

Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments

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Abstract. Under oxygenated conditions, sulfate is relatively non-toxic to aquatic plants. However, in water-saturated soils, which are usually anoxic, sulfate can be reduced to toxic sulfide. Although the direct effects of sulfate and sulfide on the physiology of a few plant species have been studied in some detail, their cumulative effects on a plant's life cycle through inhibition of seed germination, seedling survival, growth, and seed production have been less well studied. We investigated the effect of sulfate and sulfide on the life cycle of wild rice (*Zizania palustris* L.) in hydroponic solutions and in outdoor mesocosms with sediment from a wild rice lake. In hydroponic solutions, sulfate had no effect on seed germination or juvenile seedling growth and development, but sulfide greatly reduced juvenile seedling growth and development at concentrations greater than 320 µg/L. In outdoor mesocosms, sulfate additions to overlying water increased sulfide production in sediments. Wild rice seedling emergence, seedling survival, biomass growth, viable seed production, and seed mass all declined with sulfate additions and hence sulfide concentrations in sediment. These declines grew steeper during the course of the 5 yr of the mesocosm experiment and wild rice populations became extinct in most tanks with concentrations of 250 mg SO₄/L or greater in the overlying water. Iron sulfide precipitated on the roots of wild rice plants, especially at high sulfate application rates. These precipitates, or the encroachment of reducing conditions that they indicate, may impede nutrient uptake and be partly responsible for the reduced seed production and viability.

Key words: hydroponics; life cycles; sulfate; sulfide; toxicity; wetlands; wild rice; *Zizania palustris*.

INTRODUCTION

Under oxygenated conditions, sulfate, the most abundant form of dissolved sulfur in aquatic systems, is relatively non-reactive, and is therefore relatively non-toxic. However, where oxygen is absent and organic matter is present, sulfate can serve as an electron acceptor for heterotrophic microbial metabolism, producing reactive reduced sulfur species. When sulfate concentrations limit the activity of sulfur-reducing microbes, an increase in sulfate can enhance the decomposition of organic matter and initiate a cascade of interrelated biogeochemical reactions (Garrels and Christ 1965) that alter the bioavailability of phosphorus and other nutrients (Lamers et al. 2002), and generate alkalinity (Giblin et al. 1990). One of the most reactive products of sulfate reduction is hydrogen sulfide, which we here term “sulfide.” If dissolved sulfide

persists in the rooting zone of aquatic plants, it can inhibit root growth and metabolism (Mendelsohn and McKee 1988, Koch and Mendelsohn 1989, Koch et al. 1990, Lamers et al. 2002, 2013, Gao et al. 2003, Armstrong and Armstrong 2005, Geurts et al. 2009, Martin and Maricle 2015) and photosynthesis (Pezeshki 2001). If root biomass and metabolism are reduced by elevated sulfide concentrations, then the plant's ability to take up limiting nutrients may be impaired (DeLaune et al. 1983, Koch et al. 1990, Gao et al. 2002, 2003, Armstrong and Armstrong 2005, Lamers et al. 2013).

Although the direct effects of sulfide on the physiology of individual plants of a few species have been studied in some detail, the cumulative effects of sulfide on a plant's life cycle through possible inhibition of seed germination, seedling survival, and seed production have been less well studied. Sulfide could affect any or all of these stages of a plant's life cycle, either directly by toxicity to seeds and seedlings or indirectly by decreasing nutrient uptake through roots during seed formation. If so, then populations may become sparser and less viable over several life cycles. Population effects could be realized rapidly in non-clonal annual aquatic emergent plant species that

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rely exclusively on seed production, germination, and seedling survival to produce the next generation of emergent shoots. A seed bank in the sediment would facilitate recovery of a population after one or two catastrophic growing seasons, but would become depleted if chronic sulfide toxicity does not allow occasional successful growth and reproduction to restock the seed bank.

Northern wild rice (*Zizania palustris* L., hereafter wild rice) is an annual graminoid (Family Poaceae, Tribe Oryzeae), which is most abundant in the rivers and lakes in the Lake Superior region. Because of its widespread distribution and tendency to form large monotypic stands, wild rice is an important component of the food supply for the aquatic and avian herbivores and seed consumers, such as muskrats and waterfowl. Reduction of these wild rice populations could, therefore, have cascading effects on diverse aquatic food webs. In addition, the native Ojibwe people of the Lake Superior and Lake Michigan region teach that they were led to this region to find “the food that grows upon the water,” which is wild rice. The Ojibwe identify their origins with wild rice and consider themselves “people of the rice” (Vennum 1998). The resource is also important to Menominee and Dakota peoples of the region. Efforts to enhance the productivity, perpetuation, and restoration of natural wild rice populations are of great importance to state and tribal natural resource agencies for both ecological and cultural reasons.

The wild rice life cycle begins when seeds from the previous year or years germinate in mid to late May. Juvenile seedlings grow through the water column in early to mid-June. Upon reaching the surface, the seedling generates a floating leaf that fixes carbon into carbohydrates for root production and nutrient uptake. By the end of June, nitrogen and other nutrients are translocated out of the floating leaf into an aerial shoot emerging from the leaf axil, and the floating leaf dies. The early stages of the vegetative growth of the aerial shoot happen during the next two weeks and vegetative growth continues until the emergence of flowering heads in late July. Seed production and ripening begins in early to mid-August with seed production completed by early- to mid-September. The productivity of wild rice is primarily limited by nitrogen and secondarily by phosphorus; increased nitrogen supply accelerates development of the life cycle and reduces allocation to roots (Sims et al. 2012a) and increases the number of inflorescences, seeds per inflorescence, and mean seed mass, resulting in more seedlings produced the following year, and hence greater fitness (Sims et al. 2012b).

Historic observations suggested that wild rice usually occurs in waters where sulfate concentrations were near or below 10 mg/L and populations are uncommon where sulfate concentrations exceeded 50 mg/L (Moyle 1944, 1945). Based on Moyle's (1944, 1945) research, the State of Minnesota sulfate standard for waterbodies supporting wild rice is 10 mg/L; Wisconsin, Michigan, and Ontario currently do not have sulfate standards for wild rice waters. For comparison, the EPA non-enforceable,

aesthetic (taste) secondary water quality sulfate standard for human consumption is 250 mg/L (*available online*).⁷

This research is part of a larger study coordinated by the Minnesota Pollution Control Agency on the effect of sulfate on wild rice, which included an extensive survey of potential wild rice waters across Minnesota containing surface water sulfate ranging from <2 mg/L to >600 mg/L. This study was carried out because of recent interest in the nature of the relationship between sulfate and wild rice, especially with respect to potential anthropogenic sulfate enhancements to wild rice ecosystems such as sewage treatment plants, agricultural runoff, and mining of ores containing metallic sulfides. The mechanisms responsible for the decreased wild rice density with increased sulfate concentrations observed by Moyle (1944, 1945) have not been investigated until this study.

Although we have a fairly extensive understanding of the general aspects of the life cycle of wild rice in natural stands in relation to nutrient availability and sediment chemistry (Keenan and Lee 1988, Day and Lee 1990, Meeker 1996, Lee 2002, Pastor and Walker 2006, Walker et al. 2010, Hildebrandt et al. 2012, Sims et al. 2012a, b), the way in which sulfate in surface water can affect the life cycle of wild rice, and hence its population dynamics, is much less well understood. The objectives of our research are to (1) determine the relative effects of sulfate and sulfide on seed germination, seedling viability, vegetative growth, and seed production; (2) determine the response of wild rice populations and population viability to sulfate in the overlying water and the production of sulfide in sediment porewaters.

METHODS

The effects of sulfate and sulfide on wild rice were tested in two different ways: (1) a laboratory hydroponic culture system and (2) an outdoor mesocosm system that better mimicked natural wild rice waters, but does not control the chemical exposures as precisely as the hydroponic experiments did. Short-term (10 or 11 days) hydroponic exposures of seeds and seedlings to sulfate and sulfide were conducted to examine effects on seed germination, seedling growth, and survival. Full life cycle tests were conducted in mesocosms where wild rice grew in sediment taken from a natural wild rice lake. These multi-year outdoor tests examined the effects of elevated surface water sulfate and the associated increased sedimentary sulfide concentrations on germination, survival, growth, and reproduction.

Hydroponic experiments

Li et al. (2009) published one of the few dose-response studies of aquatic macrophytes (*Typha* and *Cladium*) to sulfide, which requires the maintenance of anaerobic

⁷ <http://water.epa.gov/drink/contaminants/secondarystandards.cfm>

conditions. Malvick and Percich (1993) developed a simple hydroponic system to investigate effects of nutrients on germination and early growth of wild rice, but their system could only be implemented under aerobic conditions. We used these two studies as starting points for the development of our methods.

Wild rice seeds used for all hydroponic experiments were collected on 30 August 2012 from Little Round Lake (Minnesota Lake ID 03-0302, 46.97° N, 95.74° W; average surface water sulfate <0.5 mg/L and porewater sulfide = 77 µg/L, $n = 5$). The seeds were stored at 4°C in polyethylene bottles in a darkened room until needed for experiments. Immediately before each experiment, a subsample of these seeds was selected that were intact, filled, not green (unripe), and not moldy. To obtain seedlings for juvenile seedling response to sulfate or sulfide, the selected seeds were allowed to germinate in aerobic deionized water until a 1–2 cm long mesocotyl shoot appeared, which usually occurred 5–7 days after germination. The mesocotyl is the embryonic stem that will develop into the mature stem.

Once the seeds or seedlings were selected, they were picked up with forceps and transferred to the appropriate test in appropriate containers. The hydroponic solution was one-fifth strength Hoagland's solution in 5 mmol/L PIPES buffer to maintain a pH of 6.8 ± 0.03 (mean \pm SD) in the solution, similar to that observed in the porewater of mesocosm experiments. Nitrogen was supplied only as ammonium (0.16 mmol/L NH_4Cl) to mimic natural concentrations of inorganic nitrogen in wild rice waters (Walker et al. 2010). The Hoagland's solution contained sulfate only in trace amounts as ZnSO_4 (0.5 µmol/L) and CuSO_4 (0.15 µmol/L). This nutrient solution was then augmented with appropriate amounts of anhydrous Na_2SO_4 or $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ to achieve desired sulfate or sulfide treatment concentrations. The one-fifth Hoagland's solution and PIPES buffer were chosen based on previous trials to determine proper strengths and buffers that would support seedling growth without adverse effects (see Appendix S1 for composition of our modification of Hoagland's Solution).

Germination of wild rice seeds under aerobic conditions subject to various concentrations of sulfate.—The selected seeds were placed into each of six numbered plastic cups to total 50 seeds each, then randomly assigned and transferred to each of six 1-pint Mason jars (1 pint = 473 mL) containing six sulfate treatment concentrations of 0 (trace), 10, 50, 100, 400, or 1600 mg SO_4/L . These sulfate treatments (trace to 1600 mg/L) bracket the large range encountered across Minnesota's geologically diverse landscape (10th and 90th percentiles of 0.2 and 285 mg/L, respectively; MPCA 2016), plus some mine pits over 1000 mg/L that may overflow into wild rice waters. This seed counting and random transfer was repeated twice more to result in six treatment levels with three replicate jars per treatment. The jars were covered with plastic covers fitted with rubber stoppers to facilitate solution

exchanges. Two holes in the plastic lids were left open to facilitate air exchange and to prevent the solutions from becoming anaerobic. The experiment proceeded in a growth chamber at 20°C in the dark to simulate conditions measured in sediments during the growing season, which we have measured in our mesocosms (see *Results*). The solutions were exchanged with fresh solution of the appropriate treatment concentration every three days. Dissolved oxygen in the solutions across all treatments was initially 8.280 ± 0.218 mg/L (mean \pm SD) and dropped to 2.85 ± 0.60 mg/L by the end of three days, still well above anoxic levels required for production of sulfide. Solution pH and sulfate were measured on each initial batch of sulfate treatment and on the exchanged solution from each jar. The germinated seedlings were harvested after 11 days. The number of successfully germinated seeds, determined as those that produced a mesocotyl at least 1 cm in length, were counted. The length of the mesocotyl was measured for each seed. The germinated seeds were then dried at 65°C for 3 d. The mesocotyl was then carefully separated from the seed hull and weighed.

Germination of wild rice seeds under anoxic conditions subject to various concentrations of sulfide.—The techniques used here were the same as for the germination trials under various sulfate concentrations, except that extra care was necessary to ensure anaerobic conditions. Fifty seeds were chosen as above and then placed in 700 mL borosilicate glass bottles capped using phenolic screw caps with chlorobutyl septa 5 mm thick. The one-fifth Hoagland's nutrient solution was deoxygenated with oxygen-scrubbed nitrogen before being added to the bottles. PIPES buffer was added to the test solution to maintain consistent pH levels of 6.8 ± 0.03 throughout an experiment. Bottles were filled completely with the deoxygenated nutrient solution and without introducing any air bubbles and then capped with the septa. Stock sulfide solutions (20–30 mmol/L) were prepared as needed by adding $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (sodium sulfide nonahydrate) to deionized and deoxygenated water. The concentration of the stock sulfide solution was checked periodically against a stock solution that had been standardized using an iodimetric titration. An appropriate amount of the stock solutions was added to each bottle with a Hamilton gas-tight glass syringe through the septa while simultaneously withdrawing an equivalent volume of the Hoagland's solution by means of a second syringe through the septum. All of the syringes used in this and other experiments were purged three times with oxygen-scrubbed ultra-pure nitrogen from a tilled PVDF gas sampling bag (Saint-Gobain No. D1075016-10), which had also been purged three times before filling. Added stock sulfide solution volumes range between 0.2 and 3.0 mL depending on target exposure concentrations and the nominal concentration of stock sulfide solution. The target sulfide concentrations were 0 (trace), 96, 320, 960, and 2880 µg/L. These sulfide treatments (trace to 2880 µg/L) bracket the range encountered across shallow

aquatic systems in Minnesota that potentially could host wild rice (5th and 95th percentiles of 26 and 1631 $\mu\text{g/L}$, $n = 108$; A. Myrbo, *unpublished data*).

The bottles were placed in a growth chamber in continuous darkness at $20^\circ \pm 1^\circ\text{C}$. Solutions were exchanged every two days if during the week or three days if over a weekend. The solution in each jar was sampled for sulfide analysis at the beginning and end of each two- or three-day cycle. The pH of the solution in each jar was measured at the end of each two- or three-day cycle. To obtain the initial pH of the solution, one additional replicate jar for each treatment but without seeds was filled with one-fifth Hoagland's solution, then the sulfide treatment was added using syringes as above and the jar was opened and pH was measured immediately. Total dissolved sulfide ($\text{H}_2\text{S} + \text{HS}^-$) was measured on a Hach DR5000 spectrophotometer using a colorimetric methylene blue method (4500 S2-D; Eaton et al. 2005) as implemented with Hach method 8131. The method was adapted for a lower detection limit ($\sim 15 \mu\text{g/L}$) using a photo cell with a 5 cm path length. All measurements of dissolved sulfide in both hydroponics and mesocosm experiments refer to the sum of all dissolved inorganic reduced sulfur ($\text{H}_2\text{S} + \text{HS}^-$). The samples of hydroponic water were added directly from the gas tight syringe to the sulfuric acid reagent, followed immediately by the potassium dichromate reagent. After 11 days, the germinated seeds were harvested and measured as described for the experiments on effects of sulfate on germination.

Growth of juvenile wild rice seedlings under aerobic conditions subject to various concentrations of sulfate.—We examined growth of juvenile seedlings at concentrations of 0, 10, 50, 100, 400, and 1600 mg SO_4/L . Twenty replicated 70-mL unsealed glass Kimax tubes (Cole-Parmer, Vernon Hills, IL, USA) were used for each test concentration. One seedling germinated and selected as described was placed with forceps into each Kimax tube, which was then filled with one-fifth Hoagland's solution and an appropriate amount of sulfate. The filled tubes (solution and seed) were placed into every other opening in Nalgene Resmer (ThermoFisher Scientific, Waltham, MA, USA) test tube holding racks so that light could penetrate to all sides of each tube. A total of six 40-tube racks, each containing 20 tubes, were used to hold the test tubes. Screw caps were placed loosely on the tubes to allow for oxygen exchange across the solution surface and thereby prevent the development of anaerobic conditions. The tubes were placed in a Percival environmental growth chamber where we measured $288 \pm 22 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of photosynthetically active radiation immediately above the plants using a Decagon PAR – 80 Ceptometer (Decagon Devices, Pullman, WA, USA). Tests were performed under a 16 h:8 h light:dark schedule. All racks were placed in the growth chamber so that the spaces between the racks were the same as the spaces within the racks and the tops of the tubes are within 30 cm of the bottom of the lights. The location of each rack in the growth chamber

remained the same for the test duration. Test solutions in the tubes were renewed every two days. Temperature was maintained at 21°C during lighted periods and 19°C during dark periods and the humidity was maintained at 85%. Plants were harvested after 10 days and the seed hull was carefully removed. Stem and leaf length was measured to the nearest millimeter by placing the stem with leaf stretched out on a flat surface next to ruler with the zero mark aligned with the point of stem-root transition. Total root lengths were measured in duplicate scans of the entire root system using the program WinRhizo (Regent Instruments, Quebec, Canada). Seedlings were weighed after drying at 100°C for 48 h. Control juvenile seedlings did not have any visible phytotoxic or developmental symptoms at any time and the controls had additional stem growth of at least 5.0 cm during the 10-d test.

Growth of juvenile wild rice seedlings under anaerobic conditions subject to various concentrations of sulfide.—Germinated seedlings were chosen using the same techniques described for aerobic conditions. Seven seedlings 1–2 cm in length that fit the criteria as described, were placed with a forceps in 125-mL borosilicate glass jars capped using phenolic screw caps with 5 mm thick chlorobutyl septa. Each sulfide concentration was replicated in this way in three separate jars. Deoxygenated Hoagland's nutrient solution was added as described above. Seedlings were grown in the same environmental growth chamber under the same temperature and light conditions as for the sulfate experiments but with solution sulfide concentrations of 0, 96, 320, 960, and 2880 $\mu\text{g/L}$. Solutions were exchanged every two days if during the week or three days if over a weekend. Sulfide concentrations were measured at the beginning and end of each two–three day solution exchange period. Because the plants were photosynthesizing and producing oxygen, the sulfide concentration declined during these two–three day periods. This was especially so for the lowest sulfide concentrations (less than $\sim 300 \mu\text{g/L}$) in which less than 10% remained after two days, but 70–90% of sulfide remained after two days for sulfide concentrations greater than 650 $\mu\text{g/L}$. We therefore used the time-weighted average sulfide concentration over the 10 days period to characterize the sulfide concentrations the plants were exposed to. Seedlings were harvested after 10 days, the seed hull was carefully removed, and the stem and leaf lengths and total plant mass were determined. Because many of the plants, especially at high sulfide concentrations, did not grow at all (see Results below) the roots and shoots were very fragile and no attempt was made to dissect the plants into subcomponents as with the experiment on the effects of sulfate on seedling growth.

Statistical analyses of hydroponic experiments.—The general procedure for each set of sulfate and sulfide exposure experiments was first to examine seed germination or seedling growth response across a wide range of concentrations spanning three orders of magnitude of either sulfate or sulfide as noted. The main effect of

sulfate or sulfide concentrations on the variable of interest was then tested with an analysis of variance using SigmaPlot (SYSTAT Software, San Jose, CA), USA. When the residuals were not normally distributed or the data did not have equal variance between treatments, then the data were transformed by taking the natural logarithms, which then passed normality and equal variance tests. If there were no effects across this wide range of concentrations in this experiment, then it was repeated to test whether the results were a false negative. If there were significant main effects, then Tukey's pairwise comparisons were performed to determine in which part of the range of concentrations significant effects occurred. Further experiments were then conducted twice using this narrower range of concentrations centered on the region of significant change to more precisely refine the range of response of seedling germination or growth to sulfate or sulfide concentrations.

If there was a significant effect of sulfide on seedling growth, then the biomass growth of seedlings (mg) over the 10-d period was regressed against the time-weighted total dissolved sulfide concentrations ($\mu\text{g/L}$) with a four-parameter sigmoidal function using SigmaPlot nonlinear regression

$$\text{Plant growth} = y_{\min} + \frac{y_{\max}}{1 + \exp\{-(S^{2-} - x_0)/b\}} \quad (1)$$

where y_{\min} is the right-side (minimum) horizontal asymptote (minimum growth response) y_{\max} is the height of the left-side horizontal asymptote (maximum growth response) above y_{\min} , S^{2-} is total dissolved inorganic sulfide ($\text{H}_2\text{S} + \text{HS}^-$), x_0 is the sulfide concentration at the inflection point of the curve, and b is a parameter that scales $\mu\text{g/L}$ of sulfide concentration to mg of biomass growth. The 50% effects concentration (EC50, the concentration of sulfide that caused a 50% reduction in change in plant mass compared to controls) was calculated from this regression.

The sulfate experiment had to be conducted under aerobic conditions while the sulfide experiment had to be conducted under anaerobic conditions. Therefore, redox statuses of the solutions were necessarily confounded with sulfur speciation. To test the effect of redox status on seedling growth, we compared the growth of plants from both the lowest concentrations of the sulfate (aerobic) and sulfide (anaerobic) experiment using a single-factor analysis of variance.

Mesocosm experiments

Experimental design.—We constructed mesocosms using the same procedures and designs previously reported by Walker et al. (2010) for a 5-yr experiment on the interaction of the nitrogen cycle and wild rice population dynamics.

In late spring of 2011, polyethylene stock tanks (400 L, $132 \times 78 \times 61$ cm; High Country Plastics, Caldwell, ID, USA) were fitted with overflow drain pipes and buried to ground level. The drain pipes are connected to 20-L polyethylene overflow buckets buried adjacent to each tank. Water tables were set by the inflow to the drain pipe at 23 cm above the sediment surface. The tanks were leveled

and then partly filled with 10 cm of clean sand washed with the same well water later added to the tanks (see next paragraph). The sand layer was then covered with 12 cm of surface sediment collected from a natural wild rice bed in Rice Portage Lake (Minnesota Lake ID 09-0037, 46.70° N, 92.70° W) on the Fond du Lac Band of Lake Superior Chippewa Reservation, Minnesota. Rice Portage Lake is approximately 337 ha, of which approximately 50 ha are wild rice beds (Minnesota Department of Natural Resources 2008). Ten to 20 cm of sediment over sand is sufficient to support the rooting depths we have observed in natural wild rice lakes. The sediments were kept saturated and then thoroughly homogenized in a large stock tank prior to distribution into the tanks. Analyses of five volumetric samples of the mixed sediment indicate a homogenous material ($\text{C} = 14.8\% \pm 1.7\%$, $\text{N} = 1.12\% \pm 0.13\%$, $\text{S}[\text{acid volatile sulfur}] = 0.005\% \pm 0.003\%$). Sediment bulk density was $0.27 \pm 0.01 \text{ g/cm}^3$ (Walker et al. 2010). These nutrient and bulk density values are similar to those of other wild rice beds (Keenan and Lee 1988, Day and Lee 1990). No new sediment has been added to the stock tanks since the mesocosms were established in 2011.

The tanks were immediately filled with water obtained from a nearby well after sediment additions to prevent the sediment from drying. Water was added cautiously from a garden hose to prevent redistribution and suspension of sediment. During the growing season, water levels were maintained at 23 cm above the sediment surface by weekly additions of water to the drain pipe heights or by allowing water to drain through the pipe into the overflow buckets. Rainfall N concentrations as $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ ranged from 0.2 to 1.99 mg/L while the $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ concentrations in the well water are always $<0.2 \text{ mg/L}$ (Walker et al. 2010). Sulfate concentrations in well water averaged $10.73 \pm 0.75 \text{ mg/L}$ ($n = 36$) and in rainwater averaged $2.13 \pm 1.02 \text{ mg/L}$ ($n = 16$). The sediments comprise a natural inoculation source for microbes and a background supply of nutrients for plant growth source. The sediments and plant litter remain submerged in the mesocosms year round with water levels set at approximately 20 cm in late fall.

Wild rice was planted once in late spring 2011 from seeds obtained from Swamp Lake (Minnesota Lake ID 16-0256, 47.85° N, 90.58° W), a 37-ha lake on the Grand Portage Band of Lake Superior Chippewa Reservation, Minnesota. Seeds from each year's crop were allowed to fall unimpeded into the tanks to provide the seed source for the next year's population; no further seeding from external seed sources occurred.

End-of-season plant density in Minnesota wild rice lakes monitored by the 1854 Treaty Authority averages 40 plants/ m^2 (Vogt 2010). Accordingly, the seedlings were thinned to this density (30 plants per tank) in late spring or early summer each year before the floating leaf stage was achieved. The seedlings removed from each tank during thinning in 2012–2015 were counted to estimate seed germination and early seedling success.

Immediately after installation and seeding, beginning in late June 2011, the tanks were treated with different amounts

of sulfate to achieve several target sulfate concentrations in the overlying water. There were five overlying water sulfate concentrations and six replicate tanks per sulfate concentration, for a total of 30 tanks. Nominal water column sulfate concentrations of 50, 100, 150, and 300 mg SO₄/L were maintained in sulfate-amended tanks. Aside from incidental sulfate in the make-up water from a well and rainwater, control tanks did not receive any sulfate amendments and overlying water concentrations ranged from 2 to 10 mg/L (average of 7 mg/L) depending on rainfall, evapotranspiration, and loss via sulfate reduction in the sediment. The overlying water sulfate concentrations in the mesocosm experiments bracket both the existing 10 mg/L Minnesota statutory standard for wild rice waters and the EPA drinking water standard of 250 mg/L. Samples of the water column were taken weekly and analyzed for sulfate concentration using a Lachat QuikChem 8000 Autoanalyzer (Method 10-116-10-1-A, Hach Co., Loveland, CO, USA). When necessary (approximately every two weeks), the sulfate concentration was adjusted to near the desired nominal concentrations with appropriate amounts of 10 g/L sodium sulfate (Na₂SO₄; Fisher Chemical S421, Thermo Fisher Scientific, Waltham, MA, USA) stock solution and well water. The sodium sulfate stock solution was first mixed in 1–2 L of water from the tank, then added back to the tank's overlying water with mild mixing.

Plant, sediment, and water sampling and analyses.—In each year from 2011 to 2015, five plants in each tank were randomly chosen in early summer for detailed measurements throughout the growing season and to be destructively sampled at the end of the growing season. In late August to September, ripe seeds from these plants were collected every two or three days by gently removing them, leaving unripe seeds behind for the next collection date. The seeds from each individual plant were placed in a paper envelope and marked with the tank identification number. The plants were then harvested for determination of biomass, root:shoot mass ratios and total seed production by counting seed peduncles along the flowering stem.

Seeds from each of the five sampled plants were separated into filled (viable) seeds and empty (nonviable) seeds, counted, and weighed. A subsample of seeds collected in all years except 2013 were dried at 60°C for determination of moisture content to convert wet mass to dry mass. The five sample plants were separated into root and shoot (stem + leaves), and then weighed. Root:shoot ratios and seed masses and numbers from the five sampled plants were applied to total aboveground population masses and total plant numbers to determine total root and seed biomass and number and total biomass in each tank.

While harvesting the plants for growth and biomass measurements, we noticed that plants in the tanks amended with sulfate had blackened roots while plants grown in the control tanks had white or light tan or orange roots. To investigate this further, a sample of roots from a plant from one control tank and a plant from one 300 mg/L amended tank were collected and placed immediately in water in

which dissolved oxygen had been purged by bubbling with oxygen-free N₂. These samples were analyzed for Fe and S concentrations by energy-dispersive X-ray spectroscopy (EDS) using a Hitachi TM-1000 scanning electron microscope (Hitachi High Technologies, Schaumburg, IL, USA) fitted with a Quantax EDS unit (Bruker Corporation, Billerica, MA, USA). The nominal spot size was 0.2 µm and the analysis volume was ~5 µm³. The sample of blackened roots was analyzed at seven points and the sample of tan/orange control roots was analyzed at five points.

All aboveground plant material was collected from each tank at the end of the growing season and weighed to determine total aboveground biomass. A subsample was taken to determine wet:dry ratios for moisture correction after drying at 60°C. All aboveground plant material except for the five sample plants were returned to each tank. All stems in each tank were counted at the time of harvesting the aboveground plant biomass to determine end of growing season plant density.

In 2013, significant seedling mortality occurred in all tanks after thinning but before the floating leaf stage. We believe this early season mortality was due to a record cold and late spring in northern Minnesota in April and May of 2013; ice stayed on lakes an average of 3 weeks later than the median ice-out date (data *available online*).⁸ The reduced overall emergence of plants in the spring of 2013 precluded the destructive sampling of five sample plants in each tank at the end of the 2013 growing season because this harvesting would have greatly decreased the number of viable seeds returned to the sediment for the following growing season. Instead, during 2013 all seeds were harvested from each and every plant in the tanks, sorted as described above on each collection day, and returned to the tanks within 24 h of collection without drying in order to maintain their viability for future populations. To determine wet-dry conversion ratios for these seeds, additional seeds were collected at the same collection times from an adjacent experiment on wild rice (Walker et al. 2010) for moisture determination after drying them at 60°C.

Polycarbonate porewater equilibrators (peepers) with sampling ports spaced 1.5 cm intervals were used to make in situ measurements of geochemical profiles of sulfur and iron species at discrete depths in the sediment porewater of a subset of tanks in August of 2013. Care was taken that the installation and extraction of the peepers did not disturb any plants. The method for collecting samples for sulfate, sulfide, and ferrous iron with peepers was modified from Koretsky et al. (2007). Sulfide and iron were quantified in samples immediately with minimal oxygen exposure using a colorimetric methylene blue method (4500 S2-D; Eaton et al. 2005) as implemented with Hach method 8131 for sulfide and a colorimetric phenanthroline method for iron (3500-Fe-B; Eaton et al. 2005). Sulfate was quantified with ion chromatography on a Dionex ICS 1100 system (Thermo Fisher Scientific, Waltham, MA, USA) after acidifying samples to pH < 3

⁸ http://climate.umn.edu/doc/journal/ice_out_recap_2013.htm

using hydrochloric acid and purging gently with oxygen-free nitrogen gas.

In August 2013 and 2015, we also used 10-cm long Rhizon samplers (Rhizosphere Research Products B.V., Wageningen, The Netherlands) to obtain porewater for sulfide analysis. The sampler was inserted vertically into the sediment and connected to an evacuated 125-mL serum bottle. Sulfide samples were prepared without removing the butyl rubber stopper for inline distillation by automated flow injection colorimetric analysis (4500 S2-E; Eaton et al. 2005).

On 6 October 2015, a 10-cm long sediment core was taken from each mesocosm and homogenized. Extractable iron was quantified following a 30-min exposure to 0.5 mol/L HCl, following Balogh et al. (2009), at the Minnesota Department of Health Environmental Laboratory. Total organic carbon was determined using the method of oxidative combustion-infrared analysis (U.S. EPA 2004), after pre-treatment with acid to remove inorganic carbon, at Pace Analytical Services in Virginia, Minnesota, USA.

Statistical analyses of mesocosm experiments.—The effects of sulfate concentrations on plant attributes were tested by repeated measures analysis of variance followed by pairwise comparisons between attributes of plants in the control tanks and each higher sulfate concentration. We also regressed each plant attribute against average annual sulfate concentration for each year. Correlations were assessed using Pearson's correlation test. This combination of both analysis of variance and regression was used as recommended by Cottingham et al. (2005). We used target sulfate concentrations as categorical variables in analyses of variance and growing season actual sulfate concentrations in regression analyses.

RESULTS

Hydroponic experiments

Effect of sulfate on seed germination.—Between 71% and 76% of the seeds pre-selected as filled and mold-free germinated at each sulfate concentration. Sulfate exposure concentrations of 0, 10, 50, 100, 400, and 1600 mg SO_4/L did not affect germination success, mesocotyl lengths, or the masses of the stem plus leaf (if any) and roots ($P > 0.10$ for each test). The experiment was repeated with the same results.

Effect of sulfide on seed germination.—Sulfide concentrations of 0, 96, 320, 960, and 2880 $\mu\text{g}/\text{L}$ did not affect germination success of seeds, mesocotyl masses, or mesocotyl lengths ($P > 0.10$ for each test). The experiment was repeated with the same results.

Effect of aerobic and anaerobic conditions on seed germination.—There were no differences in germination rates under anaerobic compared with aerobic conditions when concentrations of sulfur were at trace ($<1 \mu\text{mol}/\text{L}$) amounts of CuSO_4 and ZnSO_4 in the Hoagland's solution. Mean

mesocotyl lengths in the anaerobic solutions (7.8 cm) were significantly reduced ($P < 0.05$) by 38% compared with mean mesocotyl lengths in the aerobic solutions (12.5 cm).

Effect of sulfate on seedling growth.—Sulfate concentrations of 0, 10, 50, 100, 400, and 1600 mg SO_4/L did not affect the growth of juvenile seedling stem length, juvenile stem mass, juvenile root mass, or total juvenile seedling mass ($P > 0.10$ for each test). Sulfate decreased juvenile root length slightly ($P < 0.02$) but only at 1600 mg SO_4/L compared with 50 mg SO_4/L . The experiment was repeated with the same results.

Effect of sulfide on seedling growth.—To examine the effects of sulfide on early seedling growth, we began by growing juvenile seedlings under a wide range of nominal sulfide exposure concentrations of 0, 96, 320, 960, and 2880 $\mu\text{g}/\text{L}$ in anoxic solutions in a first trial. Both roots and stems of control plants (no added sulfide) increased significantly ($P < 0.05$) over the exposure, approximately doubling in size compared with initial lengths and masses. In seedlings exposed to sulfide concentrations 320 $\mu\text{g}/\text{L}$ or more, stem and leaf masses ($P < 0.01$) and total plant masses ($P < 0.001$) were significantly depressed by an average of 60% and 75%, respectively, relative to controls. Root lengths were only weakly depressed with increasing sulfide concentration ($P < 0.10$).

To narrow the range of toxicity, we then conducted two additional trials focusing on the effects of sulfide on juvenile seedling growth at concentrations less than 1600 $\mu\text{g}/\text{L}$ sulfide. The second trial examined growth at exposure concentrations of 0, 200, 400, 800, 1600 $\mu\text{g}/\text{L}$ sulfide and the third trial examined growth at exposure concentrations of 0, 160, 320, 640, and 1280 $\mu\text{g}/\text{L}$ sulfide. Consistent with the first trial, the biomass of all control plants increased significantly ($P < 0.05$) during the 10 d of

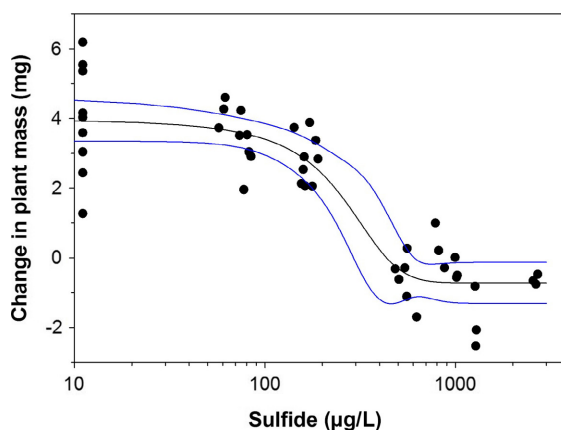


FIG. 1. Growth of wild rice seedlings declines with increasing sulfide concentrations in hydroponic solutions. Individual data points are from three separate experimental runs (see *Methods* and *Results* sections). Fitted sigmoidal response curve (Eq. 1) is shown in black, 95% confidence intervals in blue; $r^2 = 0.80$, $y_{\min} = -0.7172$, $y_{\max} = 5.1353$, $x_0 = 245.9051$, $b = -103.8853$. μ (Color figure can be viewed at wileyonlinelibrary.com.)

exposure, approximately doubling in size compared with initial lengths and masses, and exposure to sulfide across these narrower ranges of concentration again significantly depressed stem plus leaf lengths and total masses of juvenile seedlings.

Because all three trials produced similar effects, we performed a pooled analysis of variance using data from all three. Exposures of seedlings to sulfide concentrations of 320 $\mu\text{g/L}$ or greater significantly reduced growth rates ($P < 0.01$) of wild rice seedlings compared to the control by 88% or greater; Fig. 1). Seedlings exposed to sulfide concentrations at 320 $\mu\text{g/L}$ or greater hardly grew at all and in some cases their mass decreased during the 10-d course of the exposure (Fig. 1). But exposures at sulfide concentrations less than 320 $\mu\text{g/L}$ did not significantly reduce growth rates ($P > 0.10$) compared with the controls (Fig. 1). There was a sigmoidal response of seedling growth to elevated sulfide concentrations, with an inflection point at approximately 245 $\mu\text{g/L}$ (Fig. 1; see figure caption for parameter values and r^2 for Eq. 1). The EC_{50} calculated from this regression was 227 $\mu\text{g/L}$ sulfide.

Effect of aerobic and anaerobic conditions on seedling growth.—Under micromolar concentrations of sulfur

from trace amounts of CuSO_4 and ZnSO_4 in the Hoaglands solution, stem lengths were 10% longer ($P < 0.02$), root lengths were 73% shorter ($P < 0.001$), and total plant masses were 16% less ($P < 0.01$) under anaerobic conditions compared to aerobic conditions.

Mesocosm experiment

Sulfate concentrations in overlying water.—The average monthly measured sulfate concentrations in amended tanks were consistently within 80–100% of nominal target concentrations of 50, 100, 150, and 300 mg/L (Table 1). The sulfate concentrations sometimes decreased after large rainfall events.

Porewater sulfide concentrations with sulfate additions.—Profiles of sulfate, sulfide, and iron in the mesocosm porewaters showed patterns consistent with sulfate diffusion from the overlying water into the surficial 5 cm of sediment with subsequent reduction to sulfide (Fig. 2). Concentrations of sulfide were typically highest in upper 3–5 cm, which is the rooting zone of seedlings. Sediment in tanks contained on average $8.3 \pm 0.8 \text{ mg/g}$ extractable iron; extractable iron did not vary with average surface

TABLE 1. Target and measured sulfate concentrations in overlying water in the mesocosm experiment.

Target sulfate concentration	Measured growing season mean sulfate concentrations (mg/L)					
	12 Jul–30 Aug 2011	6 Jun–28 Aug 2012	5 Jun–27 Aug 2013	27 May–26 Aug 2014	5 May–4 Sep 2015	Average over all years
0	8.05 (0.34)	8.0 (0.31)	7.05 (0.18)	5.8 (0.16)	6.16 (0.25)	7.01 (0.45)
50	50.0 (1.58)	34.0 (1.26)	37.2 (1.02)	43.3 (0.8)	41.7 (1.26)	41.2 (2.73)
100	97.7 (4.33)	77.1 (1.76)	79.7 (1.41)	87.2 (1.29)	85.3 (2.03)	85.4 (3.58)
150	135.0 (3.73)	126.0 (2.08)	127.0 (1.55)	131.0 (1.68)	132.0 (2.56)	130.0 (1.57)
300	254.0 (7.35)	263.0 (3.32)	268.0 (2.37)	273.0 (2.52)	272.0 (4.08)	266.0 (3.50)

Note: Values in parentheses are SE.

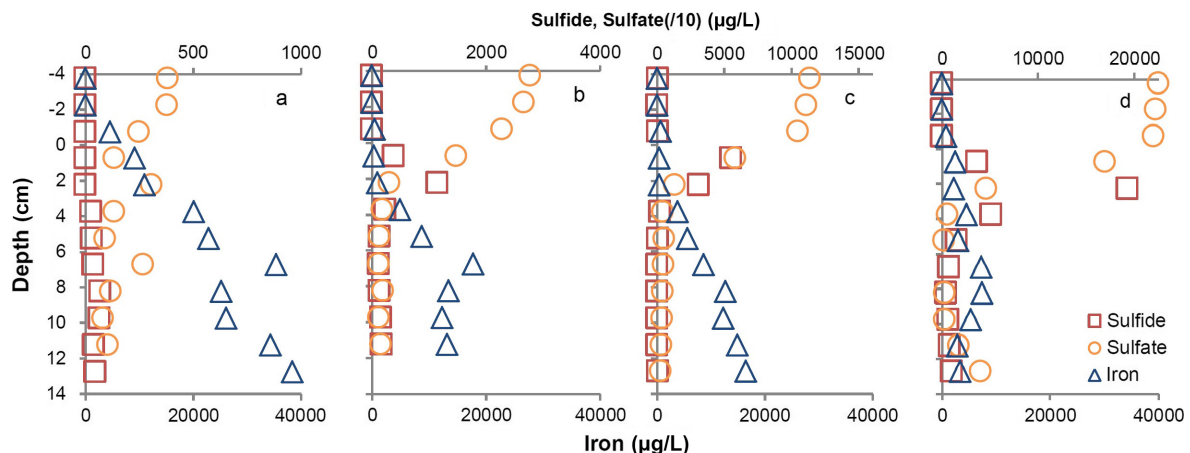


FIG. 2. Vertical profiles of sulfate, sulfide, and iron in mesocosms with different measured sulfate concentrations in the overlying water measured during August 2013. Average annual overlying water sulfate concentrations were (a) 7.05 mg/L , (b) 37.2 mg/L , (c) 127 mg/L , and (d) 268 mg/L . Note different scales for sulfate and sulfide in panels b, c, and d. (Color figure can be viewed at wileyonlinelibrary.com.)

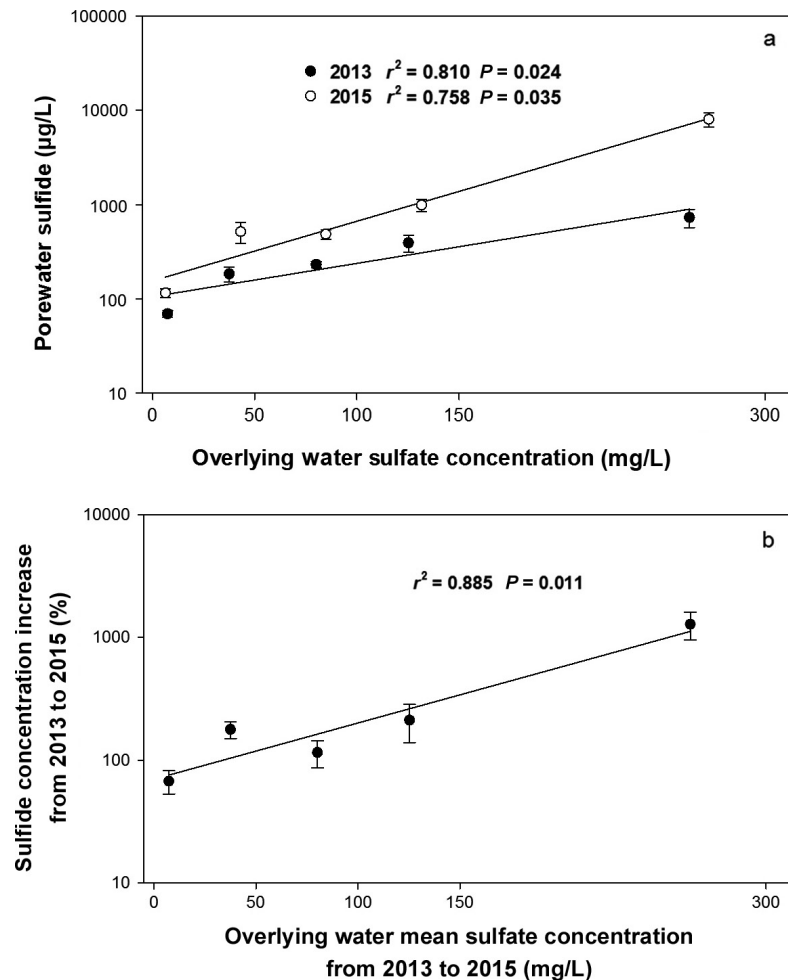


FIG. 3. (a) Porewater sulfide concentrations are strongly correlated with measured concentrations of sulfate in overlying water in the mesocosms and (b) the sulfide concentrations increased from 2013 to 2015 in proportion to sulfate concentrations. Symbols are means and standard errors.

water sulfate concentration (linear regression $r^2 = 0.02$). Sediment in control tanks contained less than 0.15 mg/g acid volatile sulfides (1 mol/L hydrochloric acid, Allen et al. 1991) while sediment in 300 mg/L sulfate tanks contained over 1.75 mg/g in 2013.

Porewater sulfide concentrations obtained from the upper 10 cm of sediment with Rhizon samplers were highly correlated with sulfate concentrations in the overlying water in both 2013 and 2015 (Fig. 3a). Concentrations were higher in 2015, and disproportionately higher in the higher sulfate treatments (Fig. 3b), which could be a consequence of progressively less precipitation with iron, which was a limited quantity.

Effects of sulfate and sulfide on seedling emergence rate and seedling survival.—In each spring after the initial planting in 2011, the number of seedlings that emerged from the sediment (Fig. 4a) declined significantly with increased sulfate concentrations ($P < 0.001$). Emergence rates differed from year to year ($P < 0.001$) but the rate

of decline in seedling emergence with amended sulfate concentrations (slopes of regressions in Fig. 4a) did not change significantly from year to year (sulfate \times year interaction $P = 0.598$).

The subsequent survival of those seedlings remaining after thinning (Fig. 4b) also declined significantly with increased sulfate concentrations ($P < 0.001$) and year ($P < 0.001$). The rate of decline in seedling survival with amended sulfate was twice as high in 2014 and 2015 than in 2012 and 2013. The number of surviving seedlings was not correlated with the number of seedlings that had been removed by thinning in any given year ($P > 0.10$), so the magnitude of thinning itself had no effect on seedling survival in the same year. The number of surviving seedlings was also not correlated ($P > 0.10$) with the production of straw litter from the previous year, so the decline in seedling survival was not an artifact of inhibition by thatch accumulation or nitrogen immobilization into fresh litter (Walker et al. 2010).

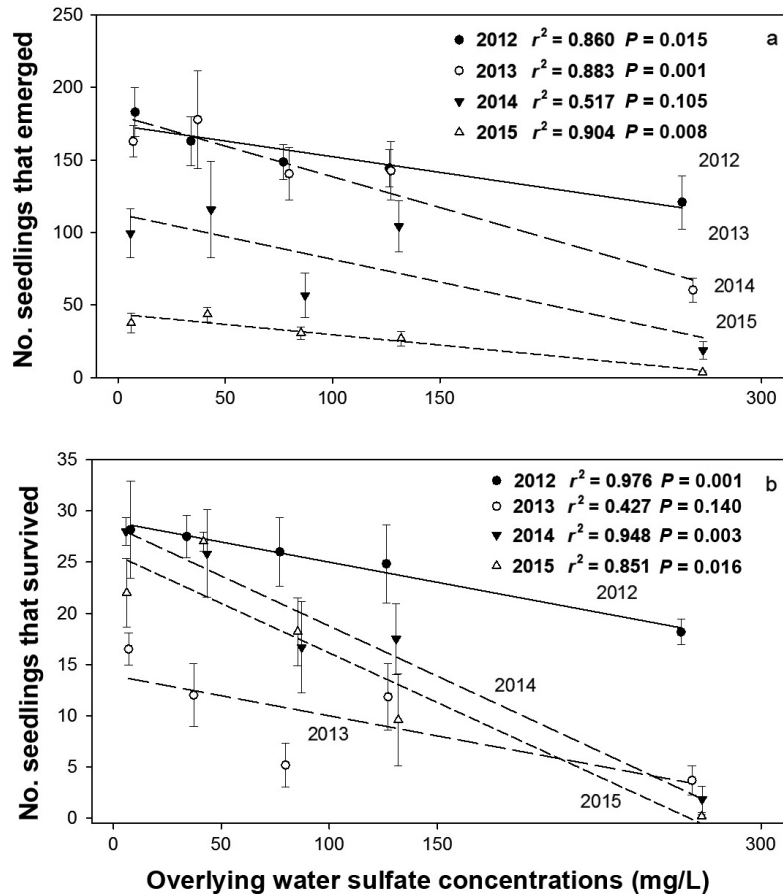


FIG. 4. Emergence (a) and survival (b) of seedlings in mesocosms declines with increasing measured sulfate concentrations in the overlying water. Symbols are means and standard errors.

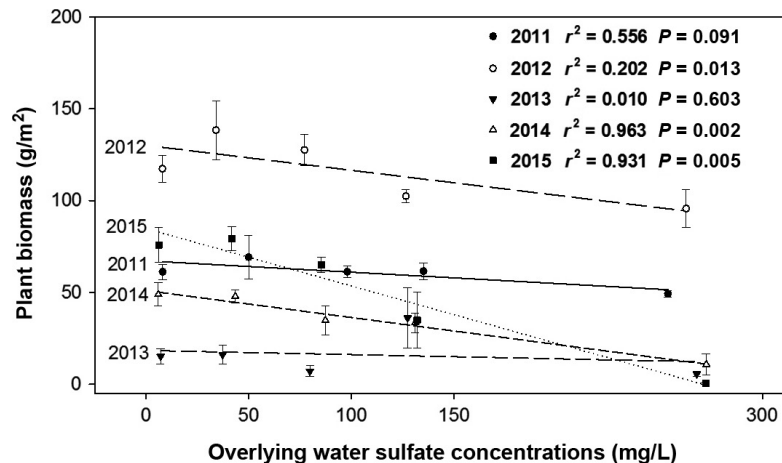


FIG. 5. Vegetative biomass in mesocosms declines with increasing measured sulfate concentrations in the overlying water. Symbols are means and standard errors.

In each year, there were no differences between control tanks and tanks amended to 50 mg/L SO_4 , but seedling emergence and survival were significantly lower ($P < 0.05$) in tanks amended to 100 mg/L SO_4 or greater compared to control tanks.

Effects of sulfate and sulfide on vegetative growth.—Elevated sulfate and presumably sulfide concentrations decreased plant biomass ($P < 0.001$) and the rate of decline increased significantly during the course of the experiment, but most especially in 2015 (sulfate \times year

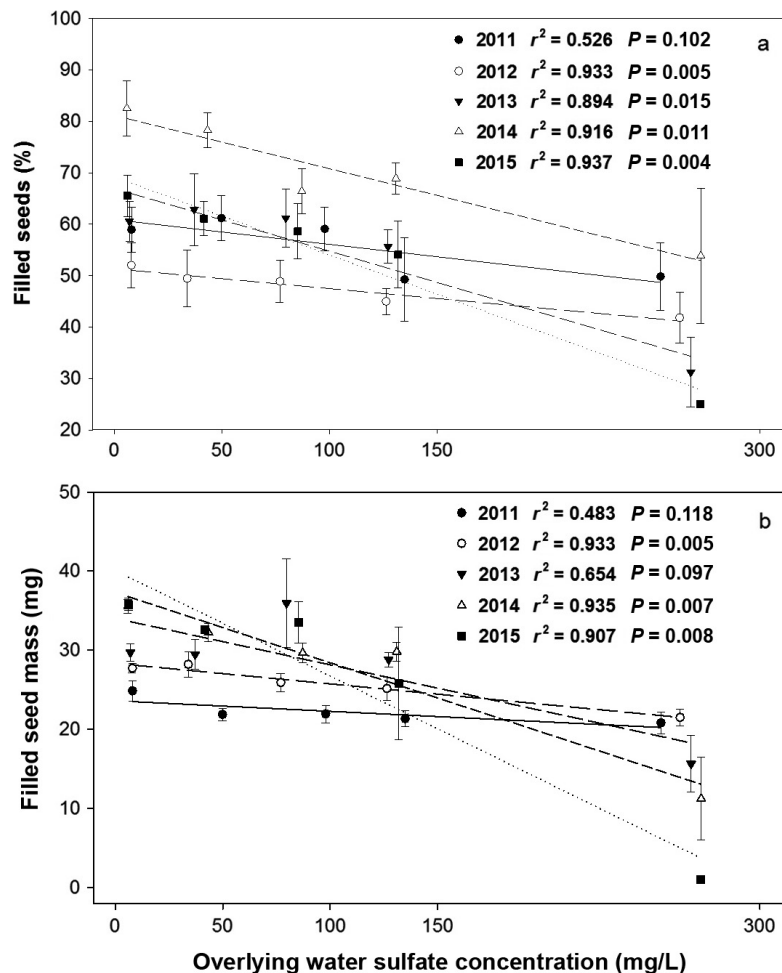


FIG. 6. (a) The proportion of seeds that were filled and (b) the mean seed mass in mesocosms both declined with increasing measured sulfate concentrations in the overlying water. Symbols are means and standard errors.

interaction statistically significant at $P < 0.001$; see Fig. 5 and the figure legend for r^2 and P levels). By 2015, wild rice was extinct in all but one replicate in the 300 mg/L treatment, which supported only two plants. Root and shoot masses of individual plants were highly correlated ($r = 0.998$, $P < 0.001$) and root:shoot ratios were nearly constant between 0.210 and 0.224. Therefore, while the amounts of root and shoot productions were significantly affected by elevated sulfate concentrations, the proportional allocation of production between roots and shoots was not.

Effects of sulfate and sulfide on seed production.—The number of seeds produced per plant (both filled and empty, as determined from peduncle counts) did not change significantly across all sulfate concentrations (not displayed), but the proportion of seeds produced that were filled declined significantly with increasing sulfate concentrations (Fig. 6a, $P < 0.001$). Although 55–80% of seeds from control plants were filled during all four years, the slopes of the regressions of the proportions of filled

seeds against sulfate concentration declined more steeply with each successive year (sulfate \times year interaction significant at $P < 0.001$). By 2015, the proportions of filled seeds were as low as 25% in the tanks with the highest sulfate concentrations.

Individual seed masses declined with increased sulfate concentrations (Fig. 6b, $P < 0.001$). The seed masses declined more steeply with increasing sulfate concentrations with each successive year (sulfate \times year interaction significant at $P < 0.001$).

In each year, seed production did not differ between control tanks and tanks amended to 50 mg/L SO_4 , but seed mass and the proportion of viable seeds were significantly lower ($P < 0.05$) in tanks amended to 100 mg/L SO_4 or greater compared to control tanks.

Blackened roots associated with elevated sulfate.—Beginning in 2012 and continuing for each subsequent year, plants in the tanks amended with sulfate had blackened roots while plants grown in the control tanks had white or light tan or orange roots when we

TABLE 2. Summary of the effects of sulfate and sulfide on the stages in the life cycle of wild rice.

Wild rice life cycle stage	Effects of increased sulfate and/or sulfide	
	Hydroponic experiments	Mesocosm experiments
Germination rate	no effect of sulfate or sulfide	not assessed
Juvenile seedling growth	significant negative effect of sulfide, no effect of sulfate	not assessed
Seedling emergence from sediment	not assessed	significant negative effect of sulfate addition, probably a result of reduced seed viability rather than direct effects of sulfide
Seedling survival	not assessed	significant negative effect of sulfate addition, most likely through sulfide production
Mature plant growth	not assessed	significant negative effect of sulfate addition, most likely through sulfide production
Seed production (number of seeds per plant)	not assessed	no effect of sulfate or sulfide
Seed viability, both individual seed mass and proportion of filled seeds	not assessed	significant negative effect of sulfate addition, most likely through sulfide production

harvested them at senescence. Visual estimates of the proportion of blackened roots increased progressively from approximately 50% in the tanks with sulfate concentrations approximately 50 mg/L to 100% in tanks with sulfate concentrations approximately 300 mg/L. These roots were pliable and white in cross sections cut with a knife, so they appeared to be still alive. In these cross sections, the blackening appeared to be crusted plaques on the root surfaces. The blackened roots from the 300 mg/L amended tank averaged $28.3\% \pm 9.8\%$ Fe and $13.4\% \pm 4.6\%$ S by mass, both much greater than tan/orange roots from the control tanks, which averaged $5.0\% \pm 3.9\%$ Fe and $0.34\% \pm 0.29\%$ S. We are investigating the chemistry of these plaques further, but our analyses thus far suggest that the blackening was caused by precipitation of some form of iron sulfide.

DISCUSSION

Table 2 summarizes the major effects of sulfate and sulfide in these experiments. In the mesocosms, the correlation between sulfate concentrations in overlying water and sulfide concentrations in porewater (Fig. 3a) is so strong within a given year that we can reasonably use sulfate concentrations in overlying water as a surrogate for increased sulfide concentrations in sediment porewater. Porewater sulfide increased substantially between 2013 and 2015 (Fig. 3a, b). The sulfide production in these sulfate-amended mesocosms will eventually overwhelm the available iron and accumulate free sulfide in the porewater, which may be responsible for the disproportionately higher sulfide in the highest treatment in 2015 (Fig. 3b). The mesocosms did not mimic the steady state that occurs in the natural environment because sulfate in overlying water was resupplied but iron was not. Mechanistic models that include the interaction between sulfide and iron (e.g., Wang and Van Cappellen 1996, Eldridge and Morse 2000) include the continuous addition of iron from the overlying to the sediment, successfully modeling the steady-state relationship between sulfate, sulfide, and iron observed in the environment.

The sedimentation of new iron to the sediment occurs in the natural environment, but was not included in this mesocosm experiment. Nevertheless, the experiment successfully exposed wild rice to progressively higher concentrations of porewater sulfide and documented the biological effects.

The porewater sulfide concentrations observed in natural waterbodies will vary depending on each site's surface water sulfate and sedimentary concentrations of organic matter and iron (Eldridge and Morse 2000). The sediment organic matter and extractable iron in this experiment (8.1% and 8.3 mg/g) are within the range of 67 Minnesota wild rice waterbodies; organic matter is lower than the median of 9.1%, and the iron is higher than the median of 4.8 mg/g (5th to 95th percentiles of 0.9–31.0% and 1.6–15.3 mg/g, respectively; A. Myrbo, unpublished data).

Upwelling groundwater through sediment would cause a waterbody to deviate from the conceptual model presented here; upward groundwater flow would not only counter downward diffusion of sulfate, but could also supply water with chemistry completely different than the overlying water. In a survey of 46 Wisconsin lakes, Nichols and Shaw (2002) found that the occurrence of wild rice is associated with areas of inflowing groundwater. In some cases, upwelling groundwater may supply sulfate to the reduction zone in littoral sediments (Krabbenhoft et al. 1998), so the effect of groundwater is unpredictable. Wild rice waters most likely to exhibit elevated porewater sulfide are those with relatively high organic matter, which allows enhanced microbial activity, and relatively low iron, which minimizes removal of porewater sulfide as a FeS precipitate (Heijs et al. 1999, Eldridge and Morse 2000).

Elevated sulfate concentrations were not directly toxic to wild rice seedlings in hydroponic solutions, in agreement with results reported by Fort et al. (2014). But adding sulfate to overlying waters in the mesocosms with wild rice sediment increased porewater sulfide concentrations most strongly in the upper 5 cm of sediment in 2013, after three field seasons of sulfate amendments (Fig. 2).

Sulfide was clearly toxic to early seedling growth in hydroponic experiments at concentrations above 320 $\mu\text{g/L}$, as indicated by slower growth or even zero or negative growth in a few cases (Fig. 1). Sulfide concentrations in excess of 320 $\mu\text{g/L}$ were observed in the upper 5 cm of sediment when sulfate concentrations in the overlying water exceeded 20–50 mg/L (depending on season, Fig. 2).

The upper 2–5 cm of sediment is where seed germination and very early seedling growth most likely takes place. Wild rice seeds are shaped like torpedoes and penetrate the sediment aided by their long awns, which act as rudders and keep the seed vertical as it falls through the water column (Ferren and Good 1977). It is likely that the seeds are buried in the upper 2–5 cm of this sediment where oxygen is low and sulfide concentrations are greatest (Fig. 2). To survive, the seedling must germinate in and grow through this zone of high sulfide concentrations. In nature, the mesocotyl may elongate up to 6 cm (Aiken 1986), allowing a buried seed to emerge through up to “3 inches of flooded soil” (Oelke et al. 1982). After emergence into the overlying oxygenated water, the mesocotyl differentiates into the mature stem. Wild rice is unusual among grasses in that the stem develops before the root, probably because the seedling may have to grow between 50 and 100 cm before reaching the water surface, at which time floating leaves supply energy for root development (Aiken 1986). This is consistent with the enhanced stem plus leaf growth of seedlings we observed under anaerobic conditions without elevated sulfide concentrations. Root growth, in contrast, was reduced by anaerobic conditions in our hydroponic experiments, as it has been previously observed for wild rice (Campiranon and Koukkari 1977) and white rice (Kordan 1972, 1974a, b).

Elevated sulfide concentrations greatly reduced shoot and leaf elongation in our hydroponic experiments, particularly at concentrations greater than 320 $\mu\text{g/L}$. The toxic effect of sulfide on shoot and leaf elongation and seedling growth (Fig. 1) overrides the enhanced growth that normally happens under anaerobic conditions. Seedlings in the mesocosms with elevated sulfate (and hence sulfide) concentrations likely were inhibited from emerging successfully from the sediment and reaching aerobic conditions higher in the water column, resulting in reduced survival in the mesocosms.

It is possible that high ionic strength or salinity in the mesocosms with the higher concentrations of elevated sulfate could be the cause of reduced seedling emergence and survival. However, the hydroponic experiments demonstrated that seeds and seedlings could withstand sulfate concentrations of up to 1600 $\text{mg SO}_4/\text{L}$ without adverse effects. This sulfate concentration is half the salinity of seawater (Schlesinger 1991). Electrical conductivity in the mesocosms was correlated with sulfate concentrations but, in 2012, we saw only small effects of sulfate on seedling emergence and survival even though electrical conductivity was high then as it was in 2015. High ionic strength alone is therefore probably not the

cause of the progressively greater declines in seedling emergence and survival in the mesocosms.

It is likely that the observed negative effects on wild rice seedling growth and survival can be directly attributed to the toxic effects of sulfide because of the coherence between the mesocosm experiments and the hydroponic experiments, which isolated the toxic effect of sulfide on seedling growth from any direct effect of sulfate. The progressive decline in seedling emergence and survival during the 5-yr course of the experiment could have resulted from increasingly greater sulfide concentrations (Fig. 3) and progressive titration of reactive forms of ferrous iron out of the system as insoluble iron sulfide. The cumulative effects of this progressive loss of reactive ferrous iron could have allowed more sulfide to remain in solution (Fig. 3) and thereby have increasingly toxic effects on seedling emergence and survival. The possible loss of reactive ferrous iron during the 5-yr course of the experiment may have been partly responsible for the declines in population densities, even to extinction at the highest sulfate concentrations.

Elevated sulfate concentrations in the mesocosm water progressively reduced vegetative production over the five years, but to much less extent than seed production was reduced. The proportion of seeds that were filled, as well as their mean masses, decreased by over 30% and as much as 50% in the 300 mg/L mesocosm treatment by year five of the experiment. Reduced seed production and seed masses followed by reduced seedling emergence and survival the following year depressed population growth in successive years eventually driving wild rice populations to extinction at high sulfate concentrations. It is likely that this extinction was driven by reduced seed production, seedling emergence, and seedling survival that depleted the seed bank over the five years of the experiment, and cumulative impacts on sediment chemistry from repeated sulfate additions could have exacerbated the decline.

The strong decline in measures of seed viability with increased sulfate concentrations at the end of the growing season (Fig. 6) compared with the weaker decline in vegetative growth in early to mid-growing season (Fig. 5) could not have been due to decreased N or P availability late in the growing season. Litter from the previous year has begun mineralizing N and P at this point in the growing season (Walker et al. 2010, Hildebrandt et al. 2012). The production of sulfide is correlated with many other chemical changes associated with the sulfate-enhanced anaerobic decay of organic matter (Lamers et al. 2002), including increased phosphate solubility. Phosphorus availability could not be controlled independent of sulfide in sediment, and sediment porewater and overlying water phosphate concentrations were elevated in sulfate amended tanks (A. Myrbo, *unpublished data*) most likely because precipitation of sulfide with reduced iron liberates phosphate (Caraco et al. 1989, Lamers et al. 2002). Since N and P availability were likely not limiting late in the growing season, it is unlikely that

reduced N or P availability were responsible for the decline in seed production with increased sulfate concentrations. Therefore, by deduction, it must have been uptake that was limiting.

Sixty percent of annual N uptake in wild rice plants occurs early in the growing season but there is a second burst of nitrogen and phosphorus uptake in August during seed filling and ripening (Grava and Raisanen 1978, Sims et al. 2012a). Even though N and P were most bioavailable in August when wild rice seeds were being developed and filled, there was coincident peak accumulation of sulfide in the sediment porewater (Fig. 2). When exposed to high sulfide concentrations, roots of white rice (*Oryza sativa*) often become suberized (Armstrong and Armstrong 2005) with subsequent possible reduction in nutrient uptake across the thicker root membranes (DeLaune et al. 1983, Koch et al. 1990, Armstrong and Armstrong 2005, Lamers et al. 2013). Suberization of roots in response to high sulfide concentrations at this stage in wild rice's life cycle might inhibit nutrient uptake, resulting in fewer and smaller filled seeds.

Another possible mechanism for impaired nutrient uptake might be the precipitation of black iron sulfide plaques on the roots of plants that grew in mesocosms with elevated sulfate and sulfide concentrations. Our EDS analyses suggest that the tan or orange coatings on roots of plants grown under low sulfate concentrations may be iron hydroxide plaques, which are often found on healthy wild rice roots (Jorgenson et al. 2012). The existence of tan or orange coatings, consistent with iron hydroxide plaques, strongly suggests that the immediate vicinity of the roots is oxidized when sulfate concentrations are low, most likely due to radial oxygen loss through the aerenchyma tissues within the roots (Stover 1928, Colmer 2003, Yang et al. 2014). Blackened roots, however, are often observed in white rice (*Oryza sativa*) populations subjected to elevated sulfate concentrations or organic carbon (Jacq et al. 1991, Gao et al. 2003, Sun et al. 2015) and our EDS observations suggest that the blackened plaques on our roots are some form of iron sulfide. Sun et al. (2015) also found that these black plaques contain substantial amounts of iron sulfides. Precipitation of iron sulfide plaques on roots, whether a direct inhibitor of nutrient uptake or a harbinger of the encroachment of reducing conditions to nearer the root tissue, may be partly responsible for the reduced proportion of filled seeds as sulfate concentrations increased (Fig. 6). Further experiments using labeled ^{15}N would be useful to determine whether reduced nutrient uptake during seed filling is the cause of reduced seed production.

Suberization of roots and precipitation of iron sulfide plaques may not be independent. Enhanced suberization when the root tissue is exposed to sulfide (Armstrong and Armstrong 2005) might cause decreased radial oxygen loss from roots of wetland plants (Joshi et al. 1975, Gao et al. 2002, Armstrong and Armstrong 2005). If radial oxygen loss from roots is essential to maintaining low concentrations of hydrogen sulfide in the immediate vicinity of roots

(Eldridge and Morse 2000), then sulfide concentrations in the rhizosphere could encroach nearer to the root surface when radial oxygen loss from roots is impaired. Iron (hydr)oxide present on or near the roots under these conditions could be reduced to iron sulfide and precipitated on the roots. Nutrient uptake during the stage of seed filling therefore might be impaired directly by suberization of roots followed by precipitation of iron sulfides on the roots if suberization reduces radial oxygen loss.

CONCLUSIONS

In our hydroponic experiments, elevated sulfide concentrations are directly toxic to seedlings. In our mesocosm experiments, sulfate amendments increased sulfide concentrations in the rooting zone, which then apparently decreased seedling emergence and survival. The reductions in seedling emergence and survival in the mesocosms are consistent with the toxic effects of sulfide on seedling growth in the hydroponic experiments.

The vegetative growth phase of wild rice's life cycle did not appear to be as strongly affected by sulfide as the production of viable seeds. The mechanisms behind reduced seed production and viability with increased sulfate and hence sulfide production in sediments are more difficult to discern, but may involve reduction of nutrient uptake during seed set by iron sulfide plaques on roots of mature plants (Jacq et al. 1991) or by increased suberization with elevated sulfide concentrations later in the summer (Armstrong and Armstrong 2005).

In natural wild rice ecosystems, the extent to which sulfate is reduced to sulfide, and to which sulfide persists in porewaters, are controlled by factors such as the sedimentary concentrations of iron and organic matter, and groundwater flow, among others, all of which may differ from the conditions in our mesocosms. But our experiments strongly suggest that the reduction of sulfate to sulfide in sediments, to the extent that it occurs in natural systems, may cause populations to decline by adversely affecting the reproductive phases of wild rice's life cycle.

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