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TOXICITY OF SULFATE AND CHLORIDE TO EARLY LIFE STAGES OF WILD RICE (*ZIZANIA PALUSTRIS*)

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Abstract: Despite the importance of wild rice (*Zizania palustris*) in the Great Lakes region of North America, its sensitivity to sulfate is not well understood. A 21-d hydroponic experiment was performed to determine the toxicity of sulfate to wild rice seeds and seedlings. Effects of 6 sulfate concentrations ranging from 10 mg/L to 5000 mg/L and of chloride salts at equivalent conductivity were evaluated to determine whether adverse effects were attributable to sulfate or to conductivity-related stress. Sulfate treatment decreased root length, shoot length, and leaf number, and increased phytotoxic effects at concentrations of 5000 mg/L relative to a 50 mg/L control. The time to 30% mesocotyl emergence decreased at 2500 mg/L sulfate, indicating a potential stimulatory effect. Sulfate exposures of $\leq$5000 mg/L had no effect on 5 additional end points. Multiple regression analysis indicated that most observed changes could be attributed to conductivity-related stress rather than sulfate per se, with the exception of shoot length and leaf number. Chloride was more toxic than sulfate, as determined by root length and phytotoxicity. In summary, sulfate concentrations below 5000 mg/L did not adversely affect early–life stage wild rice during a 21-d period, and effects at 5000 mg/L sulfate were attributable to conductivity-related stress rather than sulfate toxicity in 2 of 4 end points. *Environ Toxicol Chem* 2014;33:2802–2809. © 2014 SETAC

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INTRODUCTION

Wild rice (*Zizania palustris* L.) is native to North America and grows extensively in the Great Lakes region of the United States and Canada. This nutritious, large-seeded grain is high in protein and carbohydrates and has been a staple of early North American diets for centuries [1]. Field studies in the 1930s and 1940s suggested that surface water sulfate may influence the geographic distribution of *Z. palustris* in Minnesota (USA) at concentrations above 10 mg/L [2]. Consequently, in 1973, the Minnesota Pollution Control Agency adopted a statewide sulfate water quality standard of 10 mg/L for the protection of wild rice. Sulfate is a required nutrient for terrestrial and aquatic plants and is necessary to assimilate or synthesize certain amino acids and coenzymes [3]. Although sulfate is a required plant nutrient, few studies have examined the effects of sulfate over a range of concentrations on wild rice under controlled conditions.

The US Environmental Protection Agency (USEPA) guidelines for derivation of water-quality criteria [4] specify developing criteria based on direct toxicity testing of a minimum of 8 families of aquatic animals. The USEPA guidelines also specify consideration of economically or recreationally important species, including plants, when toxicity testing of animals may not be protective of other potentially sensitive species. Wild rice is sacred to Ojibwe tribes in the upper midwestern United States and Canada and is important to portions of the upper midwestern US economy. Wild rice also provides food for waterfowl and other wildlife and is an important ecological component of lakes and streams in the Great Lakes region. Thus, protection of wild rice is a priority, and understanding causal factors negatively impacting wild populations is desirable. Controlled studies to determine wild rice sensitivity to sulfate are not currently available in peer-reviewed literature. Consequently, we conducted a hydroponic study to test the impact of sulfate on seed germination and early seedling development of wild rice under controlled laboratory conditions. We hypothesized that sulfate would not adversely affect germination and early development of wild rice at concentrations below 5000 mg/L over the 21-d hydroponic exposure and that effects induced at high sulfate concentrations were the result of conductivity-related stress. Dose–response relationships of sulfate concentrations and wild rice germination, growth, and developmental parameters were used to determine the toxicity of sulfate in early life stages of *Z. palustris*. Seedlings also were subjected to osmotic conditions similar to those created by the sulfate treatments using chloride salts to determine whether sulfate was uniquely toxic to wild rice or if observed toxic effects were primarily the result of conductivity-related stress.

MATERIALS AND METHODS

Preliminary studies and study design

Preliminary studies were conducted to determine a suitable standard test medium in which to grow wild rice hydroponically for definitive sulfate toxicity testing; to identify reproducible, ecologically relevant test end points and associated test acceptability criteria; and to determine appropriate sulfate test concentrations for the definitive study. Three different growth media were tested, each at 3 different ammonium:nitrate ratios.

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Based on these preliminary experiments, a daily-renewal hydroponic system utilizing a modified Hoagland's solution (HS-1 [5,6]) was used to test the effects of sulfate and chloride salts on 10 biological end points in wild rice seeds and seedlings over 21 d. The modified HS-1 solution contained 25% ammonium (molar basis) in a mixture of ammonium and nitrate and served as the base medium and diluent for all test exposures in the definitive study. Deionized water was used to prepare all solutions and routinely tested to ensure the absence of various organic and inorganic contaminants. The HS-1 macronutrients consisted of 2.55 mM NO_3^-, 0.92 mM NH_4^+, 0.12 mM $H_2PO_4^-$, 1.10 mM K^+, 0.75 mM Ca^{2+}, 0.50 mM Mg^{2+}, and 0.50 mM SO_4^{2-}. Micronutrients included 46.3 μM B, 14.9 μM Fe, 0.76 μM Zn, 0.31 μM Cu, 9 μM Mn, and 0.50 μM Mo. A combination of sodium, potassium, calcium, and magnesium salts of sulfate and chloride was used to prepare the stock solutions with monovalent:divalent salt ratios of 2:1. All salts were reagent-grade, obtained from Sigma-Aldrich (>98% pure).

Hydroponic sulfate and chloride exposures

All test solutions were based on modifications to the HS-1 control medium, which contained 50 mg/L sulfate. Sulfate and chloride exposures were prepared with a balanced mixture of mono- and divalent cationic salts to create equivalent conductivity at each exposure level and ensure that Ca^{2+} and Mg^{2+} deficiencies did not occur during culture (Table 1). Chloride exposures were included to determine whether any biological responses to sulfate were attributable to conductivity-related stress or specifically to sulfate. A boron exposure (100 mg/L boron from boric acid) was used as a positive control to document plant response to a known toxicant. Nominal sulfate concentrations of 10 mg/L, 250 mg/L, 1000 mg/L, 2500 mg/L, and 5000 mg/L (0.8 meq/L, 4.2 meq/L, 19.8 meq/L, 51.1 meq/L, and 103.2 meq/L, respectively) were evaluated for effects on wild rice relative to the HS-1 control (0.8 meq/L). Sulfate (USEPA method 375.4) was measured in the test media in 2 replicates of each treatment at study days 0 (initiation), 10, and 21 (conclusion). Five chloride exposures with the same sulfate concentration as the HS-1 control and the same osmotic strength as the 4 highest sulfate exposures were developed (Table 2). Specific conductance (conductivity) of all sulfate and chloride exposures was measured on study days 0, 10, and 21 with USEPA method 120.1 [7].

Wild rice seeds

Wild rice seeds were hand-harvested from Little Round Lake in Becker County, Minnesota, and sieved through a 4-mm mesh and then a 2-mm mesh sieve to remove debris. Seeds were stored at 4 °C in the dark prior to test initiation. A visual inspection was conducted as seeds were loaded into the test system, and damaged, discolored, or deformed seeds were discarded. Seeds were placed in baskets with inert mesh to support seeds and seedlings and housed in 10-L glass aquaria equipped with temperature and photoperiod control. Each test replicate consisted of 2 baskets, each containing 30 seeds (i.e., 60 seeds/replicate). On study day 0, seeds were randomly placed in the test system, with 5 seeds added to each basket in accordance with a randomized design chart until each basket contained 30 seeds. From each test replicate, 1 basket of 30 was harvested at study day 10 and the other at study day 21. Each treatment (i.e., each sulfate and chloride concentration) was replicated 4 times in separate aquaria, with all aquaria placed randomly.

Table 1. Composition of sulfate treatments for hydroponic study

Concentration SO_4 (mg/L)	Concentration (mg/L HS-1)			
	Na_2SO_4	K_2SO_4	$CaSO_4$	$MgSO_4$
10[a]	—	—	—	—
50[b]	—	—	—	—
250	123.3	151.3	74.8	52.3
1000	492.9	604.7	298.7	208.8
2500	1232.2	1511.8	746.8	522.1
5000	2464.4	3023.5	1493.6	1044.2

[a]Prepared as modified Hoagland's solution (HS-1) medium.
[b]HS-1 (1:4) medium.

Table 2. Composition of chloride treatments for hydroponic study

Estimated conductivity[a] (μmhos/cm)	Concentration (mg/L HS-1)			
	NaCl	KCl	$CaCl_2$	$MgCl_2$
770	20.7	25.8	12.8	8.9
1890	104.8	128.6	63.6	44.5
2830	365.4	514.0	253.9	177.5
4100	1047.4	1285.0	634.8	443.8
8350	2094.7	2570.0	1269.6	887.6

[a]Based on an experimentally derived conductivity conversion factor of 0.85 to convert from sulfate to chloride salts.

Hydroponic culture and test conditions

Test solutions were administered using a static-renewal hydroponic system consisting of 10-L chambers containing 4 L of media. All test solutions were supplied from a common sulfate stock with 70% solution volume exchanged daily, providing consistent exposure concentrations and water-quality conditions throughout the study. Hydroponic chambers were cleaned daily to remove debris. Following a 10-d dark germination phase, a combination of incandescent and fluorescent plant growth lights was used to provide a 16:8-h light:dark photoperiod at an intensity of 5000 ± 1000 lux at the water surface. Water temperature was maintained at 21 ± 2 °C (light) and 12 ± 2 °C (dark). In each replicate hydroponic chamber, temperature and light intensity were measured daily. Dissolved oxygen (USEPA 360.1), oxidation/reduction potential, and pH were measured in 1 replicate per treatment 3 times per week. Measurements of dissolved oxygen and oxidation/reduction potential confirmed that reducing conditions did not exist in test solution, and pH was consistently maintained within physiologically acceptable ranges for wild rice (6.1–7.2 standard units). Total hardness (USEPA method 130.2), total alkalinity (USEPA method 310.1), ammonia-nitrogen (USEPA method 350.2), nitrate-nitrogen (USEPA method 353.2), and phosphate-phosphorus (USEPA method 365.2) concentrations were measured in the media of 2 replicates of each treatment at study initiation and conclusion [7].

Biological end points

Ten wild rice seed or seedling end points were assessed at study day 10 and study day 21. Seed activation was defined as sufficient absorption of water by the seed to result in seed coat disruption (assessed using a magnification lens). Mesocotyl emergence was defined as the appearance of plant tissue from the germinated seed for purposes of assessing percent emergence. Mesocotyl emergence was also expressed as the time to 30% emergence in days (ET30) for a given exposure. Seedling mortality was applied only to germinated seeds and defined as

degradation of emerged plant tissue with no additional signs of growth. Shoot biomass consisted of the combined dry mass of the mesocotyl, coleoptile, primary leaf, and all secondary leaves. Root biomass consisted of the combined dry mass of the seminal root and associated rootlets. Seminal root and shoot lengths were determined to the nearest 0.1 mm by digitizing a photograph of the root and shoot using commercial software to determine linear length (SigmaScan Pro 5.0; SPSS). Leaf number was based on the number of secondary leaves formed per seedling during the exposure period. Phytotoxicity, which included leaf chlorosis, darkening of the plant tissue, wilting, and deformity, was assessed visually and expressed as a percent of emerged seeds. Visual assessments were also performed by a second analyst to verify results.

One randomly selected basket within each replicate was sacrificed from all replicates per exposure at study day 10 to evaluate all test end points. Plant tissue was rinsed to remove any external salt deposits, and then digital photographs were taken. Plants were dried overnight at 105 °C for biomass determinations. The remaining basket in each replicate was evaluated for all test end points at study day 21. Based on preliminary testing, mesocotyl emergence was not expected to be 100%, so test acceptance criteria of ≥95% seed activation, ≥30% mesocotyl emergence, and ≥90% seedling survival in the HS-1 control were established. The boron-positive control had a test acceptance criterion of ≥80% phytotoxicity.

Data analysis

Statistical analyses utilized SigmaStat 11.2 (Systat Software) and Minitab® 17.1.0 (Minitab), with $\alpha = 0.05$ for all tests. Seed activation and seedling survival were 100% in all treatments, so these end points were excluded from analysis. The remaining 8 end points at study day 21 were each subjected to normality testing and Johnson transformation if not normally distributed. If data could not be successfully transformed (ET30, root biomass, and phytotoxicity), the end point was subjected to nonparametric testing. Tests for outliers (Grubbs's test) and equivalence of variances (Levene's test) also were conducted on normalized data for parametric end points. No outliers were identified, and additional data transformation was applied to generate equivalent variances where possible.

Initial *t* tests were used to compare the boron-positive control against HS-1 in end points for which parametric data were available, and Kruskal-Wallis tests were used for nonparametric end points (i.e., ET30, root biomass, and phytotoxicity). Next, one-way analyses of variance (ANOVA) were performed on the 5 parametric biological end points to identify whether any significant differences existed across the 6 sulfate treatments overall or in each end point. For end points exhibiting statistical significance in ANOVA, a Dunnett's post hoc separation of means test was applied using HS-1 as the control. In lieu of ANOVA, Kruskal-Wallis median tests were used to identify any significant differences across sulfate treatments for the 3 nonparametric end points.

The lowest concentration of sulfate at which a given end point exhibited a significant difference from HS-1 in a manner that indicated toxicity (i.e., significantly lower for growth measures or significantly higher for phytotoxicity) was designated as the lowest-observed-effect concentration (LOEC), and the next lower end point was designated as the no-observed-effect concentration (NOEC). Given the good agreement between nominal and measured sulfate concentrations (i.e., ± 10% as subsequently discussed), nominal sulfate concentrations were used in NOEC and LOEC determinations. To determine toxicity based on osmotic effects, 25% inhibition concentration (IC25) values for emergence and phytotoxicity based on conductivity were determined from probit regression separately for sulfate- and chloride-treated plants. For other end points, IC25 values and 25% stimulatory concentrations (SC25) were determined using linear interpolation of conductivity data. Ninety-five percent fiducial intervals were calculated for each point estimate. Mean measured conductivity values were used in making the IC25 and SC25 determinations.

Determinations of whether observed effects in the above analysis were attributable to sulfate or conductivity-related stress were made by carrying out multiple regression analyses on biological end points using data from HS-1 and all higher sulfate concentrations along with their chloride osmotic potential equivalents. Conductivity data were used as the independent variable and modeled using both quadratic ($Y = [A \times \text{Conductivity}^2] + [B \times \text{Conductivity}] + C$) and linear ($Y = [B \times \text{Conductivity}] + C$) models, where Y represents the predicted mean value of the end point varying with changes in conductivity, A is the quadratic coefficient, B is the linear coefficient or slope, and C represents the constant. Ion type (sulfate or chloride) and conductivity multiplied by the ion type interactions were also included. Thus, for each end point, the multiple regression tests determined 2 values for A, B, and C and 1 each for sulfate and chloride; whether these values significantly differed from 0; and whether the 2 values significantly differed between sulfate and chloride treatments. Changes in end points were attributable to conductivity-related effects alone if neither the Conductivity^2 × Ion nor the Conductivity × Ion interaction was statistically significant and if the quadratic and/or linear coefficients were statistically significant. The first scenario indicated that the shapes of the regression curves differed significantly between the 2 ion types, whereas the second scenario indicated that conductivity significantly influenced the end point. For each end point, only the model with the higher adjusted R^2 value is presented.

RESULTS

Analyses indicated strong similarity between study day 10 and study day 21 results. More specifically, a greater response for each individual end point was not observed at study day 21 than was observed at study day 10; thus, study day 10 results are not reported.

Physicochemical exposure conditions

The average measured sulfate concentration in the HS-1 control solution was 52 ± 1.2 mg/L (Table 3). The average measured sulfate concentrations were 90% to 110% of the nominal concentrations in sulfate exposures of 50 mg/L and above, indicating good agreement between nominal and measured sulfate concentrations. Similarly, average measured sulfate concentrations for the chloride treatments ranged from 50 mg/L to 54 mg/L (100–108% of the nominal concentration). Acceptable agreement between average conductivity in the chloride treatments and their corresponding osmotic potential sulfate exposure was also obtained, as was a high degree of precision within treatments. The average measured test solution alkalinity, nitrate, and phosphate concentrations were generally similar across all sulfate and chloride exposures. Based on the content of the standard media HS-1 [6], alkalinity was expected to be low and variability may have been influenced by plant growth.

Control and positive control performance

The control (HS-1) minimum performance criteria established during method validation were 95% seed activation, 30%

Table 3. Physicochemical characteristics of sulfate and chloride hydroponic treatments

Nominal concentrations				Measured concentrations[a]					
Salt treatment	(meq/L)	SO_4^{2-} (mg/L)	Cl^- (mg/L)	SO_4^{2-} (mg/L)	Conductivity (μmhos/cm)	Hardness (mg/L)	Alkalinity (mg/L)	Nitrate-N (mg/L)	Phosphate-P (mg/L)
SO_4	0.8	10	71	13 (0.4)	554 (13.2)	160 (22.6)	10 (1.2)	59.3 (0.3)	5.0 (0.1)
	0.8	50	10	52 (1.2)	572 (15.5)	146 (19.9)	19 (1.0)	66.5 (0.6)	4.70 (0.2)
	4.2	250	10	239 (3.7)	1729 (12.2)	248 (45.4)	9 (1.5)	61.8 (4.3)	4.8 (0.1)
	19.8	1000	10	1044 (8.1)	2797 (18.0)	589 (91.0)	8 (1.8)	57.0 (3.0)	4.6 (0.1)
	51.1	2500	10	2545 (12.6)	4423 (76.2)	1348 (10)	10 (0.8)	61.3 (1.3)	4.9 (0.1)
	103.2	5000	10	4980 (19.1)	8507 (59.3)	2240 (352.9)	12 (2.4)	63.0 (1.8)	4.7 (0.2)
Cl^-	0.8	50	40	53 (1.3)	699 (11.0)	259 (56.6)	10 (0.8)	55.8 (0.9)	4.9 (0.1)
	4.2	50	199	50 (1.2)	1960 (26.6)	410 (75.2)	9 (1.3)	56.5 (1.2)	4.8 (0.2)
	19.8	50	763	50 (1.7)	2906 (20.9)	539 (94.4)	8 (0.8)	56.0 (3.0)	4.9 (0.1)
	51.1	50	1995	54 (1.1)	4224 (28.2)	795 (173.6)	7 (1.0)	59.0 (0.4)	4.8 (0.0)
	103.2	50	3940	53 (1.3)	8509 (59.8)	1468 (335.6)	6 (0.5)	56.0 (4.1)	4.6 (0.1)

[a]Mean with standard error of the mean in parentheses.

mesocotyl emergence, and 90% control survival. All of these criteria were met (data not shown). The HS-1 control plants were compared against those grown in a 100-mg/L boron-positive control known to induce phytotoxicity. The occurrence of 100% phytotoxicity indicated compliance with the predetermined test acceptability criterion of 80% or greater for this end point in the positive control; in contrast, HS-1 plants exhibited 0% phytotoxicity ($p = 0.008$). Statistically significantly reduced emergence (*t* test, $p = 0.002$) and root length (*t* test, $p = 0.018$), along with an increased ET30 value (Kruskal-Wallis test, $p < 0.013$ using ET30 values of 21 d for boron-treated plants), further demonstrated the toxic effects of boron on developing wild rice. Root biomass (Kruskal-Wallis test, $p = 0.240$,), shoot biomass (*t* test, $p = 0.086$), shoot length (*t* test, $p = 0.843$), and leaf number (*t* test, $p = 0.186$) did not differ significantly between boron-treated plants and the HS-1 control.

Sulfate toxicity and sulfate concentration-based end points

Of the 5 end points meeting the requirements for ANOVA, 3 (root length, shoot length, and leaf number) exhibited significant toxic effects at 5000 mg/L sulfate relative to the HS-1 control ($p \pm 0.004$ for all 3 tests; Table 4 and Figure 1). Root length was also significantly increased at 10 mg/L sulfate compared with the HS-1 control. In contrast, mesocotyl emergence ($p = 0.062$) and shoot biomass ($p = 0.073$) showed no significant effect of sulfate concentration overall. Based on Kruskal-Wallis tests, phytotoxicity was significantly increased at 5000 mg/L sulfate relative to HS-1 ($p < 0.001$), whereas the ET30 value was significantly reduced at 2500 mg/L sulfate (i.e., fewer days were required to achieve 30% emergence; $p = 0.036$; Table 5). Root biomass ($p = 0.301$) was not significantly influenced by sulfate concentration overall. Thus, 4 of the 10 end points assessed exhibited toxic effects attributable to sulfate exposure at the 2 highest concentrations.

The subsequent sulfate NOEC and LOEC determinations (Table 3) indicated toxic effects attributable to exposure to 5000 mg/L sulfate (i.e., LOEC 5000 mg/L) for shoot length, root length, leaf number, and phytotoxicity. Seed activation, mesocotyl emergence, emergence time, seedling survival, shoot biomass, and root biomass were not adversely affected by sulfate exposures of 5000 mg/L. The ET30 was decreased by exposure to sulfate at 2500 mg/L, indicating a stimulatory effect. Based on these test results, shoot length, root length, leaf number, and phytotoxicity were the end points most sensitive to sulfate exposure.

Sulfate and chloride point estimates

The IC25 and SC25 values based on conductivity were determined separately for sulfate and chloride treatments (Table 6). Results of this analysis were consistent with LOEC determinations for sulfate, indicating that toxicity was observed only for the shoot length, root length, leaf number, and phytotoxicity end points, with conductivity-based IC25 values ranging from 5590 μmhos/cm to 7385 μmhos/cm in sulfate-treated plants. Root length and shoot length were the most sensitive end points to sulfate-induced conductivity in these treatments (respective IC25 values of 5590 μmhos/cm and 5780 μmhos/cm). Based on overlapping 95% fiducial limits, root length, and shoot length, IC25 values for sulfate-treated plants were not significantly different; however, both were significantly lower than IC25 values for leaf number and phytotoxicity. Based on nonoverlapping 95% fiducial limits for IC25 values, sulfate was more toxic than chloride as determined by shoot length, whereas chloride was significantly more toxic than sulfate as determined by root length and phytotoxicity.

The sulfate SC25 value of 1771 μmhos/cm for mesocotyl emergence corroborates the finding of sulfate stimulation of

Table 4. Summary of concentration-based end points for *Zizania palustris* exposed to sulfate

Measurement end point	ANOVA p[a]	NOEC (mg SO_4^{2-}/L)	LOEC (mg SO_4^{2-}/L)
Seed activation	ND	5000	>5000
Mesocotyl emergence	0.062	5000	>5000
30% emergence time[b]	0.036	5000	>5000
Seedling survival	ND	5000	>5000
Shoot biomass	0.073	5000	>5000
Shoot length	0.004	2500	5000
Root biomass	0.301	5000	>5000
Root length[c]	<0.001	2500	5000
Leaf number	<0.001	2500	5000
Phytotoxicity	<0.001	2500	5000

[a]Analysis of variance (ANOVA), Dunnett's test, $\alpha = 0.05$ with the exceptions of time to 30% emergence, root biomass, and phytotoxicity, which used Kruskal-Wallis test, $\alpha = 0.05$.
[b]Decreased time to 30% emergence relative to Hoagland's solution (HS-1) was found only in 2500 mg/L sulfate.
[c]Increased root length relative to HS-1 was found in 10 mg sulfate/L, Dunnett's test, $p < 0.001$.
ND = not determined because of 100% activation and 100% survival in all sulfate treatments; NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration.

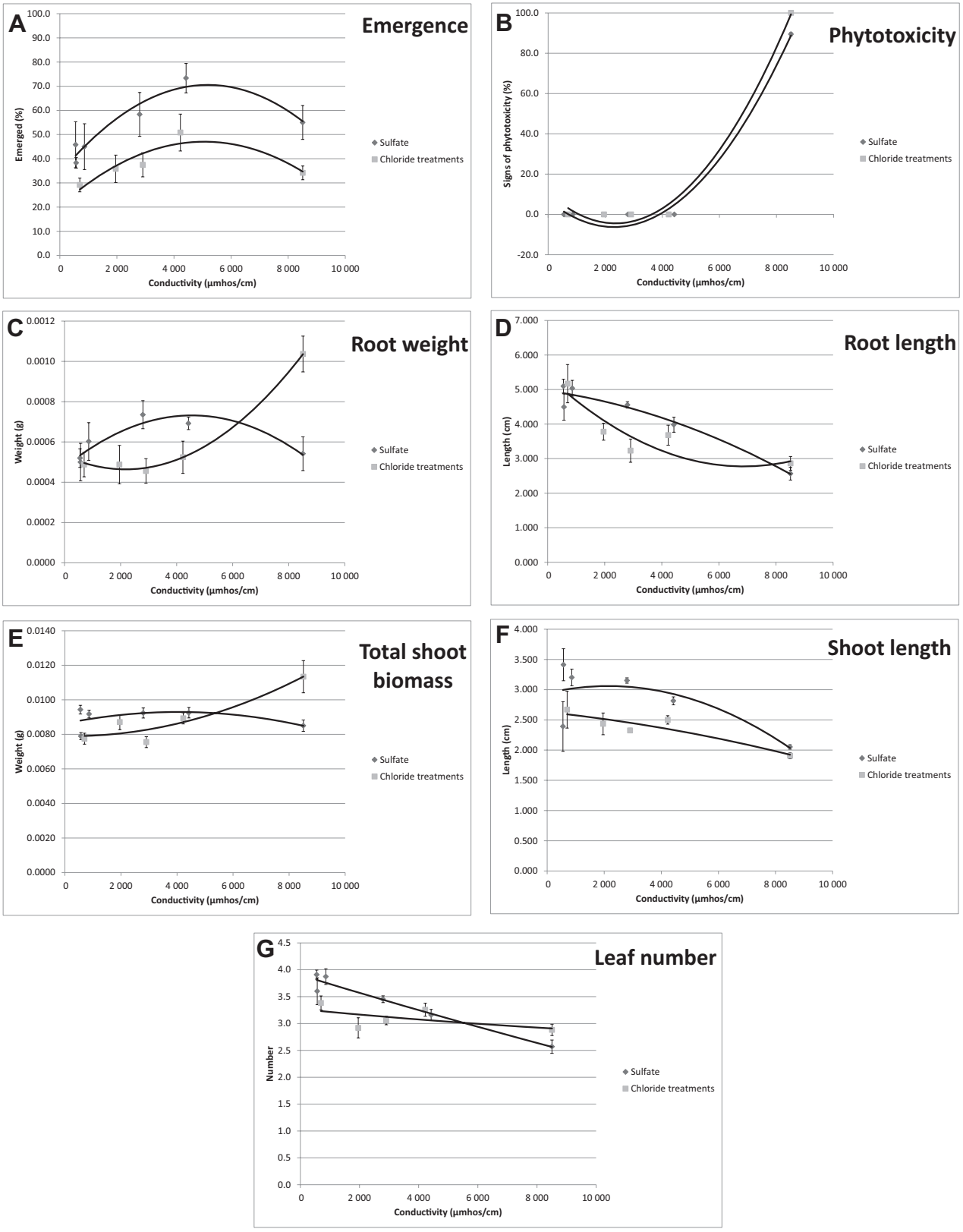

Figure 1. Conductivity–response relationships for each respective measurement end point at study day 21 for sulfate and chloride exposure: (**A**) mesocotyl emergence (percent), (**B**) phytotoxicity (percent), (**C**) root weight (grams), (**D**) root length (centimeters), (**E**) total shoot biomass (grams), (**F**) shoot length (centimeters), (**G**) leaf number. Conductivity–response curves are based on treatment means and standard errors of the mean.

ET30 at 2500 mg/L sulfate. An equivalent stimulatory effect on mesocotyl emergence was not observed in chloride-treated plants. Values for the ET30 end point could not be determined because of high interreplicate variability in the chloride exposures and lack of a concentration–response relationship (Table 5). However, 4 of 5 chloride exposures had increased ET30 values relative to the HS-1 control, and all were greater than their sulfate osmotic equivalent exposures.

Table 5. Effect of sulfate and chloride exposure on time to 30% mesocotyl emergence (ET30) in *Zizania palustris*

Sulfate		Chloride	
Concentration (mg/L)	ET30 (d)[a]	Concentration (meq/L)	ET30 (d)[a]
10	4.0 (0.6)	NA	NA
50	7.8 (1.6)	0.8[b]	>13.8 (>4.2)
250	4.5 (0.5)	4.2[b]	>12.5 (>4.9)
1000	4.3 (0.6)	19.8[c]	>10.8 (>4.0)
2500	3.0 (0.0)	51.1	6.0 (0.9)
5000	5.3 (0.6)	103.2[c]	>11.8 (>3.5)

[a]Mean with standard error of the mean in parentheses.
[b]Two replicates failed to reach 30% emergence by study day 21; calculations based on use of 21 d as the value for these replicates.
[c]One replicate failed to reach 30% emergence by study day 21; calculations based on use of 21 d as the value for this replicate.
NA = not applicable.

Root biomass and shoot biomass were stimulated by increased chloride concentrations (SC25 values, 4600 μmhos/cm and 7054 μmhos/cm, respectively), but sulfate-treated plants did not exhibit a response for these test end points (SC25 value >8507 μmhos/cm). Seed activation and seedling survival end points exhibited IC25 and SC25 values greater than their highest mean conductivity condition (8507 μmhos/cm and 8509 μmhos/cm for sulfate and chloride exposures, respectively), indicating the low sensitivity of these test end points.

Overall, these findings indicated that sulfate toxicity to wild rice occurred only at the highest sulfate exposure (5000 mg/L) and only for 4 of the test end points assessed. Three of these 4 test end points also exhibited chloride toxicity, indicating the possibility that conductivity-related effects are the cause of toxicity rather than sulfate toxicity per se. Toxic effects on wild rice were likely sulfate-specific for shoot length, whereas chloride exhibited greater toxicity than sulfate based on root length and, to a lesser extent, phytotoxicity. Sulfate-specific stimulatory effects occurred for mesocotyl emergence, whereas chloride-specific stimulatory effects occurred for root biomass and shoot biomass.

Multiple regression—Chloride versus sulfate responses

To further evaluate whether the effects of high sulfate concentrations on wild rice were specifically attributable to sulfate or more generally to conductivity-related stress, regression analysis of conductivity and ion type was carried out for 7 biological end points previously assessed for sulfate toxicity. For all 7 end points, quadratic and/or linear regression models were overall statistically significant (Table 7). Quadratic models accounted for a greater proportion of variation than linear models in 5 end points, whereas variation in shoot length and leaf number was better described using linear models. The statistically significant components of each model differed by end point.

The ET30 end point was not evaluated because of numerous chloride replicates having nonquantitative ET30 values (>21 d; Table 5). However, both sulfate and chloride exposures generally exhibited decreasing trends in ET30 up to 51.1 meq/L (2500 mg/L sulfate), then increased slightly at 103.2 meq/L (5000 mg/L sulfate). The exception was the 10–mg/L sulfate treatment, which had a lower ET30 value than HS-1 (4 d vs 7.5 d). As previously stated, chloride-treated plants also generally had higher ET30 values as their sulfate equivalents.

Variations in mesocotyl emergence and phytotoxicity were better fit by quadratic equations for conductivity, with all model coefficients (quadratic coefficient A, linear coefficient B, and *y*-intercept C) significantly differing from 0, suggesting that observed changes in these end points were attributable at least partly to conductivity-related effects. Ion type (chloride vs sulfate) was not significant overall, and the regression coefficients did not differ significantly by ion type, indicating no sulfate-specific effect on mesocotyl emergence or phytotoxicity (Figure 1A and B). Thus, observed changes in mesocotyl emergence and phytotoxicity were attributed to conductivity-related effects alone. As seen for mesocotyl emergence and phytotoxicity, root biomass and root length data were also better fit by quadratic equations, with A and C significant for both end points. However, the values of A and B also significantly differed by ion type for root biomass and root length. Thus, sulfate exposure affected root biomass and root length in a manner that appeared to be sulfate-specific (Figure 1C and D). Variation in shoot biomass data was also better fit by a quadratic equation; however, A and B were not significantly different from 0 when data from sulfate and chloride treatments were considered together. Ion type also had no significant effect overall or on the value of the linear coefficient B. However, coefficient A differed significantly by ion type, specifically in that shoot biomass of chloride-treated plants increased in response to increasing conductivity, whereas sulfate-treated plants' shoot biomass was negatively affected at high conductivity (Figure 1E).

Table 6. Summary of conductivity-based point estimates for *Zizania palustris* exposed to sulfate and chloride

	Sulfate		Chloride	
Measurement end point	Point[a]	Estimate (μmhos/cm)	Point[a]	Estimate (μmhos/cm)
Seed activation	SC/IC25	>8507[b]	SC/IC25	>8509[b]
Mesocotyl emergence[c]	SC25	1771 (1431–2076)	SC/IC25	>8509[b]
30% emergence time	SC/IC25	ND[d]	SC/IC25	ND[d]
Seedling survival	SC/IC25	>8507[b]	SC/IC25	>8509[b]
Shoot biomass	SC/IC25	>8507[b]	SC25	7054(6367–7741)
Shoot length	IC25	5780 (5491–6069)	IC25	7690 (7306–8075)
Root biomass	SC/IC25	>8507[b]	SC25	4600 (4370–4830)
Root length	IC25	5590 (5311–6290)	IC25	1870 (1777–1964)
Leaf number	IC25	6810 (6470–7151)	SC/IC25	>8509[b]
Phytotoxicity[c]	IC25	7385 (7271–7485)	IC25	6476 (6387–6555)

[a]Stimulatory concentration (SC) or inhibitory (IC) concentration estimate, with 95% fiducial interval in parentheses. Determined by linear interpolation.
[b]Value reflects mean conductivity value at highest exposure concentration with no effect.
[c]Emergence and phytotoxicity point estimates determined by probit analysis due to percentage data.
[d]Not determined (ND), as data could not be fit to linear interpolation model.

Table 7. Multiple regression of measurement end points on conductivity and ion type for *Zizania palustris* exposed to sulfate and chloride[a]

	Emergence	Root biomass	Root length	Total shoot biomass	Shoot length	Leaf number	Phytotoxicity
Model	Quadratic	Quadratic	Quadratic	Quadratic	Linear	Linear	Quadratic
Adjusted R^2 (%)	47.8	51.4	58.5	40.5	72.2	53.7	98.9
C	0.015[b]	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
Sulfate	25.8	0.000426	4.72	0.00763	3.55	3.89	9.54
Chloride	20.6	0.000501	5.40	0.00799	2.69	3.24	10.8
B	0.025[b]	0.463	0.002[b]	0.480	0.001 [b]	0.140	<0.001[b]
Sulfate	0.0167	1.23×10^{-7}	2.43×10^{-6}	8.25×10^{-7}	−0.000172	0.000154	−0.0120
Chloride	0.0104	-4.20×10^{-8}	−0.000769	-3.13×10^{-7}	-8.74×10^{-5}	-3.79×10^{-5}	−0.0129
A	0.028[b]	0.036[b]	0.022[b]	0.069	NA[c]	NA[c]	<0.001[b]
Sulfate	-1.55×10^{-6}	-1.28×10^{-11}	-3.32×10^{-8}	-9.01×10^{-11}	NA	NA	2.50×10^{-6}
Chloride	-1.03×10^{-6}	1.25×10^{-11}	5.63×10^{-8}	8.34×10^{-11}	NA	NA	2.74×10^{-6}
Ion	0.636	0.596	0.242	0.733	<0.001[b]	<0.001[b]	0.733
B × ion	0.314	0.041[b]	0.018[b]	0.067	0.016[b]	0.002[b]	0.644
A × ion	0.414	0.003[b]	0.010[b]	0.008[b]	NA[c]	NA[c]	0.253

[a]Analysis based on individual replicate data from all treatments with sulfate concentrations ≥50 mg/L. All models consider ion type as a categorical variable (sulfate vs chloride) and conductivity as a continuous variable. Quadratic and linear models were tested, with results for the model with the higher adjusted r^2 value shown. C is the *y*-intercept value; B is the coefficient to the linear term conductivity; A is the coefficient to the quadratic term conductivity^2.
[b]Statistically significant *p* value ($\alpha = 0.05$).
[c]Not applicable because of model type.

Shoot length and leaf number data were both better fit by linear models in which ion type was significant overall. These results indicated significant differences between sulfate- and chloride-treated plants across conductivity levels independent of conductivity-related effects, with sulfate-treated plants generally producing longer shoots and more secondary leaves. Although coefficient C was also significantly different from 0 in both cases, B overall was significantly different from 0 only for shoot length. This response suggested that observed effects on shoot length, but not necessarily leaf number, were at least partially attributable to conductivity-related effects. Nevertheless, for both shoot length and leaf number, the value of B differed significantly by ion type, indicating a distinct (stimulatory) effect of sulfate that could be only partially attributed to conductivity-related effects. However, this response created a greater negative impact on wild rice shoot length and leaf number as sulfate and conductivity-related stress increased when chloride and dissolved solids increased (Figure 1F, G), although the trend for shoot length may have been an artifact of the sulfate-treated plants having greater shoot lengths than their chloride-treated equivalents at low dissolved solid levels.

In summary, multiple regression analyses indicated that the wild rice end points assessed often responded as a result of increased conductivity regardless of ion type. Toxic effects as a result of conductivity regardless of ion type were reflected for mesocotyl emergence and phytotoxicity. Five of 7 end points (i.e., root biomass, root length, shoot biomass, shoot length, and free leaf number) showed some indication of possible sulfate-specific response in addition to the expected conductivity-related effects, whereas effects on emergence percentage and phytotoxicity could be attributed to conductivity-related effects alone. These sulfate-specific responses were sometimes stimulatory (i.e., shoot length and leaf number) and sometimes inhibitory.

DISCUSSION

Results from the present study indicated that increased sulfate concentrations did not induce an adverse response in wild rice seedlings under hydroponic conditions at concentrations below 5000 mg/L. Based on ET30 assessments, a more rapid germination time occurred at 2500 mg/L, an apparently anomalous effect given the lack of stimulation or toxicity in all sulfate exposures. Assessing toxic effects based on IC25 values for conductivity indicated that sulfate toxicity occurred in the same 4 test end points identified as responding to sulfate based on LOEC assessments: shoot length, root length, leaf number, and phytotoxicity. The IC25 determinations also indicated that 3 of these 4 end points were responsive to chloride toxicity, with root length and phytotoxicity showing greater sensitivity to chloride than to sulfate. Overall, this suggested that shoot length and root length were the most sensitive wild rice end points for toxic effects due to sulfate and that root length was also an especially sensitive end point by which to asses chloride toxicity.

Typically, plant response to conductivity-related stress is a function of osmotic potential, with increasing conductivity producing greater growth inhibition. In the present study, IC25 values for wild rice based on conductivity in sulfate-treated plants ranged from 5590 μmhos/cm to 7385 μmhos/cm, with similar conductivity-based IC25 values for chloride solutions observed for 3 of the 4 test end points. This is generally consistent with other studies indicating similar adverse effects at equivalent osmotic potentials regardless of the salt composition [8–10]. Yield reductions in conventional rice (*Oryza sativa*) grown to maturity have been found at field water salinities exceeding 1900 μmhos/cm [11]. This is in good agreement with the chloride-based salts' IC25 value of 1870 μmhos/cm observed for wild rice root length observed in the present study.

Plants respond to increased salinity in 2 ways: by conductivity-related effects and by ion-specific effects [8]. Ion-specific effects include direct toxicities and nutritional disorders. Seedlings in the present study generally responded in similar ways with increases in the electrical conductivity of the test solution regardless of ion source, but some differences were observed between different ion types for some end points. For example, phytotoxicity had a lower IC25 value for chloride-treated than for sulfate-treated plants, and multiple regression analysis showed phytotoxicity to be a quadratic function of conductivity regardless of ion type. Such effects are indicative of salt damage, not sulfate-specific toxicity. Overall, the results indicate that tissue length end points may be more sensitive measures of growth than biomass end points. Neither root biomass nor shoot biomass was significantly influenced by sulfate concentrations of 5000 mg/L based on one-way

ANOVA, although multiple regression results indicated that a combination of conductivity-related and sulfate-specific toxic effects may have occurred in these end points. In contrast, shoot length and root length both were significantly reduced at 5000 mg/L sulfate, and multiple regression analysis for both end points suggested that both conductivity-related and sulfate-specific effects were likely. Previous work has shown that expansive growth in plants, controlled largely by cell elongation, is a very salt-sensitive process [12]. Therefore, it is not surprising that growth parameters in wild rice involving tissue expansion (increased shoot and root length) were more sensitive to increased osmotic potential than parameters affecting biomass.

In the case of shoot length, the IC25 value was significantly lower for sulfate-treated wild rice than for chloride-treated plants, suggesting that the sulfate-specific effect was predominant. At low conductivities, wild rice seedling shoot lengths were greater in sulfate treatments than in the equivalent chloride treatments. This finding is not surprising because plants have been found to perform better under sulfate-based salinity than chloride-based salinity at the same osmotic potential [13]. It is possible that modest increases in sulfate at low concentrations (10–250 mg/L) may be somewhat stimulatory by correcting a sulfur deficiency.

The IC25 value for root length was significantly lower for chloride-treated plants than for sulfate-treated plants, and the regression curve for sulfate indicated a possible toxicity-mitigating effect at moderate sulfate concentrations. This suggested that conductivity-related stress predominantly affected root length. It should be noted, however, that the root length in the 10–mg/L sulfate treatment was markedly greater than in the HS-1 control. A plant with higher root length can have a developmental advantage for acquisition of nutrients over those with smaller root length. Plants with high root length density (total root length per volume of soil) are better able to absorb nutrients with low solubility, which reach the root surface via diffusion compared with plants with the same root mass but lower root length densities [14]. Chloride was also more phytotoxic than sulfate, based on IC25 determinations.

Leaf number was reduced at 5000 mg/L sulfate. Unlike shoot and root length, multiple regression analysis did not show a significant effect of conductivity overall, but the slope of the regression lines was significantly influenced by ion type, with sulfate-treated plants performing somewhat better than chloride-treated plants at low conductivity and somewhat worse at high conductivity. Conductivity-related stress is known to reduce leaf emergence rates in grasses [15], so the weak response in chloride-treated wild rice is somewhat unexpected.

Four wild rice end points were adversely affected by exposure to 5000 mg/L sulfate; emergence, however, showed some indications of being stimulated by high sulfate concentrations. Although the proportion of mesocotyl emergence on study day 21 did not significantly differ across sulfate treatments, multiple regression analysis indicated that conductivity had a significant influence on this end point, with a stimulatory effect at moderate conductivity and a toxic effect at high conductivity. Furthermore, the median time required to reach 30% emergence was significantly shorter for 2500 mg/L sulfate (3 d) than for HS-1 seeds (7.5 d). This may be an artifact of the HS-1 treatment as it was the only 1 in the sulfate series having any replicates with ET30 >7 d. Conversely, most chloride treatments had at least 1 replicate with ET30 values >21 d, suggesting a possible sulfate-specific stimulatory effect on germination that was not necessarily dependent on conductivity. The previously mentioned sulfur deficiency hypothesis does not account for stimulatory effects at high sulfate concentrations. Typically, germination is delayed, not stimulated, as the concentration of salts in the soil solution increases [7,12]. However, a previous study demonstrated that wild rice seeds stored at 3 °C for 120 d followed by a 7-h exposure to 43% ethanol increased the germination percentage 4-fold [16]. The success of this chemical treatment in breaking dormancy suggests that sulfate could similarly stimulate germination.

CONCLUSION

Overall, the results from the present study suggest that sulfate did not adversely affect germination and early development of wild rice at concentrations below 5000 mg/L over the 21-d hydroponic exposure. Some effects induced at high sulfate concentrations also were observed in osmotically equivalent chloride treatments, and some sulfate-specific stimulatory effects may be attributable to the effects of sulfate as a plant nutrient. Two end points, shoot length and leaf number, appeared to have sulfate-specific toxic responses; however, the remainder of the observed responses likely were the result of a general salinity-induced, conductivity-related stress and not specifically sulfate toxicity. Root length appeared to be an especially sensitive end point to conductivity-related stress induced by chloride-dominated salt solutions.

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