copper and arsenic, and may indicate competitive uptake between these elements in *A.*
caroliniana. Addition of silicon had no apparent effect on copper uptake by *A. caroliniana*, similar to results reported for uptake of copper by Arabidopsis (Li, et al. 2008).

Copper concentrations in *L. minor* ranged from 19.7 to 451.7 mg Cu kg\(^{-1}\) in the control and Cu treatments, respectively (Table 5-2). These values are lower than those reported by Razinger, et al. (2006) in *L. minor* (500 mg Cu kg\(^{-1}\)) grown in 10 \(\mu\)M Cu for 24 h. Interestingly, arsenic had no direct effect on copper uptake by *L. minor*, possibly indicating that there is no competition for uptake between the two elements in this species, at these concentrations. However, when arsenic was not included in the treatment solution (Cu+Si), silicon inhibited uptake of copper. While not observed in *A. caroliniana*, silicon is known to reduce toxicity of some metals by inhibiting uptake (see references in Liang, et al. 2007).

*A. caroliniana* and *L. minor* might be useful for removing silicon from contaminated waters. In *A. caroliniana*, silicon concentrations ranged from 77.4 to 3190 mg Si kg\(^{-1}\) in the As and As+Si treatments, respectively (Table 5-2). In *L. minor*, silicon concentrations ranged from 132.4 to 6995 mg Si kg\(^{-1}\) in the control and As+Si treatments, respectively (Table 5-2). Some plant species (e.g. *O. sativa*) accumulate as much as 10 – 15% silicon in their dry biomass (Epstein 1999). Although the plant species examined here had comparatively lower tissue concentrations (< 0.7% in dry biomass), they could still be included in the group of species that are considered silicon accumulators.

At these concentrations in tissues, silicon apparently affected uptake of arsenic and copper in *A. caroliniana* and *L. minor*. Copper inhibited silicon uptake in both species in the Cu+Si treatment only. This coincides with reduced copper uptake in *L.*
minor, and may indicate competitive uptake between copper and silicon and/or general stress caused by copper stress.

Table 5-2. Concentrations of arsenic, copper, and silicon in *A. caroliniana* and *L. minor*.

<table>
<thead>
<tr>
<th></th>
<th>Total As (mg kg(^{-1}))</th>
<th>Total Cu (mg kg(^{-1}))</th>
<th>Total Si (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. caroliniana (n = 3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>nd</td>
<td>61.3 ± 1.9 (a)</td>
<td>81.7 ± 13.6 (a)</td>
</tr>
<tr>
<td>Si</td>
<td>nd</td>
<td>57.5 ± 4.8 (a)</td>
<td>3 124 ± 147.0 (b)</td>
</tr>
<tr>
<td>Cu</td>
<td>nd</td>
<td>327.9 ± 43.2 (b)</td>
<td>106.7 ± 8.3 (a)</td>
</tr>
<tr>
<td>Cu + Si</td>
<td>nd</td>
<td>331.6 ± 5.7 (b)</td>
<td>1 968 ± 132.8 (c)</td>
</tr>
<tr>
<td>As</td>
<td>76.8 ± 12.4 (a)</td>
<td>52.8 ± 3.6 (a)</td>
<td>77.4 ± 1.5 (a)</td>
</tr>
<tr>
<td>As + Si</td>
<td>50.8 ± 7.6 (a,b)</td>
<td>58.1 ± 1.6 (a)</td>
<td>3 190 ± 376.2 (b)</td>
</tr>
<tr>
<td>As + Cu</td>
<td>53.8 ± 8.8 (a,b)</td>
<td>229.2 ± 29.4 (c)</td>
<td>115.1 ± 8.9 (a)</td>
</tr>
<tr>
<td>As + Cu + Si</td>
<td>33.7 ± 1.1 (b)</td>
<td>257.7 ± 6.2 (b,c)</td>
<td>3 006 ± 343.7 (b)</td>
</tr>
<tr>
<td><strong>L. minor (n = 3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>nd</td>
<td>19.7 ± 8.4 (a)</td>
<td>132.4 ± 15.1 (a)</td>
</tr>
<tr>
<td>Si</td>
<td>nd</td>
<td>32.1 ± 5.6 (a)</td>
<td>6 134 ± 1 041 (b)</td>
</tr>
<tr>
<td>Cu</td>
<td>nd</td>
<td>451.7 ± 54.7 (b)</td>
<td>321.8 ± 81.4 (a)</td>
</tr>
<tr>
<td>Cu + Si</td>
<td>nd</td>
<td>294.3 ± 37.7 (c)</td>
<td>2 446 ± 443 (c)</td>
</tr>
<tr>
<td>As</td>
<td>140.5 ± 7.5 (a)</td>
<td>31.4 ± 1.5 (a)</td>
<td>195.2 ± 48.7 (a)</td>
</tr>
<tr>
<td>As + Si</td>
<td>166.3 ± 9.8 (a)</td>
<td>31.0 ± 3.4 (a)</td>
<td>6 995 ± 355 (b)</td>
</tr>
<tr>
<td>As + Cu</td>
<td>323.5 ± 20.0 (b)</td>
<td>413.8 ± 38.3 (b,c)</td>
<td>356.5 ± 56.6 (a,c)</td>
</tr>
<tr>
<td>As + Cu + Si</td>
<td>450.6 ± 20.8 (c)</td>
<td>447.5 ± 37.9 (b)</td>
<td>6 151 ± 242.8 (b)</td>
</tr>
</tbody>
</table>

Values represent means ± standard error. Values followed by different letters are significantly different (p < 0.05).

Arsenic was not detected (nd) in some tissue samples. The minimum level of detection for arsenic was 13.7 mg As kg\(^{-1}\) in plant tissue.

5.3.2. *Effects of arsenic, copper, and silicon on plant growth*

The effects of arsenic, copper, and silicon on plant growth varied between the plant species. Arsenic had a negative effect on dry biomass of *A. caroliniana*, while
copper and silicon alone had no significant effect (Figure 5-1a). The combination of arsenic and silicon, however, caused the greatest reduction in biomass in *A. caroliniana*. In fact, the As+Si and As+Cu+Si treatments resulted in dry biomass values approximately 50% less than the control. This observation was unexpected because, as previously stated, silicon is generally thought to alleviate harmful effects of some metals. In fact, detrimental effects of silicon, when combined with arsenic, have not been previously reported in the literature.

Unlike *A. caroliniana*, dry biomass of *L. minor* was affected by copper, but arsenic and silicon alone had no effect. Dry biomass of *L. minor* was lowest in the Cu and As+Cu treatments at 0.33 and 0.32 g, respectively (Figure 5-1b). Dry biomass values of *L. minor* in the Cu+Si and As+Cu+Si treatments were not significantly different from controls, indicating a possible mitigating effect of silicon. While the exact mechanism of the effect of silicon cannot be determined from our work, it likely was not a result of inhibited uptake of copper. As stated earlier, copper levels remained consistent in *L. minor* when exposed to the high level of copper.

Exposure to arsenic and copper affected RGR in *A. caroliniana* and *L. minor*, respectively (Figure 5-2). The average RGR for *A. caroliniana* treated without arsenic was 111.1 mg g⁻¹ d⁻¹ and with arsenic was 69.1 mg g⁻¹ d⁻¹. Although silicon compounded the negative effect of arsenic on dry biomass, it did not have the same effect on RGR. The average RGR for *L. minor* in the Cu and As+Cu treatments was 52.7 mg g⁻¹ d⁻¹; for all other treatments, average RGR was 121.7 mg g⁻¹ d⁻¹. Like it did for dry biomass, silicon mitigated the negative effect of copper on RGR.
Figure 5-1. Dry mass of (a) *A. caroliniana* and (b) *L. minor* treated with combinations of arsenic (20 µM), copper (78 µM), and silicon (1.8 mM). Bars represent means ± standard error (n = 3). Significant differences within each species are represented by different letters (p < 0.05).
Figure 5-2. Relative growth rates (RGR) of (a) *A. caroliniana* and (b) *L. minor* treated with combinations of arsenic (20 µM), copper (78 µM), and silicon (1.8 mM). Bars represent means ± standard error (n = 3). Significant differences within each species are represented by different letters (p < 0.05).
5.3.3. Effects of arsenic, copper, and silicon on chlorophyll and anthocyanin content

The concentration of total chlorophyll (a+b) in *A. caroliniana* was not strongly affected by any of the contaminants, but chlorophyll content in *L. minor* was again affected by copper (Figure 5-3). In *A. caroliniana*, the only significant difference in total chlorophyll content was between the As+Cu and As treatments – 328.8 and 474.2 µg g\(^{-1}\), respectively (Figure 5-3a). More importantly, the fact that chlorophyll content was maintained might indicate some level of tolerance to the contaminants used in this study. The chlorophyll content of some plants species decreases when exposed to arsenic (Jain & Gadre 1997; Azizur Rahman, et al. 2007), possibly reducing their ability to perform photosynthesis. In contrast, total chlorophyll (a+b) concentrations in *L. minor* were lowest when treated with copper, regardless of arsenic or silicon availability. Total chlorophyll (a+b) content ranged from 274.7 to 579.1 µg g\(^{-1}\) in the As+Cu and Si treatments, respectively (Figure 5-3b). This is another indication of the toxicity of copper to *L. minor*. There were no significant changes in chlorophyll a to b ratio in either species. Chlorophyll a to b ratios ranged from 3.7 to 5.2 in *A. caroliniana* and from 6.5 to 11.5 in *L. minor*.

Anthocyanins are antioxidant compounds sometimes produced in response to metal stress, and were detected in all *A. caroliniana* treated with arsenic (Figure 5-4). As expected, based on preliminary experiments, *A. caroliniana* treated with the other contaminants lacked detectable anthocyanins. The lowest anthocyanin content was in plants in the As treatment (1.36 OD units g\(^{-1}\) fw). Anthocyanin contents increased with the addition of any of the other contaminants, possibly indicating increased stress, not yet manifested in the growth of *A. caroliniana*.
Figure 5-3. Total chlorophyll (a+b) content of (a) *A. caroliniana* and (b) *L. minor* treated with combinations of arsenic (20 µM), copper (78 µM), and silicon (1.8 mM). Bars represent means ± one standard error (n = 3). Significant differences within each species are represented by different letters (p < 0.05).
Figure 5-4. Anthocyanin content in *A. caroliniana* treated with combinations of arsenic (20 µM), copper (78 µM), and silicon (1.8 mM). Anthocyanins were not detected (nd) in some *A. caroliniana* samples. Preliminary tests indicated *L. minor* did not produce detectable amounts of anthocyanins, and so were not analyzed here. Bars represent means ± one standard error (n = 3). Significant differences within each species are represented by different letters (p < 0.05).

5.5. Conclusions

The ultimate goal of this research is to develop wetlands for remediation of contaminated water. Both plant species accumulated each contaminant when it was available, but when combined, interactions between arsenic and silicon (in *A. caroliniana*) and between arsenic and copper (in *L. minor*) altered uptake. Based on analysis of growth and stress responses, *A. caroliniana* was particularly sensitive to arsenic, while copper was more detrimental to *L. minor*. Based on our observations, in combination with previously published results, it is apparent that the effects of arsenic,
copper, and silicon are plant species-specific, in terms of tolerance and uptake. It would be prudent to consider the effects of mixed contaminants prior to selecting plant species for engineered wetlands.

**Acknowledgements**

We would like to acknowledge the United States Department of Agriculture (Grant No. 2006-38894-03732) for funding; Jonathan Frantz, Defne Apul, Alison Spongberg, and Michael Weintraub for suggestions during preparation of the manuscript; and Doug Sturtz for operation of the ICP-OES.
References


Chapter Six

Designing a Wetland for Northwest Ohio

The results obtained during the course of this dissertation could have implications for selecting plants for treatment wetlands in northwest Ohio:

i. Native, wetland plant species can be used in treatment wetlands and can maintain the ability to accumulate arsenic and continue growth during extended periods of arsenic stress. The degree to which they tolerate arsenic is specific to each species and, for judicious selection of plants, should be investigated prior to the construction of a treatment system.

ii. A mixture of plant species could be used to maximize arsenic uptake throughout the growing season in a temperate climate. Some species might have higher uptake rates during spring while others are better during summer. Utilizing a variety of overlapping species could maximize uptake for the duration of the growing season.

iii. A mixture of plant species could be used for remediation of mixed contaminants. The effects of combined contaminants appear to be specific to
each plant species, but utilizing a variety of plant species could result in concurrent phytoextraction of all contaminants.

We will use these conclusions to select plant species for a treatment wetland at an arsenic-contaminated site in northwest Ohio. The purpose of the wetland is to remediate water contaminated with arsenic (1500 μg L⁻¹) to a level that meets guidelines for discharge into surface water (150 μg L⁻¹). The wetland will be designed according to the conceptual model presented in Chapter 1 (Figure 1-1). The primary treatment step (a sedimentation basin) will be used to remove insoluble arsenic. The water, along with dissolved arsenic, will then flow through the subsurface of a wetland cell to maximize contact with soil particles and plant roots. A variety of native plant species will be used in this portion of the system to maximize arsenic uptake for the duration of the growing season. Aboveground portions of the plants will be harvested in the fall, when the greatest portion has been transferred to leaves, to permanently remove the contaminant from the system. Uptake will be minimal outside of the growing season, so removal during the winter months will be primarily due to adsorption to soil or co-precipitation with iron or sulfur. Effluent from the wetland will enter another basin for final polishing. Floating macrophytes and possibly other treatment technologies (i.e. zero-valent iron) will be used to ensure that the final effluent meets the standard for water quality.