LABORATORY- AND FIELD- SCALE BIOASSAYS FOR PREDICTING RESPONSES OF WILD RICE (*ZIZANIA PALUSTRIS* L.) TO EXPOSURES OF SITE-SPECIFIC SEDIMENTS AND WATERS

A DISSERTATION PRESENTED TO
THE FACULTY OF GRADUATE STUDIES
OF

LAKEHEAD UNIVERSITY

THUNDER BAY, ONTARIO, CANADA

BY

O'NIELL ROY TEDROW

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY, BIOTECHNOLOGY
OCTOBER 2020

EXAMINING COMMITTEE:
DR. PETER F. LEE, COMMITTEE CHAIR
DR. KAM LEUNG, COMMITTEE MEMBER
DR. NANDAKUMAR KANAVILLIL, COMMITTEE MEMBER
DR. PAUL BLOOM, EXTERNAL EXAMINER

ABSTRACT

LABORATORY- AND FIELD- SCALE BIOASSAYS FOR PREDICTING RESPONSES OF WILD RICE (*ZIZANIA PALUSTRIS* L.) TO EXPOSURES OF SITE-SPECIFIC SEDIMENTS AND WATERS

O'NIELL ROY TEDROW
DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY
DEPARTMENT OF GRADUATE STUDIES
LAKEHEAD UNIVERSITY
2020

Four bioassays were used to expose wild rice (WR) to site-specific waters and sediments within the boundaries of site-specific conditions. Mesocosm- and microcosm- scale bioassays were developed to measure responses of WR to sediment exposures. In mesocosms, WR height (HT), dry weight biomass (DWB), and seed production (SP) were statistically lower for Cleaver and Unnamed Lake sediment-grown plants compared to Rat River Bay sediment-grown plants. An accelerated-growth microcosm-scale bioassay accurately represented the mesocosm-scale bioassay, while decreasing overall time, sediment, water, and space. WR developed to near reproductive maturity. Significant differences were not identified between mesocosm:microcosm ratios for WR DWB or SP, which appeared to be primarily influenced by sediment ammonia-nitrogen concentrations.

Rafts were deployed in two select aquatic systems: three in the Seine River (non-industry-influenced); and two rafts in each of three legacy-mine influenced pits to determine the life stage (aerial, floating leaf, submerged) more sensitive to water depth and water depth increases. Based on data obtained during this study, floating leaf plants were determined more sensitive to depth increases; aerial stage was least sensitive to depth increases. WR developed to aerial stage in 20 and 40 cm water depth treatments in all legacy-mine influenced pits indicating no adverse responses to pit waters.

Two flow-through WR paddies were constructed adjacent to separate legacy-mine influenced pits with elevated sulphate concentrations. Seeded WR in each paddy developed according to typical phenology during each of multiple successive growing seasons. In the Pit A paddy, no statistical decreases in HT or DWB were observed between growing seasons; average SP statistically increased in 2019; and seed DWB remained statistically similar between growing seasons. Over two successive growing seasons, WR stem density remained statistically similar in the Pit C paddy. Despite conditions potentially conducive to iron sulphide root coating

formation, this was not identified via SEM-EDX characterization. No adverse WR responses observed throughout this study were determined resultant of Pit A or Pit C water exposures.

Representativeness of natural WR areas is paramount to bioassay data defensibility. Bioassays described herein were designed to represent field conditions to the extent possible given the scale of the bioassay.

DEDICATION

I dedicate this to my family: Mom, Dad, Brother, Rocky Dog and Jack Dog.

‘Every silver lining’s got a touch of grey. I will get by; I will survive.’ Robert Hunter.

ACKNOWLEDGEMENTS

First and foremost, I want and need to thank my Graduate Advisor Dr. Peter Lee. His efforts throughout the entirety of this Degree have been nothing short of extraordinary. Without his calming conversations and logical approach to disasters which never ever seemed to stop, I never would have made it this far. Much gratitude is given to Dr. Kam Leung and Dr. Nandakumar Kanavillil for their exceptional patience over the past few years, and critical Dissertation reviews. Thank you so much. And my gratitude goes to Dr. Paul Bloom for serving as the External Examiner, and providing a detailed and critical Dissertation review. Much appreciated.

Matching industry partner and National Science and Engineering Research Council (NSERC) of Canada financial support was critical to completion of this work. Much thanks in particular to Shane Hayes of Barrick Gold, Hemlo Mine; and Tracy Anderson of First Quantum Minerals.

A great 'Thank you' to so many folks who provided assistance at Lakehead University; specifically, Johane Joncas, Kristi Dysievick, Mike St. James, Adam Pun, Keri Pidgen, Paige Perrons, and all of the analysts at the Lakehead University Environmental Laboratory (LUEL). Could not have done much, if any, of this without you all. Chris and Bernie Clein were critical for the success of the mesocosm bioassay. My sincere gratitude for use of their farm, and for the daily inspections and periodic maintenance. They were exceptionally gracious and kind. Dr. Brenda Magajna's assistance and patience have been so critical throughout this process. Thank you.

My colleagues at Northeast Technical Services, Inc. (NTS), and Vermilion Community College (VCC) could not have been more supportive. Specifically, I thank Rick Crum (NTS) for his insightful conversations, patience, and time allowances; Kurt Doran (NTS) for time-intensive document reviews; and VCC Provost Shawn Bina and all of the outstanding VCC students for allowing my class absences in pursuit of this Degree.

This would never have been anywhere near possible without the assistance and patience of so many friends and others. Specifically: Tim and Amy Jo for always being amazing friends; Dr. B. Maurice and Sheree Duke for being such awesome friends, and for so much meaningful support; and Jenny for sacrificing so much of her everything in non-countable ways. And all the best scratches to the dogs through the years, with whom I have been allowed such loving company.

Thank you all so…very…much.

TABLE OF CONTENTS

ABBREVIATIONS

µg = Microgram.

µg g^{-1} = Micrograms per Gram.

µg L^{-1} = Micrograms per Liter.

µmol g^{-1} = Micromoles per Gram.

µS cm^{-1} = Micro Siemens per Centimeter.

BD = Below Detection.

Bicarb. Alk. = Bicarbonate Alkalinity.

CL = Cleaver Lake.

cm = Centimeter.

Diss. = Dissolved; referring to the measurable fraction of 'analyte,' which passed through a 0.45 µM pore-size filter.

EDX = Energy Dispersive X-Ray.

FeS = Iron Sulphide.

H_2S = Hydrogen Sulphide.

Kg = Kilogram.

Kg ha^{-1} = Kilograms per Hectare.

L = Liter.

LOI = Loss on Ignition.

mg = Milligram.

mg L^{-1} = Milligrams per Liter.

MN DNR – Minnesota Department of Natural Resources.

MPCA = Minnesota Pollution Control Agency.

ND = Non-Detect.

NM = Not Measured.

ON = Ontario.

RRB = Rat River Bay.

SEM = Scanning Electron Microscopy.

SO_4 = Sulphate.

TDS = Total Dissolved Solids.

UL = Unnamed Lake.

WPL = Wild Potato Lake.

WQS = Water Quality Standard.

WR = Wild Rice.

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.0 WILD RICE DEVELOPMENT

Weir and Dale (1960) describe the phenological development of WR and its relationship to water depth. Three forms of leaves develop – submerged, floating, and aerial – which differ in anatomical characteristics likely related to their physiological environment. The submerged leaves are thin and lack a cuticle, the outer waxy covering present on leaves of terrestrial plants; and no stomata are present for gas exchange. These leaves also have abundant air passages at this time which are known as lacunae, and greatly reduced conducting / vascular tissue which transports both water and carbohydrates. In late May to early June, the floating leaves appear. These differ from the submerged leaves by having a cuticle with stomata on the upper surface of the leaf and a well-developed mid vein on the lower surface, which does not have a cuticle (Hawthorn and Stewart 1970). Most biomass production occurs in the aerial stage of development. The ratio of root:shoot biomass also increases at this stage of development as the plant becomes better anchored (Thomas and Stewart 1969). Another occurrence at this stage is that the shoot apex (vertical growing tip) changes from vegetative to reproductive growth, triggering the plant to begin seed formation (Weir and Dale 1960). The timing for this event varies depending on environmental characteristics (water depth, nutrient availability) of individual sites and the depth tolerance of the seed source involved (Counts and Lee 1988). Timing of the developmental change between vegetative growth to reproductive growth may be delayed depending on how early in the growing season the plant was able to begin floating leaf and aerial stage growth.

1.1 AQUEOUS SULPHATE AND WILD RICE

Wild rice (WR; *Zizania palustris* L.) is a freshwater grass, which is native to North America, and is highly valued for its cultural, recreational, and economic benefits. During the mid-1940s, initial field research focused on WR distribution in Minnesota (MN), USA, was completed (Moyle 1944, 1945, 1956). Of interest are statements focusing on associations of WR presence / abundance and aqueous sulphate (SO_4) concentration. Initial conclusions stated were that WR thrives more effectively in water 30 – 90 cm in depth, a SO_4 concentration of 10 mg L^{-1} or less, and in areas with an organic-rich sediment substrate. However, Moyle also stated that WR was observed growing in waters containing greater than 10 mg SO_4 L^{-1}. In Moyle (1945), a more

empirical-data based presentation of WR and SO_4 association is detailed; specifically, that WR was observed in waters with a SO_4 concentration ranging from 3 – 282 mg L^{-1} (the single 282 mg L^{-1} site is described as likely a pond planted with WR) with a median concentration of approximately 21 mg L^{-1}; and that no known large stands of WR exist in waters with a SO_4 concentration greater than 10 mg L^{-1} (Moyle 1944). Moyle's observational and empirical research has in part led to additional studies focused on WR ecology, distribution, density, and characteristics of areas in which WR was typically present, with a general focal component of aqueous SO_4 influences. Paulishyn and Stewart (1970) reported WR growing in Manitoba waters with SO_4 concentrations ranging from 2 – 170 mg L^{-1}. In a study completed in the Mississippi River in Minnesota (MN), Lee and Stewart (1971) reported WR growing well in water containing 30 mg SO_4 L^{-1}; and that seasonal SO_4 concentrations fluctuated from 5 – 120 mg L^{-1}. Rogalsky (1971) suggested that an aqueous SO_4 concentration of 200 mg L^{-1} was acceptable for WR paddies. Others focused on using specific growth media for WR studies.

Malvich and Percich (1993) recommended a growth medium for hydroponic studies containing 48 mg SO_4 L^{-1}. Using this growth medium, Lee and Hughes (2000) observed adverse WR developmental-characteristic responses at amended SO_4 exposure concentrations ranging from 1,200 – 1,500 mg L^{-1}. Vicario and Halstead (1968) used an artificial culture medium for *in vitro* WR growth studies ranging from 0 – 8,800 mg SO_4 L^{-1}. They observed decreases in dry weight biomass and plant height responses at SO_4 exposure concentrations exceeding 220 mg L^{-1}. More recently, Fort et al. (2014) completed a detailed hydroponic study of WR responses to SO_4 and chloride exposures. They concluded that adverse WR responses to 2,500 – 5,000 mg SO_4 L^{-1} were likely due to osmotic pressure disruptions similar to those observed in side-by-side osmotically equivalent chloride exposures. Observations by Fort et al. (2014) tend to support those observed by Lee and Hughes (2000) in terms of influences on WR during the seedling (submerged) developmental stage. Overall, increased SO_4 exposures tended to correlate with adverse WR responses, specifically during the seedling developmental stage, but at much higher concentrations than have been observed under field conditions. However, laboratory studies such as these, although valuable for understanding detailed phenology of WR, were completed under conditions not representative of aquatic systems; specifically, lacking the sediment matrix for

rooting. As a result, a need for additional detailed studies focused on influences of aqueous SO_4 on WR phenology, distribution, and density within WR waters was identified.

The Minnesota Pollution Control Agency (MPCA) completed a nearly State-wide multi-year survey of waters listed as containing WR. This effort resulted in a detailed update to the MPCA WR database in 2015. Eighteen surveyed locations produced densities of WR of at least 100 stems m^{-2} with an aqueous SO_4 range of non-detect – 198 mg L^{-1} at the time of survey; and seven locations with aqueous SO_4 concentrations ranging from 175 – 838 mg L^{-1} produced 11 – 121 stems m^{-2} (MPCA Wild Rice Waters Database – July 19, 2016). Although, 'stem density' does not always equate to 'plant density' due to tillering (additional potential grain-producing stems from a single root mass). Based on these data, aqueous SO_4 does not appear to be directly causative of adverse influences on WR phenology, distribution, density, or productivity in field-relevant concentrations. As this conclusion became more clearly defined, WR study foci shifted from aqueous SO_4 to sediment pore water-associated hydrogen sulphide.

1.2 SEDIMENT AND PORE WATER HYDROGEN SULPHIDE

As a result of the recognized conclusion that aqueous SO_4 is more benign than initially hypothesized, sediment-associated pore water became more intensely studied; in particular, transformation of sulphur (S^{+6}) to sulphide (S^{-2}), and ultimately hydrogen sulphide (H_2S). Hydrogen sulphide toxicity to aquatic plants is well studied. Lamers et al. (2013) provide a review of multiple types of aquatic systems in which H_2S has been concluded as causative of phytotoxicity. Recent studies used stock-tank mesocosms (Myrbo et al. 2017; Pastor et al. 2017) or bucket-style mesocosms (LaFond-Hudson et al. 2018, 2020) as containers in which to expose WR to concentrations of aqueous SO_4 up to 300 mg L^{-1}. In these studies, a particular focus was placed on sediment and pore water H_2S, and its likely causation of adverse WR responses. General conclusions were that 1) increasing concentrations of aqueous SO_4 results in increased pore water H_2S; 2) over multiple growing seasons, decreases in multiple plant responses (dry weight biomass, filled seeds, surviving seedlings, filled seed dry weight biomass, grain nitrogen concentration) coincided with increased aqueous SO_4 and pore water H_2S; and 3) H_2S was a more likely causative agent of these measured adverse WR responses than aqueous SO_4. In exposure replicates of 300 mg SO_4 L^{-1}, iron-containing coatings (iron (III) oxide/hydroxides, iron-sulphide) on WR roots (LaFond-Hudson et al. 2018) were determined causative of

decreased plant and seed nitrogen concentrations compared to non- SO_4 amended controls (LaFond-Hudson et al. 2020). These were fairly thorough investigations of WR phenology using bucket-style mesocosms; however, the overall design decreased representation of field conditions and natural systems supportive of self-sustaining populations of WR.

1.3 WATER DEPTH

Although not specifically related to SO_4 / S^{-2} concentration concerns, water depth as a critical influence on WR phenological development, distribution, density, and productivity must be considered. Increasing water depth in some WR waters has been detrimental to the localized (WR) population. Substantial research has been completed focused on influences of water depth and water depth increases on WR phenological development, distribution and density, and productivity.

Water depth as a critical influence on WR growth, development, and maturation, has been well described (Aiken et al. 1988; Stevenson and Lee 1987; Tucker et al. 2011). Water depths of 15 – 90 cm are more conducive to WR growth and propagation (MN DNR 2008); however, given appropriate sediment nutrient element availability (i.e., nitrogen, phosphorus, potassium), in general the shallower the water the better for WR phenological development and productivity. Since water depth is a primary determinant of WR growth, development, distribution, and ultimately productivity, periodic changes (sudden or more gradual) could result in adverse influences on one or more characteristics of WR phenology.

Influences on WR from water depth increases depend on its phenology at the time of increase. If the increase is sudden during the submerged or floating leaf stage, the less developed roots may not be able to anchor the plant, which may then be uprooted (Chambliss 1940; Thomas and Stewart 1969). If the water depth increase is more gradual and the plant is still in the submerged stage, it will take longer to reach the surface with corresponding decreases in seed production due in part to decreased tillering, or complete loss of reproductive success due to mortality. If the plant has achieved floating leaf stage and is then submerged, the plant is placed under additional stress due to atmospheric gas exchange interruption; at this stage, the cuticle may have already formed further exacerbating WR plant stress due to decreased gas exchange capacity while submerged (Hawthorne and Stewart 1970). Using rafts with suspended tubs containing WR

plants, Stevenson and Lee (1987) concluded that WR was able to tolerate water depth increases of up to 50 cm; however, increases in water depth of 15 – 30 cm caused decreases in total dry weight, number of tillers, and seed production. The more severe observed responses to depth increases were attributed to decreased / decreasing sediment nutrient element concentrations.

Depending on the configuration of the WR area, large sections of WR in deeper water areas may be adversely influenced well into the summer from increases in water depths that may not adversely influence WR in shallower water. Decreased light penetration due to increased water depths also decreases tillering and ultimately seed production. Bloom et al. (2001) reported that WR seed production yields in the same transects in Rice Lake, MN, decreased > 8x from approximately 1,567 Kg ha^{-1} in year 2000 to 192 Kg ha^{-1} in year 2001 as the average water depth increased from 66 cm to 109 cm. They also reported that, in both years, seed production and biomass were negatively correlated to water depth increases. Marcum and Porter (2006) reported that mature seed per panicle and total seed production decreased in controlled experiments as water depth increased from 15 – 30 cm to 46 – 61 cm. Under those conditions, no seedlings were able to reach the water surface when planted at a water depth of 76 cm. In a recent multi-year study focused on determining potential re-introduction of WR into lakes previously supportive of harvestable densities, long-term (several years to decades) water depth increases from 60 – 90 cm to > 150 cm, due in part to beaver activity, were determined as the more likely cause of harvestable WR loss in these systems (personal communications). All else being appropriate for typical WR phenology, maintaining a water depth conducive to WR growth throughout the growing season is critical to also maintaining a self-sustaining WR population.

1.4 Rationale and Need for Research

Representativeness of conditions in natural WR areas within recent studies focused on WR responses to exposures of aqueous SO_4 has become a more focused critique. However beneficial for identifying specific potential adverse influences on WR phenology, distribution, density, and productivity, their overall design may have been conducive to these influences; specifically, H_2S production and its associated effects on WR. Therefore, a distinct need existed for development of defensible bioassays, from which empirical WR exposure-response data may be obtained under conditions more representative of field conditions. Furthermore, WR exposure-response data may be desired or required using water and / or sediment from aquatic systems which are

not currently conducive to WR growth (i.e., water depth exceeds WR tolerance; general lack of sediment; lack of power for equipment such as pumps; general remoteness or difficult access; vandalism, personal injury concerns). Therefore, a scaled range of bioassays were needed to obtain WR exposure-response data more representative of field conditions, while accommodating site-specific conditions, resource availability, accessibility, and data use objectives.

For the current studies, four distinct bioassays were developed and used to expose WR to site-specific waters and sediments within the boundaries of site-specific conditions, while maintaining representation of field conditions to the extent possible. An *in-situ* mesocosm-scale bioassay, designed as a flow-through system, was used to measure WR responses to site-specific sediments throughout one entire growing season. Based on data and observations obtained during this mesocosm-scale bioassay, a flow-through laboratory / greenhouse microcosm-scale bioassay using WR was developed and used to represent mesocosm-scale bioassay conditions, while conserving resources (time, water, sediment, footprint). To better accommodate needs for WR exposure-response data in sufficiently remote locations, those not conducive to WR growth, and / or lacking power for powered equipment, a raft assembly bioassay was used to measure responses of WR to *in-situ* exposures of site-specific waters and sediments. Finally, under unique conditions of systems which were remote and not conducive to WR growth (deep water; lack of sediment), but sufficient space and resources were available, flow-through paddies were developed and used to measure responses of WR to site-specific waters and sediments. In their totality, these bioassays represent a scaling progression from smaller-scale to (essentially) field-scale, while retaining field representativeness to the extent possible.

1.5 DISSERTATION ORGANIZATION

This Dissertation is organized into five chapters, including introductory information as Chapter One and summary of conclusions as Chapter Five. Chapters Two, Three, and Four are formatted as journal-specific submissions. As a result of the intent to submit Chapters Two through Four for independent peer-review publication, some replication of information was necessary. The organization of this Dissertation follows the progression of bioassay scale; specifically, from small(er)-scale to larger-scale, and their independent and compounded benefits. Basically, examining site-specific applications and benefits of smaller- and / or larger- scale bioassays

using wild rice (*Zizania palustris* L.) as the indicator organism for these exposure-response scenarios.

- **CHAPTER TWO: INFLUENCE OF SEDIMENT QUALITY ON *ZIZANIA PALUSTRIS* L. GROWTH: A MESOCOSM AND MICROCOSM STUDY**

Site-specific sediments were selected for this study based on their 1) previous use as a control sediment (i.e., non-industry influenced) for WR study from a system typically producing exceptional harvestable densities of WR; 2) status as a legacy-mine influenced sediment; or 3) status as an active-mine influenced sediment. Two scales of bioassays were developed and used for this study: one a larger, mesocosm-scale bioassay using approximately 420-litre stock tanks, requiring an entire growing season (approximately three months) for completion; an approximately nine m^2 footprint; hundreds of thousands of liters of water; and approximately 120 liters of each sediment. The other a smaller, microcosm-scale bioassay required approximately seven weeks for completion based on the data use objectives; an approximate two m^2 footprint; in the low-thousands of liters of water (over the course of seven weeks); and approximately 12 liters of each sediment.

Critical for development and use of these bioassays were general considerations of resources such as time, water, sediment, space, and data use objectives. The *in-situ* mesocosm-scale bioassay was specifically developed and used due to sediment sampling locations' access limitations; the need for frequent, detailed observations of the WR life cycle throughout an entire growing season; the capability to represent a flow-through system; and vandalism concerns. Based on the ability for detailed, near-daily, observations during the mesocosm-scale bioassay, a need was identified for an accelerated, smaller-scale bioassay.

- **CHAPTER THREE: MEASURING *IN-SITU* RESPONSES OF WILD RICE (*ZIZANIA PALUSTRIS* L.) TO EXPOSURES OF VARIABLE WATER DEPTHS AND SITE-SPECIFIC SEDIMENTS IN NON-MINE AND LEGACY-MINE INFLUENCED WATERS**

Development and use of these *in-situ* raft-style bioassays was critical to obtain the necessary WR exposure-response data in locations selected for this study. Two overall locations were selected for raft-style bioassay use: Seine River near Mine Centre, Ontario; and three legacy-mine

influenced pits in Minnesota. In the case of the Seine River portion of the study, the remoteness, water depth, and lack of available power-source options were the primary considerations for use of raft-style bioassays. In the case of the legacy-mine influenced portion of the study, water depth and lack of sediment were the primary considerations for use of raft-style bioassays. Additionally, aqueous SO_4 in each mine pit was used as a selection criterion. Overall, each deployment location was specifically chosen in an effort to better represent field conditions which were the focus of the study.

The substrate sediment chosen for the Seine River portion of this study was selected due to its previous use as a control sediment in studies of WR growth and productivity. In question were water depth manipulations at specific life stages. Site-specific waters (Pits A, B, and C) chosen for the portion of this study completed in legacy-mine influenced pits were selected based on their chemical characteristics; specifically, their legacy-mine influence resulting in atypically high aqueous SO_4 concentrations. Sediment chosen for the portion of this study completed in legacy-mine influenced pits was chosen due to its proximity to raft deployment locations, and chemical characteristics indicating appropriate nutrient concentrations for supporting WR growth. Water depths chosen for both portions of this study were selected based on their classification as supportive of typical WR phenology, or sufficiently deep to likely cause adverse responses. Water depth manipulations were chosen based conclusions of previous studies focused on responses of WR to increased / increasing water depth in terms of WR phenology and reproductive potential.

- **CHAPTER FOUR: DEVELOPMENT AND USE OF AN *IN-SITU* FLOW-THROUGH PADDY-SCALE BIOASSAY FOR EXPOSURES OF WILD RICE (*ZIZANIA PALUSTRIS* L.) TO SITE-SPECIFIC WATERS**

The need for development and use of this bioassay cannot be overstated. More accurately representing characteristics of natural WR areas was critical to increasing the understanding of responses of WR to exposures of waters containing concentrations of SO_4 previously suggested as adversely influential to (WR) phenology, distribution, and productivity. Use of these larger-scale, *in-situ*, flow-through systems throughout multiple, successive growing seasons allowed

detailed study of how WR is more likely to respond to these types of exposures under field conditions.

Two of the legacy-mine influenced pits used for the previous raft-style bioassay (Pits A and C) were selected for this paddy-scale bioassay. Their selection was based on chemical characteristics of water in each pit, available space for paddy development, and their ease of access. The same sediment was used in each paddy; and was selected due to its nutrient concentrations typically supportive of WR phenology, and proximity and ease of collection. It was also the organic sediment used in the raft study in these two pits.

REFERENCES

Aiken, S.G., Lee, P.F., Punter, D., Stewart, J.M. 1989. Wild rice in Canada. NC Press, Toronto. 130 pp.

Bloom, P., Schumer, H.J., Carson, T. 2001. Factors limiting yield of wild rice in paddies and natural stands. (IN) Minnesota Wild Rice Research 2001. Univ. of Minnesota.

Chambliss, C.E. 1940. The botany and history of *Zizania aquatica* L. ("wild rice"). Journal of the Washington Academy of Sciences 30 (5), 185-205.

Fort, D.J., Mathis, M.B., Walker, R., Tuominen, L.K., Hansel, M., Hall, S., Richers, R., gratten, S.R., Anderson, K. 2014. Toxicity of sulfate and chloride to early life stages of wild rice (*Zizania palustris*). Environ. Toxicol. Chem. 33(12):2802-2809.

Hawthorn, W. R., Stewart, J.M. 1970. Epicuticular wax forms on leaf surfaces of *Zizania aquatica*. Can. J. Bot. 48, 201-205.

LaFond-Hudson, S., Johnson, N.W., Dewey, B. 2020. Interactions between sulfide and reproductive phenology of an annual aquatic plant, wild rice (*Zizania palustris*). Aquatic Botany 164, 103230.

LaFond-Hudson, S., Johnson, N.W., Pastor, J., Dewey, B. 2018. Iron sulfide formation on root surfaces controlled by the life cycle of wild rice (*Zizania palustris*). Biogeochemistry. https://doi.org/10.1007/s10533-018-0491-5.

Lamers, L.P.M., Govers, L.L., Janssen, C.J.M., Geurts, J.J.M., Van der Welle, M.E.W., Van Katwijk, M.M., Van der Heide, T., Roelofs, J.G.M., Smolders, A.J.P. 2013. Sulfide as a soil phytotoxin – A review. Frontiers in Plant Science 4 (268), 1-14.

Lee, P.F., Hughes, P.C. 2000. The effects of sulfate levels on the early development of wild rice. Prepared for U.S. Steel Corp. (Minntac) Minnesota.

Lee, P.F., Stewart, J.M. 1981. Ecological relationships of wild rice, *Zizania aquatica* L. 1. A model for among-site growth. Can. J. Bot. 59, 2140-2151.

Malvick, D.K., Percich, J.A. 1993. Hydroponic culture of wild rice (*Zizania aquatica* L.) and its application to studies of silicon nutrition and fungal brown spot disease. Can. J. Plant Sci. 73, 969-975.

Marcum, D.B., Porter, R.A. 2006. Influence of water depth upon plant development, yield and seed size of modern wild rice cultivars. ASA Annual Meeting.

Minnesota Department of Natural Resources (MN DNR). 2008. Natural Wild Rice in Minnesota: A Wild Rive Study document submitted to the Minnesota Legislature by the MN DNR, February 15, 2008.

Moyle, J. B. 1944. Wild rice in Minnesota. J. Wildlf. Manage. 8, 177-184.

Moyle, J.B. 1945. Some chemical factors influencing the distribution of aquatic plants in Minnesota. Amer. Midl. Nat. 34, 402-420.

Moyle, J.B. 1956. Relationships between the chemistry of Minnesota surface waters and wildlife management. J. Wildlf. Manage. 20: 303-320.

Myrbo, A., Swain, E. B., Engstrom, D. R., Coleman Wasik, J., Brenner, J., Dykhuizen Shore, M., Blaha, G. (2017). Sulfide generated by sulfate reduction is a primary controller of the occurrence of wild rice (*Zizania palustris*) in shallow aquatic ecosystems. J. Geophys. Research: Biogeosciences, 122, 2736–2753. https://doi.org/10.1002/2017JG003787.

Pastor, J., Dewey, B., Johnson, N.W., Swain, E.B., Monson, P., Peters, E.B., and Myrbo, A. 2017. Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments. Ecological Applications 27(1), pp. 321-336.

Paulishyn, W., Stewart, J.M. 1970. Sulfate ion concentrations and wild rice distribution in Manitoba. Agassiz Research Abstract, University of Manitoba.

Rogalsky, J.K., Clark, K., Stewart, J.M. 1971. Wild rice paddy production in Manitoba. Man. Dept. of Agr. Pub. 527.

Stevenson, S.C., Lee, P.F. 1987. Ecological relationships of wild rice, *Zizania aquatica*. 6. The effects of increases in water depth on vegetative and reproductive growth. Can. J. Bot. 65, 2128-2132.

Thomas, A.G., Stewart, J.M. 1969. The effect of different water depths on the growth of wild rice. Can. J. Bot. 47, 1525 – 1531.

Tucker, R.C., Zanis, M.J., emery, N.C., Gibson, K.D. 2011. Effects of water depth and seed provenance on the growth of wild rice (*Zizania palustris* L.). Aquatic Botany 94, 113-118.

Vicario, B.T., Halstead, E.H. 1968. Progress report on wild rice research. Univ. of Saskatchewan, Dept. of Soil Science.

CHAPTER TWO: INFLUENCE OF SEDIMENT QUALITY ON *ZIZANIA PALUSTRIS* L. GROWTH: A MESOCOSM AND MICROCOSM STUDY

ABSTRACT

During the 2018 growing season, wild rice (*Zizania palustris* L.; WR) was used as the experimental species for development of two bioassays, in this case using sediment obtained from Rat River Bay (non-mine-influenced system; RRB), Cleaver Lake (CL; legacy-mine influenced lake), and Unnamed Lake (UL; active-mine influenced lake). Notable sediment characteristic differences were ammonia-nitrogen (N), approximately 56-66% lower in CL and UL sediments, respectively; and sulphur, ≥ 10x higher in CL and UL sediments. A mesocosm-scale bioassay was developed to measure responses of WR to sediment exposures during an entire growing season. By the end of the season, ammonia-N was below detection in CL and UL sediments, and decreased by 93% in RRB sediment. Wild rice plant height (HT), dry weight biomass (DWB), and seed production (SP) were statistically lower for CL and UL sediment-grown plants; however, visual differences in WR growth between sediments were documented on July 28, 2018. Based on these observations, and using the same source sediments, an accelerated-growth microcosm-scale bioassay was developed to accurately represent the mesocosm-scale bioassay in terms of WR HT, DWB, and SP (measured as female pistilate inflorescence per panicle), while decreasing the time-to-response and overall resource use (sediment, water, footprint). Over the course of approximately seven weeks, WR developed to near reproductive maturity, with similar visual differences in size and reproductive capacity between sediments as observed during the mesocosm-scale bioassay. WR HT, DWB, and SP were statistically lower in UL than CL and RRB; and were significantly higher in RRB than in either CL or UL sediment-grown plants. Significant differences were not identified between mesocosm:microcosm ratios for WR DWB or SP; HT ratios were significant. Responses of WR to these sediment exposures appeared to be more influenced by ammonia-N concentration than other sediment constituents. Based on data obtained during this study, this accelerated-growth microcosm-scale bioassay accurately represented the mesocosm-scale bioassay in terms of WR DWB and SP. Therefore, the microcosm-scale bioassay was representative of the mesocosm-scale bioassay in these two critical response characteristics, both of which can typically be visually recognized. Use of this microcosm-scale bioassay to obtain accurate representations of WR responses to site-specific sediment, or water, exposures can save critical water resource managers time, money, and overall resources.

2.1 INTRODUCTION

Some early studies of northeast Minnesota aquatic systems in which wild rice (*Zizania palustris* L.; WR) was observed focused on characteristics of overlying water potentially indicative of WR preferences, and those that may cause adverse influences on WR populations (Moyle 1944, 1945). In particular, aqueous sulphate was suggested as a potential adverse influence on density and distribution in concentrations higher than 10 mg L^{-1}; although, WR was observed growing in waters of higher sulphate concentration. Recently, concern about adverse influences on WR phenology and distribution has resulted in multiple studies focused on WR responses to exposures of aqueous sulphate and sediment / pore-water (hydrogen) sulphide (Fort et al. 2014, 2017; Myrbo et al. 2017; Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020). Although the aqueous exposure conditions within these studies were extensively described, specific adverse influences on WR due to overlying water sulphate exposures were not discernable. Fort et al. (2014) in particular concluded that observed adverse WR responses to sulphate exposures ≤ 5,000 mg L^{-1} were more likely due to general osmotic stress, and were not discernable from similar WR responses to osmotically / conductivity equivalent chloride exposures. Other observed adverse responses (germination, growth, density, and overall reproductive fitness) described in Pastor et al. (2017) were attributed to sediment pore water characteristics, which were concluded as having been influenced by overlying water characteristics (e.g., increased aqueous sulphate resulted in increased pore water hydrogen sulphide). Pastor et al. (2017) and LaFond Hudson et al. (2018, 2020) used non-flow-through mesocosms as exposure chambers for WR to increasing concentrations of aqueous sulphate; others (Fort et al. 2014, 2017) exposed WR seeds and seedlings to increasing concentrations of aqueous sulphate and sulphide under laboratory-scale hydroponic conditions. Although Pastor et al. (2017) used mesocosms to test WR responses to increasing overlying water sulphate concentrations, conclusions were based on exposure of WR to sediment pore water hydrogen sulphide as a function of overlying water sulphate concentration in formulated water. An investigation by Fort et al. (2017) exposing WR to laboratory formulations of increasing sulphide and iron concentrations concluded that although sulphide adversely influenced WR development, increasing concentrations of iron tended to mitigate sulphide toxicity to WR. Based on conclusions from these studies, overlying water sulphate concentrations appear to be of lesser importance than previously suggested. Rather, responses of WR measured in these studies are more substantially influenced by sediment characteristics. Hydroponic laboratory studies concluded that more field-relevant concentrations of aqueous sulphate (Fort et al. 2014) and sulphide (Fort et al. 2017) are not

adversely influential to WR germination, or growth and development into the seedling stage as has been suggested. As such, the possibility exists for bioavailable sediment nutrient elements (i.e., nitrogen) to be more of an influence on WR growth and development than these forms of sulphur, which is also a required plant nutrient element. Sims et al. (2012a,b) and Grava and Raisanen (1978) provide reviews of changing nutrient requirements and uptake / accumulations throughout a growing season. It was with this knowledge of WR phenology-associated nutrient requirements that the current bioassays were designed.

As the focus shifted from overlying water to sediment as the medium of greater influence on WR phenology, a need developed for empirical data obtained via experimentation with sediment characteristics as the focus. Previous studies (Fort et al. 2014, 2017) concluded that field-relevant aqueous sulphate and pore water sulphide concentrations are less adversely influential on WR phenology than suggested. Others (Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020) used sulphate-amended water in semi-static mesocosms as the source of influence on sediment pore water characteristics.

For the current study, flow-through mesocosms were used to measure responses of WR to exposures of non-mine, legacy- and active- mine influenced sediments following an entire growing season (June-September 2018). Amounts of water and sediment required for completion were in the hundreds-of-thousands and hundreds of liters, respectively. These types of mesocosm-scale studies can provide defensible and valuable information; however, they are time and resource intensive, and tend to require outdoor deployment, which limits the experimental duration to the specific growing season.

Due to the need for a more time- and resource- efficient method for determining influences on WR growth, development, and productivity from industry-influenced sediments, a microcosm-scale bioassay was designed. In an effort to decrease the time-to-response, a method used by WR growers in California (Dr. Dan Marcum, personal communication), and also observed by Tucker et al. (2011), was employed to accelerate aerial and flowering stage development. Tucker et al. (2011) observed statistically higher root, stem, leaf, and inflorescence dry weight biomass from WR plants grown in ‘zero’ water depth (saturated sediment) in contrast to -15, 23, and 46 cm water depths. In the current study, water depth in microcosm-scale exposure chambers was maintained at near-zero (cm). This resulted in germination, followed by immediate development into the aerial stage, bypassing the floating leaf developmental stage. Achieving this accelerated development was critical to the success of this microcosm-scale bioassay.

Although these bioassays may use either water or sediment as the testable exposure medium, for purposes of this current study site-specific sediment was the tested medium for both the mesocosm- and microcosm- scale bioassays. The overall objective of the current study was to develop a microcosm-scale bioassay that accurately represents a mesocosm-scale bioassay, while using less time, sediment, and water. Specific objectives were to: 1) characterize mine- and non-mine- influenced sediment exposures under mesocosm- and microcosm- scale scenarios; 2) measure WR responses to these sediment exposures in terms of height, dry weight biomass, specific elemental plant and seed content, and seed production (or female pistilate inflorescence); and 3) determine similarities of mesocosm:microcosm WR responses to these sediment exposures.

2.2 MATERIALS AND METHODS

2.2.1 STUDY SITE DESCRIPTIONS

Rat River Bay (RRB; 48°38'01.17" N, 92°38'56.10" W) located near the Seine River First Nation Community was selected as a control sediment source since it supports a large natural stand of WR, and has no influence from mines or other industries. Two aquatic systems were chosen as mine-influenced sediment sampling locations for this study. Cleaver Lake (CL; 48°58'21.96" N, 87°22'45.83" W) located near Schreiber, ON, was chosen due to its status as legacy-mine influenced. Historically, discharge associated with, and influenced by, an up-gradient nickel-zinc mine entered into the north end of CL. Unnamed Lake (UL; 48°41'25.17" N, 85°56'18.95" W) located near Hemlo, ON, was chosen due to its status as a lake currently influenced by mine activity. Currently, neither CL nor UL contain populations of WR, but do support aquatic plant assemblages similar to those of other nearby lakes.

2.2.2 MESOCOSM-SCALE BIOASSAY

Approximately 400 liters of sediment was obtained from RRB, CL, and UL for use in a mesocosm-scale aquatic plant bioassay. Wild rice was used as the experimental aquatic plant species for this bioassay. Seeds were harvested from Whitefish Lake (48°13'40.78" N, 90°03'46.94" W) near Kakabeka Falls, ON, during 2017. Previous research using sediment from these locations resulted in a germination rate of > 90% using seeds harvested from Whitefish Lake. Therefore, WR seeds were germinated to the seedling developmental stage for use in this bioassay under controlled light:dark (16h:8h) and temperature (20°C) conditions.

Mesocosm experiments were initiated during June 2018 at a secure outdoor location approximately 10 kilometers west of Lakehead University campus (Thunder Bay, ON).

Mesocosms were approximately 60 cm tall, with an internal diameter of approximately 150 cm (**Figure 2.1**). Ten, 12-liter tubs were approximately 75% filled (nine liters) with each sediment and evenly spaced within their respective mesocosm. Non-industry influenced water from the Neebing River was pumped into a 1,550-liter holding tank and gravity-fed into each mesocosm at a rate of approximately 500 mL per minute for the duration of this study. Five healthy-appearing seedlings were randomly selected and planted (evenly spaced) in sediment contained in tubs in each respective mesocosm. Above-sediment water depth and sediment depth in each tub was approximately 20 cm (Stevenson and Lee 1987). Competing vegetation was removed if and when observed in WR tubs to mitigate potential adverse influences from this variable (Elakovich and Wooten 1989; Quayyum et al. 1999). Approximate weekly inspections were completed to document WR development.

During July 2018, potential growth differences were visually observed between WR plants growing in CL and UL sediments contrasted to RRB sediment. Therefore, a mid-season harvest event was completed in an effort to characterize any potential differences. WR plants in three randomly selected tubs were harvested. Sediment was sampled from a randomly selected tub from which WR was harvested.

The mesocosm-scale bioassay was concluded following verification of filled seeds on WR plants in each sediment exposure tub. On September 08, 2018, remaining WR plants in each tub in each mesocosm were harvested. Due to plant sizes, multiple tissues were sampled. Lower leaf, flag leaf, and seed samples from individual plants were obtained. Plants in CL sediment exposures were combined, as were plants from UL sediment exposures, to achieve sufficient dry weight biomass for total N, P, K, S, Ca, Mg, Na, Fe, Al, Cu, Mn, and Zn measurement replication. Sediment samples were obtained from randomly selected tubs in each mesocosm.

2.2.3 MICROCOSM-SCALE BIOASSAY

During June 2019, approximately 100 liters of sediment was sampled from RRB, CL, and UL. This bioassay was initiated during August 2019, and was concluded in September 2019. The set-up location was the main greenhouse at Lakehead University. Twelve, one-liter replicate containers were filled with 750 mL of sediment from each site. Grab samples of RRB, CL, and UL sediment were obtained from bulk sediment containers prior to experiment initiation. Two groups of six replicates of each sediment were randomly placed within a flow-through water bath to maintain sediment saturation and temperature (**Figure 2.2**). Four WR seeds were planted in each exposure replicate at experiment initiation. Inflow water (*p*H-adjusted tap water) was

contained in a 440-liter tank beneath the sediment exposure containers, and was automatically and evenly distributed to each exposure replicate chamber three times daily to maintain sediment saturation. An accelerated rate of WR development was promoted in an effort to decrease the time to observed response by limiting the overlying water depth in each replicate to near-zero (cm) (Tucker et al. 2011). Natural sunlight was augmented with artificial solar irradiation to allow for a 16h:8h light:dark cycle, and more evenly distributed and consistent irradiance. The experimental duration was approximately seven weeks. Following germination, WR density in each replicate was decreased to one seedling; in each case, the healthier appearing seedling was retained in each replicate. This experiment was concluded following observations of WR plants achieving formation of female pistilate inflorescence. All WR plants were harvested from each sediment exposure container. Total plant height and the number of observed female pistilate inflorescences were measured at the time of harvest. Similar to the mesocosm-scale bioassay, plants from CL sediment exposures were combined, as were plants from UL sediment exposures, to achieve sufficient dry weight biomass for P, K, S, Ca, Mg, Na, Fe, Al, Cu, Mn, and Zn measurement replication; however, no dried plant tissue remained for total nitrogen measurement. Only plants grown in RRB sediment contained sufficient dry weight biomass for total nitrogen measurement. Therefore, RRB plant total nitrogen measurements in this microcosm bioassay were compared / contrasted to RRB, CL, and UL sediment-grown plants at a similar developmental stage sampled on July 28, 2018 (mid-season, early flowering stage), during the mesocosm-scale bioassay. Grab samples of each sediment were obtained from randomly selected replicates at experiment conclusion.

2.2.4 LABORATORY ANALYTICAL METHODS

Quality controls for sediment, plant, and seed samples characterized by LUEL included reference standards and replicate samples, and follows procedures required by the Canadian Association of Laboratory Accreditation (CALA). All sediment samples from the mesocosm- and microcosm-scale bioassay experiments were frozen and delivered to the Lakehead University Environmental Laboratory (LUEL) for processing and analyses. Sample preparation and analytical procedures followed those described in Lee and McNaughton (2004), the LUEL Quality Assurance Manual, and reference Forest Canada, ASTM, and USEPA methods. Total nitrogen (when possible) was measured on dry sediment using a combustion ELVario cube carbon-hydrogen-nitrogen-sulphur (CHNS) analyzer. Sediment *p*H was measured as a 1:1 sediment:deionized water mixture using a Mettler Toledo meter with an InLinePro *p*H sensor. Bulk density was measured by weighing 20mL of wet sediment, drying at 80°C, and re-weighing. Phosphate was determined using the

BRAY P2 method – Al and Fe phosphates are dissolved in ammonium fluoride, and measured colorimetrically using a SKALAR analyzer. Al, Cu, Fe, Mn, S, and Zn were extracted in 0.1 N HCl, while Ca, K, Mg, and Na were extracted in ammonium-acetate at *p*H 7.0; all of which were measured using ICP. Sediment samples were processed as wet samples to better represent field conditions. Measured sediment analyte concentrations were corrected for bulk density.

Plants from each mesocosm- and microcosm- scale bioassay were refrigerated in Ziploc® bags following harvest. Seeds harvested during the mesocosm-scale bioassay were removed from individual plants and stored in separate Ziploc® bags. No filled seeds were observed on plants grown during the microcosm bioassay; therefore, the entirety of each plant was submitted to LUEL for processing and characterization. Plant analytical methods followed those described in Lee and McNaughton (2004) and the LUEL Quality Manual. WR plants and seeds were dried at 50°C and kept frozen until processing. Analyses were based on those described by the American Soil and Plant Council. Plant, or seed, biomass (≥ 0.5g) was digested using a CEM Mars Express microwave in Express Teflon closed vessels in one mL of trace metal grade HNO_3 and three mLs of trace metal grade HCl, and diluted using 25mL of deionized water. Al, Ca, Cu, Fe, Mg, Mn, Na, S, and Zn in this dilution were measured using a Varian ICP-AES. Total nitrogen and phosphorus were digested using trace metal grade HNO_3 / HPO_4 in a stepwise process at 400°C, and measured using the SKALAR analyzer.

2.2.5 STATISTICAL ANALYSES

All sediment, WR plant, and seed data were organized using Microsoft® Excel® or SigmaPlot-SigmaStat® v14.0 (Systat Software, Inc.) for table and graphical representation. Statistical analyses described below were used to test for differences between WR plant characteristics at the aerial stage and at the end of the growing season. Statistical analyses and treatment of WR plant data were completed using SigmaPlot-SigmaStat® v14.0. t-Tests or one-way analysis of variance (ANOVA) were used to compare two or more groups; specifically, WR responses to sediment exposures measured as plant height, final dry weight biomass, seed production (mesocosm-scale bioassay), and number of female pistilate inflorescence (microcosm-scale bioassay). Holm-Sidak multiple comparison was used to discern significant differences between three or more groups if data met normality of distribution and variance. Data were natural logarithmic transformed if normality of distribution and / or variance equality assumptions were violated. If data transformation failed to correct non-normal distribution and / or variance, raw data were used for statistical treatment. ANOVA on ranks was used when significant differences

were identified between three or more groups, followed by Dunn's multiple comparison to discern specific between-group differences, and a Mann-Whitney Rank Sum test was used to discern significant differences between groups used for t-test comparisons, when data were not corrected for normality of distribution or variance equality through natural logarithmic transformation. Level of significance was set for all statistical analyses at 0.05. Non-transformed data were used for table and figure display purposes.

t-Tests (alpha = 0.05) were used to discern if differences between mesocosm:microcosm ratios of WR plant height, dry weight biomass, and seed / female pistilate inflorescence production were significant. A lack of significant difference between mesocosm:microcosm height, dry weight biomass, and / or seed / female pistilate inflorescence production ratios indicated similarity between the two bioassays.

2.3 RESULTS

2.3.1 SITE-SPECIFIC SEDIMENT

Characteristics of sediment used in the mesocosm-scale bioassay are detailed in **Table 2.1**. Non-mine-influenced sediment (RRB) differed from the mine-influenced sediments (CL and UL) in multiple characteristics; of interest are ammonia-nitrogen, phosphate-phosphorus, potassium, and sulphur. Sediment characteristics which increased in concentration at the end of the experiment could be due to influences from source water, variability within sediment sampling, plant biomass in sediment samples, or a combination of these sources.

Initial sediment ammonia-nitrogen in both CL and UL sediments was approximately 56 and 66% less, respectively, compared to RRB sediment. In all three sediments, ammonia-nitrogen decreased throughout the duration of the mesocosm-scale bioassay. In CL and UL sediments, ammonia-nitrogen was below detection at bioassay conclusion; and decreased by ~ 96% in RRB sediment. Phosphate also tended to decrease in RRB, CL, and UL sediment throughout the mesocosm-scale bioassay; potassium tended to remain at a similar concentration throughout this study. Sulphur, an element of interest within this current study, was approximately 10x higher in both CL and UL sediments contrasted to RRB sediment, and did not decrease throughout this bioassay.

Similar sediment characteristic trends were observed following the microcosm-scale bioassay; sediment characteristics at bioassay initiation and conclusion are detailed in **Table 2.2**. Ammonia-nitrogen decreased by approximately 47, 75, and 62% in RRB, CL, and UL sediments,

respectively, but remained above detection. Concentrations of other measured analytes remained similar between bioassay initiation and conclusion, and / or followed similar trends as observed in the mesocosm-scale bioassay.

2.3.2 WILD RICE

One-way ANOVAs during the mesocosm-scale bioassay on WR plant samples from July 28, 2018, identified significant differences between RRB, CL, and UL sediment-grown WR (height $F_{(2,28)} = 60.120$, $p < 0.001$; dry weight biomass $F_{(2,28)} = 40.855$ $p < 0.001$). Paired comparison tests indicated that WR grown in CL and UL sediments were statistically smaller (RRB vs UL $p < 0.001$; RRB vs CL $p < 0.001$) and contained significantly less dry weight biomass than plants grown in RRB sediment (RRB vs UL $p < 0.001$; RRB vs CL $p < 0.001$). One-way ANOVAs on WR plant samples at the conclusion of the mesocosm-scale bioassay also identified significant differences between RRB, CL, and UL sediment-grown WR (height $F_{(2,46)} = 33.319$ $p < 0.001$; dry weight biomass $F_{(2,46)} = 47.758$ $p < 0.001$). Paired comparison tests indicated that WR plants grown in CL and UL sediments remained statistically smaller (RRB vs UL $p < 0.001$; RRB vs CL $p < 0.001$) than plants grown in RRB sediment, and also contained significantly less dry weight biomass (RRB vs UL $p < 0.001$; RRB vs CL $p < 0.001$) (**Figure 2.3**).

Similar WR responses were observed during the microcosm-scale bioassay. One-way ANOVAs at the conclusion of the microcosm-scale bioassay indicated significant differences between RRB, CL, and UL sediment-grown WR (height $F_{(2,21)} = 61.183$ $p < 0.001$; dry weight biomass $F_{(2,21)} = 73.054$ $p < 0.001$). Paired comparison tests indicated that WR grown in RRB sediment was statistically taller (RRB vs CL $p < 0.001$; RRB vs UL $p < 0.001$) and contained significantly higher dry weight biomass than those grown in CL and UL sediments (RRB vs CL $p < 0.001$; RRB vs UL $p < 0.001$), with CL sediment-grown plants containing statistically less than RRB and UL plants (**Figure 2.4**).

Despite the significant size and dry weight biomass differences, plants grown during the mesocosm-scale bioassay in CL and UL sediments contained higher concentrations of nitrogen in plant tissues and filled seeds at bioassay conclusion than WR grown in RRB sediment. One-way ANOVA identified significant differences between seed percent nitrogen content between RRB, CL, and UL sediment-grown WR ($F_{(2,43)} = 7.145$ $p = 0.002$). Paired comparison tests indicated that seeds harvested from plants grown in CL sediment contained statistically higher nitrogen content than seeds from plants grown in RRB sediment ($p = 0.003$). Sulphur content of plant tissues was higher in plants grown in CL and UL sediments for the mesocosm-scale

bioassay; however, sulphur content of seeds harvested from plants grown in all three sediments was nearly identical, but was higher in plants grown in CL and UL sediments at conclusion of the microcosm-scale bioassay. Furthermore, despite CL and UL sediment containing several times higher sulphur concentration than RRB, WR grown in CL and UL sediment did not contain a proportionally higher sulphur concentration in plant tissues or seeds compared to WR grown in RRB sediment (**Table 2.3**). Ultimately, with the exception of Al and Mn, plant tissue characteristics were similar regardless of sediment characteristics. Filled seeds harvested from mesocosm-scale bioassay plants contained similar concentrations of all measured analytes regardless of sediment or other plant tissue characteristics.

At microcosm-scale bioassay conclusion, the number of female pistilate inflorescence was determined in lieu of filled seed production. One-way ANOVA identified significant differences between female pistilate inflorescence production between RRB, CL, and UL sediment-grown WR ($F_{(2,17)} = 31.067$ $p < 0.001$). Paired comparison tests indicated that WR grown in UL sediment produced significantly fewer female pistilate inflorescence than both RRB ($p < 0.001$) and CL ($p = 0.045$) sediment grown WR. Overall, RRB sediment-grown WR was significantly higher in all three measured response characteristics than CL or UL sediment-grown WR (**Figure 2.4**).

Ratios of wild rice plant dry weight biomass, seed / female pistilate inflorescence production, and height were used to determine if similar plant responses were observed between mesocosm and microcosm bioassays. t-Tests were used to determine significant differences between ratios of these response measures. Statistically, mesocosm:microcosm ratios of WR dry weight biomass (RRB:CL $t_{(16)} = 0.178$ $p = 0.861$; RRB:UL $t_{(16)} = -0.850$ $p = 0.408$; CL:UL $t_{(16)} = -1.739$ $p = 0.101$) and seed production / female pistilate inflorescence production were not significantly different between sediment treatments (RRB:CL $t_{(13)} = 1.530$ $p = 0.150$; RRB:UL $t_{(9)} = 0.150$ $p = 0.884$; CL:UL $t_{(9)} = -0.932$ $p = 0.375$) (**Table 2.4**), which tends to indicate that WR plants responded similarly in terms of dry weight biomass and seed / female pistilate inflorescence production between the mesocosm and microcosm bioassays. Wild rice plant height ratios were statistically different between all three sediments (RRB:CL $t_{(16)} = -2.414$ $p = 0.028$; RRB:UL $t_{(16)} = -4.822$ $p < 0.001$; CL:UL $t_{(16)} = -2.581$ $p = 0.020$), which tends to indicate that WR plants responded differently in terms of height between the mesocosm and microcosm bioassays.

2.4 DISCUSSION

2.4.1 MESOCOSM-SCALE BIOASSAY

Throughout the duration of the mesocosm-scale bioassay, daily observations during inflow tank refilling and weekly inspections of all three mesocosms allowed for identification of the developmental stage in which WR plants began to display visually different physical characteristics between sediment exposures. Previous studies have concluded that aqueous sulphate is responsible for increased sediment hydrogen sulphide, which causes adverse influences on WR overall biomass; and seed production, dry weight, and nutrient element (nitrogen) concentration (Myrbo et al. 2017; Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020). In an earlier study, Jorgenson et al. (2013) specifically characterized iron-containing root coatings on WR plants harvested from wetland locations in Ontario, Canada, using energy-dispersive x-ray (EDX) spectroscopy. Elemental characteristics of iron coatings on WR roots in their study indicated the primary forms were iron (III) oxide / hydroxide species. However, iron-sulphide root coatings as suggested by LaFond-Hudson et al. (2018, 2020) were not identified by Jorgenson et al. (2013).

Responses of WR to exposures of CL and UL sediments in the current study tended to follow the decreased later-season growth differential as described by LaFond-Hudson et al. (2020) due to increased sediment H_2S as a result of increased aqueous sulphate; however, CL and UL sediments also contained multi-fold lower concentrations of ammonia-nitrogen than RRB sediment. In a larger-scale, multi-paddy WR study (Tedrow 2020), although the paddy sediment contained a higher sulphur content than CL and UL sediments used for this study, WR plants grew and developed phenologically typical during the growing season. Paddy sediment also contained an initial ammonia-nitrogen concentration of approximately 24.5 ug g^{-1}, which is higher than RRB sediment, and maintained a measurable concentration at the end-of-season harvest and sampling event. Iron-containing coatings identified as iron (III) oxide / hydroxide species were documented on roots of plants harvested from these paddies; however, despite these root coatings, and the sediment H_2S content, no adverse influences on WR were observed. Rather, new root growth was documented on all harvested plants from each paddy. Given these associations, the distinct possibility exists that documented differences between RRB, CL, and UL sediment-grown plants was due to overall lower, and potential deficiency of, ammonia-nitrogen in CL and UL sediments, not the sediment sulphur concentration.

Sims et al. (2012a,b) and Grava and Raisanen (1978) provide reviews of nutrient requirements and accumulations throughout a growing season; in particular, the changing requirements and accumulation amounts of bioavailable nitrogen. The initial concentration of ammonia-nitrogen in CL and UL sediments was multi-fold lower than RRB; and only RRB sediment retained a measurable amount of ammonia-nitrogen at the time of final harvest and sampling. As detailed in previous studies, nitrogen requirements increase for WR during later growth stages, specifically during developing reproductive maturity (Grava and Raisanen 1978; Lee and Stewart 1983; Simmonds 1995; Oelke et al. 1982, 1997; Lee 2002; Sims et al. 2012a,b). Concurrently, much of the nutrient element content of the WR plant is obtained from the sediment, with a reported 70% of total nitrogen accumulated during the late boot / early flowering stage (Grava and Raisanen 1987; Oelke et al. 1997). Observations of WR plant growth and sediment ammonia-nitrogen concentrations in the mesocosm- and microcosm- scale bioassays tend to support the conclusion that during mid- to late- WR phenology available nitrogen is critical for proper plant growth. In the current study, the potential development of a nitrogen deficiency in CL and UL sediments (Day and Lee 1990), in particular nearing / during the time WR plants were flowering, is a potential cause of observed growth and productivity differences. Measured concentrations of other macro- and micro- nutrients such as PO_4, K, Fe, S, Zn, Ca, Mg, and Na appeared to be of sufficiently high concentration for typical WR growth and development. Based on previous studies of WR phenological nitrogen uptake (Grava and Raisanen 1978; Oelke 1997) bioavailable ammonia-nitrogen concentrations in sediments in this study, particularly near the end of the experiment, appeared to be a more critical determinant of WR growth and productivity than other sediment constituents.

2.4.2 MICROCOSM-SCALE BIOASSAY

The overall objective of developing the microcosm-scale WR bioassay was to test if a smaller-scale (sediment, water, time, foot print) bioassay using the same sentinel species and source sediments could predict responses similar to a larger-scale (mesocosm) bioassay. Responses of WR to exposures of RRB, CL, and UL sediments under microcosm-scale bioassay conditions were similar to the mesocosm-scale bioassay. As described previously, WR assimilates the majority of nitrogen during the early flowering stage (Grava and Raisanen 1978; Oelke et al. 1982, 1997; Sims et al. 2012a,b); with low nitrogen, or nitrogen deficiencies, presenting as decreased growth and reproductive capacity. In the microcosm-scale bioassay, WR grown in CL and UL sediments was significantly smaller, contained less dry weight biomass, and produced fewer female pistilate inflorescences than WR grown in RRB sediment, with WR grown in UL

sediment significantly lower than both RRB and CL sediment grown WR. Visual observations of microcosm-scale grown WR in all sediments were similar to those documented in the mesocosm-scale bioassay. These data tend to indicate that an insufficient concentration of ammonia-nitrogen was available in CL and UL sediments during the microcosm-scale bioassay since ammonia-nitrogen decreased by 75% and 62%, respectively, by experiment conclusion (**Table 2.2**). Different sediments resulted in plant tissues such as lower leaves and flag leaves to have substantially different concentrations of specific elements. However, nutrient characteristics of seeds were similar and appeared independent of sediment or other plant tissue characteristics (**Table 2.3**).

2.4.3 MICROCOSM:MESOCOSM BIOASSAY REPRESENTATIVENESS

WR plant height, dry weight biomass, and seed / female pistilate inflorescence production per panicle ratios between plants harvested at conclusion of the mesocosm and microcosm bioassays were used as measures of microcosm-scale bioassay similarity to the mesocosm bioassay. Overall, WR plants responded similarly between mesocosm and microcosm RRB, CL, and UL sediment exposures. As determined by t-Tests, average ratios of dry weight biomass and seed / female pistilate inflorescence production were not statistically different between mesocosm- and microcosm- grown WR plants. However, average RRB:CL plant height ratios were statistically different (**Table 2.4**). Sediment nitrogen appeared to be limiting plant growth and development in the absence of mortality. Although the microcosm-scale bioassay was concluded prior to development of filled seeds, reproductive phenology was achieved; and the overall objective of designing a bioassay requiring a shorter time-to-response, and a decreased amount of sediment, water, and space, while providing WR exposure-response data representative of the mesocosm-scale bioassay, was achieved.

2.5 CONCLUSIONS

The two mine-influenced sediments (CL and UL) used for this study were chemically different from each other, and differed greatly from the non-mine-influenced sediment (RRB) in multiple ways, but specifically in terms of measurable ammonia-nitrogen and sulphur. The two mine-influenced sediments contained multi-fold lower ammonia-nitrogen and higher sulphur concentrations than RRB sediment. Lower overall growth and seed production was observed from WR grown in mine-influenced sediments contrasted to RRB sediment. However, a direct link between these responses of WR to exposures of mine-influenced sediment specifically containing higher concentrations of sulphur was not identified. Rather, the appearance of

ammonia-nitrogen concentration as the likely more influential sediment characteristic for WR growth and productivity was identified. During both the mesocosm- and microcosm- scale bioassays, visual differences between WR grown in RRB, CL, and UL sediments were not identified until mid-growing season, as WR plants became fully aerial (early tillering – boot stages; Oelke 1982). Sufficiently high sediment sulphur is suggested to interfere with WR nitrogen uptake at this stage (Pastor et al. 2017; LaFond et al. 2020). Observations from the current study are supported by previous studies detailing WR nitrogen requirements and uptake (Grava and Raisanen 1978; Oelke et al. 1982, 1997; Sims et al. 2012a,b). Based on data and observations from the current study, more research is needed to discern whether addition of ammonia-nitrogen to CL and UL sediments would mitigate decreased WR growth and reproductive potential compared to RRB sediment.

Data obtained during development of these bioassays indicates the microcosm-scale bioassay accurately predicted WR responses to RRB, CL, and UL sediment exposures measured as dry weight biomass and seed production, both of which are generally visually observable. Development of WR plants during early tillering – boot stages was indicative of differing responses to CL and UL sediment exposures contrasted to RRB sediment exposures. The primary visual observations included decreased plant height, overall smaller appearing leaves, and few developing tillers. The more likely source of these observations is an initially lower concentration of ammonia-nitrogen, followed by development of bioavailable nitrogen deficiencies in CL and UL sediments. Increased bioavailable nitrogen is required during early tillering – boot stages for optimal growth and reproductive capability (Grava and Raisanen 1978; Oelke et al. 1982, 1997; Sims et al. 2012a,b). Based on the lack of statistical differences between mesocosm and microcosm WR dry weight biomass and seeds per panicle ratios, this microcosm-scale WR bioassay may be substituted for the mesocosm-scale bioassay for predictions of these WR responses to sediment exposures. Use of this microcosm-scale bioassay will decrease overall resource use (sediment, water, time, foot print), resulting in more timely and defensible data for decisions focused on predicting responses of WR to exposures of site-specific water and / or sediment; and management of existing waters in which it thrives. Use of this microcosm-scale bioassay could be critical to further discern specific water and / or sediment influences on characteristics of early life stage development of WR.

ACKNOWLEDGEMENTS

The authors would like to thank Barrick Gold (Shayne Hayes in particular), Hemlo Mine, First Quantum Minerals (Stacy Anderson in particular), Ltd., and the National Science and Engineering Research Council of Canada (NSERC) for providing matching funding for this research. We also thank Johane Joncas and Lakehead University Environmental Laboratory (LUEL) personnel for analytical support. Much gratitude goes to Chris and Bernie Clein for allowing space for the mesocosm-scale bioassay at their farm and time committed to maintaining system inflow; and Kristi Dysievick, Paige Perrons, and Keri Pidgeon (Lakehead University Greenhouse Manager), for their assistance with monitoring and maintaining the status of both bioassays.

REFERENCES

Day, W.R., Lee, P.F. 1990. Mineral deficiencies of wild rice grown in flocculent sediments. J. Aquat. Plant Manage. 28, 84-88.

Elakovich, S.D., Wooten, J.W. 1989. Allelopathic potential of sixteen aquatic and wetland plants. J. Aquat. Plant Manage. 27, 78-84.

Fort, D.J., Mathis, M.B., Walker, R., Tuominen, L.K. Hansel, M., hall, S. Richards, R., Gratten, S.R., Anderson, K. 2014. Toxicity of sulfate and chloride to early life stages of wild rice (*Zizania palustris*). Environ. Toxicol. Chem. 33(12), 2802-2809.

Fort, D.J., Todhunter, K., Fort, T., Mathis, M.B., Walker, R., Hansel, M., Hall, S., Richards, R., Anderson, K. 2017. Toxicity of sulfide to early life stages of wild rice (*Zizania palustris*). Environ. Toxicol. Chem. 36(8), 2217-2226.

Grava, J., Raisanen K.A. 1978. Growth and nutrient accumulation and distribution in wild rice. Agronomy Journal 70(6), 1077-1081.

Jorgenson, K.D., Lee, P.F., Kanavillil, N. 2013. Ecological relationships of wild rice, *Zizania* spp. 11. Electron microscopy study of iron plaques on the roots of northern wild rice (*Zizania palustris*). Botany 91, 189-201.

LaFond-Hudson, S., Johnson, N., Pastor, J., Dewey, B. 2018. Iron sulfide formation on root surfaces controlled by the life cycle of wild rice (*Zizania palustris*). 2018. Biogeochemistry. https://doi.org/10.1007/s10533-018-0491-5.

LaFond-Hudson, S., Johnson, N.W., Pastor, J., Dewey, B. 2020. Interactions between sulfide and reproductive phenology of an annual aquatic plant, wild rice (*Zizania palustris*). Aquatic Botany 164, 103230.

Lee, P.F., Stewart, J.M. 1983. Ecological relationships of wild rice, *Zizania aquatica*. 2. Sediment-plant tissue nutrient relationships. Can. J. Bot. 61, 1775-1784.

Lee, P.F. 2002. Ecological relationships of wild rice, *Zizania* spp. 10. Effects of sediment and among-population variations on plant density in *Zizania palustris*. Can. J. Bot. 80, 1283-1294.

Lee, P.F., McNaughton, K.A. 2004. Macrophyte induced microchemical changes in the water column of a northern boreal lake. Hydrobiologia 522, 207-220.

Moyle, J.B. 1944. Wild rice in Minnesota. J. Wildlf. Manage. 8, 177-184.

Moyle, J.B. 1945. Some chemical factors influencing the distribution of aquatic plants in Minnesota. Amer. Midl. Nat. 34, 402-420.

Myrbo, A., Swain, E.B., Engstrom, D.R., Coleman Wasik, J., Brenner, J., Dykhuizen Shore, M., Blaha, G. 2017. Sulfide generated by sulfate reduction is a primary controller of the occurrence of wild rice (*Zizania palustris*) in shallow aquatic ecosystems. J. Geophys. Research: Biogeosciences 122, 2736–2753. https://doi.org/10.1002/2017JG003787.

Oelke, E.A., Grava, J., Noetzel, D., Barron, D., Percich, J., Schertz, C., Strait, J., Stucker, D. 1982. Wild rice production in Minnesota. Univ. Minn. Ext. Bull. 464. 40 pp.

Oelke, E.A., Teynor, T.M., Carter, P.R., Percich, J.A., Noetzel, D.M., Bloom, P.R., Porter, R.A., Schertz, C.E., Boedicker, J.J., Fuller, E.I. 1997 (last update). Alternative Field Crops manual: Wild Rice. UW and UMN Extension Services.

Pastor, J., Dewey, B., Johnson, N.W., Swain, E.B., Monson, P., Peters, E.B., Myrbo, A. 2017. Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments. Ecological Applications 27(1), 321-336.

Quayyum, H.A., Mallik, A.U., Lee, P.F. 1999. Allelopathic potential of aquatic plants associated with wild rice (*Zizania palustris*): I. Bioassay with plant and lake sediments. Journal of Chemical Ecology 25(1), 209-219.

Simmonds, N.W. 1995. The relationship between yield and protein in cereal grain. J. Sci. Food Agric. 67, 309-315.

Sims, L., Pastor, J., Dewey, B. 2012a. Nitrogen, phosphorus, and light effects on growth and allocation of biomass and nutrients in wild rice. Oecologia 170, 65-76.

Sims, L., Pastor, J., Lee, T., Dewey, B. 2012b. Nitrogen, phosphorus, and light effects on reproduction and fitness of wild rice. Botany 90, 876-883.

Stevenson, S.C., Lee, P.F. 1987. Ecological relationships of wild rice, *Zizania aquatica*. 6. The effects of increases in water depth on vegetative and reproductive production. Can. J. Bot. 65, 2128-2132.

Tedrow, O.R. 2020. Laboratory- and Field- Scale Bioassays for Predicting Responses of Wild Rice (*Zizania palustris* L.) to Exposures of Site-Specific Sediments and Waters. Ph. D. Dissertation, Lakehead University, Thunder Bay, Ontario.

Tucker, R.C., Zania, M.J., Emery, N.C., Gibson, K.D. 2011. Effects of water depth and seed provenance on the growth of wild rice (*Zizania aquatica* L.). Aquatic Botany 94, 113-118.

FIGURES

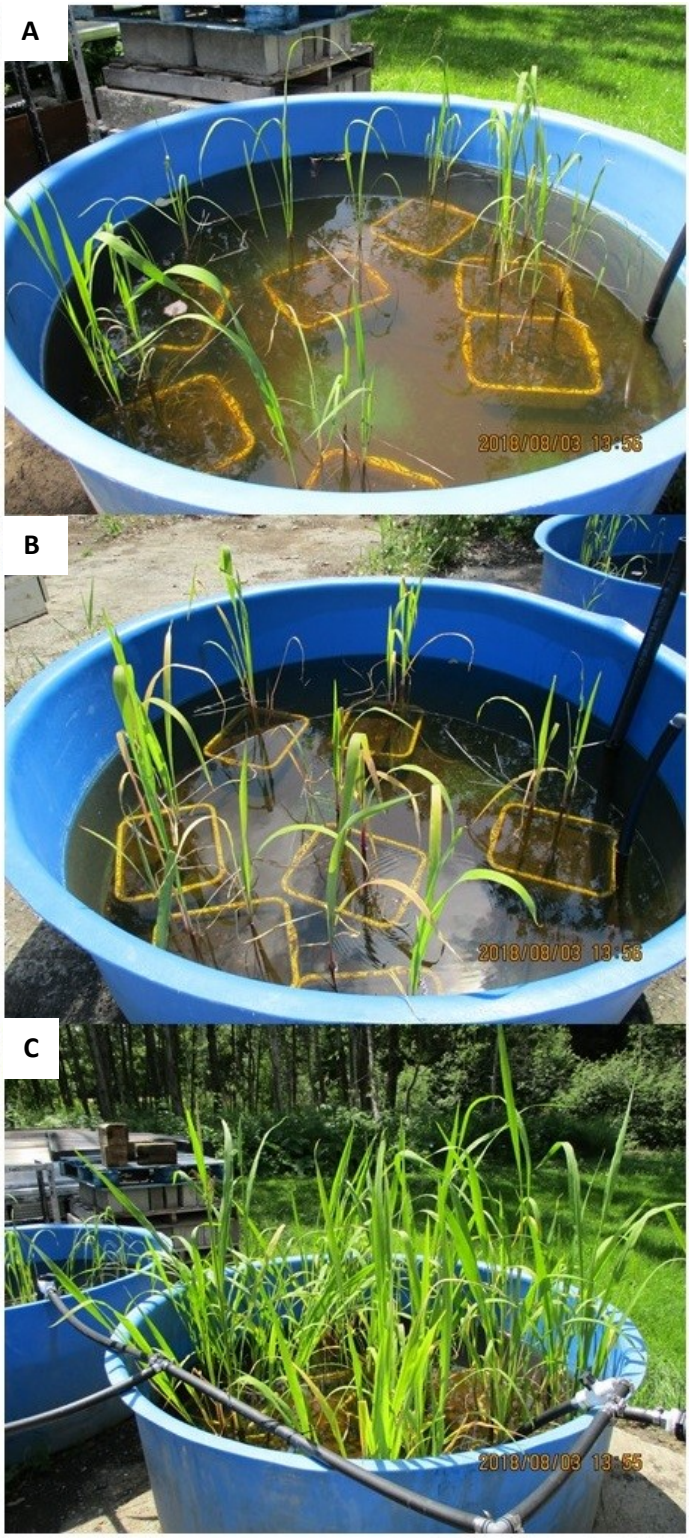

Figure 2.1. August 03, 2018. Mesocosm-scale WR sediment exposure chambers: A) Cleaver Lake; B) Unnamed Lake; and C) Rat River Bay.

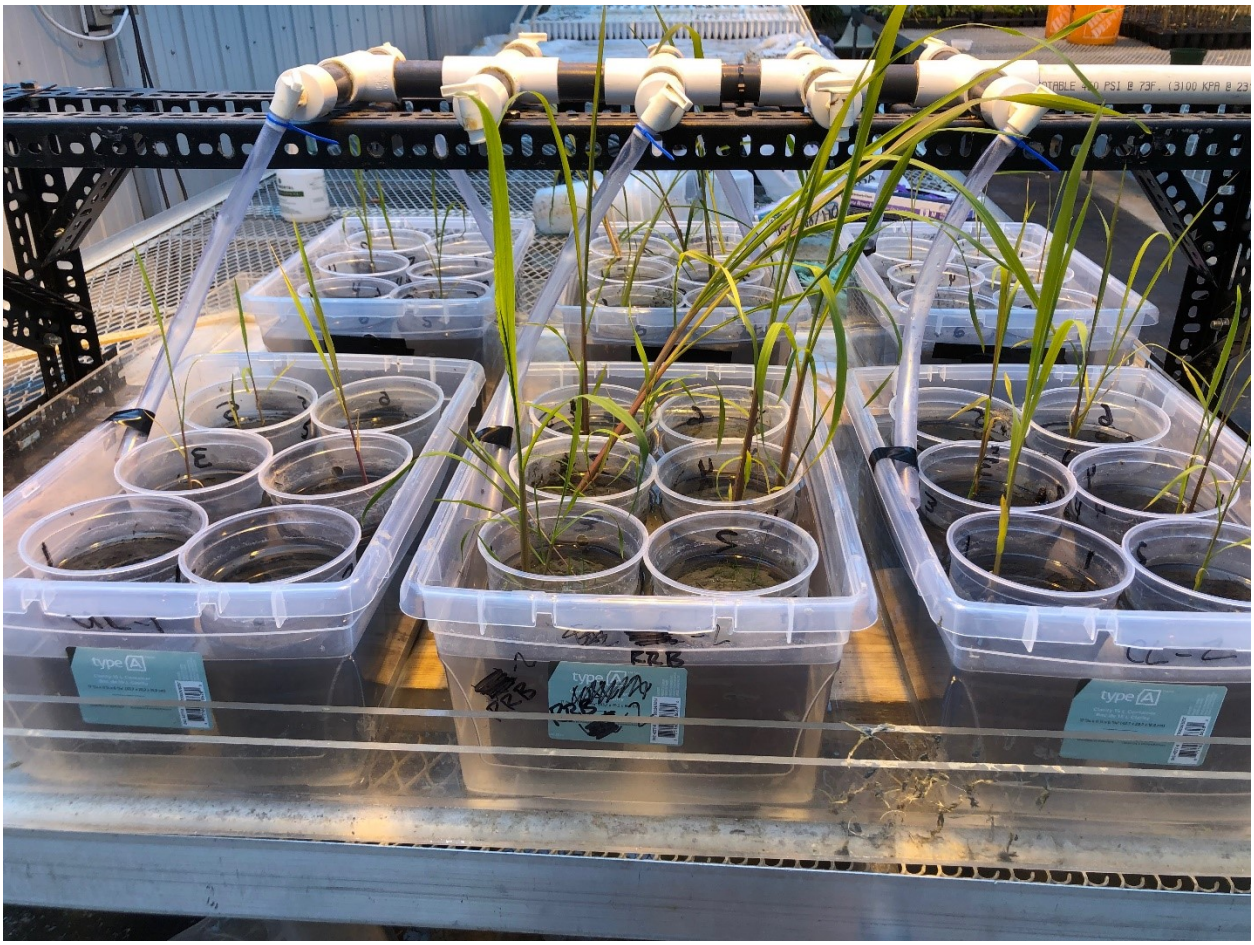

Figure 2.2. August 21, 2019. Microcosm-scale WR sediment exposure chambers. Left to Right: six replicates of Unnamed Lake; Rat River Bay, and Cleaver Lake. Six additional replicate exposures of each sediment were placed on the opposing side of the water bath.

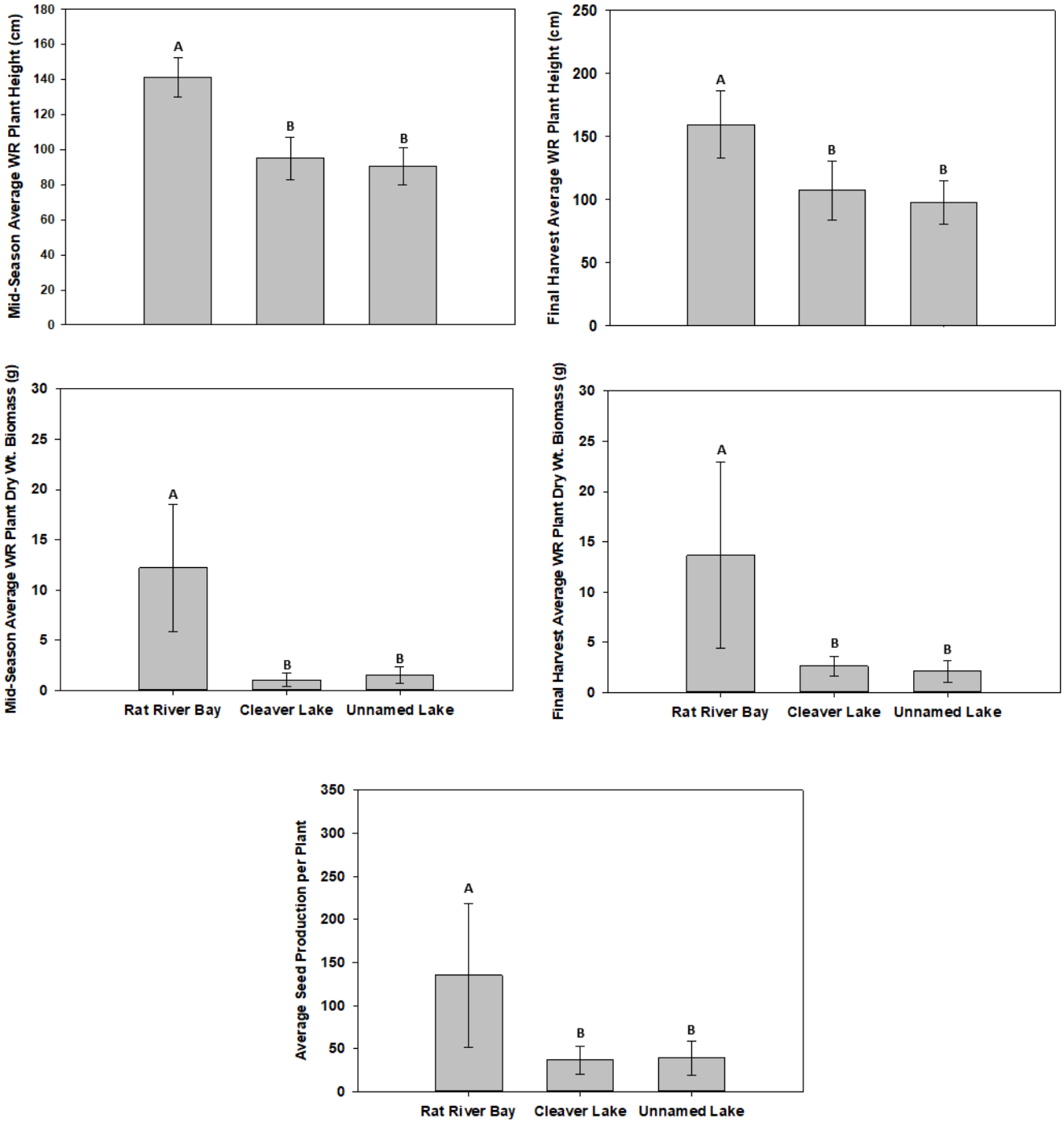

Figure 2.3. Mesocosm-scale bioassay average WR plant height and dry weight biomass from the mid-season harvest and final harvest; and seed production at final harvest. Error bars represent one standard deviation. Differing letters (A and B) indicate statistical significance between sediment types.

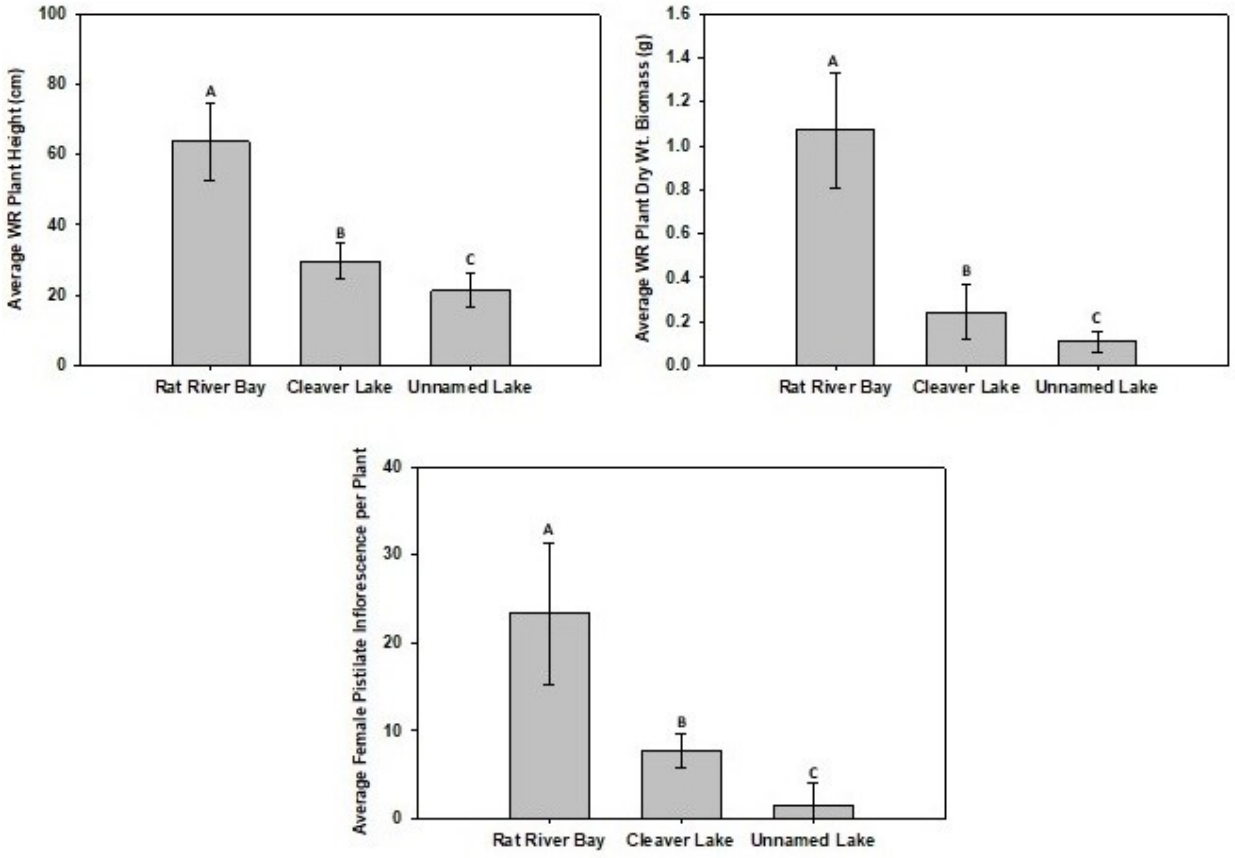

Figure 2.4. WR responses to RRB, CL, and UL sediments under microcosm-scale bioassay conditions. Plants grown in UL sediment were statistically smaller with a statistically lower reproductive potential than plants grown either CL or RRB sediment. Error bars represent one standard deviation. Differing letters (A, B, C) indicate statistical significance.

TABLES

Table 2.1. Characteristics of RRB, CL, and UL sediment used for the mesocosm-scale bioassay at experiment initiation, July 28, 2018 (approximate mid-point of experiment), and experiment conclusion (n = 1 for each sediment sampling event). Units for NH_3 through Na are µg g^{-1}.

	RRB			CL			UL		
	Initial	Mid	Conclusion	Initial	Mid	Conclusion	Initial	Mid	Conclusion
*p*H (SU)	6.00	6.05	5.49	5.81	5.84	5.66	6.97	7.01	6.85
Bulk Density (g cm^{-3})	0.60			0.47			0.30		
NH_3+NH_4-N (µg g^{-1})	14.35	4.89	0.31	6.31	1.33	BD	4.84	2.06	BD
PO_4	0.98	0.34	0.64	5.60	1.19	2.01	2.30	4.06	1.35
Al	160	224	236	197	323	262	167	183	121
Cu	1.31	0.78	1.13	0.13	0.03	1.18	0.09	0.12	0.21
Fe	243	515	484	109	314	173	30	66	31
Mn	46	55	53	12	19	12	23	30	19
S	1	2	2	13	89	63	17	26	27
Zn	NM	6	4	NM	154	153	NM	3	3
Ca	527	612	651	287	478	337	491	569	498
K	9	12	13	3	3	7	9	8	8
Mg	144	198	216	19	48	46	21	25	28
Na	2	5	9	2	BD	9	47	21	16

BD = below detection; NM = not measured.

Table 2.2. Characteristics of RRB, CL, and UL sediment sampled at initiation and conclusion of the microcosm-scale bioassay (n = 1 for each sediment sampling event). Units for NH_3 through Na are µg g^{-1}.

	RRB		CL		UL	
	Initial	Conclusion	Initial	Conclusion	Initial	Conclusion
*p*H (SU)	6.00	5.48	5.81	5.49	6.97	6.86
Bulk Density (g cm^{-3})	0.60		0.47		0.30	
NH_3+NH_4-N (µg g^{-1})	14.35	7.64	6.31	1.59	4.84	1.85
PO_4	0.98	2.30	5.60	5.46	2.30	2.34
Al	160	186	197	182	167	105
Cu	1.31	1.47	0.13	0.79	0.09	BD
Fe	243	379	109	90	30	48
Mn	46	55	12	9	23	16
S	1.35	1.25	13	16	17	22
Zn	2	3	40	43	3	2
Ca	527	550	287	285	491	393
K	9	14	3	3	9	6
Mg	144	147	19	19	21	13
Na	2	5	2	4	47	21

BD = below detection.

Table 2.3. Characteristics of plant tissue and seed sampled during and following the mesocosm- and microcosm- scale bioassays (n). Note similarities between concentrations of measured elements in seed samples regardless of sediment or other plant tissue characteristics. Units for N through Fe are %; units for Al through Zn are µg g^{-1}.

	Mesocosm												Microcosm		
	Mid-Season Plant			Final LL (3)			Final FL (3)			Seeds			Final Plant		
	RRB (10)	CL (9)	UL (10)	RRB	CL	UL	RRB	CL	UL	RRB (24)	CL (12)	UL (9)	RRB (4)	CL (3)	UL (2)
N (%)	1.85	2.52	1.14	0.83	1.33	0.9	1.1	1.81	1.24	1.19	1.58	1.44	1.05	NM	NM
P	0.34	0.15	0.26	0.1	0.08	0.13	0.13	0.15	0.16	0.32	0.3	0.31	0.32	0.1	0.2
K	1.15	2.12	2.29	0.49	1.3	0.78	0.6	2.17	1.29	0.42	0.5	0.43	1.18	1.26	0.75
S	0.1	0.19	0.19	0.09	0.13	0.15	0.12	0.16	0.13	0.11	0.12	0.1	0.11	0.37	0.29
Ca	0.3	0.35	0.43	1.14	0.9	1.02	1.19	1.01	1.33	0.02	0.02	0.02	0.49	0.48	0.72
Mg	0.13	0.1	0.09	0.23	0.17	0.15	0.23	0.25	0.18	0.1	0.09	0.09	0.18	0.1	0.15
Na	0.97	1.04	0.72	0.6	0.9	0.96	0.41	0.46	0.66	0.01	0.01	0.01	0.6	0.69	1.24
Fe	0.07	0.09	0.03	0.12	0.05	0.02	0.06	0.02	0.02	0.01	0.01	0.01	0.08	0.07	0.08
Al (ug g^{-1})	403	178	73	804	176	70	367	28	38	22	4	4	233	101	186
Cu	25	14	56	19	4	2	2	5	2	5	6	4	15	15	29
Mn	388	721	1,154	1,434	862	1,951	1,347	641	1,968	25	21	30	898	496	1,668
Zn	28	63	32	21	63	20	21	39	25	32	33	27	44	116	92

LL = lower leaves; FL = flag leaves; NM = not measured.

Table 2.4. Ratios and statistical ratio comparison (t-Test; alpha = 0.05) between physical characteristics of WR plants harvested at mesocosm- and microcosm-scale bioassay conclusion.

	Mesocosm Bioassay			Microcosm Bioassay			Mesocosm:Microcosm *p*-Value		
	DWB (g)	Height (cm)	Seeds per Panicle	DWB (g)	Height (cm)	*Seeds per Panicle	DWB (g)	Height (cm)	Seeds per Panicle
RRB:CL	5.596	**1.485**	6.1	5.239	**2.011**	3.0	0.861	**0.028**	0.150
RRB:UL	8.639	**1.600**	6.0	11.964	**2.927**	5.3	0.408	**< 0.001**	0.884
CL:UL	1.565	**1.097**	1.1	3.069	**1.506**	1.5	0.101	**0.020**	0.375

DWB = dry weight biomass. **Bolded** values indicate statistical significance.

*Microcosm bioassay seeds per panicle measured as number of female pistilate inflorescence per panicle.

CHAPTER THREE: MEASURING *IN-SITU* RESPONSES OF WILD RICE (*ZIZANIA PALUSTRIS* L.) TO EXPOSURES OF VARIABLE WATER DEPTHS AND SITE-SPECIFIC SEDIMENTS IN NON-MINE AND LEGACY-MINE INFLUENCED WATERS

ABSTRACT

Water depth and water depth increases are critically influential on wild rice (WR) growth, development, and ultimately reproductive potential. Determination of life stages more sensitive to water depth increases can be critical for water resource management decisions. Development and use of raft-style bioassays to obtain these data from remote areas, and areas not conducive to WR growth, was completed. Rafts were deployed in two select aquatic systems: The Seine River, a non-industry-influenced location; and three legacy-mine influenced pits. In the Seine River, tubs containing formulated sediment were suspended within the water column at 40 (control), 40, 60, and 80 cm. The 40, 60, and 80 cm treatments were lowered by 30 cm over 15 days in 10 cm increments at aerial (Raft 1), floating leaf (Raft 2), or submerged (Raft 3) life stages. Fewer plants remained on Raft 2 than Rafts 1 or 3. WR on Raft 2 was significantly shorter and contained significantly less dry weight biomass than Raft 1 plants in all depth increase treatments. No plants remained in 60+30 cm treatments on Raft 2; and only the Raft 2 40 control depth treatment contained plants achieving seed production. Only WR in the 40+30 cm depth treatment on Raft 3 achieved seed production. WR in Raft 1 (aerial stage) 40 control, 40+30, and 60+30 cm depth treatments achieved seed production. Based on data obtained during this study, floating leaf plants were determined more sensitive to depth increases than submerged-stage WR, with plants already having achieved aerial stage less sensitive to depth increases. The study involving deployment of rafts in the legacy-mine influenced pits was designed to conclude when WR achieved aerial stage development; the stage identified in the Seine River study, that, once achieved, is more likely to result in seed production. Two rafts were deployed in each of three pits; one raft to evaluate WR responses to water depths (20, 40, 80, 160 cm), and one to evaluate responses to sediment formulations at a 20 cm water depth. Aerial WR development was observed in 20 and 40 cm water depth treatments. No WR achieved aerial stage in 80 or 160 cm depth treatments during the course of this study. Critically, no adverse WR responses were observed due to exposures of legacy-mine influenced pit waters. Furthermore, water depths exceeding 80 cm are not likely to be conducive for WR achieving reproductive

maturity. Raft-style bioassays allowed for these *in-situ* exposure-response data to be obtained at remote locations not conducive of typical WR growth under conditions more representative of natural WR areas. These data are critical for informed water resource, and WR resource, management decisions.

3.1 INTRODUCTION

Wild rice (*Zizania palustris* L.; WR) is an annual grass native to North America, and is an important cultural, recreational, and nutritional grain. Fluctuations in WR density in natural systems can be problematic for all intended resource uses, and are often attributed to water depth fluctuations. Moyle (1944) concluded that approximately every four years, crop yields will fail during one season, and be fair to high during the three other seasons. Water depth as a critical influence on WR growth, development, and maturation, is well described (Weber and Simpson 1967; Aiken et al. 1988; Stevenson and Lee 1987; MN DNR 2008; Tucker et al. 2011; Vennum 1988; Pillsbury and McGuire 2009). Although WR can germinate and grow under conditions of water depth beyond typical upper limits, this increases the time needed to achieve more mature phenological life stages, thus decreasing the likelihood of reproductive success (Thomas and Stewart 1969). Tucker et al. (2011) concluded that total biomass, dry weight biomass of inflorescences, and number of tillers decreased as water depth increased from 'zero' cm to 23 and 46 cm. Bloom et al. (2001) reported yields in the same transects in Rice Lake, MN, decreased > 8x from approximately 1,567 Kg ha^{-1} in year 2000 to 192 Kg ha^{-1} in year 2001 as the average water depth increased from 66 cm to 109 cm. They also reported that, in both years, seed production and biomass were negatively correlated to water depth increases. Marcum and Porter (2006) reported that mature seed per panicle and total seed production decreased in controlled experiments as water depth increased from 15-30 cm to 46-61 cm. Under those conditions, no seedlings were able to reach the surface of the containers when planted at a water depth of 76 cm.

Other studies have concluded that WR crop yields are specifically dependent on water depth fluctuations, particularly during more sensitive WR life stages (Stevenson and Lee 1987). If the increase is sudden during the submerged or floating leaf stage, the less developed roots may not be able to anchor the plant, which may then be uprooted (Chambliss 1940; Thomas and Stewart 1969). If the water depth increase is more gradual while plants are in the submerged stage, it will

take longer to reach the surface with subsequently decreased yields due in part to decreased tillering, or complete loss of reproductive success due to mortality. If the plant has achieved floating leaf stage when the cuticle has formed and is then submerged, the plant will be placed under additional stress due to decreased gas exchange ability (Hawthorne and Stewart 1970; Stevenson and Lee 1987).

However, although it is generally accepted that water depth and water depth fluctuations are the main causes of reductions in wild rice growth and development, there are suggestions that water and sediment quality can also exert considerable influence. For example, sulphate has long been suggested as a causative factor in wild rice production in Minnesota (Moyle 1944, 1945). More recently, sulphate has been implicated as an adverse influence on WR growth, development, and reproductive capability in part due to its transformation into hydrogen sulphide under anoxic and chemically reductive sediment conditions (Myrbo et al. 2017; Pastor et al. 2017; Pollman et al. 2017; LaFond-Hudson et al. 2018, 2020). Determining the effects of these modified chemical conditions on wild rice needs to be examined under controlled conditions that accurately reflect the environmental conditions in terms of water and sediment in an impacted area.

Previous studies used non-flow-through mesocosms as aqueous sulphate exposure chambers with periodic water additions to maintain water depth and sulphate concentration (Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020). Although useful for obtaining specific WR exposure-response and water-sediment chemistry interaction data, these types of systems tend not to represent field conditions which have overlying water refresh via flow-through conditions. Therefore, a critical need for *in situ* WR:sulphate exposure:response data has developed that is based on examining the concurrent effects of water depth and chemical characteristics on wild rice growth and development.

The approach used in this study was to examine the growth and development of wild rice under controlled *in-situ* conditions in a remote non-contaminated wild rice area in order to develop an *in-situ* bioassay for examining the production of wild rice in industrially influenced areas. Raft assemblies based on those described by Stevenson and Lee (1987) were developed and deployed in two specific water resources. Three rafts were deployed in a protected cove on the Seine River in northwestern Ontario (non-contaminated site) and wild rice plants were grown to maturity. Additionally, pairs of rafts were deployed in each of three legacy-mine influenced pits. Wild rice

in the mine pit component of this study was allowed to develop until aerial phenology was observed.

The overall purpose for designing these raft-style bioassays was multi-fold: 1) to allow for *in-situ* observational and empirical characterization of WR exposure-response data to site-specific water and / or sediment with minimal maintenance and attention; and 2) to allow for these *in-situ* evaluations in areas of aquatic systems not typically conducive to WR growth (i.e., water depth exceedances; lack of appropriate sediment; remoteness precludes powered equipment for flow-through bioassay testing; onsite design, build, and deployment of testing structures is required), in water which may be discharged and affect WR downstream. Raft-style bioassay assemblies as designed and used herein mitigated for all of these considerations, and allowed completion of two evaluations of WR exposure-response scenarios where *in-situ* data were critical to successful completion of the study.

Specific objectives of these studies were to 1) measure responses of WR to variable water depth exposures at distinct phenological stages in terms of shoot height, plant dry weight biomass (shoots+leaves), and seed production in the non-contaminated site to determine the phenological stage more sensitive to water depth and water depth increases; and 2) use results from the non-contaminated site as the basis of a bioassay for examining phenological responses of WR to water depth, and sediment and water type, exposures under *in situ* conditions in legacy-mine influenced pits. Since the raft study completed in support of Objective 1 determined the more sensitive WR phenology to water depth increases, inherently the less sensitive stage was also determined. In this application, Objective 2 was accomplished by developing a raft-style bioassay which concluded following observance of the less sensitive WR phenological stage.

3.2 MATERIALS AND METHODS

Raft assemblies were designed and built as described in Stevenson and Lee (1987). Nine, 12-liter plastic tubs were suspended within each quadrat; 36 tubs per raft. Each tub contained approximately 10 liters of sediment. Three rafts were deployed in the Seine River near Mine Centre, Ontario (48°44’12.55” N, 92°21’15.12” W; **Figure 3.5**) for the 2014 growing season, in a natural wild rice area; and two rafts in each of three legacy-mine influenced pit locations in Minnesota for the 2017 growing season (**Figure 3.6**).

3.2.1 RAFT ASSEMBLIES – SEINE RIVER

During May 2014, three rafts were constructed at the Seine River First Nation Community, and transported to the deployment location. All tubs were pre-filled at Lakehead University with black earth soil and five (5) grams of Smartcote® (Spectrum Brands IP, Inc.) slow-release pelletized fertilizer (14N:14P:14K), resulting in amendment of 700 mg N, P, and K to each tub, and transported to the Seine River First Nation Community. Wild rice seed was germinated and allowed to grow to 10-15 cm seedlings in controlled photoperiod (16h light:8h dark) and temperature (20°C) conditions at Lakehead University. Following raft deployment, nine tubs containing substrate were anchored in each raft quadrat and submerged 40 cm below the water surface. Ten seedlings were planted in each tub following substrate saturation with site water, which were culled to five to seven healthier-appearing plants five days after planting. After culling, tubs in each quadrat were set or lowered to their assigned initial water depth (40, 60, or 80 cm). Tubs in one quadrat on each raft were assigned as the 40 cm control (40 Ctl) treatment. The three initial depths were held constant until a specific phenological stage was achieved on individual rafts. Each individual raft was assigned a WR phenological stage for initiating water depth increases; raft assignments were as follows: Raft 1 – emergent / aerial; Raft 2 – floating leaf; and Raft 3 – submerged. Following observance of one of these stages, the tubs in a specific raft quadrat were lowered 10 cm every five days until a 30 cm depth increase was achieved. The final depths for three quadrats on each raft were 70, 90, and 110 cm; henceforth referred to as 40+30, 60+30, and 80+30, respectively. Near weekly inspections were completed in an effort to document WR development throughout the experimental duration. All remaining WR plants were harvested approximately four weeks following the final water depth increase. The total duration of this study was from June-September 2014; approximately four months, which corresponds to the length of a typical WR growing season.

Plants were separated into shoot, leaf, root, and seed components for height / length and dry weight biomass measurement and stored on ice in Ziploc® bags. Although length and dry weight biomass measurements were obtained from root samples, root biomass removal during harvest was neither complete nor consistent. Therefore, root data were not used as a response measure for this study. Sediment was sampled from one randomly selected tub within each quadrat, from which plants were also harvested, for measurement of parameters listed in **Table 3.5**.

3.2.2 RAFT ASSEMBLIES – LEGACY-MINE INFLUENCED PITS

During May 2017, two rafts were deployed in each of the three selected legacy-mine influenced pits: Pit A, Pit B, and Pit C. Deployment of rafts in these pits resulted in exposure of WR seeds and substrate to sulphate concentrations ranging from approximately 420, 170, and 1,300 mg L^{-1} in Pits A, B, and C, respectively. Water clarity in each of the selected pits was high, with light penetration reaching the bottom sediment. Sediment of two general types obtained from an onsite creek were used for this current study: one a sediment with higher sand content, and one sediment with a higher organic content. To maintain representative field conditions, no fertilizer (i.e., Smartcote®) was added to sediment in this study. One raft was used to test WR responses to specific water depths (20, 40, 80, and 160 cm) in 100% organic sediment. These water depths were chosen based on the depths used for the Seine River portion of the study and that are likely to result in reproductive success (20, 40 cm), and one (160 cm) which is not likely to result in WR reproductive success. The other raft was used to test WR responses to 100% sandy sediment, and three sandy:organic sediment formulations (v/v; 25, 50, 75%) at a single water depth (20 cm below surface). Sediments were mixed in these ratios in an effort to determine if a sediment-associated gradient of influence on WR phenology was measurable. Tubs were filled with 10 litres of the assigned sediment formulation and transported to the raft deployment locations. Wild rice seeds were harvested from lakes in northern Minnesota, and were distributed within the top two cm of substrate in each tub at a rate of approximately 28 Kg ha^{-1}. Field measurements of *p*H, dissolved oxygen, temperature, and specific conductance were obtained at each of the four depth treatments during each visit using a calibrated YSI® ProPlus® or a Hach® MS5 HydroLab®. This raft study was initiated in May 2017, and was concluded mid-July 2017 after WR achieved aerial phenological development.

3.2.3 LABORATORY ANALYTICAL METHODS

Quality controls for sediment, plant, and seed samples characterized by LUEL included reference standards and replicate samples, and follows procedures required by the Canadian Association of Laboratory Accreditation (CALA). All sediment samples from the Seine River and mine pit raft studies were stored frozen in Ziploc® bags, and delivered to the Lakehead University Environmental Laboratory (LUEL) for processing and measurement of total nitrogen, *p*H, bulk density, phosphate, and extractable metals. Sample preparation and analytical procedures followed those described in Lee and McNaughton (2004), the LUEL Quality Assurance Manual,

and reference Forest Canada, ASTM, and USEPA methods. Total nitrogen (when possible) was measured on dry sediment using a combustion ELVario cube carbon-hydrogen-nitrogen-sulphur (CHNS) analyzer. LOI was measured by drying 20 mL of sediment at 80°C, weighing, ashing at 600°C, and re-weighed. Sediment *p*H was measured as a 1:1 sediment:deionized water mixture using a Mettler Toledo meter with an InLinePro *p*H sensor. Bulk density was measured by weighing 20mL of wet sediment, drying at 80°C, and re-weighing. Phosphate was determined using the BRAY P2 method – Al and Fe phosphates are dissolved in ammonium fluoride, and measured colorimetrically using a SKALAR analyzer. Al, Cu, Fe, Mn, S, and Zn were extracted in 0.1 N HCl, while Ca, K, Mg, and Na were extracted in ammonium-acetate at *p*H 7.0; all of which were measured using ICP. Sediment samples were processed as wet samples to better represent field conditions. Measured sediment analyte concentrations were corrected for bulk density.

All plant and seed samples obtained during the course of the Seine River raft studies were stored refrigerated in Ziploc® bags, and delivered to LUEL for processing immediately after harvest. Analytical methods for sediment, plant, and seed samples followed those described in Lee and McNaughton (2004) and the LUEL Quality Manual. WR plants and seeds were dried at 50°C and kept frozen until processing. Analyses were based on those described by the American Soil and Plant Council. Plant, or seed, biomass (≥ 0.5g) was digested using a CEM Mars Express microwave in Express Teflon closed vessels in one mL of trace metal grade HNO_3 and three mLs of trace metal grade HCl, and diluted using 25mL of deionized water. Al, Ca, Cu, Fe, Mg, Mn, Na, S, and Zn in this dilution were measured using a Varian ICP-AES. Total nitrogen and phosphorus were digested using trace metal grade HNO_3 / HPO_4 in a stepwise process at 400°C, and measured using the SKALAR analyzer. No plant tissue or seed samples were obtained during the raft study focusing on legacy-mine influenced pit waters.

Quality controls for water samples characterized by Pace Labs included reference standards, matrix spikes, and conformed to The National Environmental Laboratory Accreditation Conference (NELAC) Standards and the laboratory's Quality Assurance Manual, where applicable. Surface water and pore water samples obtained during the course of the mine pit raft study were stored on ice and delivered to Pace Analytical Laboratories (Pace) in Virginia, Minnesota, for processing immediately following sampling. Analytical methods for water and pore water followed those described in Pace (2020a,b). Specifically, Ca, As, Co, Mg, and Fe

were measured using ICP following acidification to *p*H < 2.0 using trace metal grade HNO_3 (USEPA method 200.7). Additionally, one mL of concentrated trace metal grade HNO_3 and 0.5 mL of concentrated trace metal grade HCl were added to 50 mL of sample. Acidified samples were heated to approximately 95°C for four to five hours. After heating, samples were brought to a 50 mL volume with double-deionized water, gently inverted, and allowed to settle overnight prior to analysis. Chloride (Cl) and SO_4 were separated from non-chemically-preserved samples through a series of ion-selective columns, and measured using IC (method 300.0). Aqueous TSS was defined as the fraction of solids retained on a 0.45 µM pore-size filter, dried at 103-105°C overnight, and measured gravimetrically. Aqueous TDS was defined as the fraction of solids remaining in the filtrate passing through a 0.45 µM pore-size filter; the filtrate was then dried at 103-105°C overnight, and the remaining solids were measured gravimetrically. Samples for sulfide (as H_2S) were preserved using sodium hydroxide / zinc acetate, and was measured according to SM 4500-S2-G.

3.2.4 STATISTICAL ANALYSES

All surface water, sediment, pore water, and WR plant and seed data were organized using Microsoft® Excel® or SigmaPlot-SigmaStat® v14.0 (Systat Software, Inc.) for table and graphical data representation. Statistical analyses and treatment of WR plant data were completed using SigmaPlot-SigmaStat® v14.0. t-Tests or one-way analysis of variance (ANOVA) were used to compare two or more groups; specifically, WR responses to water depth exposures measured as plant height, final dry weight biomass, and seed production. Holm-Sidak multiple comparison was used to discern significant differences between three or more groups if data met normality of distribution and variance. Data were natural logarithmic transformed if normality of distribution and / or variance assumptions were violated. If data transformation failed to correct non-equal distribution and / or variance, raw data were used for statistical treatment. ANOVA on ranks was used when significant differences were identified between three or more groups, followed by Dunn's multiple comparison to discern specific between-group differences. A Mann-Whitney Rank Sum test was used to discern significant differences between groups used for t-test comparisons when data were not corrected for equality of normality or variance through natural logarithmic transformation. Non-transformed data were used for table and figure display purposes.

3.3 RESULTS

3.3.1 SEINE RIVER

Overall, WR plants survived in all water depth treatments; however, not all tubs contained surviving WR at harvest (**Figure 3.7**). Wild rice grown in the Raft 2 (floating leaf) 40 Ctl treatments was significantly taller than Raft 1 (emergent) 40 Ctl WR ($t_{(30)} = -4.261$ $p < 0.001$) (**Figure 3.7**). One-way ANOVA identified significant differences in average plant heights between rafts ($F_{(3,62)} = 9.329$ $p < 0.001$). Paired comparison tests indicated that average shoot height of plants from the 40+30 cm depth treatment on Rafts 1 and 3 were statistically higher than the 60+30 (R1 $p < 0.001$; R3 $p = 0.013$) and the 80+30 water depth treatment on Raft 1 (R1 $p = 0.001$). In general, as water depth treatments increased on Rafts 2 (floating leaf) and 3 (submerged), WR height tended to decrease when compared to Raft 1 (aerial) plants. This indicates that floating leaf and submerged life stages are more sensitive to water depth increases of this magnitude than the aerial stage. Although substantial differences exist between water depth treatments in terms of dry weight biomass, identifying statistical differences was confounded by intra-treatment variability. For example, although Raft 1 60+30 and 80+30 depth treatments contained measurable amounts of WR dry weight biomass, no statistical difference was identified between Rafts 1 and 3 within these treatments (60+30 $p = 0.150$; 80+30 $p = 0.119$) (**Figure 3.8**). These data primarily indicate that the greater the initial water depth in which WR plants were grown, followed by a 30 cm water depth increase, the less dry weight biomass those plants accumulated, regardless of height. Seed production between water depth treatments tended to indicate that not only does increasing water depth decrease seed production, but increasing water depths during specific life stages resulted in a lack of seed production at water depth treatments of 110 cm (Raft 1, aerial stage); 70, 90, and 110 cm (Raft 2, floating leaf stage); and 90 and 110 cm (Raft 3, submerged stage) (**Figure 3.9**).

Based on WR plant height, dry weight biomass, and seed production data obtained during this study, the floating leaf stage was determined more critically sensitive to water depth increases than submerged and aerial stages under these conditions. Since this Seine River study was intended to be used as a bioassay framework for determining the more sensitive life stages, specifically to water depth increases, it also functioned as a framework to determine the less sensitive life stage to water depth increases. In this case, WR aerial phenology was less sensitive

than floating leaf and submerged stages based on observations of reproductive success following water depth increases from 40 to 70 cm, and 60 to 90 cm. It was with this application that the legacy-mine influenced pit raft study was developed.

Measured concentrations of nutrient elements in sediment samples obtained at the end-of-season harvest appeared sufficiently high to have sustained WR in all sediment treatments throughout the growing season. Ammonia-nitrogen concentrations ranged between 9-40 μg g^{-1} at the time of harvest. Additionally, potassium (34.0-70.5 ug g^{-1}) and phosphate (2.8-8.3 ug g^{-1}) do not appear to have been growth or development limiting during the growing season (**Table 3.5**). No evidence of nutrient deficiency or disease (Day and Lee 1990; Oelke et al. 1997) was observed throughout the course of this raft study. However, shoot height and dry weight biomass of harvested WR were significantly different within and between raft water depth treatments.

3.3.2 LEGACY-MINE INFLUENCED PITS

Characteristics of legacy-mine influenced pit waters in which rafts were deployed remained consistent between 2016 and 2017 (**Table 3.6**). Specific conductance substantially differed between the three selected pits, but remained consistent within each pit throughout the study duration; temperature, *p*H, and dissolved oxygen remained similar within and between the three pits (**Figure 3.10**). This raft study was concluded following observation of aerial WR phenology in 20 and 40 cm water depth treatments on rafts deployed in all three pits.

The two sediment types used for this raft study contained similar concentrations of potassium and phosphate, but differed by an order of magnitude in ammonia-nitrogen (**Table 3.7**), with the organic sediment containing approximately 25 μg g^{-1} and the sandy sediment containing 2.6 μg g^{-1}. Measured concentrations of hydrogen sulphide in peeper pore water samples were often below detection (< 0.078 mg L^{-1}), or below the suggested 0.165 mg L^{-1} protective level for WR. Only three measurable H_2S concentrations exceeded 0.165 mg L^{-1}: 0.313 and 0.249 mg L^{-1} in the Jul. 31, 2017, Pit A 20 cm (25:75% sandy:organic sediment) and the Pit A 20 cm (100% organic sed.) samples, respectively; and 0.193 mg L^{-1} in the Pit A 160 cm (100% organic sed.) treatment on Aug. 31, 2017.

Wild rice seeds germinated in all tubs suspended at all water depths in all sediment formulations in each of the three legacy-mine influenced pits. WR at 20 and 40 cm water depths developed temporally similar to WR in local natural areas (personal observations) (**Table 3.8**). This

indicates that WR phenology was not delayed due to water (or sediment) type exposures under these conditions. However, a general phenology differential was observed between the four tested water depths. Plants growing at 20 and 40 cm depths achieved floating leaf and aerial phenology nearly simultaneously (between raft inspections). WR at 80 and 160 cm depths did not achieve floating leaf or aerial stages during this study, but did grow to an overall submerged height of 60-90 cm. Furthermore, plants grown at 80 and 160 cm were not likely to achieve one or both stages within typical temporal WR phenology. This was not unpredicted, and has been well documented (Moyle 1944; Weber and Simpson 1967; Stevenson and Lee 1987; Tucker et al. 2011).

Based on conclusions of the raft study completed in the Seine River indicating that achieving aerial phenology results in an overall increased likelihood of reproductive success, even following a 30 cm water depth increase to 90 cm total depth, the mine pit raft study was concluded following observation of aerial phenology on rafts deployed in each mine pit. Plants growing at 20 and 40 cm depths were initially observed to have developed aerial phenology on June 27, 2017; however, achieving this stage occurred between raft inspections. Plants growing at 80 and 160 cm depths showed exceptional growth; however, these plants were ≥ approximately 20 cm beneath the water surface when plants at 20 and 40 cm depths had achieved aerial phenology. It is unlikely that WR at 80 and 160 cm depths would have achieved reproductive success due to growing season length and physical plant limitations (Thomas and Stewart 1969). Based on data obtained during this mine pit raft study, overall water depth was more influential and critical for typical WR phenology than water and / or sediment type.

3.4 DISCUSSION

3.4.1 SEINE RIVER

Growth and development of WR in response to water depth has been detailed by Weir and Dale (1960), Atkins (1983), Thomas and Stewart (1969), and Tucker et al. (2011); and water depth increases by Stevenson and Lee (1987). In the current study, differential sensitivities of WR to water depth increases based on WR phenology at the time of increase were documented. Depth increases for WR plants on Raft 2 (floating leaf stage) tended to result in a higher rate of plant loss than on Rafts 1 (aerial) or 3 (submerged). This may be due to uprooting as suggested by Thomas and Stewart (1969). Plants remaining on Rafts 1 and 3 tended to be taller than plants

remaining on Raft 2. One possible explanation for these observations is described by Stevenson and Lee (1987). In their raft study, they observed height increases following submersion of floating leaf and aerial stage plants after they had achieved their initial height. They suggested that this continued growth is likely due to internodal growth, as described by Weir and Dale (1960). Dry weight biomass decreases, regardless of plant height, following water depth increases is likely due to decreased aerial plant growth. Aerial leaves are reported to be more heavily lignified (Weir and Dale 1960), which would tend to increase plant dry weight biomass. Failure to achieve aerial stage, and therefore the absence of flowering, would also result in subsequent losses in seed production.

No seed production was observed on plants in the 80+30 depth treatment on any raft, on plants grown in Raft 2 and Raft 3 60+30 depth treatments, and only four plants produced seeds in the Raft 1 60+30 depth treatment. Similar decreases in reproductive potential results were observed by Tucker et al. (2011) and Stevenson and Lee (1987) in response to increased water depth and water depth increases, respectively. In the current study, aerial WR phenology was less sensitive to water depth increases with more plants remaining at harvest, and generally higher plant height, dry weight biomass, and seed production in Raft 1 40 Ctl, 40+30, and 60+30 depth treatments.

Although WR in the Raft 1 60+30 water depth treatment achieved aerial stage, and ultimately reproductive success, this occurred after the plant had already achieved aerial phenology. As described in Weir and Dale (1960), WR growing in shallower water or saturated sediment produce few, if any, floating leaves; and tend to have "more heavily lignified tissue in the stem." than WR growing in deeper waters. This may be due to earlier, if not immediate, development of the more structurally rigid aerial leaves, and / or development of a more extensive root system in shallower water. Previous studies have concluded that the majority of nitrogen is assimilated as phenology progresses through aerial and into reproductive maturity (Grava and Raisanen 1978; Oelke et al. 1997; Sims et al. 2012a,b). In the Seine River study, WR plants allowed to achieve aerial stage at a constant water depth prior to depth increases had higher survival, growth, biomass accumulation, and reproductive success rates contrasted to floating leaf and submerged stages experiencing the same water depth increases (**Figure 3.7**, **Figure *3.8***, **Figure *3.9***). This could be due to a more well established and extensive root zone that better anchored the plant,

and assimilated nutrient elements, during a potentially high stress event when the plant entered reproductive phenology.

In this study, water depth in the raft deployment location exceeded that which is more conducive to WR growth, and the location was sufficiently remote to reasonably collect and transport site water in sufficient volume for in-lab testing. Furthermore, remoteness precluded use of any powered equipment for flow-through system testing applications. Therefore, *in situ* evaluation of WR responses to site water exposures using raft assemblies was a viable and defensible tactic. Onsite design and build simplified transport and deployment; and the lack of daily maintenance allowed continued deployment in the absence of field personnel. Successful completion of this study was dependent on these benefits of using these raft assemblies throughout the growing season.

Ranking of WR phenological stages in terms of sensitivity to water depth increases is not phenologically progressional. Based on data obtained during the Seine River raft study, the floating leaf stage is the more sensitive, followed by submerged, and finally the aerial stage (less sensitive). Based on these data, use of raft assemblies as described, as an *in-situ* bioassay, may defensibly use development of WR to aerial stage as an endpoint for determining likely reproductive success.

3.4.2 LEGACY-MINE INFLUENCED PITS

Concerns focused on chemical characteristics of water, specifically aqueous sulphate, have dominated recent WR research foci (Fort et al. 2014, 2017; Myrbo et al. 2017; Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020). However, a general lack of *in situ* WR response data to industry-influenced water exposures exists. The Seine River raft study served as a template to obtain these data more time efficiently – to determine the stage following which WR is more likely to continue development to reproductive success. Using similar raft assemblies, Stevenson and Lee (1987) reported that the aerial stage is less sensitive than floating leaf and submerged stages to water depth increases. Data and observations from the Seine River raft bioassay supported the same conclusion.

Based on the Seine River study, a similar raft assembly bioassay was initiated to test *in situ* WR responses to legacy-mine influenced water exposures, with development to aerial phenology as the endpoint. Similar to the Seine River study, remoteness precluded collection of site water of

sufficient volume to complete in-lab bioassay testing as described in Chapter Two (this Dissertation); and onsite personnel completed design and build, which simplified and minimized raft transport and deployment. Although water depth is a primary determinant of typical WR phenology and distribution, water in the selected pits was sufficiently clear to allow WR plant growth at a depth far exceeding that which would result in reproductive success (≥ three meters). However, only WR plants in 20 and 40 cm depth treatments (more appropriate for typical WR phenology) (**Table 3.8**) developed to aerial stage within approximately 47 days (late June 2017), a typical developmental duration within a typical growing season. This indicated that water quality in the three selected pits did not adversely influence typical WR phenology; and that WR in these depth treatments would have likely achieved reproductive success under these conditions. Although WR grew to be 60-90 cm tall in 80 and 160 cm depths, aerial stage was not achieved within a typical duration during this growing season. Visually, WR responded similarly to all sediment formulations used in the mine pit raft bioassay in terms of germination, growth, and development.

This raft style of bioassay allowed measurement of *in situ* responses of WR to these exposures, while also testing the influence of water depth concurrent with water and sediment type. These critical data may be used by industrial water resource managers to more effectively, efficiently, and environmentally plan and schedule flow-rate and volume of aqueous outflows in an effort to manage and preserve receiving system WR populations.

3.5 CONCLUSIONS

Based on data obtained during the Seine River raft study, the more sensitive WR developmental stage to water depth increases was determined to be floating leaf (Raft 2). In general, WR remaining on Raft 2 was smaller, less developed, and less productive than WR on Rafts 1 or 3 following all water depth increases. Wild rice allowed to achieve aerial stage (Raft 1) prior to water depth increases was more likely to re-achieve aerial phenology and continue development to reproductive maturity. Increased and increasing water depth at more sensitive developmental stages (e.g., floating leaf and submerged) was more adversely influential on WR survival and development into reproductive maturity. Therefore, WR in aerial phenology at an appropriate water depth is more likely to continue development through reproductive maturity resulting in seed production.

Chemical characteristics of overlying water in each of the three legacy-mine influenced pits chosen for this study did not appear to adversely influence WR germination, growth, or development during this study. This is evidenced by typical WR phenological development in appropriate water depths (20 and 40 cm) into aerial phenology. Based on data and observations obtained during the Seine River raft study, additional development of reproductively mature WR in the 20 and 40 cm depth treatments would have been observed; specifically, in organic sediment, which contained the higher ammonia-nitrogen concentration. WR plants in the 80 and 160 cm depth treatments were not likely to have achieved reproductive maturity during this study. The overall WR growing season duration is not likely sufficient to allow WR plants growing at these depths to achieve reproductive success. Furthermore, the overall plant height would have increased susceptibility to (plant) breakage (Thomas and Stewart 1969).

Under more optimal and stable water depth conditions, the earlier within the growing season WR plants can achieve aerial phenology, the more likely they are to achieve reproductive maturity and success. Therefore, maintaining appropriate water depths for WR growth, development, and maturation within WR areas throughout the growing season is vital to sustaining more stable populations of WR in critical water resources. Under typical growing season conditions, using development of WR into aerial stage as an end point for determining reproductive success is appropriate.

Additionally, the overall value of using this raft-style bioassay for *in-situ* deployment in remote areas, and areas not typically conducive to WR growth and development (deep water; lack of appropriate sediment), specifically in waters which may be discharged, cannot be overstated. Development and use of these raft-style bioassays was critical to obtaining the required exposure-response data in support of the study objectives. Water resource managers require accurate, representative, and defensible data with which to make critical water resource use decisions. Using raft-style bioassays can provide such data in areas not conducive to typical bioassay testing; and can save time, money, and other resources, while providing observational and empirical data of nearly any frequency for use with critical water resource decisions.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the International Joint Commission for providing funding for this project; and the Seine River First Nation and Councilor John Kabatay for the

opportunities provided by working on, and completing, this project. We also acknowledge Johane Joncas, Cassandra Soulias, Greg Vahaaho, Kristi Dysievick, Megan Clark, and all LUEL personnel for assistance with WR planting, raft maintenance, and analytical support.

REFERENCES

Aiken, S.G., Lee, P.F., Punter, D., Stewart, J.M. 1988. Wild rice in Canada. NC Press, Toronto. 130 pp.

Bloom, P., Schumer, H.J., Carson, T. 2001. Factors limiting yield of wild rice in paddies and natural stands. (IN) Minnesota Wild Rice Research 2001. Univ. of Minnesota.

Chambliss, C.E. 1940. The botany and history of *Zizania aquatica* L. ("wild rice"). Journal of the Washington Academy of Sciences 30(5), 185-205.

Counts, R.L. Lee, P.F. 1988. Patterns of variations in Ontario wild rice (*Zizania aquatica* L.) 3. Similarity between source and introduced population pairs under experimental and field conditions. Aquat. Bot. 31, 37-55.

Day, W.R., Lee, P.F. 1990. Ecological relationships of wild rice, *Zizania aquatica*. 9. Production in organic-flocculent sediments. Can. J. Bot. 68(7): 1542–1548. doi:10.1139/b90-197

Hawthorn, W.R., Stewart, J.M. 1970. Epicuticular wax forms on leaf surfaces of *Zizania aquatica*. Can. J. Bot. 48, 201-205.

LaFond-Hudson, S., Johnson, N.W., Dewey, B. 2020. Interactions between sulfide and reproductive phenology of an annual aquatic plant, wild rice (*Zizania palustris*). Aquatic Botany 164, 103230.

LaFond-Hudson, S., Johnson, N.W., Pastor, J., Dewey, B. 2018. Iron sulfide formation on root surfaces controlled by the life cycle of wild rice (*Zizania palustris*). Biogeochemistry. https://doi.org/10.1007/s10533-018-0491-5.

Lakehead University Environmental Laboratory (LUEL). 2020. Lakehead University Environmental Laboratory Parameter List / Reference Methods: Sediment Analysis. Lakehead University, Thunder Bay, Ontario.

Lee, P.F., McNaughton, K.A. 2004. Macrophyte induced microchemical changes in the water column of a northern Boreal Lake. Hydrobiologia 522, 207-220.

Marcum, D.B., Porter, R.A. 2006. Influence of water depth upon plant development, yield and seed size of modern wild rice cultivars. ASA Annual Meeting.

Minnesota Department of Natural Resources (MN DNR). 2008. Natural Wild Rice in Minnesota: A Wild Rive Study document submitted to the Minnesota Legislature by the MN DNR, February 15, 2008.

Moyle, J.B. 1944. Wild rice in Minnesota. J. Wildlf. Manage. 8, 177-184.

Myrbo, A., Swain, E.B., Engstrom, D.R., Coleman Wasik, J., Brenner, J., Dykhuizen Shore, M., Blaha, G. 2017. Sulfide generated by sulfate reduction is a primary controller of the occurrence of wild rice (*Zizania palustris*) in shallow aquatic ecosystems. J. Geophys. Research: Biogeosci. 122, 2736–2753. https://doi.org/10.1002/2017JG003787.

Oelke, E.A., Teynor, T.M., Carter, P.R., Percich, J.A., Noetzel, D.M., Bloom, P.R., Porter, R.A., Schertz, C.E., Boedicker, J.J., Fuller, E.I. 1997 (last update). Alternative Field Crops manual: Wild Rice. UW and UMN Extension Services.

Pace Analytical Laboratories (Pace). 2020. Minnesota Department of Health Environmental Laboratory Accreditation Program Accreditation Certificate. State Lab ID: 027-137-152 (Duluth, Minnesota).

Pace Analytical Laboratories (Pace). 2020. Minnesota Department of Health Environmental Laboratory Accreditation Program Accreditation Certificate. State Lab ID: 027-137-445 (Virginia, Minnesota).

Pastor, J., Dewey, B., Johnson, N.W., Swain, E.B., Monson, P., Peters, E.B., Myrbo, A. 2017. Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments. Ecological Applications 27(1), pp. 321-336.

Pillsbury, R.W., McGuire, M.A. 2009. Factors affecting the distribution of wild rice (*Zizania palustris*) and the associated macrophyte community. Wetlands 29, 724-734.

Stevenson, S.C., Lee, P.F. 1987. Ecological relationships of wild rice, *Zizania aquatica*. 6. The effects of increases in water depth on vegetative and reproductive growth. Canadian Journal of Botany 65, 2128-2132.

Thomas, A.G., Stewart, J.M. 1969. The effect of different water depths on the growth of wild rice. Canadian Journal of Botany 47, 1525-1531.

Tucker, R.C., Zanis, M.J., Emery, N.C., Gibson, K.D. 2011. Effects of water depth and seed provenance on the growth of wild rice (*Zizania palustris* L.). Aquatic Botany 94, 113-118.

Vennum, T. 1988. *Wild rice and the Ojibwe people*. Minnesota Historical Society Press.

Weber, R.P., Simpson, G.M. 1967. Influence of water on wild rice (*Zizania aquatica* L.) grown in a prairie soil. Canadian Journal of Plant Science 47, 657-663.

Weir, C.E., Dale, H.M. 1960. A developmental study of wild rice, *Zizania aquatica* L. Canadian Journal of Botany 38, 719-739.

FIGURES

Figure 3.5. Configuration of raft assemblies used at the Seine River deployment location. Wire-mesh encased quadrats were attached between the Styrofoam floats.

Figure 3.6. Configuration of raft assemblies deployed in pairs in the three legacy-mine influenced pits. No wire-mesh encasements for individual quadrats were used on these rafts.

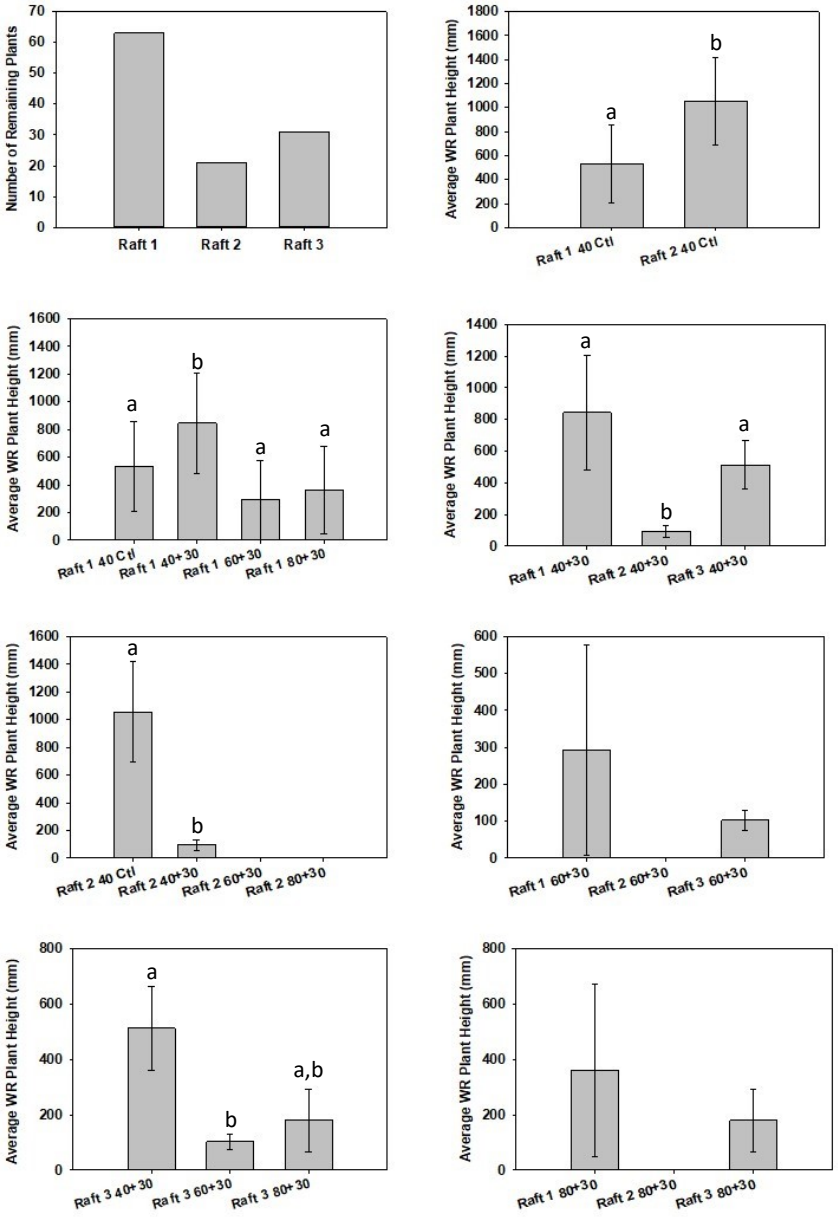

Figure 3.7. Water depth increase developmental stages: Raft 1 – Emergent Stage; Raft 2 – Floating Leaf Stage; Raft 3 – Submerged Stage. Overall surviving plants at harvest, and responses of WR (total plant height) between and within individual raft water depth treatments initiated at specific life stages. Raft 3 40 Ctl plant data were not included due to analytical inconsistencies. Error bars represent one standard deviation. Differing letters (a and b) indicate statistical significance.

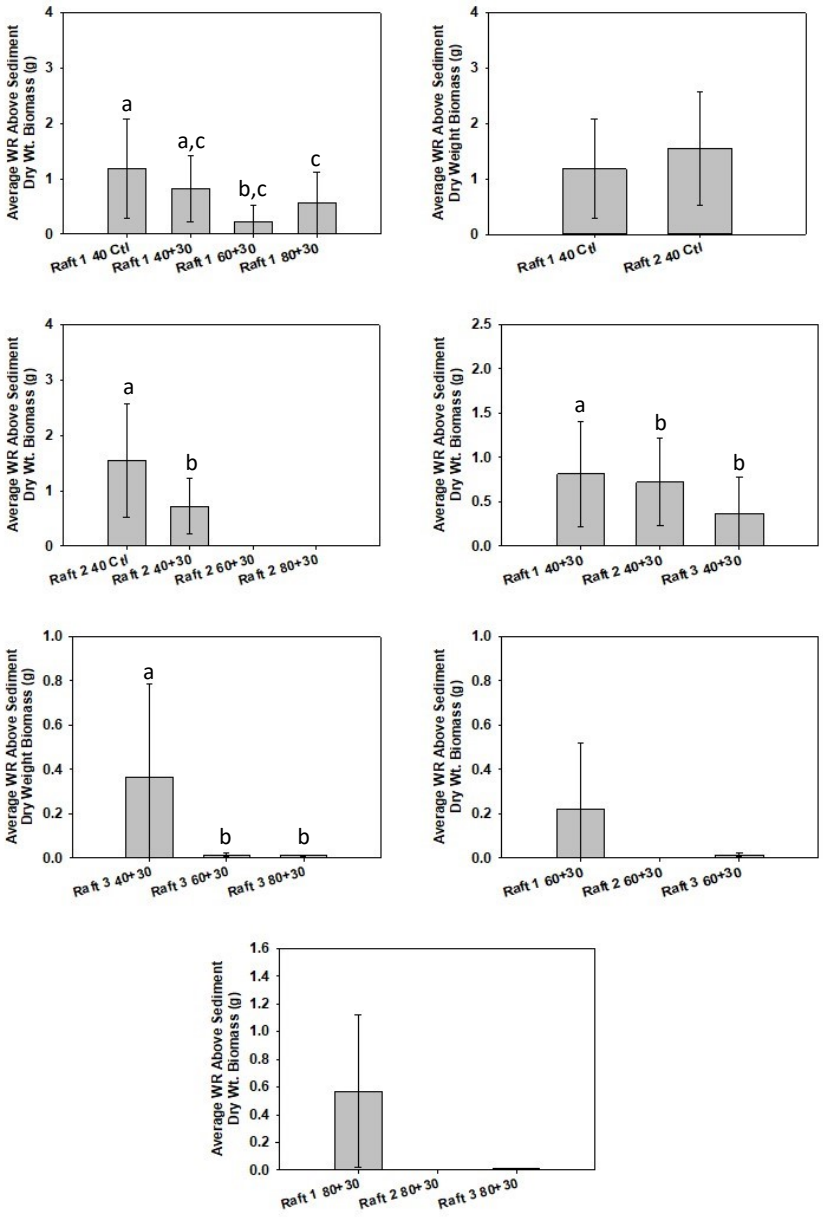

Figure 3.8. Water depth increase developmental stages: Raft 1 – Emergent Stage; Raft 2 – Floating Leaf Stage; Raft 3 – Submerged Stage. Responses of WR (above sediment dry weight biomass) between and within individual raft water depth treatments. Error bars represent one standard deviation. Differing letters (a, b, c) indicate statistical significance.

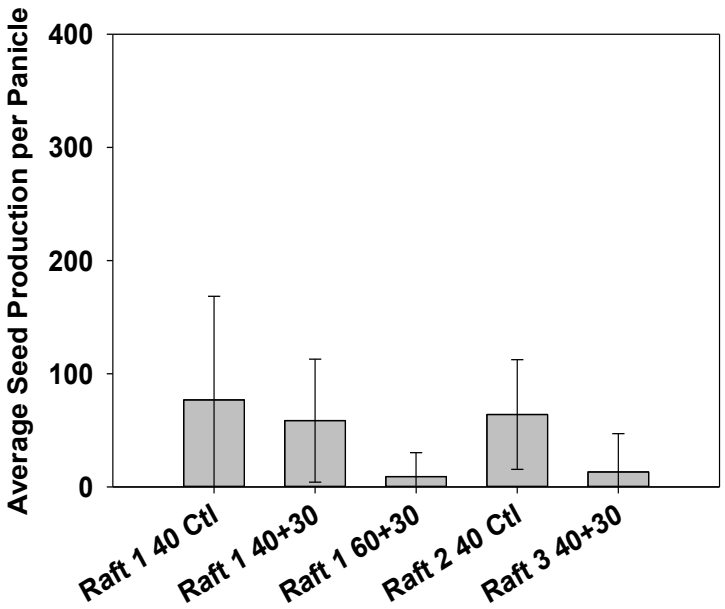

Figure 3.9. Average seed production from raft depth treatments which produced reproductively mature WR plants. No statistical differences were identified between seed productivity of WR plants remaining in these water depth treatments. Error bars represent one standard deviation.

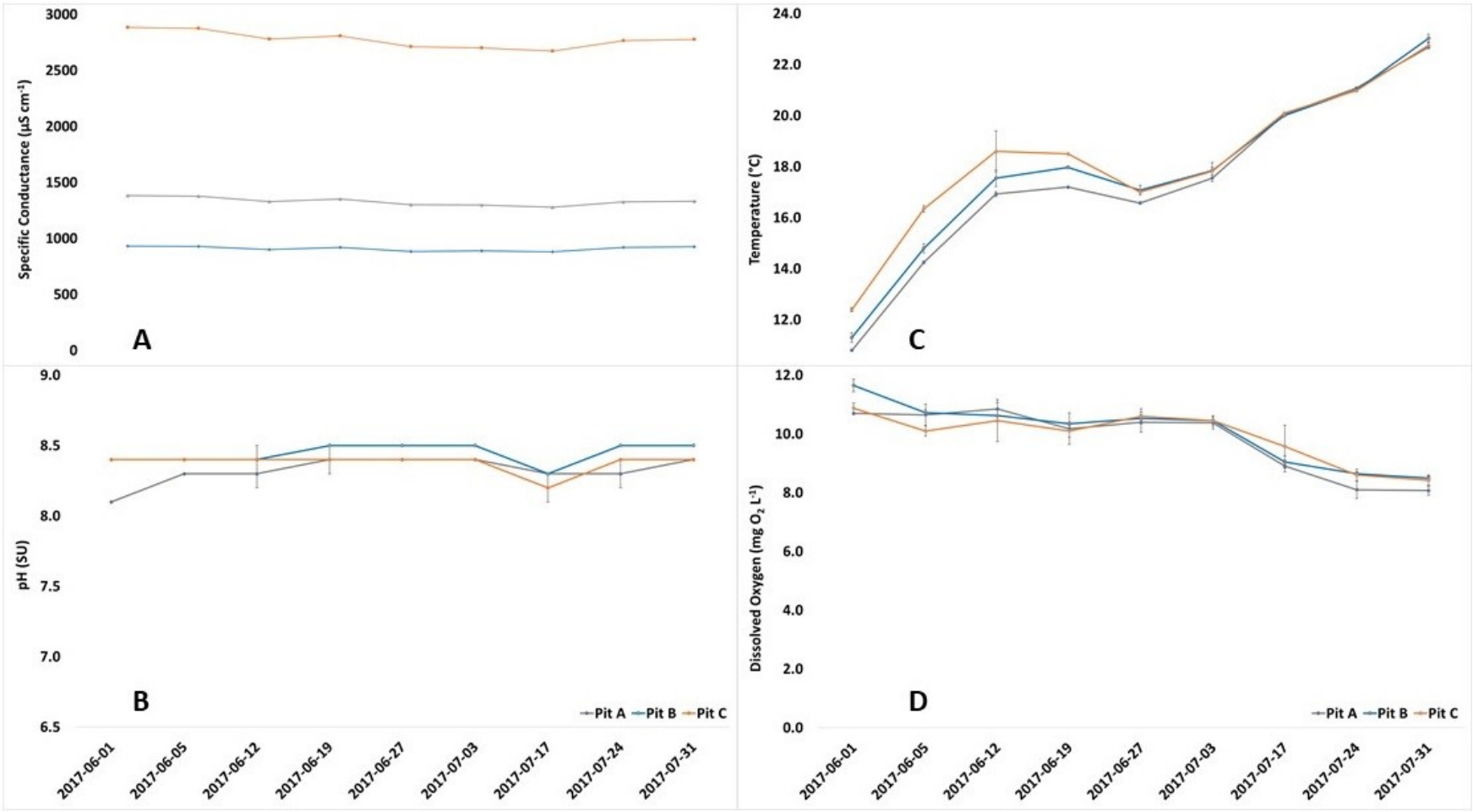

Figure 3.10. Field measurements of **A)** specific conductance; **B)** *p*H; **C)** temperature; and **D)** dissolved oxygen at depths used for the legacy-mine pit raft study. Measurements of these characteristics at each of the four depths within each pit were averaged on each measurement date due to minimal observed variation with depth. Error bars represent one standard deviation; however, not all are visible. The primary difference between these waters is specific conductance as a function of TDS; specifically, aqueous sulphate.

TABLES

Table 3.5. Measured characteristics of black earth used in the Seine River raft study at experiment conclusion. Emergent, floating leaf, and submerged are the WR developmental stages at which water depth increases were initiated (n = 1 for each Depth Treatment). Units for NH_3-N through PO_4 are $\mu g\ g^{-1}$.

	*Depth Treatment (cm)	Conductance ($\mu S\ cm^{-1}$)	*p*H (SU)	NH_3+NH_4-N ($\mu g\ g^{-1}$)	K	PO_4
Raft 1 Emergent Stage	40 Ctl.	816	5.9	35.9	34.0	4.6
	40+30	760	5.9	15.5	37.6	4.7
	60+30	883	6.1	41.2	70.5	8.3
	80+30	745	5.9	17.9	40.9	3.8
Raft 2 Floating Leaf Stage	40 Ctl.	698	5.9	37.5	69.2	5.9
	40+30	571	6.0	17.7	47.9	3.7
	60+30	599	5.9	14.3	39.9	3.4
	80+30	556	5.9	10.8	38.9	2.8
Raft 3 Submerged Stage	40 Ctl.	918	6.0	31.4	65.1	4.0
	40+30	839	6.0	17.5	47.2	5.7
	60+30	787	5.9	19.0	41.7	3.4
	80+30	792	5.9	31.5	59.9	5.2

*All tubs contained black earth soil amended with five (5) grams of slow-release pelletized fertilizer (Smartcote®; 14N:14P:14K).

Table 3.6. 2016-2017 Measured characteristics of water contained within the three legacy-mine influenced pits selected for these raft studies (average ± one standard deviation). Units for Bicarb. Alk. through SO_4 are mg L^{-1}; As and Co are µg L^{-1}.

	Pit A		Pit B		Pit C	
	2016	2017	2016	2017	2016	2017
Bicarb. Alk. (mg L^{-1})	305 ± 34	323 ± 24	311 ± 23	349 ± 19	519 ± 54	643 ± 69
Ca	56 ± 4	54 ± 3	27 ± 1.8	27 ± 2	42 ± 11	49 ± 17
Cl	21 ± 3	20 ± 1	13 ± 1	13 ± 1	9 ± 1	8 ± 2
Total Hardness	744 ± 45	711 ± 44	475 ± 21	445 ± 32	1,914 ± 120	1,826 ± 152
Mg	147 ± 9	140 ± 7	99 ± 5	92 ± 7	439 ± 25	414 ± 31
TDS	928 ± 48	899 ± 53	531 ± 20	553 ± 49	2,189 ± 111	2,261 ± 161
TSS	1.2 ± 0.4	1.2 ± 0.3	1.0 ± 0.1	1.6 ± NC	2.9 ± 1.3	2.0 ± 0.7
SO_4	435 ± 32	420 ± 22	163 ± 9	186 ± 17	1,325 ± 50	1,303 ± 59
As (µg L^{-1})	1.0 ± NC	0.9 ± NC	1.4 ± 0.1	1.4 ± NC	NM	NM
Co	0.11 ± NC	0.13 ± NC	BD	BD	NM	NM

NC = not calculable (n = 1); NM = not measured; BD = below detection.

Table 3.7. Measured characteristics of sediment sampled May 2017; and used in tubs suspended from rafts deployed in legacy-mine influenced pits (n = 1 for each sediment type). Units for NH_3 through Na are µg g^{-1}.

	Sediment Type	
	Sandy	Organic
LOI (%)	2.7	12.3
*p*H (SU)	6.60	6.60
Bulk Density (g cm^{-3})	1.50	0.42
NH_3+NH_4-N (µg g^{-1})	2.60	24.80
PO_4	9.03	5.10
S	166	214
Al	285	357
Cu	2.29	2.06
Fe	881	1,819
Mn	49	267
Zn	2.64	6.95
Ca	1,382	3,235
K	90	86
Mg	425	1,246
Na	33	23

Table 3.8. Wild rice development in rafts deployed in the three legacy-mine influenced pits at 20 and 40 cm depth treatments, regardless of sediment type or formulation. Adapted from Oelke et al. (1982).

Stages of Wild Rice Development		*Date	**Growth Days	Year Day
Vegetative Growth Phase ↓	Planting	May 11-12	0	131-132
	Germination	May 22, 2017	11	142
	Emergence	June 12, 2017	32	163
	Floating Leaf	June 15, 2017	35	166
	Aerial Leaf	June 27, 2017	47	178

* Dates on which these developmental stages were observed during weekly inspections.
** Based on observation of developmental stage. Achieving specific developmental stages would have preceded the date on which it was observed.

CHAPTER FOUR: DEVELOPMENT AND USE OF AN *IN-SITU* FLOW-THROUGH PADDY-SCALE BIOASSAY FOR EXPOSURES OF WILD RICE (*ZIZANIA PALUSTRIS* L.) TO SITE-SPECIFIC WATERS

ABSTRACT

Wild rice (*Zizania palustris* L.; WR) has recently been used in multiple laboratory- and mesocosm- scale studies focused on measuring and responses of WR to exposures of aqueous sulphate and hydrogen sulphide (H_2S). For the current study, two flow-through paddies were constructed adjacent to separate legacy-mine influenced mine pits (Pit A, Pit C), which have partially filled with water. These pits are not hydraulically connected; however, each contains water with an aqueous sulphate (SO_4) concentration previously concluded to be detrimental to WR phenology: approximately 350 mg L^{-1} (Pit A), and approximately 1,300 mg L^{-1} (Pit C). Sediment for each paddy was obtained from a nearby creek; and paddy inflow water was pumped directly from the adjacent pit. WR seed was broadcast at a rate of 28 Kg ha^{-1} when initiated (May 2017 for Pit A; May 2018 for Pit C). Pit A paddy received an additional 0.25 Kg wet wt. seed broadcast during October 2017. No additional seed was broadcast in the Pit C paddy. Pore water H_2S varied between < 0.078-3.07 mg L^{-1} within the Pit A paddy; and from < 0.078-23.5 mg L^{-1} within the Pit C paddy. Over the course of three growing seasons (2017-2019) for the Pit A paddy, initial WR stem density significantly increased from approximately 60-70 to > 400 m^{-2}. No statistical decreases in stem height or dry weight biomass (DWB) were observed between growing seasons; average seeds per panicle statistically increased in 2019; and seed DWB remained statistically non-changed. Seed nutrient element characteristics between growing seasons were similar, and did not indicate adverse influences from Pit A water exposures. Over the course of two growing seasons in the Pit C paddy (2018-2019), initial WR stem density remained statistically non-changed; however, due to repeated extensive goose herbivory during July-August 2019 average stem height and DWB (2018 = approximately 140 cm, 6.0g; 2019 = approximately 60 cm, 0.5g), and average seeds per panicle [approximately 50 (2018) vs. 20 (2019)] significantly decreased. The average 2019 Pit C paddy seed DWB was contrasted to 2017 and 2019 Pit A Seed DWB. The average 2019 Pit C seed DWB was statistically less than the 2017 Pit A seed DWB. Regardless of inflow water characteristics, or other plant tissue characteristics, and with the exception of zinc which was 2.5x higher in Pit A WR seeds, inter-

and intra- paddy seed chemical characteristics were similar between growing seasons. Despite conditions within each paddy potentially conducive to iron sulphide root coating formation, this was neither visually observed nor identified via SEM-EDX characterization on WR harvested from either paddy during July 2018. Overall, no adverse WR responses observed or measured throughout the course of this study resulted from Pit A or Pit C water, or water-related, exposures. Based on data and observations obtained during this study, aqueous SO_4 and pore water H_2S do not appear to adversely influence WR phenology, density, or productivity.

4.1 INTRODUCTION

Early research describing wild rice (*Zizania palustris* L.; WR) distribution and density in relation to water chemistry in Minnesota was completed by Moyle (1944; 1945; 1956). Moyle suggested that WR was primarily found in waters with a total alkalinity less than 40 mg L^{-1}, *p*H between 6.8-7.0 SU, and a sulphate concentration of less than 10 mg L^{-1}. However, WR was also observed growing in waters with an aqueous sulphate concentration exceeding 200 mg L^{-1} (Moyle 1945). Moyle's observational work has led to a number of studies with the overall objective of more accurate and thorough discernment any influence, whether beneficial or adverse, on WR growth, development, and reproductive potential resulting from increased or increasing aqueous sulphate exposure(s). Paulishyn and Stewart (1970) reported WR growing in Manitoba in waters with sulphate ranging from 2-170 mg L^{-1}. Rogalsky et al. (1971) advised that a concentration of 200 mg L^{-1} sulphate was acceptable for WR paddies. In a study in the Mississippi River in Minnesota, Lee and Stewart (1981) showed that WR grew well in waters with levels of 30 mg L^{-1} sulphate; and that sulphate in the water seasonally varied (5-120 mg L^{-1}) at one sampling location. In a comprehensive field study completed by the Minnesota Pollution Control Agency (MPCA) during 2011-2013, wild rice was observed growing in surface waters ranging from < 0.5-838 mg L^{-1} sulphate.

Controlled experiments that measured influences of sulphate have also been completed. The hydroponic solution recommended by Malvich and Percich (1993) uses a sulphate concentration of 48 mg SO_4 L^{-1}. Using this culture medium, Lee and Hughes (2000) found that early WR development was adversely influenced at sulphate concentrations ranging from 1,200-1,500 mg SO_4 L^{-1}. Vicario and Halstead (1968) conducted experiments with rice in culture solutions with sulphate that ranged from 0 to 8,800 mg SO_4 L^{-1}. They observed decreases in weight and height when sulphate in the culture media exceeded 220 mg SO_4 L^{-1}. Fort et al. (2014) concluded that under laboratory hydroponic test conditions WR seeds would germinate and develop into seedlings in 5,000 mg SO_4 L^{-1}, with the overall conclusion that any observed WR adverse responses were more likely due to osmotic pressures than sulphate exposure. A current debate has been initiated that adverse influences to WR are not necessarily due to aqueous sulphate, but rather H_2S in the WR rhizosphere. Grava and Rose (1975) suggested that H_2S could form in WR paddies and adversely affect WR when sulphate was added as a fertilizer as ammonium sulphate, or as an algicide as copper sulphate (Grava 1977).

Other water quality characteristics have also been described for waters in which WR grows. In a survey of WR lakes in Minnesota and Ontario, Lee (1979) observed that most lakes supporting WR had softer water with average alkalinities of 40 mg L^{-1}, and *p*H levels of 6.9. Pip (1984) examined the distribution of 59 species of aquatic macrophytes, including WR, outside and inside the Precambrian shield of central Canada. She detailed the more important chemical water characteristics associated with WR distribution to be *p*H, total dissolved solids, and total alkalinity. Chloride, phosphorus, and sulphate concentrations were reported as of minor importance in both areas. Pillsbury and McGuire (2009) attributed losses of WR in Minnesota and Wisconsin to residential and agricultural developments that increased nutrient element levels. Ammonia and pH changes were specifically implicated. Decreased WR range has been attributed to multiple sources of human disturbance (Bennet et al. 2000; Meeker 1996). Jorgenson et al. (2013) showed that WR could grow in both eutrophic waters with seasonal total phosphorus concentrations reaching 1,500 µg L^{-1}, and non-eutrophic waters with total phosphorus concentrations of 170 µg L^{-1}. Although WR distribution may be at least correlated to water chemistry, WR also influences water chemistry in which it lives. Lee and McNaughton (2004) showed that water surrounding WR stands contained lower sulphur, and higher conductivity, calcium, and iron concentrations than open water areas. Conclusions in more recent studies suggest that total aqueous sulphate may not be a primary chemical characteristic adversely influencing WR plant development; rather, sediment-associated H_2S may be more influential on WR plant development in some areas (Myrbo et al. 2017; Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020).

One proposed approach to help protect WR is to apply a site-specific calculation-based limit on sulphate concentrations in aqueous discharges which, based on more recent data, would be less likely to result in adverse influences on WR (MPCA, March 24, 2015). The two variables included in the proposed equation(s) are sediment total organic carbon (TOC), and sediment extractable iron – a more loosely bound fraction of iron theorized to be more available for complexation with sulphide to form iron sulphide, a more insoluble form of both sulphur and iron. The reasons for including these two sediment characteristics were that it was theorized that the higher the sediment organic carbon concentration, the higher the rate of microbially facilitated sulphur (as sulphate) conversion to sulphide; and the higher the extractable iron concentration, the higher the potential to complex with sulphide as H_2S into less soluble iron

sulphide, and therefore mitigate potential H_2S toxicity (Lamers et al. 2013) to WR. This approach to site-specific water quality standard (WQS) determination also resulted in calculation of a suggested sediment H_2S protective level of 0.165 mg L^{-1} for WR. Sediment TOC and extractable iron function antagonistically re: the sediment pore water H_2S concentration – the lower the sediment TOC and the higher the sediment extractable iron, the less H_2S will be present in pore water. Fort et al. (2017) exposed WR seeds to varying concentrations of sulphide (sodium sulphide) concurrent with variable concentrations of iron (ferric chloride). Overall observations from this study indicated a mitigatory influence from iron on adverse WR responses to these sulphide-iron exposures. Under field conditions, iron available for complexation with sulphide is more likely to result in a decreased, if not mitigated, adverse influence on WR development in WR areas receiving inputs of water with an increased aqueous sulphate concentration. This particular association between overlying-water sulphate concentration, sediment pore water H_2S concentration, and adverse WR responses has not been specifically supported under larger-scale, flow-through exposure conditions more representative of natural WR areas. Efforts to apply these calculation-based site-specific WQS are not currently pursued in part due to lack of supporting field evidence for their veracity. However, transforming sulphide into iron sulphide (FeS) may not eliminate its risk to WR. Based on inflow water characteristics to each paddy, development of iron-containing coatings on WR roots appeared possible. In a study of WR from wetlands in Ontario, Jorgenson et al. (2013) measured iron (III) oxide / hydroxide coatings on WR roots using scanning electron microscopy (SEM) and energy-dispersive x-ray (EDX) techniques. In a bucket-style mesocosm study in Minnesota, LaFond-Hudson et al. (2018, 2020) inferred FeS coatings on WR roots, which ultimately may have resulted in decreased plant height, dry weight biomass, and seed characteristics including overall per-plant seed production. However, other more critical physical water resource characteristics conducive to WR growth such as an appropriate water depth have been more extensively studied and reported (Stevenson and Lee 1987; Marcum and Porter 2006; MN DNR 2008; Tucker et al. 2011; Dysievick 2019; Tedrow 2020).

Weir and Dale (1960) describe the phenological development of WR and its relationship to water depth. Three forms of leaves develop – submerged, floating, and aerial – which differ in anatomical characteristics likely related to their physiological environment. The submerged leaves are thin and lack a cuticle, the outer waxy covering present on leaves of terrestrial plants;

and no stomata are present for gas exchange. These leaves also have abundant air passages at this time which are known as lacunae, and greatly reduced conducting / vascular tissue which transports both water and carbohydrates. In late May to early June, the floating leaves appear. These differ from the submerged leaves by having a cuticle with stomata on the upper surface of the leaf and a well-developed mid vein on the lower surface, which does not have a cuticle (Hawthorn and Stewart 1970). Most biomass production occurs in the aerial stage of development. The ratio of root:shoot biomass also increases at this stage of development as the plant becomes better anchored (Thomas and Stewart 1969). Another occurrence at this stage is that the shoot apex (vertical growing tip) changes from vegetative to reproductive growth, triggering the plant to begin seed formation (Weir and Dale 1960). The timing for this event varies depending on the environmental characteristics (water depth, nutrient availability) of the individual sites and the depth tolerance of the seed source involved (Counts and Lee 1988). Timing of this developmental change may be delayed depending on how early in the growing season the plant was able to begin floating leaf and aerial stage growth.

In a 2017 (growing season) study using *in situ* raft assemblies to expose WR to legacy-mine influenced pit water (Tedrow 2020), WR plants were able to achieve aerial stage development in 20 and 40 cm depth treatments; and plants at 80 and 160 cm depths grew to 60-90 cm in height, but did not achieve floating leaf stage prior to study conclusion in July 2017. Pit water was sufficiently clear to allow WR plant growth at 80 and 160 cm depths, but plants would not have achieved reproductive maturity prior to the end of the growing season. Water characteristics, specifically aqueous sulphate concentrations, of legacy-mine influence in the selected pits did not appear to adversely influence WR phenology, growth, or density.

During May 2017 and May 2018, flow-through WR paddies were constructed adjacent to two of the legacy-mine influenced pits in Minnesota used in the raft study (Pit A and Pit C) in an effort to better represent conditions under which WR may be exposed to legacy-mine influenced waters. Water contained within the two pits is sufficiently different to encompass a range of aqueous sulphate **[**approximately 350 mg L^{-1} (Pit A) to 1,300 mg L^{-1} (Pit C)**]** theorized to be adversely influential on overall WR phenology and reproductive potential. In both the raft bioassay and this paddy bioassay, water depth and competing vegetation were controlled to eliminate these as adverse influence variables (Stevenson and Lee 1987; Elakovich and Wooten

1989; Quayyum et al. 1999). More accurately representing characteristics of natural WR areas was critical to increasing the understanding of responses of WR to exposures of waters containing concentrations of SO_4 previously suggested as adversely influential to (WR) phenology, distribution, and productivity. Use of these larger-scale, *in-situ*, flow-through systems throughout multiple, successive growing seasons allowed detailed study of how WR is more likely to respond to these types of exposures under field conditions.

Specific objectives were to **1)** observe WR phenological responses to exposures of Pit A and Pit C water; **2)** measure responses of WR to these waters in terms of plant height, dry weight biomass, and seed productivity; and **3)** measure specific chemical characteristics of harvested plant and seed samples. In addition to focusing on these objectives, WR was harvested in July 2018 from each paddy for the purpose of characterization of any iron-containing coatings on roots. Concurrent with these objectives overlying water, sediment, and sediment pore water samples were characterized in an effort to better identify any constituents which may have contributed to any observed or measured adverse WR responses to Pit A and / or Pit C water exposures.

4.2 MATERIALS AND METHODS

Two specific legacy-mine influenced pits were chosen for this study. With the exception of aqueous sulphate and associated characteristics (i.e., hardness, alkalinity, total dissolved solids, conductance), Pit A and Pit C waters are similar based on existing data. The method used to measure how WR responds to exposures of these waters was designed as a flow-through rice paddy immediately adjacent to each pit. These were designed to mimic a nearby creek flow regime with the overall objective of measuring potential influences from water, sediment, and pore water characteristics on WR density, development, productivity, and chemical characteristics of plant and seed tissues grown in each paddy.

Source sediment used in each paddy was obtained from a nearby creek. Sediment from this location was generally high in organic content [12.26% loss on ignition (LOI)]; and available forms of nutrient elements such as ammonia-N (24.80 $\mu g\ g^{-1}$), PO_4-phosphorus (5.09 $\mu g\ g^{-1}$), and potassium (86.06 $\mu g\ g^{-1}$). Concentrations of other macro- and micro- nutrients were typical of sediment producing yearly harvestable densities of WR (Day and Lee 1989, 1990).

4.2.1 PADDY DESIGN, AND INFLOW / OUTFLOW AND SEDIMENT SAMPLING

During April-May 2017, an approximate 55 m^2 paddy was constructed adjacent to Pit A. Pit A was selected as the water source due to its specific water characteristics and ease of access. The paddy was constructed using onsite materials as impermeable 90 cm tall perimeter berms, with an impermeable liner as the base. Sediment and water depth at initial seed distribution were approximately 25 and 20 cm, respectively. Source seeds were harvested from lakes in northern Minnesota. Seeds were broadcast at a rate of approximately 56 Kg per ha (1.8 Kg wet weight). Additional seed was distributed (0.45 Kg) throughout this paddy during October 2017. This paddy was visited approximately weekly throughout the 2017-2019 growing seasons. **Figure 4.11** shows typical temporal Pit A paddy observations during the 2017-2019 growing seasons.

During April-May 2018, an approximate 150 m^2 paddy was constructed adjacent to Pit C. Pit C was selected for the same two reasons as Pit A. Onsite materials were used to form perimeter berms, with an impermeable liner as the base. Sediment and water depth at initial seed distribution were approximately 50 and 25 cm, respectively. Source seeds were harvested from lakes in northern Minnesota, and were broadcast at a rate of approximately 28 Kg per ha (2.3 Kg wet weight). This system was visited approximately weekly throughout the 2018-2019 growing seasons. **Figure 4.12** shows typical temporal Pit C paddy observations during the 2018-2019 growing seasons.

In an effort to more closely mimic a natural WR area, neither of these paddies were drained at the end of, or between, growing seasons. Rather, inflow water from their respective pits was maintained until freeze-up, and was initiated as early as possible each spring; typically, late-March / early-April. Additionally, no fertilizer was amended to sediment used in either paddy. The planted area of each paddy was delineated into one-square-meter quadrats for randomized sediment, plant, and seed sampling purposes. Water was directly sampled from the inflow and outflow of each paddy, stored on ice, and delivered to Pace Analytical Laboratories (Pace) in Virginia, Minnesota, for characterization when WR germination was first observed, and at the time of final harvest. Sediment samples from the top 10 cm of each paddy were obtained from randomly selected quadrats using a 4.8 cm internal diameter sediment core sleeve. All sediment samples were frozen and delivered to the Lakehead University Environmental Laboratory (LUEL) for characterization.

4.2.2 PORE WATER SAMPLING

Pore water samples were obtained approximately every 30 days from inflow, middle, and outflow areas within each paddy throughout each growing season of this study. Three specific pore water sampling methods were used during this study: traditional peepers, Rhizons, and a modified peeper design. All pore water representation and direct aspiration samples were stored on ice and delivered to Pace for characterization.

Traditional peepers were designed as 50 mL centrifuge tubes filled (no headspace) with double-distilled water, with a 0.45 µM pore-size filter sealed into the centrifuge tube cap (Jorgenson 2013). These rely on diffusive processes to capture a representation of dissolved pore water characteristics. Rhizons are filter assemblies (0.12-0.18 µM pore-size) designed to be inserted into the sediment and connected to a vacuum device (syringe or vacuum tube / bottle). Pore water is directly aspirated (or transferred) into the sample container protected from atmospheric exposure. All traditional peepers and Rhizon assemblies were deployed at a 10 cm sediment depth. Sodium hydroxide / zinc acetate was used as the preservative for H_2S samples. Field measurements of *p*H, dissolved oxygen, temperature, and specific conductance were obtained immediately prior to pore water sampling events from at least the inflow and outflow of each paddy using a calibrated YSI® ProPlus® or a Hach® MS5 HydroLab®.

Initially for the Pit A paddy study, Rhizon filter assemblies were attached to the traditional peeper tube with an aspiration tube connected and anchored on the paddy berm. Pore water was aspirated through the Rhizon tubing, into the sample container, immediately prior to traditional peeper retrieval. New Rhizon assemblies were used for each traditional peeper re-deployment. This avoided sediment disturbance immediately prior to pore water aspiration. Due to Rhizon filter pore size (0.12-0.18 µM) limitations, clogging was an observed problem. Periodically, this resulted in lower than desired / needed sample volume, and use of more intense vacuum than desired to obtain pore water samples.

In an effort to avoid difficulties associated with Rhizons, a modified peeper design was developed for use in 2019. This modified peeper was constructed using a 50 mL centrifuge tube filled with double-distilled water (no headspace), with an in-cap 0.45 µM pore-size filter. A luer-lock valve was sealed into the beveled end of the tube. Tubing attached to this luer lock valve was anchored on the paddy berm. This modified peeper was deployed within one meter of the

traditional peeper in each paddy area at a 10 cm sediment depth. Following removal of one tubing volume (20 mLs), 50 mLs were aspirated for H_2S measurement at the time of traditional peeper retrieval after the 30-day deployment. Modified peepers remained in position throughout their use in an effort to avoid sediment disturbance and possible atmospheric contamination of sediment and pore water around the modified peeper. Rhizon use for direct aspiration of pore water was discontinued following development of modified peepers.

A method of pore water sampling was also tested which involved obtaining a sediment core inside a 4.8 cm internal diameter plastic core sleeve. Sediment cores from the Pit A paddy were obtained; each core contained approximately 20-25 cm of sediment. Overlying water was removed from within the core sleeve. A new Rhizon filter was slowly inserted into the top 10 cm of sediment. Pore water was directly aspirated into an evacuated sample container containing the sodium hydroxide / zinc acetate preservative. This method was only briefly used during 2018 for pore water sampling in the Pit A paddy; and was discontinued due to an increased likelihood of porewater atmospheric contamination via sediment disturbance as a result of inserting the Rhizon and overlying water removal, and difficulties associated with filter clogging.

4.2.3 Plant Tissue and Seed Sampling

Groups of plants were sampled from randomly selected quadrats in the Pit A paddy during 2018 and 2019, and the stem count was completed in a 0.1 m^2 area inside each selected quadrat. Stem density per square meter was calculated from the counted 0.1 m^2 area. Stem density in the Pit C paddy was approximately 10x less than the Pit A paddy; therefore, all individual stems were counted, and random plants were sampled, from randomly selected quadrats in the Pit C paddy during 2018 and 2019. However, due to repeated extensive goose herbivory in the Pit C paddy during 2019, plants were sampled on August 01, and seeds were sampled on August 23, 2019.

With the exception of 2019 Pit C plant and seed sampling, multiple WR panicles were inspected prior to each harvest event to verify seeds had been filled. Panicles were obtained at the conclusion of all growing seasons from each paddy from plants in randomly selected quadrats to measure seed production. Filled seeds were harvested from randomly selected plants in randomly selected quadrats within the Pit A paddy in 2017. Plant tissue and seed samples were obtained from each paddy following the 2018 and 2019 growing seasons.

On July 10, 2018, during the early flowering stage, whole WR plants (including roots) were harvested from randomly selected quadrats within each paddy. Roots were rinsed in paddy surface water to remove sediment and other materials. Plants were stored refrigerated in Ziploc® bags, transported to LUEL, and prepared for SEM and EDX characterization. Scanning electron micrographs were obtained from root surface areas visually suspect of 1) typical root tissue; and 2) iron-containing coatings [Fe-O(OH) and FeS]. SEM-EDX characterization was completed as described in Jorgenson et al. (2013). Points for EDX characterization were chosen based on visual appearance and SEM imagery of the WR root surface.

4.2.4 LABORATORY ANALYTICAL METHODS

Quality controls for water samples characterized by Pace Labs included reference standards, matrix spikes, and conformed to The National Environmental Laboratory Accreditation Conference (NELAC) Standards and the laboratory's Quality Assurance Manual, where applicable. Surface water and sediment pore water samples were transported to Pace for characterization. Analytical methods for water and pore water followed those described in Pace (2020a,b). Specifically, Al, B, Ca, Fe, Mg, Mn, and Na were measured using ICP (EPA 200.7); and Cu was measured using ICP-MS (EPA 200.8) following acidification to *p*H < 2.0 using trace metal grade HNO_3. Additionally, one mL of concentrated trace metal grade $HNO_3$and 0.5 mL of concentrated trace metal grade HCl were added to 50 mL of sample. Acidified samples were heated to approximately 95°C for four to five hours. After heating, samples were brought to a 50 mL volume with double-deionized water, gently inverted, and allowed to settle overnight prior to analysis. Chloride (Cl) and SO_4 were separated from non-chemically-preserved samples through a series of ion-selective columns, and measured using IC (method 300.0). Samples for sulfide (as H_2S) were preserved using sodium hydroxide / zinc acetate. Sulfide was measured according to method 4500-S2-G.

Quality controls for sediment, plant, and seed samples characterized by the Lakehead University Environmental Laboratory (LUEL) included reference standards and replicate samples, and followed procedures required by the Canadian Association of Laboratory Accreditation (CALA). Prior to transport to LUEL, all sediment samples were frozen, plant tissues were dried and pulverized (shoots separated from roots), and seed samples were dried. Preparation and analytical procedures for sediment, plant, and seed samples followed those described in Lee and

McNaughton (2004), the LUEL Quality Assurance Manual, and reference Forest Canada, ASTM, and USEPA methods. Total nitrogen (when possible) was measured on dry sediment using a combustion ELVario cube carbon-hydrogen-nitrogen-sulphur (CHNS) analyzer. LOI was measured by drying 20 mL of sediment at 80°C, weighing, ashing at 600°C, and re-weighing. Sediment *p*H was measured as a 1:1 sediment:deionized water mixture using a Mettler Toledo meter with an InLinePro *p*H sensor. Bulk density was measured by weighing 20mL of wet sediment, drying at 80°C, and re-weighing. Phosphate was determined using the BRAY P2 method – Al and Fe phosphates are dissolved in ammonium fluoride, and measured colorimetrically using a SKALAR analyzer. Al, Cu, Fe, Mn, S, and Zn were extracted in 0.1 N trace metal grade HCl, while Ca, K, Mg, and Na were extracted in ammonium-acetate at *p*H 7.0; all of which were measured using ICP. Sediment samples were processed as wet samples to better represent field conditions. Measured sediment analyte concentrations were corrected for bulk density.

All plant and seed samples were stored refrigerated in Ziploc® bags, and delivered to LUEL for processing immediately after harvest. Analytical methods for sediment, plant, and seed samples followed those described in Lee and McNaughton (2004) and the LUEL Quality Manual. WR plants and seeds were additionally dried at 50°C and kept frozen until processing. Analyses were based on those described by the American Soil and Plant Council. Plant, or seed, biomass (≥ 0.5g) was digested using a CEM Mars Express microwave in Express Teflon closed vessels in one mL of HNO_3 and three mLs of trace metal grade HCl, and diluted using 25mL of deionized water. Al, Ca, Cu, Fe, Mg, Mn, Na, S, and Zn in this dilution were measured using a Varian ICP-AES. Total nitrogen and phosphorus were digested using trace metal grade HNO_3 / HPO_4 in a stepwise process at 400°C, and measured using the SKALAR analyzer.

4.2.5 STATISTICAL ANALYSES

All surface water, sediment, WR plant and seed, and pore water data were organized using Microsoft® Excel® or SigmaPlot-SigmaStat® v14.0 (Systat Software, Inc.) for table and graphical representation. Statistical analyses and treatment of WR plant data were completed using SigmaPlot-SigmaStat® v14.0. t-Tests or one-way analysis of variance (ANOVA) were used to compare two or more groups; specifically, WR responses to sediment exposures measured as stem density, stem height, shoot dry weight biomass, seed production, and seed dry weight

biomass. Holm-Sidak multiple comparison was used to discern significant differences between three or more groups if data met of normality and variance. Data were natural logarithmic transformed if normality of distribution and / or variance equality assumptions were violated. If data transformation failed to correct non-normal distribution and / or variance equality, raw data were used for statistical treatment, followed by ANOVA on ranks between three or more groups and Dunn's multiple comparison to discern significant differences between groups. A Mann-Whitney Rank Sum test was used to discern significant differences between groups used for t-test comparisons when data were not corrected for normality of distribution or variance equality through natural logarithmic transformation. Non-transformed data were used for table and figure display purposes.

4.3 RESULTS

4.3.1 PIT A PADDY WATER, SEDIMENT, AND PORE WATER CHARACTERISTICS

Characteristics of inflow and outflow water during 2017-2019 are detailed in **Table 4.9**, which remained consistent during the 2017-2019 growing seasons. In particular, aqueous sulphate, calcium, and magnesium remained similar between and during the 2017-2019 growing seasons, and between inflow and outflow from this paddy. With the exceptions of ammonia-nitrogen, iron, and sulphur, sediment characteristics remained similar between growing seasons (**Table 4.10**). Pore water representations and samples obtained during the 2017-2019 growing seasons are detailed in **Table 4.11**, **Table *4.12***, and **Table *4.13***. Substantial variability was observed between measured traditional peeper and Rhizon H_2S concentrations; pore water H_2S concentrations ranged from < 0.078-3.04 mg L^{-1} in Pit A traditional peeper samples; from 0.135-3.07 mg L^{-1} in Pit A Rhizon samples; and from 0.334-12.1 mg L^{-1} in Pit A modified peeper samples. Importantly, measured concentrations of H_2S exceeded the suggested 0.165 mg L^{-1} protective level for WR in nearly all pore water samples; frequently, H_2S concentrations were several times, if not orders of magnitude, higher. On only four occasions in 2017, and five occasions in 2018 were H_2S concentrations below this suggested protective level; all of which tended to occur early or late in the growing season. Little to no variability within field measurements of *p*H, dissolved oxygen, temperature, and specific conductance was documented within this paddy during the 2017-2019 growing seasons.

4.3.2 PIT A PADDY PLANT PHENOLOGY AND PRODUCTIVITY

Documentation of WR plant phenological development was similar between 2017-2019 growing seasons. **Figure 4.11** shows WR phenological stages during 2017, and is very similar to temporal phenological development during 2018 and 2019; dates of observed WR phenology during 2017, 2018, and 2019 growing seasons are listed in **Table 4.14**. Additionally, 2017, 2018, and 2019 plant and seed harvest dates were within two weeks of each other during August indicating no adverse influences on WR temporal phenological development from Pit A water-associated exposures under these conditions.

Similar to observations during the 2018 growing season, no WR plants were observed with tillers in 2019. Also similar to the 2017 and 2018 growing seasons, no evidence of WR plant diseases was observed during the 2019 growing season despite a continued plant density of approximately 400 m^{-2}. However, some yellow discoloration of WR plant leaves was observed during the 2019 growing season, indicating a potential deficiency of one or more nutrient elements (Day and Lee 1990). In contrast, also during 2019, in one documented area of lower stem density (approx. 25 m^{-2}), WR plants generally appeared healthier and were visibly taller.

One-way ANOVAs were used to discern differences between stem density, height, dry weight biomass, and seed production. Wild rice stem density throughout the paddy differed significantly between growing seasons ($F_{(2,40)} = 365.362$ $p < 0.001$) (**Figure 4.13**). Specifically, stem density increased by approximately 10x between 2017-2018, and remained at this density through 2019. Paired comparison tests indicated that, similar to the 2018 growing season, the average 2019 stem density was statistically higher than in 2017 ($p < 0.001$), but was not significantly different from 2018 ($p = 0.106$). Average 2018 stem heights were significantly higher ($F_{(2,68)} = 8.560$ $p < 0.001$) than both 2017 ($p = 0.016$) and 2019 ($p = < 0.001$). However, t-test indicated no statistical difference was identified between average 2018 and 2019 WR plant dry weight biomass ($t_{(21)} = -1.876$ $p = 0.075$) (**Figure 4.13**). No statistical difference ($F_{(2,26)} = 5.615$ $p = 0.010$) was identified in average seed production per panicle between 2017 and 2018 ($p = 1.000$), despite the 10x increase in stem density. However, paired comparison tests indicated that the average number of seeds per panicle during 2019 was significantly higher than in 2017 ($p = 0.046$) and 2018 ($p = 0.030$) (**Figure 4.13**). t-Tests indicated no statistical difference between seed dry weight biomass between 2017 and 2019 growing seasons ($t_{(13)} = 1.467$ $p = 0.166$).

4.3.3 PIT A PADDY PLANT AND SEED CHARACTERISTICS

Chemical characteristics of WR plant and seed tissue are listed in **Table 4.15**. With the exception of calcium and zinc, which were higher in plants harvested in 2019 than 2018, chemical characteristics of WR between these growing seasons were similar; specifically, measured concentrations of total plant nitrogen averaged 0.74% (± 0.10) for each season. Percent nitrogen and sulphur content of seeds harvested during 2017 averaged 1.24 (± 0.12) and 0.12 (± 0.013), respectively. During 2019, plant tissue and seed samples were concurrently processed and characterized. The average percent nitrogen content of seeds harvested in 2019 increased to 1.55 (± 0.05); the average percent sulphur content remained at 0.12 (± 0.004) (**Table 4.15**). Regardless of surface water, sediment, pore water, or plant tissue characteristics, seed characteristics were similar between growing seasons.

4.3.4 PIT A PADDY SEM-EDX WR ROOT-SURFACE CHARACTERIZATION

Overall, roots of WR harvested from the Pit A paddy did not contain identifiable coatings indicative of iron sulphide. Rather, following washing visual appearance of root surfaces did not suggest the presence of iron sulphide coatings – all Pit A paddy plants lacked the 'black' coatings more typically indicative of iron sulphide coating. Points chosen for EDX characterization were selected based on visual appearance: white appearance may be more indicative of typical root tissue, and orange appearance may be more indicative of iron (III) oxide / hydroxide coating. Carbon, oxygen, and calcium were the more dominant elements in areas suspect of typical root tissue, and carbon, oxygen, and iron were the more dominant elements in areas suspect of iron-containing coatings (**Figure 4.15**). Despite an inflow water sulphate concentration of ≥ 350 mg L^{-1}, pore water H_2S concentrations between approximately 0.5-2.0 mg L^{-1}, and dissolved iron concentrations ranging from approximately 6-15 mg L^{-1}, iron sulphide root coatings were neither visually observed nor identified via EDX characterization. Iron-containing coatings on WR roots were characterized by SEM and EDX elemental as described by Jorgenson et al. (2013). The primary elements identified via EDX characterization in their study were iron, oxygen, aluminum, potassium, silicon, and carbon, all of which typically resulted in EDX signals of higher intensity than other elements such as sulphur. EDX elemental characterization of WR roots from the Pit A paddy tend to agree with those reported by Jorgenson et al. (2013); specifically, 1) primary elements were carbon, oxygen, potassium, and

iron; and 2) the lack of identifiable iron sulphide root coatings, such as those inferred by LaFond-Hudson et al. (2018, 2020).

4.3.5 Pit C Paddy Water, Sediment, and Pore Water Characteristics

Characteristics of inflow and outflow water are detailed in **Table 4.16**, and were representative of Pit C water. Specifically, measured concentrations of constituents of interest such as sulphur / sulphate, calcium, and magnesium remained consistent between and during the 2018 and 2019 growing seasons. Sediment characteristics from 2018 and 2019 harvest events are listed in **Table 4.17**. Overall, concentrations of sediment nutrient elements such as ammonia-nitrogen, phosphate-phosphorus, potassium, and magnesium decreased between the 2018 and 2019 growing seasons.

Pore water representation samples were obtained throughout 2018 and 2019 growing seasons using traditional and modified peepers. Pore water characteristics are listed in **Table 4.18** and **Table *4.19***. Pit C pore water H_2S concentrations ranged from 0.221-6.03 mg L^{-1} in traditional peeper samples; < 0.078-0.314 mg L^{-1} in Rhizon samples; and 0.101-23.5 mg L^{-1} in modified peeper samples. With the exception of multiple Rhizon pore water samples during 2018, and the June 04, 2019, modified peeper samples, H_2S concentrations in pore water samples from this paddy exceeded the suggested 0.165 mg L^{-1} protective level for WR. At times, measured H_2S was over two orders of magnitude higher than this suggested WR protective level.

4.3.6 Pit C Paddy Plant Phenology and Productivity

t-Tests were used to discern differences between stem density, height, dry weight biomass, and seed production and dry weight biomass. WR phenological development was similar between growing seasons; dates of observed WR phenology during 2017, 2018, and 2019 growing seasons are listed in **Table 4.20**. On July 09, 2019, goose herbivory was observed throughout the paddy. Also documented on this date were initial observations of flowering; male flowers were present on several plants throughout this paddy. Despite extensive and repeated goose herbivory, t-test indicated no statistical difference between average 2018 and 2019 stem density ($t_{(22)}$ = -0.877 p = 0.390) (**Figure 4.14**). However, the 2019 average stem height was significantly less than in 2018 ($t_{(34)}$ = 11.995 $p < 0.001$). Additionally, the average WR shoot dry weight biomass ($t_{(19)}$ = 16.971 $p < 0.001$) and seeds per panicle ($t_{(16)}$ = 8.060 $p < 0.001$) were significantly less than in 2018 (**Figure 4.14**).

Due to a freezer failure in 2018, all harvested seeds were lost. Therefore, the Pit C 2019 average seed dry weight biomass was contrasted to average 2017 and 2019 Pit A seed dry weight biomass. ANOVA indicated that the average 2019 Pit C seed dry weight biomass was significantly less than the average 2017 Pit A seed dry weight biomass ($F_{(2,20)} = 8.679$ $p = 0.002$). Significantly decreased stem height, seed production, shoot dry weight biomass, and seed dry weight biomass (**Figure 4.14**) is likely resultant from goose herbivory during 2019.

4.3.7 PIT C PADDY PLANT AND SEED CHARACTERISTICS

Shoot characteristics from 2018 and 2019 are listed in **Table 4.21**. Shoots of plants harvested in 2019 contained higher concentrations of nitrogen, phosphorus, and sulphur, potentially due to nutrient uptake into plant tissues and internodal re-growth following herbivory prior to plant harvest. Plant harvest occurred prior to seed harvest during 2019 due to observed goose herbivory.

During 2019, t-tests indicated that seeds harvested from the Pit C paddy contained significantly higher concentrations of nitrogen ($t_{(10)} = -4.116$ $p = 0.002$) and sulphur ($t_{(10)} = -3.449$ $p = 0.006$) when contrasted to seeds harvested from the Pit A paddy. No significant difference was identified between concentrations of phosphorus ($t_{(10)} = -0.175$ $p = 0.865$) or potassium ($t_{(10)} = 0.376$ $p = 0.715$) in Pit C seeds contrasted to Pit A paddy seeds. Despite goose herbivory in the Pit C paddy, harvested plants appeared generally healthy (no observations of nutrient deficiencies or WR diseases), seeds had been filled, and overall WR did not appear to have been adversely influenced by chemical characteristics of Pit C water or water-associated exposures under these conditions.

4.3.8 PIT C PADDY SEM-EDX WR ROOT SURFACE CHARACTERIZATION

Overall, roots of WR harvested from the Pit C paddy did not contain identifiable coatings indicative of iron sulphide (**Figure 4.16**). The 'black' coating more typical of iron sulphide was not observed on roots of WR harvested from this paddy. Areas visually indicative of typical root tissue and iron (III) oxide / hydroxide coatings were observed. Locations in these areas were characterized using EDX. Carbon, oxygen, sulphur, and at times potassium, were the more dominant elements in areas suspect of typical root tissue, and carbon, oxygen, and iron (at times, multi-peak) were the more dominant elements in areas suspect of iron (III) oxide / hydroxide root coatings (**Figure 4.16**). Despite an inflow water sulphate concentration of ≥ 1,300 mg L^{-1},

pore water H_2S concentrations ranging from approximately 0.2-0.8 mg L^{-1}, and dissolved iron concentrations ranging from approximately 5-15 mg L^{-1}, root coatings indicative of iron sulphide were neither visually observed nor identified via EDX characterization. Similar to roots on WR grown in the Pit A paddy, EDX elemental characterization of Pit C paddy WR roots tend to agree with results reported by Jorgenson et al. (2013); specifically, the absence of identifiable iron sulphide containing root coatings as suggested by LaFond-Hudson et al. (2018, 2020).

4.4 DISCUSSION

Multiple recent investigations have focused on measuring responses of WR to well-defined exposures of sulphate and sulphide (Fort et al. 2017; Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020), but a general lack of field representativeness has resulted in a distinct need for field relevant and representative *in-situ* WR sulphate / sulphide exposure-response studies. Development and use of these paddy-scale bioassays was critical to better understanding larger-scale, and more field relevant, *in-situ* responses of WR to these waters. By designing these paddy-scale bioassays to include direct inflow exposures of legacy-mine influenced pit waters, a 'highest exposure possible' scenario was realized. If future removal of water from legacy-mine influenced pits into waters containing, or potentially containing, WR is considered, direct exposure-response data obtained using these paddies may be used to inform removal rates, periodicity, and monitoring regimes.

Regardless of inflow water characteristics, WR plants in the paddies developed normally, suggesting the growing conditions within the paddies were suitable. Sediment characteristics were suitable for WR growth and development. As detailed in Lee (2002) and Lee and McNaughton (2004), sufficiently high sediment-associated bioavailable nutrient elements such as nitrogen and phosphorus are of critical importance to WR growth, development, and distribution. Water depth was maintained, at approximately 20 cm throughout the growing season. Competition from other aquatic vegetation (cattails in particular) was minimized by removing any observed non-WR plants. This is critical due to the potential for allelopathic influences on WR from other aquatic vegetation (Elakovich and Wooten 1989; Quayyum et al. 1999) and competition for scarce resources. Mitigating potential adverse influences on WR development associated with these physical paddy conditions therefore limited the primary variable that might affect wild rice growth performance to inflow water characteristics.

Nutrient element concentrations of WR plant tissue have been reported by Hildebrandt et al. (2012). Total nitrogen (%) in shoot and root tissue was reported as 0.85 and 0.86, respectively. In the current study, Pit A paddy WR shoot nitrogen (%) content was 0.68 and 0.75 in 2018 and 2019; and Pit C paddy WR shoot nitrogen (%) was 0.74 and 1.89 in 2018 and 2019, respectively. The higher Pit C paddy WR % plant tissue nitrogen in 2019 can likely be attributed to nitrogen uptake and internodal re-growth following repeated goose herbivory throughout the paddy (Weir and Dale 1960). LaFond-Hudson et al. (2020) reported WR seed nitrogen (%) content of their non-sulphate amended exposures as 1.89, and 2.28 for the sulphate exposed (300 mg L^{-1}) WR. In the current study, seeds harvested from the Pit A paddy (approximately 350 mg SO_4 L^{-1}) WR were 1.24 and 1.55% nitrogen in 2017 and 2019, respectively. Seeds harvested from the Pit C paddy (approximately 1,300 mg SO_4 L^{-1}) WR were 1.86% nitrogen in 2019. These values are close to the nitrogen content of the non-sulphate amended WR characterized by LaFond-Hudson et al. (2020). Additionally, average seed dry weight biomass was similar between non-sulphate amended WR (LaFond-Hudson et al. 2020) and Pit A paddy WR in the current study (15.26 and approximately 15.00 mg, respectively).

During the 2017-2019 WR growing seasons, WR seedlings were observed during May of each season, with the end-of-season harvest event occurring between August 15-31. LaFond-Hudson et al. (2020) observed delayed development in WR grown in sulphate-amended (300 mg L^{-1}) water. In the current study, delayed WR phenology was not observed. Rather, WR plants tended to germinate and mature earlier in the growing season than in local natural WR areas (personal observations). This is likely due to providing growing conditions (e.g., water depth, minimal competing vegetation, appropriate sediment characteristics, larger-scale system) more conducive to WR development.

As suggested in Oelke et al. (1997), a plant density of four plants per square foot (40 m^{-2}) is recommended in an effort to avoid adverse competitive and disease influences. Initial (2017) Pit A paddy stem density was approximately 80 m^{-2}; 2018 and 2019 stem densities were approximately five-fold higher. The increase in Pit A stem density from 2017 into 2018 and 2019 was likely due to a combination of the seed amendment in October 2017, germination of seeds produced during the 2017 growing season, and sediment characteristics favorable for WR growth. These densities did not appear to adversely influence development or distribution. Pit C

paddy stem density remained at approximately 30 m^{-2} during 2018 and 2019. Although not measured during early-season growth, stem density did not appear to temporally decrease in either paddy throughout growing seasons as a result of self-thinning (Atkins 1983; Lee 2002). Under less favorable conditions for WR development (i.e., presence of competing vegetation, low nutrient levels), WR plant density has been observed to decrease throughout the growing season (Atkins 1983; Lee 2002).

Hydrogen sulphide can become problematic for aquatic plants at sufficiently high exposure concentrations. Lamers et al. (2013) provide a review of aquatic plants' responses to exposures of H_2S in multiple types of aquatic systems. Based on their review, concentrations of H_2S ranging from 10-1,500 µM (0.34-51 mg L^{-1}) in freshwater wetlands can cause decreased photosynthetic yields, leaf elongation rates, radial oxygen loss (a characteristic of WR) rates, above-ground biomass, and ultimately above- and below- ground die-off in sedges, rushes, cattails, and white rice. Armstrong and Armstrong (2005) documented that exposures of 0.174 mM H_2S (approximately 5.6 mg L^{-1}) decreased rates of radial oxygen loss in *Oryza sativa* by approximately 89% following a 10-16 day exposure duration. This was likely due to lipid-based thickening (suberisation), lignification of cell walls, and / or blockages of vascular and aeration pathways in lateral and adventitious root tissues. Following a large-scale field study including dozens of natural WR areas, a pore water H_2S concentration not to exceed 0.165 mg L^{-1} was recommended in Minnesota for wild rice production (MPCA, March 24, 2015). This conclusion was based on using Rhizons inserted into sediment cores to obtain porewater H_2S samples. This level was intended to protect against a 10% decrease in the probability of WR presence defined as two stems m^{-2}. In the current study, H_2S concentrations varied depending on the method of porewater sampling. Due to the increased potential for atmospheric contamination of porewater during sampling, we chose to not use the Rhizon-sediment core method following testing in the Pit A paddy during the 2018 growing season. Sediment pore water H_2S concentrations ranged from 0.135 (Rhizon attached to traditional peeper) up to 12.1 (modified peeper) mg L^{-1} in the Pit A paddy (> 300 stems m^{-2}); and < 0.078 (Rhizon attached to traditional peeper) up to 23.5 (modified peeper) mg L^{-1} in the Pit C paddy (30-40 stems m^{-2}). These are within the range of sulphide concentrations concluded to result in multiple adverse influences to aquatic plants including *Oryza* spp. (Armstrong and Armstrong 2005; and Lamers et al. 2013); and at times orders of magnitude higher than the suggested 0.165 mg L^{-1} protective level for WR in

Minnesota. In the current study, potential sulphide toxicity to WR may have been mitigated by relatively high concentrations of available iron for FeS complexation, and / or sediment compartments of sulphur oxidizing / reducing bacteria associated with antithetical proximity to the WR rhizosphere (Stubner et al. 1998). During their field study, Myrbo et al. (2017) also observed WR growing in areas with H_2S concentrations > 0.165 mg L^{-1}. Since H_2S can be produced via intracellular SO_4 reduction (Wang 2012), increased H_2S concentrations in Pit A and Pit C paddy pore water samples obtained using modified peepers may have been due to aspiration of sulphur reducing bacteria (SRB), SRB cellular contents, or a combination of these potential sources. One possible mitigatory influence of sulphide toxicity within these paddies is available iron in the sediment and pore water for complexation with sulphide (S^{2-}) to form iron sulphide (FeS).

Throughout the growing season, porewater dissolved iron and H_2S concentrations trended similarly to those reported by Myrbo et al. (2017) in both the Pit A and Pit C paddies; porewater dissolved iron decreased throughout the growing season, while porewater H_2S increased through mid-season, and decreased near growing season conclusion within each paddy (**Table 4.12, Table *4.13*, Table *4.18*, Table *4.19***). 'Detoxification' of H_2S to WR can be achieved in the presence of available iron (Fort et al. 2017). In their hydroponic study WR mesocotyl emergence rates increased when available iron (ferric chloride) was amended to exposure media containing higher sulphide (hydrated sodium sulphide) concentrations. Seed activation and seedling survival were less sensitive endpoints, even at the high-end sulphide exposure of 12.5 mg L^{-1}. In the current study, dissolved iron (0.45 µM pore size filtered) ranged from < 1.0-76.7 mg L^{-1} in pore water samples; and appeared to decrease throughout the growing season. This could be due to complexation with S^{2-} (FeS), iron (III) oxide / hydroxide complexation, plant uptake, or other transfer / transformation processes. Previous studies by LaFond-Hudson et al. (2018, 2020) and Pastor et al. (2017) concluded that iron-containing coatings on WR roots, potentially FeS coatings, can result in decreased plant growth and development rates, and decreased reproductive potential. Experimental conditions described for those studies could have created an environment more favorable to H_2S production (Hem 1960), FeS complexation, and adherence to WR roots. Exposure chambers were semi-static, four-litre pails inside 12-litre pails, with periodic water additions to maintain water depth and sulphate concentrations; and were set-up outdoors. Increased / increasing temperature and lack of water turbulence in exposure chambers could have

decreased gas (e.g., O_2) solubility and diffusion, resulting in decreased / decreasing near- and within- sediment Eh activities. Although root surface Eh was calculated via a modified Nernst equation, Eh and *p*H conditions favorable for FeS complexation (Hem 1960) within the sediment pore water were likely under these experimental conditions. However, as suggested in LaFond-Hudson et al. (2018), development of FeS root coatings could be a function of WR phenology; specifically, during mid-season peak growth when root metabolic activity is higher causing increased radial oxygen loss and FeS root coatings are less likely. Although root samples for SEM and EDX characterization were obtained during July 2018 (peak mid-season growth) for the current study, WR growth and development to that point was exceptional; and WR displayed no indications of delayed, slowed, or decreased growth during development. Additionally, an approximate 20-L min^{-1} flow rate was initiated in each paddy prior to the growing season (at paddy 'ice-out'), and maintained until freeze-up. Cooler and constant pit water inflow likely assisted with maintaining water column oxygenation in each paddy throughout each growing season, potentially mitigating conditions more favorable to H_2S production within the WR rhizosphere. Since potentially problematic FeS root coatings were observed under smaller-scale static experimental conditions, and not in the current study, it is likely that under experimental conditions more representative of field conditions, and appropriate for WR growth, FeS coatings on WR roots do not accumulate to a problematic extent; specifically, not during life stages of higher vegetative growth.

Jorgenson et al. (2013) observed Fe-containing coatings on roots of WR harvested from wetlands in Ontario, Canada. However, EDX characterization indicated the dominant elements in these coatings were iron, oxygen, aluminum, and potassium. No FeS WR root coatings were observed in their field study. In this current study, WR root coatings visually characteristic of iron (III) oxide / hydroxide were observed on WR harvested from both paddies. EDX characterization indicated the dominant elements in these coatings were carbon, oxygen, and iron in areas visually suggestive of iron (III) oxide / hydroxide coatings, and carbon, oxygen, sulphur, and calcium in areas suggestive of typical root tissue, at times on the same plant. Additionally, new root growth was evident on all WR plants; and, despite a similar sulphate exposure in the Pit A paddy ($\geq$ 350 mg L^{-1}) and a higher sulphate exposure in the Pit C paddy ($\geq$ 1,300 mg L^{-1}), seed nutrient-element characteristics (e.g., %N) and productivity were the same as, if not higher than, respectively, non-sulphate-amended WR characterized by LaFond-Hudson et al. (2018, 2020).

Furthermore, WR developed into successive life stages earlier in each paddy than in local natural WR areas. No delay in WR phenology was observed during current study growing seasons.

4.5 CONCLUSIONS

Throughout multiple consecutive growing seasons legacy-mine influenced waters of substantially different chemical characteristics did not appear to adversely influence WR development, temporal or otherwise, under conditions of this study. Likely due to controlled water depth, competing vegetation, and sediment conditions more optimal for WR growth and development, WR in both paddies developed and produced seed over successive growing seasons. Based on data and observations obtained during this study, water characteristics such as aqueous sulphate and pore water H_2S, and accumulation of potentially problematic iron-containing WR root coatings (FeS or otherwise) appear to play a less important role in WR phenology, distribution, and productivity, than previously suggested, under experimental conditions more representative of local, natural WR areas. Although statistical differences were identified between multiple measured WR characteristics, no adverse influences on WR phenology, density, or productivity could be determined resultant of Pit A or Pit C inflow water or water-associated exposures. Development and use of these paddy-scale bioassays allowed more accurate and field-relevant predictions of WR responses to these legacy-mine influenced waters. Additional research using these paddy-scale bioassays will be critical to discern specific water and / or sediment influences on WR nutrient uptake and allocation during specific life stages throughout successive growing seasons.

ACKNOWLEDGEMENTS

The authors would like to thank Johane Joncas and all personnel at LUEL and Pace Analytical Laboratories (Virginia, MN) for analytical support. We also thank colleagues at Northeast Technical Services, Inc., (Virginia, MN) for assistance with managing the WR paddies throughout multiple, successive growing seasons.

REFERENCES

Armstrong, J., Armstrong, W. 2005. Rice: Sulfide-induced barriers to root radial oxygen loss, Fe^{2+} and water uptake, and lateral root emergence. Annals of Botany 96, 625-638.

Atkins, T.A. 1983. The aquaculture of wild rice. Progress Year 2 Addendum. Lakehead University, Thunder Bay.

Bennett, J.P., Chiriboga, E., Coleman, J., Walker, D.M. 2000. Heavy metals in wild rice from northern Wisconsin. Sci. of the Total Env. 246, 261-269.

Counts, R.L., Lee, P.F. 1988. Patterns of variations in Ontario wild rice (*Zizania aquatica* L.) 3. Similarity between source and introduced population pairs under experimental and field conditions. Aquat. Bot. 31, 37-55.

Day, W.R., Lee, P.F. 1989. Ecological relationships of wild rice, *Zizania aquatica* L. 8. Classification of sediments. Can. J. Bot. 67, 1381-1386.

Day, W.R., Lee, P.F. 1990. Ecological relationships of wild rice, *Zizania aquatica*. 9. Production in organic-flocculent sediments. Can. J. Bot. 68(7), 1542–1548. doi:10.1139/b90-197.

Dysievick, K. 2019. Wild rice (*Zizania palustris* L.) re-establishment through mechanical removal of invasive cattails (*Typha angustifolia* L.). Lakehead University, M. Sc. Thesis.

Elakovich, S.D., Wooten, J.W. 1989. Allelopathic potential of sixteen aquatic and wetland plants. J. Aquat. Plant Manage. 27, 78-84.

Fort, D.J., Mathis, M.B., Walker, R., Tuominen, L.K. Hansel, M., hall, S. Richards, R., Gratten, S.R., Anderson, K. 2014. Toxicity of sulfate and chloride to early life stages of wild rice (*Zizania palustris*). Environ. Toxicol. Chem. 33(12), 2802-2809.

Fort, D.J., Todhunter, K., Fort, T.D., Mathis, M.B., Walker, R., Hansel, M., Hall, S., Richards, R., Anderson, K. 2017. Toxicity of sulfide to early life stages of wild rice (*Zizania palustris*). Environmental Toxicology and Chemistry 36(8), 2217-2226.

Grava, J., Rose, K. 1975. Fertility of paddy soils and fertilization of wild rice. (IN) Minnesota Wild Rice Research 1974. Univ. of Minnesota.

Grava, J. 1977. Wild rice fertilization research. (IN) Minnesota Wild Rice Research 1976. Univ. of Minnesota.

Hawthorn, W.R., Stewart, J.M. 1970. Epicuticular wax forms on leaf surfaces of *Zizania aquatica*. Can. J. Bot. 48, 201-205.

Hem, J.D. 1960. Some chemical relationships among sulfur species and dissolved ferrous iron: Chemistry of iron in natural water. USGS water-Supply Paper 1459-C.

Hildebrandt, L.R., Pastor, J., Dewey, B. 2012. Effects of external and internal nutrient supplies on decomposition of wild rice, *Zizania palustris*. Aquatic Botany 97, 35-43.

Jorgenson, K.D., Lee, P.F., Kanavillil, N. 2013. Ecological relationships of wild rice, *Zizania* spp. 11. Electron microscopy study of iron plaques on the roots of northern wild rice (*Zizania palustris*). Botany 91, 189-201.

LaFond-Hudson, S., Johnson, N.W., Dewey, B. 2020. Interactions between sulfide and reproductive phenology of an annual aquatic plant, wild rice (*Zizania palustris*). Aquatic Botany 164, 103230.

LaFond-Hudson, S., Johnson, N.W., Pastor, J., Dewey, B. 2018. Iron sulfide formation on root surfaces controlled by the life cycle of wild rice (*Zizania palustris*). Biogeochemistry. https://doi.org/10.1007/s10533-018-0491-5.

Lamers, L.P.M., Govers, L.L., Janssen, C.J.M., Geurts, J.J.M., Van der Welle, M.E.W., Van Katwijk, M.M., Van der Heide, T., Roelofs, J.G.M., Smolders, A.J.P. 2013. Sulfide as a soil phytotoxin – A review. Frontiers in Plant Science 4(268), 1-14.

Lee, P.F. 2002. Ecological relationships of wild rice, Zizania spp. 10. Effects of sediment and among-population variations on plant density in *Zizania palustris*. Can. J. Bot. 80, 1283-1294.

Lee, P.F. 1979. Biological, chemical and physical relationships of wild rice, *Zizania aquatica* L. in northwestern Ontario and northeastern Minnesota. Ph.D. Thesis, Univ. of Manitoba.

Lee, P.F., Stewart, J.M. 1981. Ecological relationships of wild rice, *Zizania aquatica* L. 1. A model for among-site growth. Can. J. Bot. 59, 2140-2151.

Lee, P.F., McNaughton, K.A. 2004. Macrophyte induced microchemical changes in the water column of a northern Boreal Lake. Hydrobiol. 522, 207-220.

Lee, P.F., Hughes, P.C. 2000. The effects of sulfate levels on the early development of wild rice. Prepared for U.S. Steel Corp. (Minntac) Minnesota.

Malvick, D.K., Percich, J.A. 1993. Hydroponic culture of wild rice (*Zizania aquatica* L.) and its application to studies of silicon nutrition and fungal brown spot disease. Can. J. Plant Sci. 73, 969-975.

Marcum, D.B., Porter, R.A. 2006. Influence of water depth upon plant development, yield and seed size of modern wild rice cultivars. ASA Annual Meeting.

Meeker, J. 1996. Wild-rice and sedimentation processes in a Lake Superior coastal wetland. Wetlands 16, 219-231.

Minnesota Department of Natural Resources (MN DNR). 2008. Natural Wild Rice in Minnesota: A Wild Rive Study document submitted to the Minnesota Legislature by the MN DNR, February 15, 2008.

Minnesota Pollution Control Agency (MPCA). 2015. March 2015 proposed approach for Minnesota's sulfate standard to protect wild rice. March 24, 2015.

Moyle, J.B. 1944. Wild rice in Minnesota. J. Wildlf. Manage. 8, 177-184.

Moyle, J.B. 1945. Some chemical factors influencing the distribution of aquatic plants in Minnesota. Amer. Midl. Nat. 34, 402-420.

Moyle, J.B. 1956. Relationships between the chemistry of Minnesota surface waters and wildlife management. J. Wildlf. Manage. 20, 303-320.

Myrbo, A., Swain, E.B., Engstrom, D.R., Coleman Wasik, J., Brenner, J., Dykhuizen Shore, M., Blaha, G. 2017. Sulfide generated by sulfate reduction is a primary controller of the occurrence of wild rice (*Zizania palustris*) in shallow aquatic ecosystems. J. Geophys. Research: Biogeosciences 122, 2736–2753. https://doi.org/10.1002/2017JG003787.

Oelke, E.A., Grava, J., Noetzel, D., Barron, D., Percich, J., Schertz, C., Strait, J., Stucker, D. 1982. Wild rice production in Minnesota. Univ. of Minn. Ext. Bull. 464. 40 pp.

Oelke, E.A., Teynor, T.M., Carter, P.R., Percich, J.A., Noetzel, D.M., Bloom, P.R., Porter, R.A., Schertz, C.E., Boedicker, J.J., Fuller, E.I. 1997 (last update). Alternative Field Crops manual: Wild Rice. UW and UMN Extension Services.

Pace Analytical Laboratories (Pace). 2020a. Minnesota Department of Health Environmental Laboratory Accreditation Program Accreditation Certificate. State Lab ID: 027-137-152 (Duluth, Minnesota).

Pace Analytical Laboratories (Pace). 2020b. Minnesota Department of Health Environmental Laboratory Accreditation Program Accreditation Certificate. State Lab ID: 027-137-445 (Virginia, Minnesota).

Pastor, J., Dewey, B., Johnson, N.W., Swain, E.B., Monson, P., Peters, E.B., Myrbo, A. 2017. Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments. Ecological Applications 27(1), 321-336.

Paulishyn, W., Stewart, J.M. 1970. Sulfate ion concentrations and wild rice distribution in Manitoba. Agassiz Research Abstract, University of Manitoba.

Pillsbury, R.W., McGuire, M.A. 2009. Factors affecting the distribution of wild rice (*Zizania palustris*) and the associated macrophyte community. Wetlands 29, 724-734.

Pip, R. 1984. Ecogeographical tolerance range variation in aquatic macrophytes. Hydrobiol., 108, 37-48.

Quayyum, H.A., Malik, A.U., Lee, P.F. 1999. Allelopathic potential of aquatic plants associated with wild rice (*Zizania palustris*): I. Bioassay with plant and lake samples. J. Chem. Ecol. 25(1), 209-220.

Rogalsky, J.K., Clark, K., Stewart, J.M. 1971. Wild rice paddy production in Manitoba. Man. Dept. of Agr. Pub. 527.

Stevenson, S.C., Lee, P.F. 1987. Ecological relationships of wild rice, *Zizania aquatica*. 6. The effects of increases in water depth on vegetative and reproductive growth. Can. J. Bot. 65, 2128-2132.

Stubner, S., Wind, T., Conrad, R. 1998. Sulfur oxidation in rice field soil: Activity, enumeration, isolation and characterization of thiosulfate-oxidizing bacteria. System Appl. Microbiol. 21, 569-578.

Tedrow, O.R. 2020. Laboratory- and Field- Scale Bioassays for Predicting Responses of Wild Rice (*Zizania palustris* L.) to Exposures of Site-Specific Sediments and Waters. Ph. D. Dissertation, Lakehead University, Thunder Bay, Ontario.

Thomas, A.G., Stewart, J.M. 1969. The effect of different water depths on the growth of wild rice. Can. J. Bot. 47, 1525-1531.

Tucker, R.C., Zanis, M.J., Emery, N.C., Gibson, K.D. 2011. Effects of water depth and seed provenance on the growth of wild rice (*Zizania palustris* L.). Aquat. Bot. 94, 113-118.

Vicario, B.T., Halstead, E.H. 1968. Progress report on wild rice research. Univ. of Saskatchewan, Dept. of Soil Science.

Weir, C.E., Dale, H.M. 1960. A developmental study of wild rice, *Zizania aquatica* L. Can. J. Bot. 38, 719-739.

FIGURES

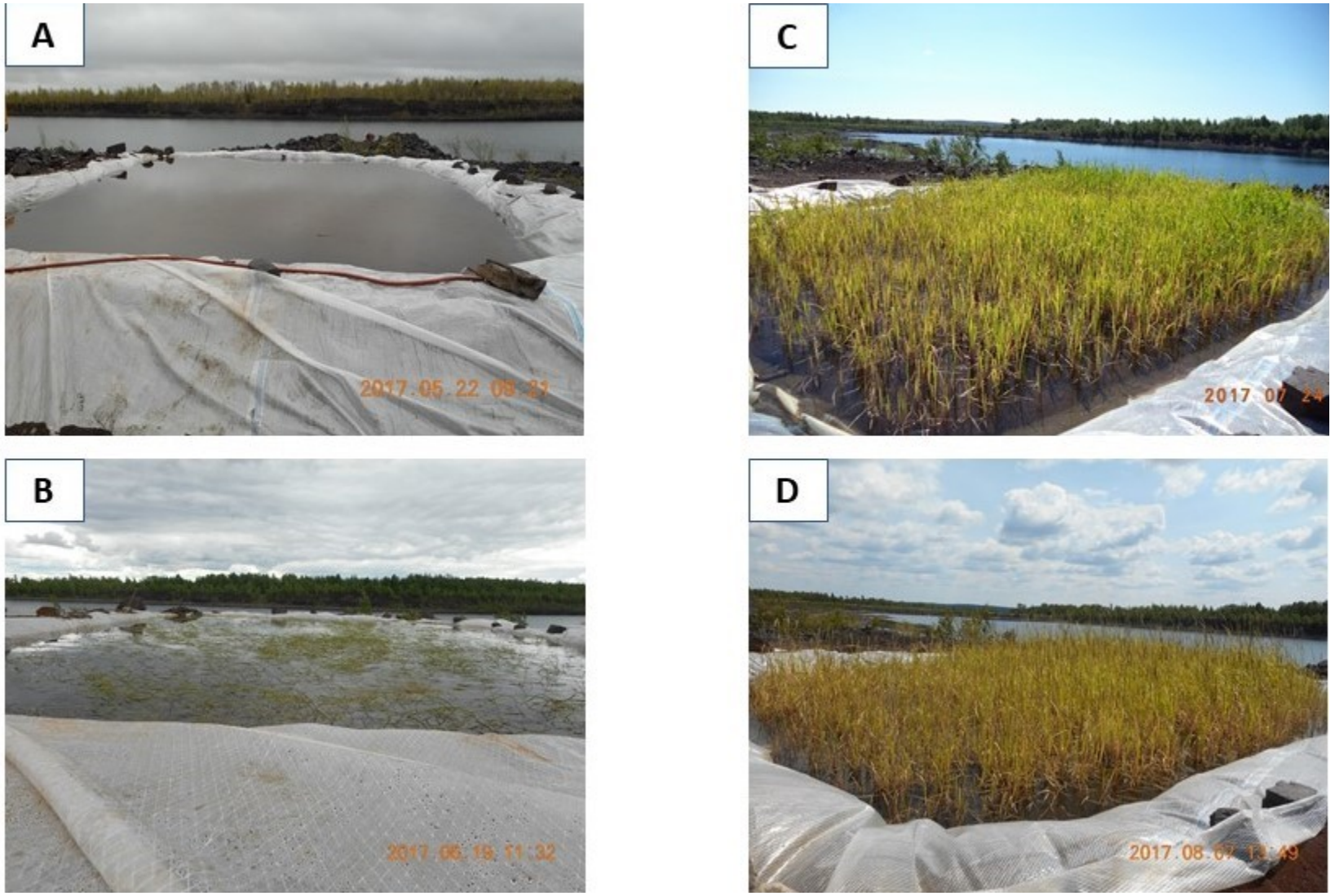

Figure 4.11. 2017 Pit A paddy. Images represent typical temporal WR phenological observations during the 2017-2019 growing seasons **A)** seed distribution date and also approximately when germination is documented (2017-05-22); **B)** floating leaf stage (2017-06-19); **C)** aerial stage (2017-07-24); and **D)** WR production of filled seeds throughout the paddy (2017-08-07).

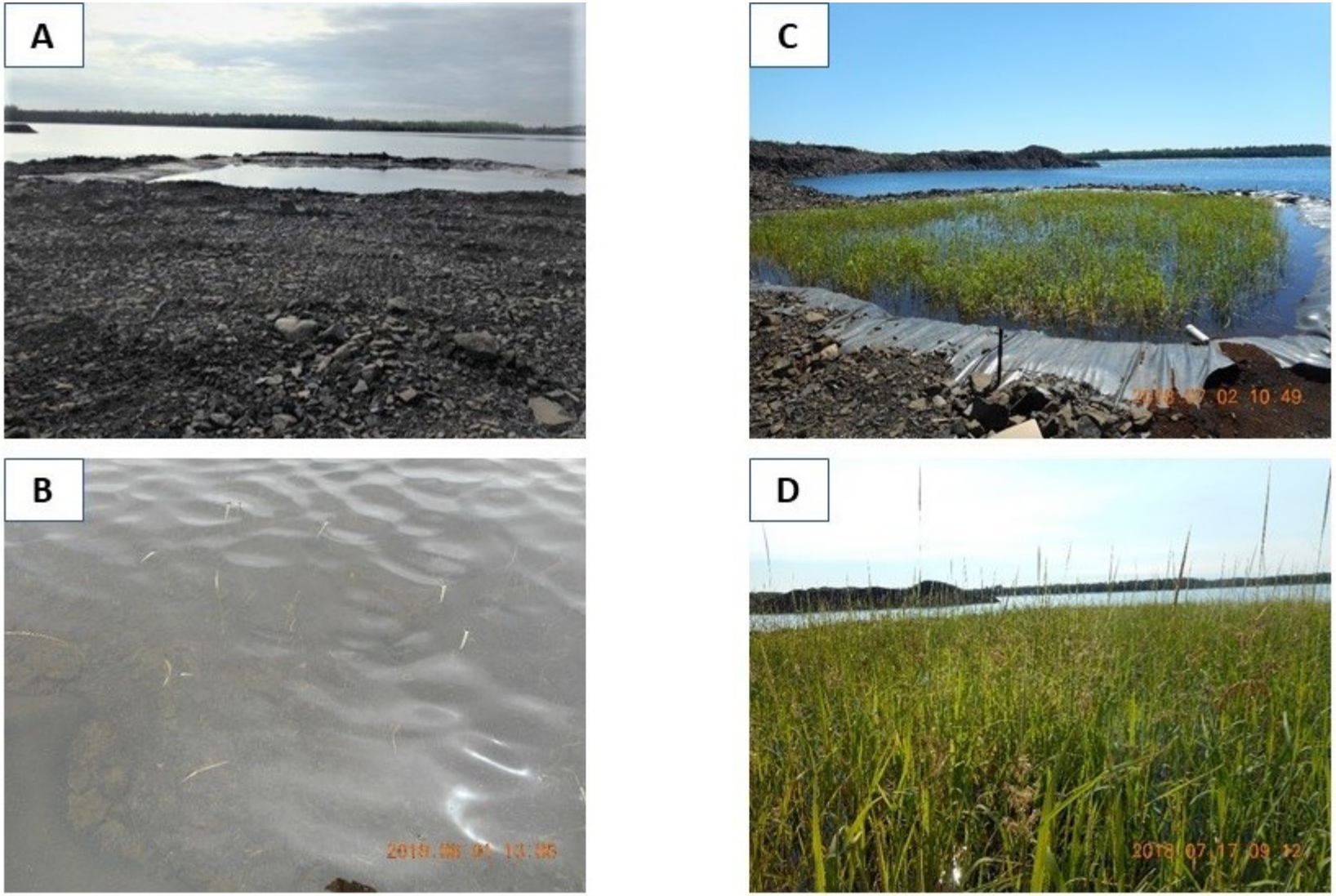

Figure 4.12. 2018 Pit C paddy. Images represent typical WR phenological observations during the 2018 and 2019 growing seasons **A)** seed distribution date (2018-05-22); **B)** floating leaf stage (2018-06-01); **C)** aerial stage (2018-07-02); and **D)** flowering and seed production (2018-07-17), although, harvest occurred in August of each year.

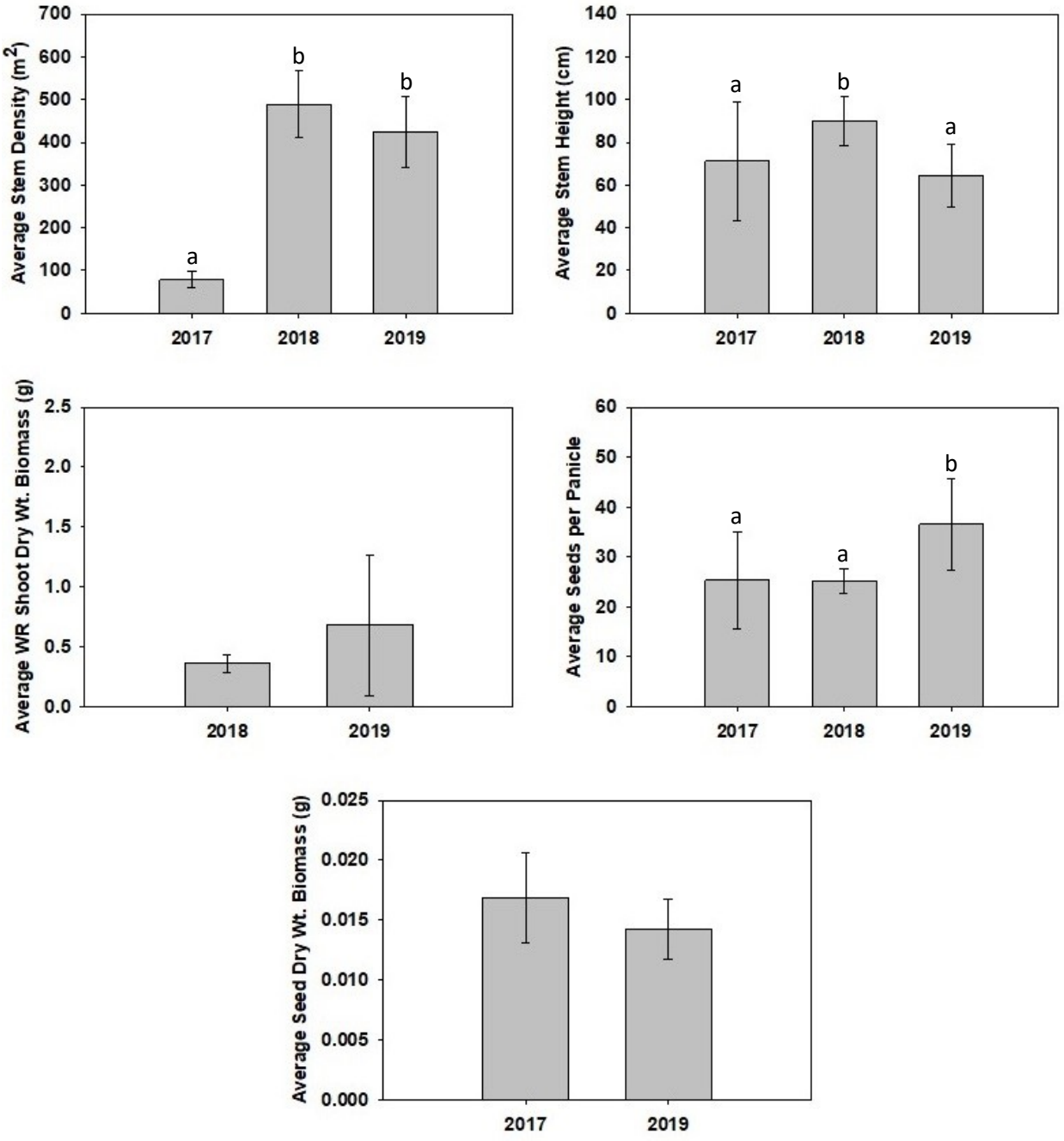

Figure 4.13. 2017-2019 Pit A paddy average stem density, stem height, plant dry weight biomass, seeds per panicle, and average seed dry weight biomass. No plant samples were obtained from the Pit A paddy during 2017. All seeds harvested during 2018 were lost due to a freezer failure. Error bars represent one standard deviation. Differing letters (a and b) indicate statistical significance at alpha 0.05.

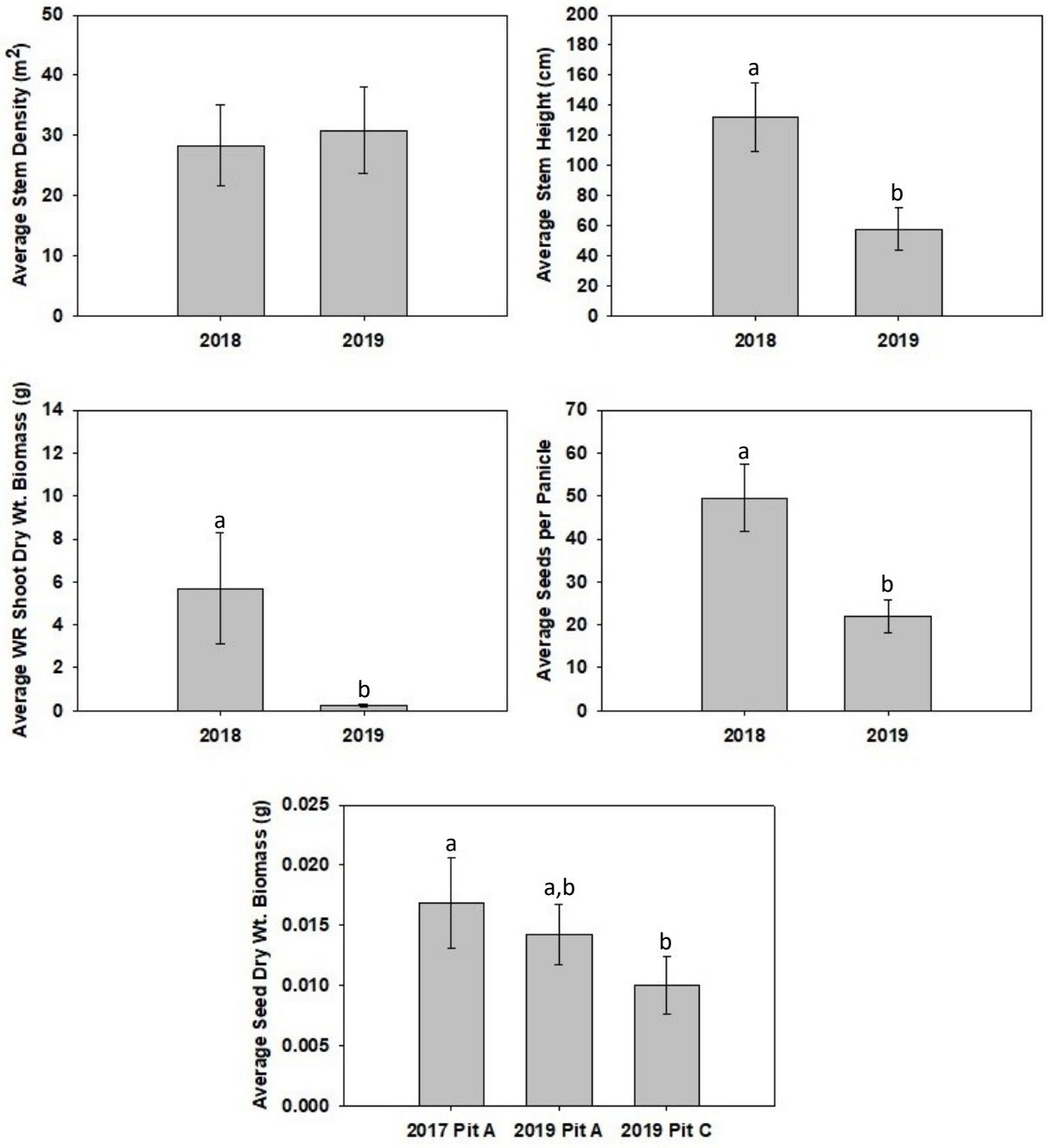

Figure 4.14. 2018-2019 Pit C paddy average stem densities, stem height, plant dry weight biomass, filled seeds per panicle, and average seed dry weight biomass. Error bars represent one standard deviation. All seeds harvested during 2018 were lost due to a freezer failure. Differing letters (a and b) indicate statistical significance at alpha 0.05.

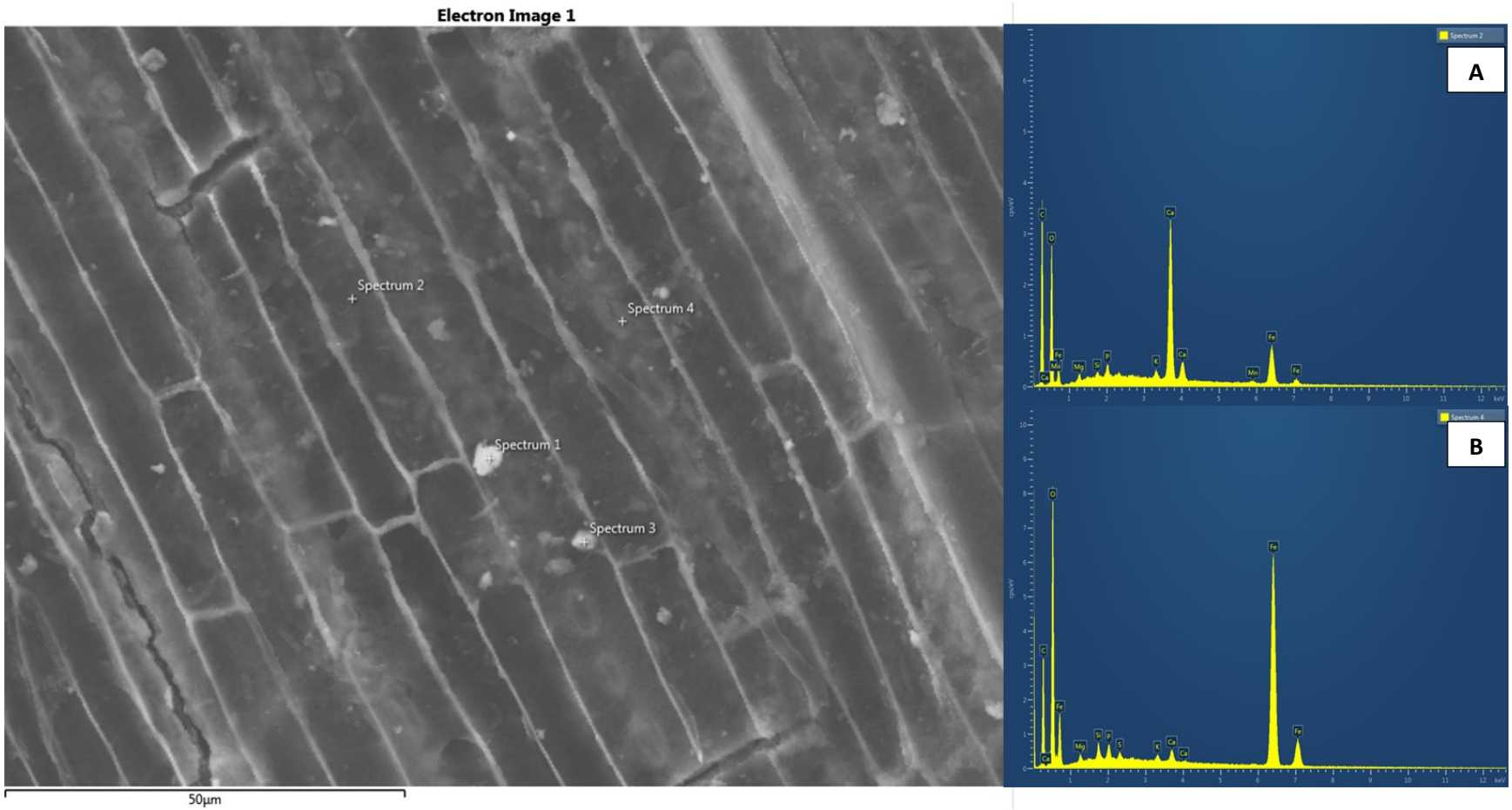

Figure 4.15. Root SEM-EDX characterization from WR harvested from the Pit A paddy (July 2018). **A)** EDX spectrum from 'Spectrum 2' location identified on SEM image representative of typical root tissue EDX spectra, with primary elements (L-R) C, O, and Ca; and **B)** EDX spectrum from 'Spectrum 4' location identified on SEM image representative of Fe(III)O-(OH) coating appearing areas, with primary elements (L-R) C, O, and Fe.

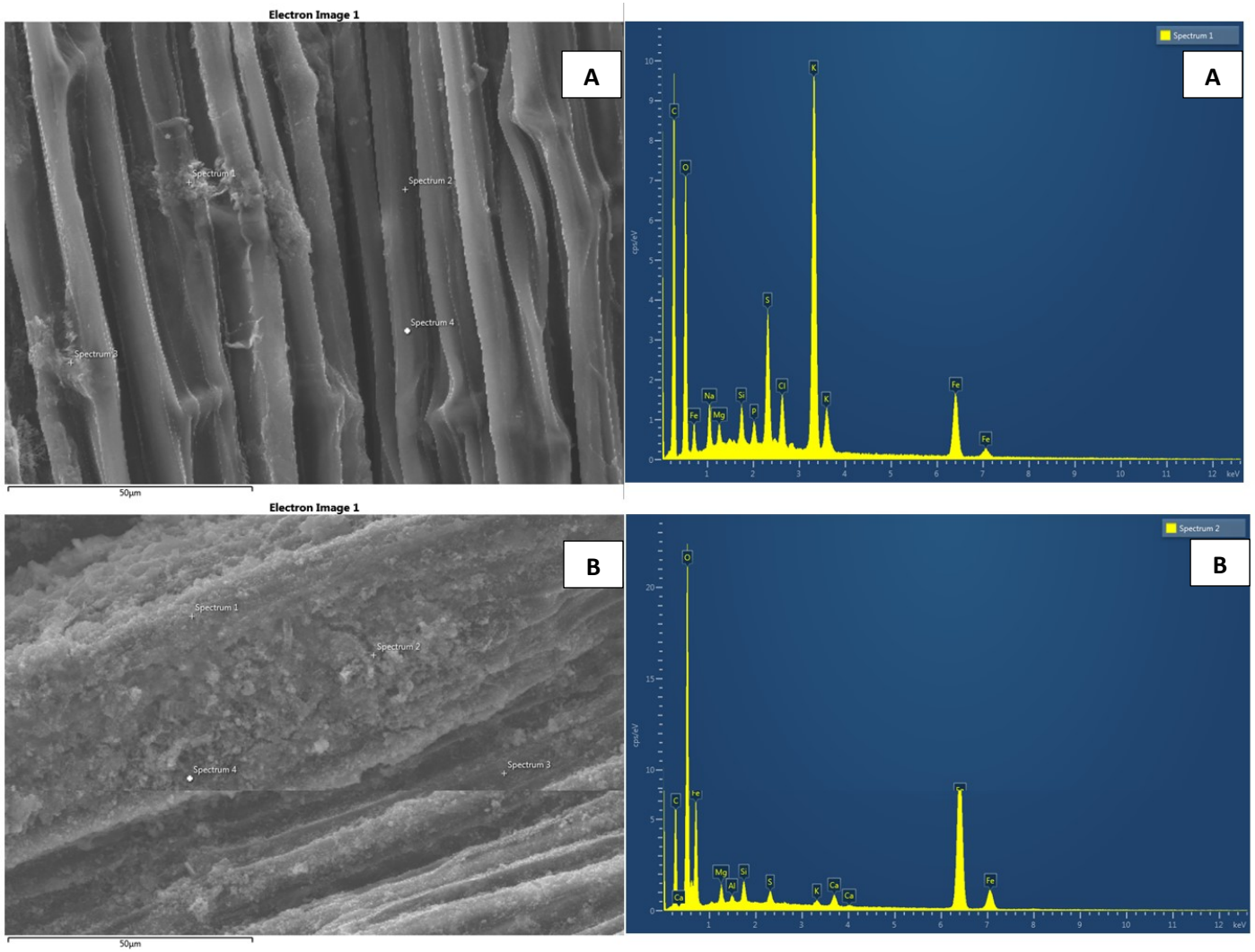

Figure 4.16. Root SEM-EDX characterization from WR harvested from the Pit C paddy (July 2018). **A)** EDX spectrum from 'Spectrum 1' location identified on SEM image representative of typical root tissue EDX spectra, with primary elements (L-R) C, O, S, and K. **B)** EDX spectrum from 'Spectrum 2' location identified on SEM image representative of Fe(III)O-(OH) coating appearing areas, with primary elements (L-R) C, O, Fe, and Fe.

TABLES

Table 4.9. 2017-2019 Pit A paddy. Measured inflow and outflow water characteristics (n = 1 for each inflow and outflow sampling event). Units for Bicarb. Alk. through SO_4 are mg L^{-1}; Al, Cu, and Mn are µg L^{-1} (n = 1 for each sampling event per location).

	Inflow			Outflow		
	Aug. 23, 2017	Aug. 16, 2018	Aug. 01, 2019	Aug. 23, 2017	Aug. 16, 2018	Aug. 01, 2019
*p*H (SU)	8.4	8.4	8.3	8.5	8.0	8.0
Conductance (µS cm^{-1})	1,225	1,210	1,185	1,211	1,219	1,198
Bicarb. Alk. (mg L^{-1})	329	288	354	328	310	364
Ca	50	47	43	49	46	40
Mg	134	126	119	131	130	121
Na	32	28	25	32	30	25
SO_4	403	368	359	405	370	360
Al (µg L^{-1})	NM	200	200	NM	200	200
Cu	NM	1.8	BD	NM	1.3	BD
Mn	NM	25	32	NM	42	14

NM = not measured; BD = below detection.
Total B and Zn were measured during 2018 and 2019, but were below detection. Total Fe was measured during 2017, but was below detection.

Table 4.10. Pit A paddy. Measured concentrations of characteristics of source sediment, and sediment sampled during the 2017, 2018, and 2019 plant harvest (average ± one standard deviation). Units for NH_3 through Na are µg g^{-1}.

	Source Sediment (n = 1)	2017 (n = 3)	2018 (n = 3)	2019 (n = 11)
LOI (%)	12.3	8.2 ± 3.8	NM	16.5 ± 6.2
*p*H (SU)	6.63	5.8 ± 0.1	7.6 ± 0.1	7.0 ± 0.2
Bulk Density (g cm^{-3})	0.4	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Total N (%)	NM	0.21 ± 0.12	0.23 ± 0.04	0.44 ± 0.19
NH_3+NH_4-N (µg g^{-1})	24.8	14.7 ± 5.4	18.1 ± 4.9	3.4 ± 1.2
PO_4	5.1	2.0 ± 0.5	6.8 ± 2.6	1.5
Al	357	149 ± 31	382 ± 26	112 ± 43
Cu	2.1	1.0 ± 0.2	3.1 ± 0.3	0.4 ± 0.4
Fe	1,819	296 ± 45	1,013 ± 106	2,181 ± 856
Mn	267	47 ± 22	75 ± 17	40 ± 31
S	214	115 ± 28	178 ± 9	402 ± 574
Zn	7.0	2.1 ± 0.3	5.34± 0.7	1.8 ± 0.9
Ca	3,235	1,031 ± 421	1,468 ± 277	766 ± 225
K	86	37 ± 6	59 ± 23	21 ± 7
Mg	1,246	573 ± 202	716 ± 136	294 ± 80
Na	23	42 ± 18	59 ± 12	24 ± 6

NM = not measured.

Table 4.11. 2017 Pit A paddy. Measured concentrations of hydrogen sulphide (H_2S; mg L^{-1}) and sulphate (SO_4; mg L^{-1}) in traditional peeper pore water representation samples (n = 1 for each sampling event per location).

	H_2S			SO_4		
	Inflow	Middle	Outflow	Inflow	Middle	Outflow
Jul. 31, 2017	BD	BD	BD	205	152	83
Aug. 31	BD	0.542	0.299	269	112	127
*Sept. 29	0.794	0.971	0.379	17	54	28
*Oct. 27	0.358	0.232	0.690	50	104	15

BD = below detection (< 0.078 mg L^{-1}).
*H_2S concentrations in Rhizon pore water samples on these dates were below detection.

Table 4.12. 2018 Pit A paddy. Measured concentrations of hydrogen sulphide (H_2S; mg L^{-1}), dissolved (Diss.) iron (mg L^{-1}), and sulphate (SO_4; mg L^{-1}) in traditional peeper pore water representation samples, Rhizon samples, and sediment core samples. 'Rh H_2S' indicates pore water H_2S measurements obtained from Rhizon samples; 'Core H_2S' indicates pore water H_2S measurements obtained from sediment core samples (n = 1 for each sampling event per location).

	Inflow				Middle				Outflow				
	Peeper H_2S	Rh. H_2S	*Diss. Fe	Peeper SO_4	Peeper H_2S	Rh. H_2S	*Diss. Fe	Peeper SO_4	Peeper H_2S	Rh. H_2S	*Diss. Fe	Peeper SO_4	Core H_2S
Jun. 01, 2018	0.244	0.172	0.33	73	0.258	0.315	NM	43	0.358	0.665	NM	211	NM
Jul. 02	0.384	0.199	13.4	12	0.513	**0.135**	15.1	109	1.89	2.09	6.09	287	NM
Aug. 02	**0.161**	1.64	1.17	58	0.940	1.12	9.84	60	0.350	1.13	1.82	13	0.590
Aug. 30	0.696	1.25	NM	NM	1.05	3.07	NM	NM	0.574	1.96	NM	NM	**0.098**
Oct. 01	1.15	**0.154**	3.78	15	1.06	0.964	0.73	28	0.941	0.433	1.72	43	**BD**

NM = not measured; BD = below detection (< 0.078 mg L^{-1}). Bolded values indicate measured concentrations of H_2S below the suggested 0.165 mg L^{-1} protection limit for WR.
*Defined as the measurable fraction of Fe, which passed through a 0.45 µM pore-size filter and acidified to *p*H < 2.0 using trace metal grade HNO_3.

Table 4.13. 2019 Pit A paddy. Measured concentrations of hydrogen sulphide (H_2S; mg L^{-1}), dissolved (Diss.) iron (mg L^{-1}), and sulphate (SO_4; mg L^{-1}) in traditional peeper pore water representation samples and modified peepers. Modified (Mod.) peepers were used to obtain direct aspirates of pore water for H_2S measurement (n = 1 for each sampling event per location).

	Inflow				Middle				Outflow			
	Peeper H_2S	Mod. Peeper H_2S	*Diss. Fe	Peeper SO_4	Peeper H_2S	Mod. Peeper H_2S	*Diss. Fe	Peeper SO_4	Peeper H_2S	Mod. Peeper H_2S	*Diss. Fe	Peeper SO_4
Jun. 04, 2019	0.280	0.452	76.7	985	0.449	0.215	12.1	41	2.68	0.643	3.13	476
Jul. 02	0.344	1.40	25.9	659	0.338	2.26	11.2	3	0.726	6.21	5.12	245
Aug. 01	0.703	12.1	14.8	234	3.04	8.22	9.59	107	0.747	12.1	1.52	204
Sept. 03	2.41	1.30	2.33	417	1.72	1.30	7.57	163	0.595	5.88	2.54	78
Oct. 03	0.670	0.334	1.92	315	0.477	2.85	5.98	175	1.54	3.10	1.07	177

Note: All measured H_2S concentrations were above the suggested 0.165 mg L^{-1} protection level for WR.
*Defined as the measurable fraction of Fe, which passed through a 0.45 µM pore-size filter and acidified to *p*H < 2.0 using trace metal grade HNO_3.

Table 4.14. Pit A paddy. Wild rice phenology as observed during 2017, 2018, and 2019 growing seasons. Adapted from Oelke et al. (1982).

		*Date of Stage Observation (Approximate Growing Days)		
Stages of Wild Rice Development		**2017	2018	2019
Vegetative Growth Phase	Germination	Jun. 01 (0)	May 17 (0)	May 07 (0)
	Emergence	Jun. 05 (4)	May 22 (5)	May 22 (15)
	Floating Leaf	Jun. 12 (11)	May 29 (12)	May 30 (23)
	Aerial Leaf	Jun. 19 (18)	Jun. 01 (15)	Jun. 04 (28)
	Early Tillering	Jul. 03 (32)	Jul. 02 (46)	Jul. 02 (56)
Reproductive Growth Phase	Jointing	Jul. 11 (40)	Jul. 02 (46)	Jul. 18 (72)
	Boot	Jul. 17 (47)	Jul. 10 (54)	Jul. 18 (72)
	Early Flowering	Jul. 24 (54)	Jul. 10 (54)	Jul. 18 (72)
	Mid Flowering	Jul. 31 (61)	Jul. 17 (61)	Jul. 18 (72)
	Grain Formation	Aug. 07 (68)	Aug. 07 (83)	Jul. 26 (80)
	Maturity	***Aug. 31 (92)	***Aug. 16 (891)	§Aug. 23 (108)

*Date on which developmental stage was observed.
**Wild rice seeds initially broadcast throughout the Pit A paddy on May 22, 2017, following approximately one week of allowing leveled sediment to saturate with Pit A water.
***Harvest and sampling date – plant, seed, and sediment.
§Harvest and sampling date – plant, seed, and sediment. However, seeds were observed dropping from WR on Aug. 15, 2019.

Table 4.15. Pit A paddy. Measured characteristics of plant and seed tissue samples obtained during harvest events of 2017, 2018, and 2019 (average ± one standard deviation). Units from Total N through Fe are %; Al through Zn are µg g^{-1}.

	Shoots		Seeds	
	2018 (n = 12)	2019 (n = 11)	2017 (n = 9)	2019 (n = 6)
Total N (%)	0.68 ± 0.08	0.75 ± 0.09	1.24 ± 0.12	1.55 ± 0.090
P	0.13 ± 0.02	0.08 ± 0.02	NM	0.243 ± 0.028
K	1.50 ± 0.23	1.18 ± 0.39	NM	0.361 ± 0.024
S	0.30 ± 0.07	0.46 ± 0.1	0.12 ± 0.01	0.118 ± 0.010
Ca	0.59 ± 0.32	1.73 ± 0.81	NM	0.038 ± 0.006
Mg	0.36 ± 0.05	0.37 ± 0.03	NM	0.102 ± 0.008
Na	0.50 ± 0.08	0.48 ± 0.10	NM	0.004 ± 0.001
Fe	0.10 ± 0.04	0.32 ± 0.20	NM	0.020 ± 0.003
Al (µg g^{-1})	80 ± 37	248 ± 314	NM	7 ± 3
Cu	1.5 ± 0.3	2.2 ± 0.7	NM	4.4 ± 0.8
Mn	1,199 ± 406	586 ± 256	NM	33 ± 7
Zn	9.6 ± 1.2	22 ± 11	NM	25± 2

NM = not measured.

Table 4.16. Pit C paddy. 2018-2019 inflow and outflow water characteristics (n = 1 for each sampling event). Units for Bicarb. Alk. through SO_4 are mg L^{-1}; B, Mn, and Cu are µg L^{-1} (n = 1 for each sampling event per location).

	Inflow		Outflow	
	Aug. 15, 2018	Aug. 01, 2019	Aug. 15, 2018	Aug. 01, 2019
*p*H (SU)	8.4	8.4	8.4	8.3
Conductance (µS cm^{-1})	2,633	2,672	2,640	2,658
Bicarb. Alk. (mg L^{-1})	461	554	487	556
Ca	29	31	32	32
Mg	383	369	405	380
Na	53	49	54	50
SO_4	1,310	1,270	1,340	1,290
B (µg L^{-1})	204	190	213	195
Mn	20	25	79	68
Cu	2.1	BD	2.3	1.1

BD = below detection.

Table 4.17. Pit C paddy. Measured concentrations of characteristics of source sediment, and sediment sampled during the 2018 and 2019 plant harvest (average ± one standard deviation). Units for NH_3 through Na are μg g^{-1}.

	Source Sediment (n = 1)	2018 (n = 3)	2019 (n = 8)
LOI (%)	12.3	5.0 ± 1.1	27 ± 10
*p*H (SU)	6.6	7.4 ± 0.1	6.9 ± 0.1
Bulk Density (g cm^{-3})	0.4	0.2 ± 0.02	0.3 ± 0.1
Total N (%)	NM	0.1 ± 0.03	0.8 ± 0.3
NH_3+NH_4-N (μg g^{-1})	24.8	6.2 ± 1.2	1.5 ± 0.5
PO_4	5.1	0.7 ± 0.1	0.6 ± 0.3
Al	357	86 ± 5	39 ± 17
Cu	2.1	0.7 ± 0.1	0.1 ± 0.1
Fe	1,819	222 ± 5	803 ± 217
Mn	267	27 ± 1	12 ± 7
S	214	175 ± 26	97 ± 62
Zn	7.0	1.0 ± 0.1	0.4 ± 0.2
Ca	3,235	271 ± 48	172 ± 72
K	86.1	11.4 ± 3.4	6.4 ± 2.9
Mg	1,246	359 ± 61	156 ± 52
Na	23	29 ± 3	11 ± 4

NM = not measured.

Table 4.18. 2018 Pit C paddy. Measured concentrations of hydrogen sulphide (H_2S; mg L^{-1}), dissolved (Diss.) iron (mg L^{-1}), and sulphate (SO_4; mg L^{-1}) using traditional peepers and Rhizons. Rhizons (columns with the 'Rh' header) were used to obtain direct aspirates of pore water for H_2S measurement (n = 1 for each sampling event).

	Inflow				Middle				Outflow			
	Peeper H_2S	Rh. H_2S	*Diss. Fe	Peeper SO_4	Peeper H_2S	Rh. H_2S	Diss. Fe	Peeper SO_4	Peeper H_2S	Rh. H_2S	Diss. Fe	Peeper SO_4
Jul. 02, 2018	0.221	**0.118**	15.8	595	0.576	BD	4.92	84	0.658	**0.150**	10.8	137
Aug. 02	0.370	**0.121**	10.1	432	0.268	**0.096**	8.47	210	0.372	**0.105**	28.8	612
Aug. 30	0.496	0.207	NM	NM	0.332	BD	NM	NM	0.377	0.314	NM	NM
Oct. 01	0.529	0.182	4.99	383	0.398	**0.134**	6.22	205	0.340	0.271	23.5	378

BD = below detection (< 0.078 mg L^{-1}); NM = not measured. Bolded values indicate measured concentrations of H_2S below the suggested 0.165 mg L^{-1} protective level for WR.
*Defined as the measurable fraction of Fe, which passed through a 0.45 µM pore-size filter and acidified to *p*H < 2.0 using trace metal grade HNO_3.

Table 4.19. 2019 Pit C paddy. Measured concentrations of hydrogen sulphide (H_2S; mg L^{-1}), dissolved (Diss.) iron (mg L^{-1}), and sulphate (SO_4; mg L^{-1}) using traditional peepers and modified (Mod.) peepers. Modified peepers were used in place of Rhizons to obtain direct aspirates of pore water for H_2S measurement (n = 1 for each sampling event).

	Inflow				Middle				Outflow			
	Peeper H_2S	Mod. Peeper H_2S	*Diss. Fe	Peeper SO_4	Peeper H_2S	Mod. Peeper H_2S	Diss. Fe	Peeper SO_4	Peeper H_2S	Mod. Peeper H_2S	Diss. Fe	Peeper SO_4
Jun. 04, 2019	0.310	**0.101**	7.55	441	1.700	0.420	12.4	574	0.700	0.730	11.9	129
Jul. 02	0.423	0.717	4.79	199	0.525	16.2	5.73	187	0.739	0.825	5.68	90
Aug. 01	0.848	3.50	0.642	468	0.706	23.5	0.974	350	6.03	5.89	0.368	358
Sept. 03	0.682	4.84	0.380	322	0.571	11.3	1.63	320	0.443	NS	4.61	326
Oct. 03	0.802	3.90	NS	383	0.898	9.90	0.436	143	1.09	NS	7.97	10

NS = no sample. Bolded values indicate measured concentrations of H_2S below the suggested 0.165 mg L^{-1} protective level for WR.
*Defined as the measurable fraction of Fe, which passed through a 0.45 µM pore-size filter and acidified to *p*H < 2.0 using trace metal grade HNO_3.

Table 4.20. Pit C paddy. Wild rice phenology as observed during 2018 and 2019 growing seasons. Adapted from Oelke et al. (1982).

	Stages of Wild Rice Development	*Date of Stage Observation (Approximate Growing Days)	
		2018	2019
Vegetative Growth Phase	Germination	May 26 (0)	May 07 (0)
	Emergence	Jun. 01 (6)	May 07 (0)
	Floating Leaf	Development of floating leaf and initial aerial stages were not documented.	May 22 (15)
	Aerial Leaf		Jun. 04 (28)
	Early Tillering	Jul. 02 (37)	§Jul. 02 (56)
Reproductive Growth Phase	Jointing	Jul 10 (45)	§§Jul. 09 (63)
	Boot	Jul 10 (45)	§§Jul. 09 (63)
	Early Flowering	Jul. 13 (48)	§§Jul. 09 (63)
	Mid Flowering	Jul 17 (58)	§§§Jul. 26 (80)
	Grain Formation	Aug. 02 (76)	‡Aug. 23 (108)
	Maturity	***Aug. 15 (87)	‡Aug. 23 (108)

*Date on which developmental stage was observed. Achievement of developmental stages would have occurred prior to observation.

**Wild rice seeds initially broadcast throughout the Pit C paddy on May 22, 2018, following approximately one week of allowing leveled sediment to saturate with Pit C water.

***Harvest and sample date – plant, seed, and sediment.

§Goose herbivory first observed.

§§Extensive goose herbivory; first flowering observed.

§§§Additional, extensive goose herbivory observed. Seed production observed on some remaining WR.

‡Seed filled and observed falling from remaining, productive plants. Immediate harvest and sampling completed.

Table 4.21. Pit C paddy. Measured characteristics of plant and seed tissue samples obtained during harvest events of 2018 and 2019 (average ± one standard deviation). Units for Total N through Fe are %; Al through Zn are μg g^{-1}.

	Shoots		Seeds
	2018 (n = 12)	2019 (n = 9)	2019 (n = 6)
Total N (%)	0.74 ± 0.34	1.89 ± 0.31	1.86 ± 0.10
P	0.10 ± 0.03	0.25 ± 0.16	0.25 ± 0.03
K	2.37 ± 0.49	2.18 ± 0.42	0.35 ± 0.05
S	0.31 ± 0.06	0.90 ± 0.17	0.14 ± 0.01
Ca	0.16 ± 0.03	0.32 ± 0.22	0.04 ± 0.01
Mg	0.43 ± 0.09	0.78 ± 0.13	0.12 ± 0.02
Na	0.42 ± 0.11	0.67 ± NC	0.01 ± 0.01
Fe	0.10 ± 0.02	0.11 ± 0.08	0.01 ± 0.01
Al (μg g^{-1})	235 ± 57	172 ± 146	6 ± 1
Cu	1.4 ± 0.4	2.4 ± 1.1	4.2 ± 0.9
Mn	333 ± 81	1,664 ± 869	37 ± 12
Zn	8.3 ± 2.2	12.1 ± 5.9	11.5 ± 2.7

NC = not calculable.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTION

Management of critical freshwater usages requires knowledge of how to scientifically evaluate both exposures within these systems and responses of indicator organisms to those exposures. Of particular current interest are responses of WR to exposures of legacy-mine influenced waters. An interest in effects of increased water depth and water depth increases has concurrently developed. Research detailed in this Dissertation provides a scientific basis for evaluating responses of WR to exposures of mine-influenced waters and sediments, be they actively influenced or legacy-influenced media. The overall questions asked within the research foci presented were: 1) can the influences of industrial discharges on WR be identified independent of water depth and water depth fluctuations; and 2) can bioassays accurately predict the influences of industrial discharges on WR. Multiple recent studies have been completed focused on investigating potential effects of changes in water quality caused by anthropogenic effects (Myrbo et al. 2017; Pastor et al. 2017; LaFond-Hudson et al. 2018, 2020); however, they were designed under conditions atypical of conditions in natural WR areas. In this thesis, these questions were approached from a toxicological perspective with the intent of ensuring representativeness of field conditions under which these exposures may occur. Based on beneficial characteristics of each bioassay, site specific strategies using small-scale (mesocosm and microcosm), and *in-situ* (raft- and paddy- scale) bioassays were used to answer these overall questions.

5.1 MESOCOSM- AND MICROCOSM- SCALE BIOASSAYS

Initially, smaller-scale mesocosm- and microcosm- scale bioassays allowing for more direct observations and management were designed and used to measure responses of WR to exposures of mine- (CL and UL) and non-mine-influenced sediments (RRB). Notable benefits of these bioassays focused on better representation of natural WR areas: use of site-specific water and / or sediment from locations not conducive for *in-situ* bioassay development (i.e., vandalism concern); flow-through conditions more representative of natural WR areas; and *in-situ* deployment (mesocosm) under access-controlled conditions when and where possible. The flow-through *in-situ* mesocosm-scale bioassay allowed for more focused evaluation of WR growth, development, and reproductive potential throughout an entire growing season. During which, a growth-rate differential between WR grown in mine (CL and UL) and non-mine (RRB) influenced sediments was visually observed during the aerial stage, although the overall

phenological developmental rate was similar between WR grown in each sediment. This suggested that the aerial stage was specifically influenced for WR grown in CL and UL sediment and not in RRB sediment. WR plants were allowed to mature through seed filling; however, the overall growth differential remained. Conclusions of previous research focused on WR nitrogen uptake at different life stages, specifically aerial prior to and during reproductive maturity, suggested a nitrogen limitation or deficiency as the potential source of the growth differential. Measured concentrations of ammonia-nitrogen in sediment samples obtained at mesocosm bioassay initiation, mid-season (when the growth differential was observed), and at bioassay conclusion, were supportive of a nitrogen limitation in CL and UL sediments contrasted to RRB sediment. WR in RRB sediment displayed typical phenological growth and development. Following these observations through reproductive maturity, the need for a smaller-scale bioassay to more rapidly discern these growth differentials was apparent.

The microcosm-scale bioassay was developed and used to evaluate WR growth and development using less time, water, sediment, and space. The ultimate goal was to design and evaluate the bioassay as a proxy (substitute) for the mesocosm-scale bioassay. Previous research detailed WR growth and development in the absence of overlying water. Therefore, this bioassay was designed to accelerate the development of WR in saturated sediment to the aerial stage at which the growth differential was previously observed. Similar WR growth responses were observed and measured, and for dry weight biomass and seed production, both generally visually observable, no statistical differences were identified between the mesocosm- and microcosm-scale bioassays. Under these conditions the microcosm-scale bioassay was determined representative of the mesocosm-scale bioassay for these two evaluation criteria and could be defensibly used as a proxy for the mesocosm-scale bioassay.

5.2 RAFT-STYLE BIOASSAYS

These *in-situ* raft-style bioassays were designed to obtain WR exposure-response data in remote areas lacking power availability, not conducive to remote-power generation (generator), a need for onsite build and deployment (simplifying transport), and not conducive to obtaining sufficient water and / or sediment for in-lab testing. An added benefit developed for the legacy-mine influenced pit study – the benefit of raft bioassay deployment in water resources which may be considered for potential discharge, but are sufficiently remote to prevent adequate sample transport for in-lab bioassay testing, and are not conducive to typical WR growth (e.g., excessive

water depth; lack of sediment). Rafts deployed in the Seine River were beneficial for multiple reasons: location remoteness, lack of power or power generation; and water depth in excess of typical WR tolerances. Conclusions of previous studies detailed how WR responded adversely to exposures of excessive water depth, or water depth increases at specific life stages. Similar observations were documented during the Seine River raft study. Developmental stages of WR were differentially sensitive to water depth increases: floating leaf appeared more sensitive, followed by the submerged stage, with the aerial stage the less sensitive stage. Basically, once WR achieved aerial stage, water depth increases of 30 cm over 15 days did not result in lack of continued growth or development; or seed production up to a 90 cm final depth. Therefore, achievement of aerial development can be determined critical to reproductive maturity specifically under these conditions.

Raft bioassay deployment in the legacy-mine influenced pits was due to excessive water depth and a general lack of sediment; and a need for *in-situ* WR exposure-response data in water suggested as detrimental to WR phenology. Specifically, aqueous sulphate in pit waters was within the range suggested to be detrimental to WR phenology. Two rafts were deployed in each of three pits representing a range of aqueous sulphate; approximately 100 (Pit B), 350 (Pit A), and 1,300 (Pit C) mg L^{-1}. One raft was designed to test WR responses to water depth exposures; and one to test WR responses to different sediment formulations. Based on data obtained during the Seine River raft study, development to the aerial stage was used as the overall endpoint for the mine pit raft study. Overall, WR developed according to typical temporal phenology in all three legacy-mine influenced pits at 20 and 40 cm water depths in all sediment formulations. A developmental differential was observed between water depths, which was expected. The greater the water depth, the longer the time between achieving subsequent developmental stages. Overall, no adverse influences from pit water exposures were observed or measured during this study; and aerial phenology was achieved at water depths conducive to typical WR phenology.

5.3 PADDY-SCALE BIOASSAYS

To date, no controlled, *in-situ* scientific studies representative of field conditions measuring responses of WR to exposures of industry-influenced waters throughout multiple, sequential growing seasons in flow-through paddies have been completed. Due to the distinct need for these data, larger-scale *in-situ* flow-through WR paddy bioassays were constructed adjacent to two of the legacy-mine influenced pits (Pit A and Pit C) selected for the previous raft study. Paddy

inflow water was directly pumped from the respective adjacent pit. For the current study, the Pit A paddy was evaluated for three growing seasons, and the Pit C paddy was evaluated for two growing seasons. Overall, WR in each paddy developed according to typical phenology. In both paddies, exposure concentrations of aqueous sulphate and pore water hydrogen sulphide exceeded those which have been suggested as detrimental to WR phenology, distribution, and productivity. Density of WR in the Pit A paddy increased nearly 10x between the first two growing seasons (approximately 60 to $\geq$ 400 m^{-2}), and remained at approximately this density through the third growing season. Although slight yellow discoloration of WR leaves was observed at approximately mid-(third) season, this was more likely due to a nitrogen limitation and / or intra-specific competition for resources than SO_4 / sulphide exposures. Density of WR in the Pit C paddy remained similar (30-40 m^{-2}) between the two growing seasons. Significant differences between seasons in terms of WR plant height, dry weight biomass, seed production, and other characteristics in the Pit C paddy can be attributed to multiple, extensive goose herbivory events beginning in early July (during boot stage) 2019.

Pore water H_2S in each paddy routinely exceeded 0.165 mg L^{-1}, a concentration suggested to be protective for WR, regardless of the sampling method (traditional peeper, Rhizon, modified peeper). Since H_2S can be produced via intracellular SO_4 reduction (Wang 2012), increased H_2S concentrations in Pit A and Pit C paddy pore water samples obtained using modified peepers may have been due to aspiration of sulphur reducing bacteria (SRB), SRB cellular contents, or a combination of these potential sources. One mechanism by which H_2S exposures may be decreased is complexation with available iron to form FeS. Although conditions within each paddy appeared conducive to formation of FeS and FeS root coatings, only Fe-O(OH) root coatings were observed and potentially verified using SEM-EDX root surface characterization. Although statistically significant within- and between- paddy and growing season differences were identified between measurements of WR plant characteristics (height, dry weight biomass, seed production), no adverse responses were due to Pit A or Pit C water, or water-related, exposures.

5.4 WILD RICE BIOASSAY BENEFITS, CURRENT CONSIDERATIONS, AND FUTURE RESEARCH FOCI

Each bioassay as developed and used is specifically designed to be as representative of field conditions as possible given site-specific limitations. These bioassays allow for more detailed

evaluation under more controlled conditions, potentially in less than one growing season, while the flow-through design tends to result in better *in-situ* representativeness. Benefits may be compounded if resources allow for concurrent use. For example: Mesocosm- and microcosm-scale bioassays are exceptionally beneficial and efficient for obtaining WR exposure-response data when resources are sufficient (water, sediment general supplies, lab / greenhouse space). For the current study, concurrent site sediment exposures of WR in mesocosm and microcosm bioassays would have resulted in approximately one year's time savings. Currently for example, research foci using this microcosm-scale bioassay are 1) amendment of nitrogen to CL and UL sediments in an effort to mitigate WR growth differentials between CL and UL vs. RRB sediments; and 2) more detailed investigation of early WR life stage development and nutrient uptake and allocation. The microcosm-scale bioassay is particularly well suited and designed for more detailed study of these research foci.

Raft-style bioassays are generally more beneficial for deployment in remote areas lacking power availability, areas of excessive water depth and / or lacking sediment, and specifically in such waters with the potential for future discharge. They are also beneficial in locations, specifically when lacking power, not conducive to development and use of larger-scale systems such as paddies. However, areas selected for deployment must be sufficiently protected from storm events. For the current legacy-mine influenced pit study, the overall challenge was maintaining rafts in their anchored position. Multiple storm events resulted in displacement of rafts in mine pits, followed by resource intense retrieval events. A more secure form of anchoring would have likely eliminated some, if not all, raft displacements and subsequent retrieval events. Furthermore, additional consideration will be given to more intense protection of tubs on rafts. Evidence of wildlife herbivory was observed on some mine-pit deployed rafts. Use of netting and / or wire mesh, as was used to protect tubs on rafts deployed at the Seine River location, is recommended.

Although the current focus is on effects of chemical characteristics of water and pore water on WR phenology, more critical data may be obtained focused on water depth influences concurrent with water and / or pore water exposure WR effects. For example: Regardless of adverse influences on WR from water and / or pore water exposures, if the water resource in question may be discharged, the receiving system may need to be carefully chosen – if supportive of WR,

increases in water depth, specifically during sensitive life stages, in particular floating leaf, may result in substantial losses within the WR population.

The overall and primary benefits of using the paddy-scale bioassay are specific to *in-situ* representativeness of conditions in natural WR areas; in particular, flow-through design on a larger, multi-growing season scale. The flow regime may be designed to simulate that of a specific creek, stream, or river using study-specific water; and may be modulated to simulate flow regime attenuations. This can be critical when accounting for water depth increases in the receiving system during specific stages of WR phenology. For the current study, paddy inflow water was directly pumped from its adjacent pit at a rate simulating that of a local stream – a potential receiving system for any future de-watering of these pits. Sediment for each paddy was obtained from this stream. Using this design, a site-specific system was developed and used to more accurately predict responses of WR to exposures of water contained within these pits under conditions as closely representative of potential pit discharge conditions as possible.

Currently, both paddies remain in use for their intended purpose. The Pit A paddy is in its fourth sequential growing season; and the Pit C paddy is in its third sequential growing season. Future research foci using these paddies include: 1) more detailed study of entire life cycle growth, development, and plant and sediment nutrient (e.g., nitrogen, phosphorus, potassium, sulphur) dynamics at specific phenological stages (e.g., seedling, floating leaf, aerial, reproductive maturity, and senescence); 2) SEM-EDX characterization of root surfaces during specific phenological stages (as above) with a particular focus on iron containing (root) coatings; and 3) continued overall characterization of WR responses to industry-influenced waters and water related exposures.

Overall, benefits of selecting one or more of these bioassays for use will depend on specific characteristics of the site and data use objectives. Larger-scale, flow-through *in-situ* paddies are the ultimate bioassay design. However, as with mesocosm, microcosm and raft-style bioassays, paddies have their requirements and therefore limitations. Representativeness of natural WR areas is paramount to data defensibility. Bioassays described and designed herein were done so to represent field conditions to the extent possible given the scale of the bioassay.

REFERENCES

LaFond-Hudson, S., Johnson, N.W., Dewey, B. 2020. Interactions between sulfide and reproductive phenology of an annual aquatic plant, wild rice (*Zizania palustris*). Aquat. Bot. 164, 103230.

LaFond-Hudson, S., Johnson, N.W., Pastor, J., Dewey, B. 2018. Iron sulfide formation on root surfaces controlled by the life cycle of wild rice (*Zizania palustris*). Biogeochemistry. https://doi.org/10.1007/s10533-018-0491-5.

Myrbo, A., Swain, E.B., Engstrom, D.R., Coleman Wasik, J., Brenner, J., Dykhuizen Shore, M., Blaha, G. 2017. Sulfide generated by sulfate reduction is a primary controller of the occurrence of wild rice (*Zizania palustris*) in shallow aquatic ecosystems. J. Geophys. Research: Biogeosci. 122, 2736–2753. https://doi.org/10.1002/2017JG003787.

Pastor, J., Dewey, B., Johnson, N.W., Swain, E.B., Monson, P., Peters, E.B., Myrbo, A. 2017. Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments. Ecological Applications 27(1), pp. 321-336.