BROWN MARSH TASK II.1: INTEGRATIVE APPROACH TO UNDERSTANDING THE CAUSES OF SALT MARSH DIEBACK - DETERMINATION OF SALT MARSH SPECIES TOLERANCE LIMITS TO POTENTIAL ENVIRONMENTAL STRESSORS

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ABSTRACT

A sudden and widespread dieback of salt marshes, dominated primarily by the perennial marsh grass, *Spartina alterniflora* (smooth cordgrass), occurred in coastal Louisiana during Spring-Summer of 2000. This phenomenon, known as the brown marsh event due to the color of the dead vegetation, coincided with the most severe drought in Louisiana in over 100 years. This record-setting event resulted in moderate to severe impact of 101,780 hectares (251,505 acres) of salt marsh in Barataria and Terrebonne basins in Louisiana. Approximately 42 % of these stressed marshes experienced die-off**.** Hence, the brown marsh event was of national importance because these marshes comprise approximately 40% of US coastal wetlands and thereby represent a major national impact to this resource. In addition, such extreme weather events, which are significant disturbances to coastal ecosystems, are predicted to increase in frequency during the 21st century due to global climate change. Thus, knowledge of the mechanisms determining the causes of environmental injury is a necessary first-step for scientifically based mitigation of impact and restoration of affected habitats.

Although the mortality of the vegetation coincided with a record setting drought, we had no empirical evidence linking the drought to the dieback. Because droughts can affect wetland environmental conditions in predictable ways, a number of plausible stressors, including hyper-salinity, water deficits, and soil acidity with resulting metal toxicity, were hypothesized as potential causative agents. These stressors may have acted singly or in combination to cause the dieback event. Our overall goal was to determine the importance of these stressors in causing the sudden salt marsh dieback of 2000. This research determined the environmental tolerance limits of *Spartina alterniflora*, the species that died during the dieback event, and those of sympatric species, *Juncus reoemerianus* and *Avicennia germinans*, that survived. These three salt marsh species were exposed in 10 separate experiments to (1) increasing stressor levels of salinity, water deficiency, acidity, and heavy metals (Al and Fe) and (2) increasing stress duration at sublethal levels of these stressors. In addition, a series of three experiments were conducted to assess the interactive effects of the stressors on plant mortality. We hypothesized that the stressor or stressors most likely responsible for sudden salt marsh dieback would be one(s) that negatively impacted *Spartina*, while not appreciably affecting *Juncus* or *Avicennia*, the two species that survived the brown marsh event.

Our findings indicate that water deficiency, acidity and metal toxicity acted in tandem to cause a stress to *Spartina* much greater than would have resulted from any of these stressors acting individually. We found that at a non-lethal pH of 3.0, a pH that could have occurred during the brown marsh event, the presence of Fe and Al, even at non-lethal concentrations, resulted in *Spartina* mortality ranging from 40 - 60 %. Further stress in the form of a sublethal water deficit resulted in *Spartina* mortality near 90 %, while having little impact on the sympatric species *Avicennia* and *Juncus*. Thus, this research supports the hypothesis that low precipitation and reduced marsh flooding during the brown marsh event induced soil oxidation, soil acidification and metal toxicity, acting in conjunction with plant water stress (possibly exacerbated by somewhat elevated soil salinities), to cause *Spartina* mortality without an effect on co-occurring plant species.

INTRODUCTION AND BACKGROUND

Extensive dieback of the perennial marsh grass, *Spartina alterniflora* (smooth cordgrass), which dominates regularly flooded salt marshes along the Gulf of Mexico and Atlantic coastlines, occurred in coastal Louisiana in 2000. This event was of national and international importance because Louisiana's coastal wetlands represent a major natural resource, accounting for 41% of the U.S. total coastal wetlands. These wetlands directly support 28% of the national fisheries harvest, the largest fur harvest in the U.S., and the largest concentration of over wintering waterfowl in the U.S. (Turner, 1990). Because dieback of marsh vegetation is often a prelude to land loss in coastal Louisiana (Mendelssohn et al., 1983), the potential economic, social, and environmental impacts involved in such a rapid and extensive alteration in the landscape are of grave concern (Turner, 1990).

Aerial surveys (McKee et al., 2001; www.brownmarsh.net/factSheets/index.htm) documented that over 121 km (75 miles) of coastline and 101,780 hectares (251,505 acres) of salt marsh in Barataria and Terrebonne basins were moderately to severely impacted. The dieback occurred over a short period of time, with first sightings of 'browning' marsh reported in May 2000 and continued through the growing season until natural senescence of vegetation in the fall (McKee et al., 2001). Extensive areas of dead salt marsh, varying in size from ~300 m^2 up to 5 km^2 in area, were observed from Point au Fer to the Mississippi River. Intensive ground surveys of the largest dieback areas in Terrebonne and Barataria Basins found 50 to 100 % mortality of plant shoots in affected zones (McKee et al. 2004)

Although fresh, intermediate, and brackish marshes may have experienced some dieback, most observations indicated that salt marsh, which accounts for ~ 157,487 hectares (389,160 acres) in Barataria and Terrebonne Basins, was the type most impacted. Two species appeared to be affected: *S. alterniflora* and to a lesser extent, *S. patens* (marshhay cordgrass). Sympatric species such as *Avicennia germinans* (Black Mangrove), *Batis maritima* (Maritime Saltwort), *Distichlis spicata* (Seashore Saltgrass), and *Juncus roemerianus* (Black Needlerush) survived the event and in many locations exhibited no visible signs of stress (McKee et al. 2004).

This rapid dieback of salt marsh in coastal Louisiana is unprecedented in the region's recorded history. Sudden salt marsh dieback has been reported previously elsewhere in the Gulf of Mexico (Carlson, 2001), but does not approach the large-scale occurrence observed in coastal Louisiana. The large extent and rapidity of this event also distinguish it from the long-term phenomenon of dieback previously described (Smith, 1970; Mendelssohn et al., 1983; Mendelssohn and McKee 1988; Turner, 1990). Historically, wetlands in coastal Louisiana have been gradually converting to open water due to a combination of natural processes related to the Mississippi River deltaic cycle (Scruton, 1960) and anthropogenic activities (Turner, 1990). Historical dieback of *S. alterniflora* occurs in interior marshes and is caused by excessive soil waterlogging, which limits aeration of plant roots and allows buildup of soil phytotoxins such as sulfide (Mendelssohn et al., 1981; Mendelssohn and McKee, 1988; Koch et al., 1990; Naidoo et al., 1992; Wilsey et al., 1992; Webb et al., 1995). However, historical dieback occurs gradually, and typically requires years for a marsh to completely die.

The cause of the recent, sudden dieback of *S. alterniflora*, however, is currently unknown. Factors such as chemical spills or outbreaks of insects or other herbivores were quickly ruled out as potential causes, due to the spatial pattern and extent of dieback and the condition of dead and dying vegetation. Although snails had heavily grazed the dead vegetation in some sites, the mortality of the vegetation clearly preceded the grazing. However, snail impacts have recently been identified as an important factor in expanding dieback areas in Louisiana by as much as 11 % (Silliman et al. 2005). No evidence of chemical residues and the large extent of the affected region were also inconsistent with a toxic spill. Although potential fungal pathogens (e.g., *Fusarium* sp. and *Macrophomia* sp.) were isolated from material collected at dieback sites, infection by these organisms usually occurs on previously stressed vegetation and is therefore unlikely to be an explanation by itself (R. Schneider, personal communication), but may work synergistically with abiotic stressors (see Task 2.1 – Fungal Pathogen Report).

Marsh dieback coincided with extreme drought conditions and unusually low freshwater discharge from the Mississippi and Atchafalaya Rivers (Grymes, 2001; Swenson, 2001). These local events are related to La Niña, a phenomenon that is characterized by drier and warmer than normal conditions across the southern US (Philander, 1990). Mean high tide levels in coastal Louisiana were also below normal during winter and early spring (Swenson 2001, McKee et al. 2004), and this factor could have limited overbank flooding of interior marshes. Drought, combined with less tidal flooding, may have decreased water availability directly and/or increased salinity by concentrating salts in the porewater. The survival of more salt tolerant species (*D. spicata*, and *A. germinans*) is consistent with this hypothesis. However, salinity of surface water prior to and during dieback (Swenson, 2001) and measured in porewater during our survey (McKee et al., 2001) did not exceed the ecological limits of *S. alterniflora* (Hester et al., 1996). Thus, it is questionable if salinity was a major driving force behind the sudden salt marsh dieback.

In addition to increases in salinity, other chemical changes may occur upon soil drying. Sediments that develop under an estuarine influence may contain pyrite or other readily oxidizable metal-sulfide compounds (Howarth, 1979). When saline sediments are oxidized, e.g., during drainage or dredging, microbial oxidation of pyrite and sulfide generates sulfuric acid, which leads to soil acidification (Nordstrom, 1982). Aeration and acidification of sediments (to pH 3-4) may also occur naturally through oxygen advection by falling water, a process observed during early spring in Louisiana coastal marshes (Feijtel et al., 1988). One field site (dominated by *S. patens*) showed evidence of soil desiccation with low soil pH (~4), high soil E_h (>600 mV), and root coatings of ferric iron, indicating oxidation (McKee et al., 2001). In addition, soils from dieback areas were found to acidify upon oxidation, whereas those from control sites did not (McKee et al. 2004). The acidification phase in early spring could have been prolonged or exaggerated by drought conditions sufficiently to negatively affect plants, either by acidity or by toxic metals released at low pH. Solubility of Fe, Mn, and Al increases several-fold with decreases in pH, and may reach toxic levels (Gambrell and Patrick, 1978). The fact that salt marsh sediments accumulate more trace and heavy metals due to mineral input from rivers compared to intermediate and freshwater marshes with more organic substrates (Feijtel et al., 1988) and show greater decreases in pH upon oxidation (Webb et al., 1984) may also help explain the prevalence of dieback in higher salinity habitats.

Thus, there are several causative agents (e.g., salinity, water deficit, pH, heavy metals) that may have singly or interactively caused the dieback event. Although these potential causative agents can occur in association with meteorological extremes such as drought, low river flow, and low water levels, we have no empirical evidence directly linking these drivers with sudden salt marsh dieback. Our goal was to develop this link by establishing the mechanism(s) of plant mortality. Knowledge of the mechanism will provide necessary information to forecast future events and to prepare appropriate restoration measures. Thus, our specific objective was to evaluate each of the potential environmental stressors and their interactions as possible causative factors in the mortality of *S. alterniflora* dominated salt marshes. In doing so, we determined the tolerance limits of *S. alterniflora*, relative to the surviving salt marsh species, to potential environmental stressors (salinity, moisture, pH, toxic metals).

This report comprises five research chapters. Each chapter investigates the effect of a different stressor or combination of stressors on the growth response and mortality of *S. alterniflora*, the plant species that experienced dieback during the brown marsh, and *Avicennia germinans* and *Juncus roemerianus*, the species that survived. The chapter titles are as follows:

Chapter 1. Salt tolerance limits of the salt marsh species *Spartina alterniflora*, *Juncus roemerianus* and *Avicennia germinans*: An assessment of hypersalinity as a cause of the Louisiana brown marsh event

Chapter 2. Drought tolerance limits of the salt marsh species *Spartina alterniflora*, *Juncus roemerianus* and *Avicennia germinans*: An assessment of plant water deficit as a cause of the Louisiana brown marsh event

Chapter 3. Acidity tolerance limits of the salt marsh species *Spartina alterniflora*, *Juncus roemerianus* and *Avicennia germinans*: An assessment of low pH as a cause of the Louisiana brown marsh event

Chapter 4. Iron and Aluminum tolerance limits and effect on salt marsh plants *Spartina alterniflora*, *Juncus roemerianus*, and *Avicennia germinans* - brown marsh case studies on increasing ferrous iron and aluminum concentration

Chapter 5. Interactive effects of drought, acidity and heavy metals on the salt marsh species *Spartina alterniflora*, *Juncus roemerianus* and *Avicennia germinans*

These five research chapters are followed by a Summary and Conclusions section.

CHAPTER 1. SALT TOLERANCE LIMITS OF THE SALT MARSH SPECIES *SPARTINA ALTERNIFLORA*, *JUNCUS ROEMERIANUS* AND *AVICENNIA GERMINANS*: AN ASSESSMENT OF HYPERSALINITY AS A CAUSE OF THE LOUISIANA BROWN MARSH EVENT

Extensive and sudden dieback, known as the brown marsh event, of the perennial salt marsh grass *Spartina alterniflora (*hereafter *Spartina)* occurred in coastal Louisiana in 2000. However, sympatric species such as the black mangrove, *Avicennia germinans* (hereafter *Avicennia*), and the black needlerush, *Juncus roemerianus* (hereafter *Juncus*) survived the event (McKee et al., 2001). This salt marsh dieback, known as the brown marsh event, coincided with extreme drought conditions, unusually low freshwater discharge from the Mississippi and Atchafalaya Rivers, and very low estuarine water levels during the 1999-2000 period (Swenson, 2001). Interstitial salinity in the marsh soil may increase via evaporation and reduced tidal leaching when drought conditions occur. Such an increase in marsh salinities are especially likely when low freshwater discharge coincides with low estuarine water levels (Swenson, 2001). Field monitoring of 18 brown marsh sites demonstrated significantly higher leaf Na/K ratios compared to control sites, although salinity of estuarine waters prior to and during dieback (Swenson 2001) and marsh porewater salinities (Mckee et al, 2001) did not exceed the ecological or physiological limits of *S. alterniflora*. Although the causes of brown marsh event are unclear, increases in salinity during the drought may have been a contributing factor.

The objective of this chapter was to investigate the effects of increasing salinity on plant growth response to determine if salinity was a probable cause of the brown marsh event. We hypothesized that if salinity was the primary cause of the dieback, an experimental increase in salinity under controlled conditions, would first affect *Spartina*, the species that died during the brown marsh event, and that only at higher salinities would *Juncus* and *Avicennia*, the species that survived the event, be affected.

Materials and Methods

Plant materials

The salt marsh plants, *Spartina alterniflora* (smooth cordgrass) and *Avicennia germinans* (black mangrove) were acquired from the Plant Materials Center of the National Resources Conservation Service (NRCS). *Spartina alterniflora* included populations E1-6, E2-3, E2-6 and E3-4. *Juncus roemerianus* (black needlerush) was collected from a salt marsh near Cocodrie, Louisiana. After acquired, all plants were planted into a substrate of commercial potting soil (Miracle Gro lawn, Inc.) and garden soil (Greenleaf Products, Inc.) (1:1) to grow to a satisfactory size before the initiation of the experiment. Because the mix of potting soil and garden soil can more readily be washed from plant roots than marsh sediment, the use of this rooting material reduced physical damage to the plants when they were transferred from the potting soil to sand, the substrate used in the experiment.

Experimental Procedures

Three weeks before the start of the experiment, the potting soil was carefully washed from the plant roots. The three marsh plant species (*Spartina, Juncus*, and *Avicennia*), free of the soil, were separately transplanted into sand filled plastic pots (20 cm height and 15 cm diameter) with 4 holes (2x2 cm) on the sidewall near the pot bottom. The holes were covered with a thin

layer of sponge to let water drain freely, but to retain the sand inside the pots. Ten ramets (stems) of *Spartina* and *Juncus* and one individual of *Avicennia* were transplanted into each plastic pot. After transplantation, three experimental units (one pot of each species) were placed in a plastic tub (30x40x25 cm in depth, width and height). Thus, all three species were treated with the same salinity and other conditions, such as nutrient and pH levels. The experiment was conducted in the growth chamber with light intensity about 800 $\mu mol/m^2/s$ at the canopy level during the daytime and 0 $\mu mol/m^2/hr$ during the nighttime. The daytime temperature was 30 °C for 12 hours and nighttime temperature was 21 °C for 12 hours in a 24-hour period. The tubs, containing the experimental units, were filled with half-strength Hoagland's nutrient solution to 0.5 cm above the soil surface. As soon as the plants recovered from initial transplant shock (about one to two weeks), salinity was increased to 7 ppt in the Hoagland's solution with sea salts (Forty Fathoms, MarineMix Corp.). Water was added to the tubs daily to maintain the 0.5 cm water level. The salinity was raised to 7 ppt one week after transplantation, 15 ppt after the second week, and 23 ppt after the third week. The experiment started with the plants acclimated to 23 ppt.

Three treatments were included in the experiment:

1. Control: Salinity of the nutrient solution maintained at 23 ppt throughout the experiment.
2. Salinity increased to 119 ppt (hereafter noted as the salinity-increase treatment): Salinity of the nutrient solution was increased from 23 ppt to 119 ppt at a rate of 12 ppt every two weeks.
3. Salinity maintained at 59 ppt (hereafter noted as the salinity-hold treatment): Salinity of the nutrient solution was increased to 59 ppt at a rate of 12 ppt every two weeks, then held at 59 ppt for 10 weeks.

Thus, salinities in the nutrient solution were 23, 35, 47, 59, 71, 83, 95, 107 and 119 ppt. The pH value of the nutrient solution was maintained at pH 6.5 with HCl. With each increase in salinity level, the experimental units were flushed with new nutrient solution containing the next salinity level until the solution that drained the experimental units reached this salinity. The drained solution was discarded. Pots were filled to 0.5 cm above the soil surface with nutrient solution at the appropriate salinity. The nutrient solutions in the control experimental units (23 ppt) were also renewed every two weeks following the same procedure. Salinity was maintained by watering with tap water daily to replace the water lost by evapo-transpiration, and pH kept at 6.5 with dilute HCl.

Plant responses to salinity were measured every 2 weeks (measurements started 7-9 days after an increase in salinity). The plant response variables included: (1) leaf elongation rate, (2) photosynthetic rate, (3) stem density, (4) shoot height and (5) percent mortality

Methodologies

<u>Leaf expansion rate</u>. The difference in leaf length from a fixed point to the tip of a young leaf over a three-day period was measured to determine the leaf growth rate per day. The unit is cm/day.

Photosynthetic rate. Photosynthetic rate was measured with an infrared gas analyzer (Li-Cor 6400). A representative leaf was selected and enclosed in a leaf chamber to analyze net carbon dioxide exchange rate. Photosynthetic active radiation (PAR) of 2000 $\mu mol/m^2/s$ and CO_2 concentration of 350 ppm were used to measure plant photosynthetic rate. The unit is μmol $CO_2/m^2/s$.

Stem density (branch numbers for *Avicennia*). Live stem numbers of *Spartina* and *Juncus* were directly counted in each experimental unit. For *Avicennia*, instead of stem number, live branch number was measured in each experimental unit because there is only one stem of *Avicennia* in each pot. Thus, the live branch numbers of *Avicennia* were considered analogous to the live stem numbers of *Spartina* and *Juncus*. The unit is number/pot.

Total live shoot height. The height of each live shoot of *Spartina* and *Juncus* were measured with a meter stick. Total live shoot height was derived by summing the heights of all live shoots in each experimental unit. For *Avicennia*, instead of live shoot height, total live leaf number was measured. The number of live leaves served as a non-destructive surrogate for live leaf biomass similar to the use of live shoot heights of *Spartina* and *Juncus*.

Percent mortality. Percentage of dead tissue was estimated ocularly every two weeks to determine mortality rate.

Statistics

Statistical analysis was conducted with the Mixed Model procedure of SAS (version 8.0) with repeated measurements. Least square means were used to compare between individual treatment-levels when interactions were significant. Saxton's macro was used for statistical separation of the means with letter groupings in Proc Mixed (SAS, 1998). The measured variables were converted to a percentage of the control for each species to account for the large differences in growth and physiology among the three plant species. The salinity-increase experiment and salinity-hold experiment were statistically analyzed separately. Statistical significance was reported at a probability of 0.05 unless otherwise stated.

Results

Effect of salinity on leaf expansion rate

Increasing salinity visually affected plant condition (Figs. 1.1A-D). At 47 ppt salinity, impact to *Juncus* and *Spartina* began (Fig. 1.1A). At 59 ppt salinity, the effect on *Juncus* and *Spartina* was dramatic, although *Juncus* mortality was greater than that for *Spartina* (Fig. 1.1B). By 71 ppt salinity, complete mortality of *Juncus* occurred (Fig. 1.1C). Only *Avicennia* survived at the 83 ppt and 95 salinity levels (Figs. 1.1D and 1.1E).

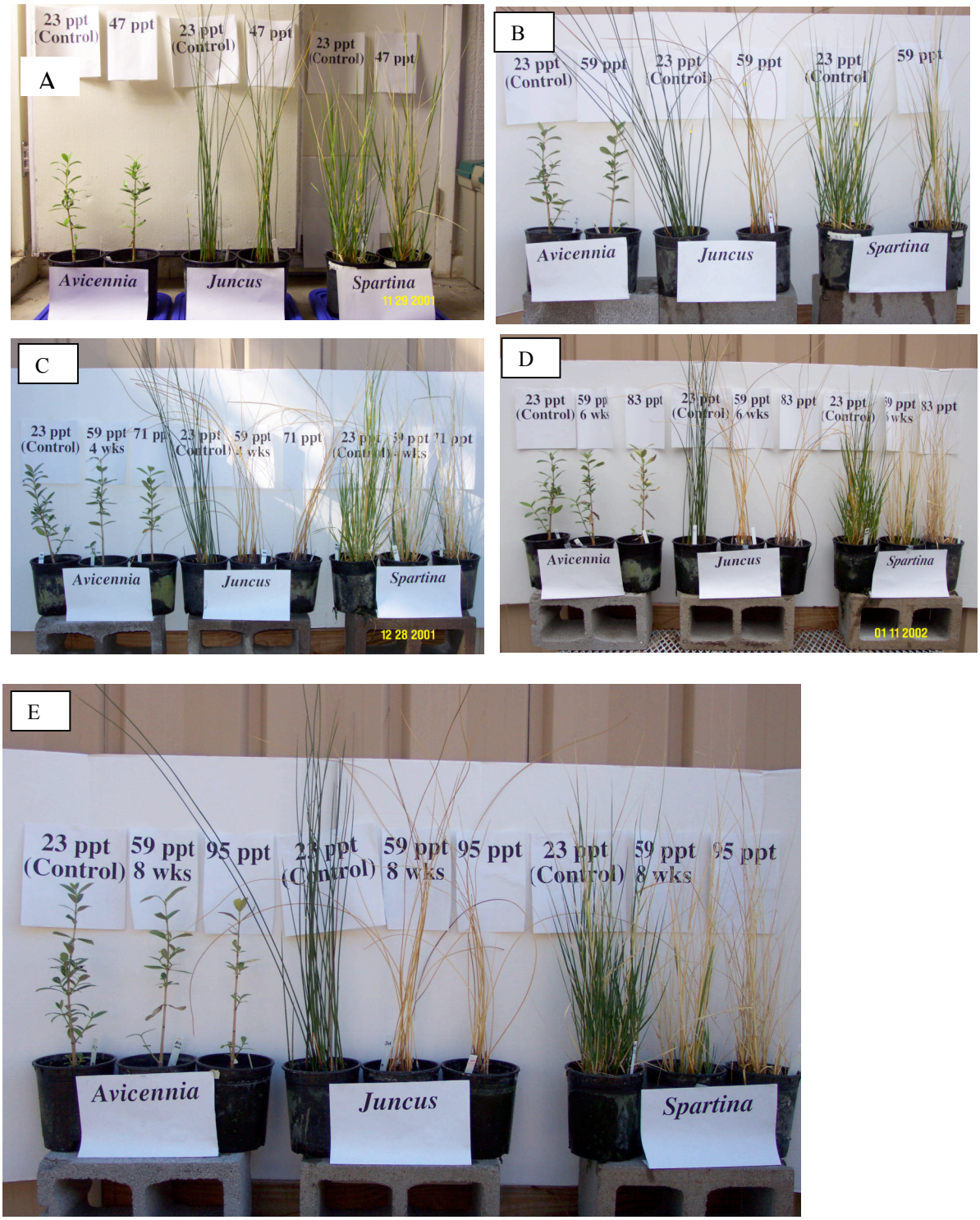


Fig. 1.1. Effect of elevated salinity on health status of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*. 1.1A: Salinity at 47 ppt; 1.1B: Salinity at 59 ppt;; 1.1C: Salinity at 59 and 71 ppt;; 1.1D: Salinity at 59 ppt for 6 weeks and at 83 ppt;. 1.1E: Salinity at 59 ppt for 8 weeks and at 95 ppt.

The salinity-increase treatment significantly reduced leaf expansion rates ($p<0.0001$), expressed as a percent of the control. In addition, averaged over salinity level, *Juncus* was significantly ($p<0.0001$) more impacted by increasing salinity than *Spartina* and *Avicennia.* However, the effect of increasing salinity on leaf expansion significantly differed with plant species (significant species x salinity interaction, $p<0.0001$). The negative effect of increasing salinity on *Juncus* leaf expansion was much greater than on the leaf expansion of *Spartina* and *Avicennia*. Leaf expansion rate of *Juncus* significantly decreased at 35 ppt, and was reduced to nearly zero at the 59 ppt salinity level (Fig. 1.2A). In contrast, leaf expansion rate of *Spartina* decreased significantly at 47 ppt and was zero at 71 ppt and greater. *Avicennia* was the most tolerant of the three species to increasing salinity with leaf expansion rate showing a significant decrease at 59 ppt and was still detectable at 95 ppt.

For the treatment with salinity held at 59 ppt (Fig. 1.2B), leaf expansion rate of *Juncus* was significantly lower than that for *Spartina* and *Avicennia*. However, leaf expansion rate was not significantly different between *Spartina* and *Avicennia*, with 30% to 40% of the control level for both *Spartina* and *Avicennia*. Leaf expansion rate of *Juncus* was zero, not surprisingly based on its mortality, when salinity was held at 59 ppt.

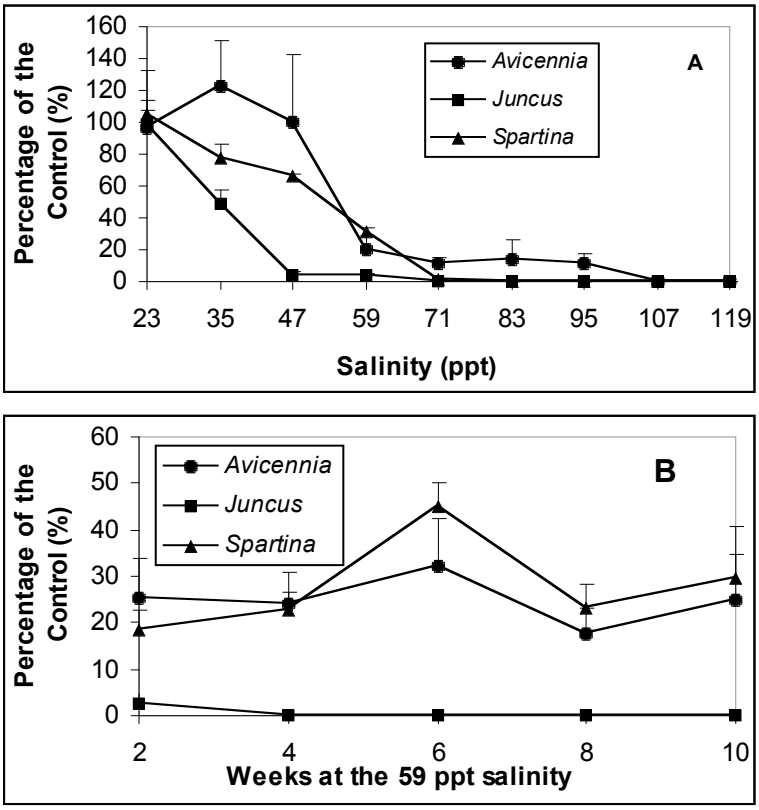


Fig. 1.2. Effects of increasing salinity (A) and the salinity held at 59 ppt (B) on the leaf expansion rates of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of salinity on the photosynthetic rate

Regardless of plant species, increasing salinity significantly reduced plant photosynthetic rate, expressed as a percent of the control. However, the photosynthetic response to increasing salinity did not significantly differ by species nor was there an overall significant species effect.

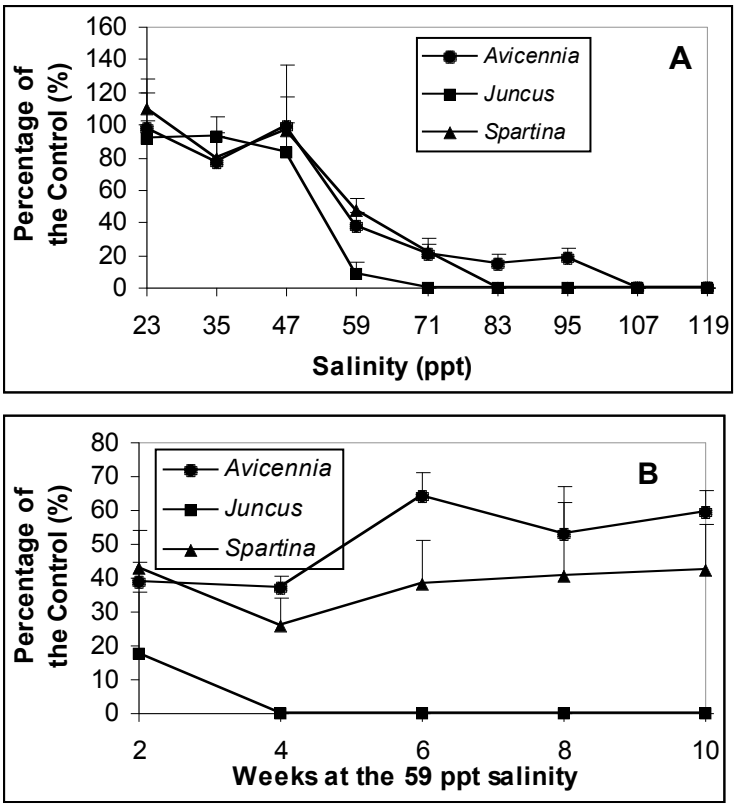


Fig. 1.3. Effects of increasing salinity (A) and salinity held at 59 ppt (B) on the photosynthetic rates of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

For the treatment with salinity held at 59 ppt (Fig. 1.3B), the photosynthetic rate of *Juncus* was significantly lower than that for *Spartina* and *Avicennia*, which did not significantly differ although *Spartina* tended to be more affected than *Avicennia*. The photosynthetic rate of *Juncus* was zero at the hold salinity of 59 ppt, a salinity resulting in complete mortality of *Juncus*.

Effect of salinity on the live stem density

Live stem density, expressed as a percent of the control, significantly decreased with increasing salinity (Fig. 1.4A) and was significantly lower for *Juncus* than for *Spartina* and *Avicennia,* averaged over all salinity levels (Fig. 1.4A). Live stem density of *Spartina* was significantly lower than that of *Avicennia*. A significant (p=0.001) interaction between salinity and species, however, indicated that the decrease in live stem number with increasing salinity was dependent on plant species. Live stem density of *Juncus* significantly decreased at the 59

ppt salinity level and became zero at the 71 ppt salinity level. Although live stem density of *Spartina* significantly decreased at a relatively low salinity level of 47 ppt, the first major decrease was at 71 ppt and no live stems occurred at the 95 ppt salinity level. Total live branch number of *Avicennia,* used as a surrogate for stem density for this species, did not significantly decrease until a salinity of 71 ppt; all branches died at 119 ppt salinity.

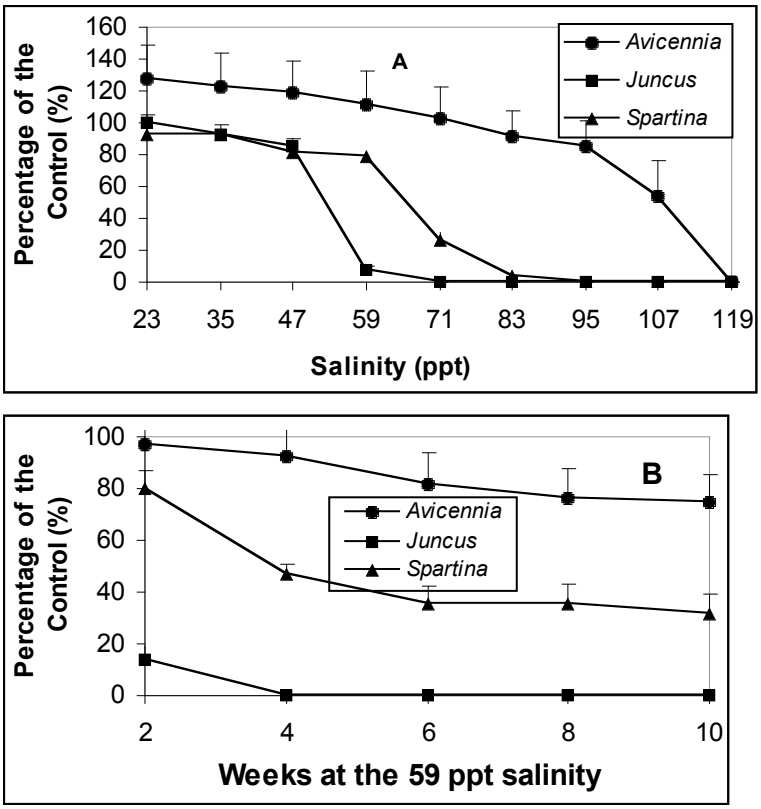


Fig.1.4. Effects of increasing salinity (A) and the salinity held at 59 ppt (B) on the live stem density of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

For the treatment with salinity held at 59 ppt (Fig. 1.4B), live branch number was significantly higher for *Avicennia* (ca. 80 to 90% of the control) than for *Spartina*. In addition, live stem density of *Spartina*, between 30 to 45% of the control, was significantly higher than that for *Juncus*, which reached zero after 4-weeks at 59 ppt

Effect of salinity on the cumulative live shoot height

Cumulative live shoot height is a relative index of live aboveground biomass. Cumulative live shoot height, expressed as a percent of the control, of the three species decreased with increasing salinity and significantly differed by species (Fig. 1.5A). However, the decrease in cumulative live shoot height with increasing salinity was dependent on plant species (significant salinity by species interaction, $p < 0.001$). The cumulative live shoot height of *Juncus* was significantly lower than that of *Avicennia* and *Spartina*, and significantly decreased at the 47 ppt salinity level. Cumulative live shoot height of *Spartina* significantly decreased at the 59 ppt

salinity level of and further dramatically decreased at salinity levels between 59 and 71 ppt, and became zero at 83 ppt salinity. For *Avicennia*, where total live leaf number was used as a surrogate for cumulative live shoot height, total live leaf number of *Avicennia* significantly decreased at the 71ppt salinity level, then gradually decreased at salinity levels between 71 to 107 ppt, and became zero at 119 ppt. These results demonstrate *Avicennia*'s greater salt tolerance to elevated salinity.

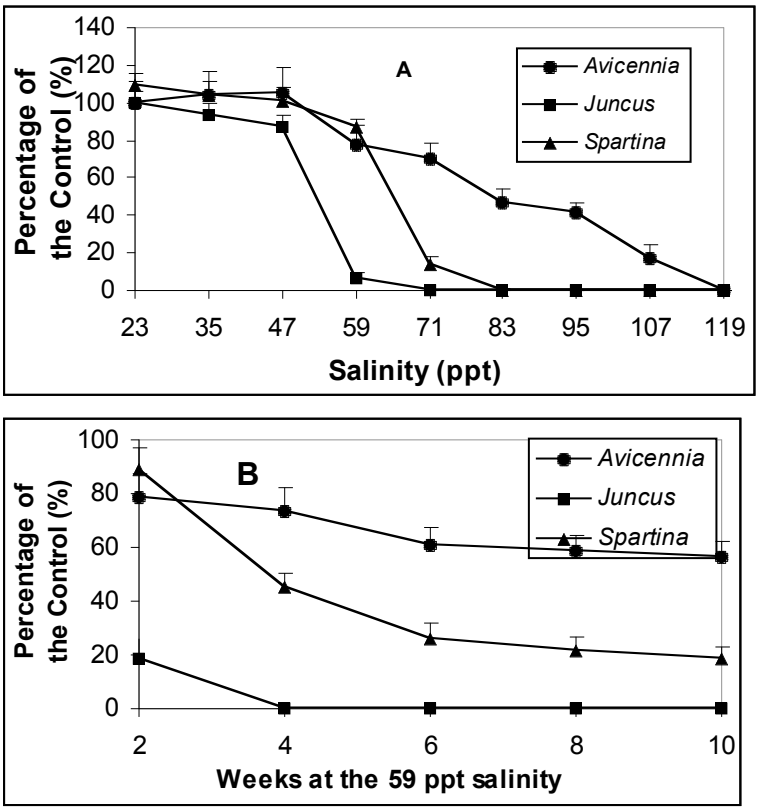


Fig. 1.5. Effects of increasing salinity (A) and the salinity held at 59 ppt (B) on the cumulative live shoot height of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

For the treatment with salinity held at 59 ppt (Fig. 1.5B), cumulative live leaf number of *Avicennia*, about 60 to 75% of its control, was significantly higher than that (cumulative live shoot height) of *Spartina*. Cumulative live shoot height of *Spartina*, about 20 to 40% of the control, was significantly higher than that of *Juncus*. The cumulative live shoot height of *Juncus* was zero when salinity was held at 59 ppt. *Avicennia* was able to maintain cumulative live leaf number with increasing salinity to a significantly greater extent than *Spartina*; Juncus was not able to maintain live stems at a hold salinity of 59 ppt (significant salinity x species interaction, $p < 0.001$).

Effect of salinity on plant mortality

The percentage dead tissue is a relative index of plant mortality rate. The mortality rate of the three plant species increased with increasing salinity, especially for *Juncus* and *Spartina* (Fig.

1.6A). Both the main effects of salinity and plant species on plant mortality were statistically significant ($p<0.0001$). However, the response of plant mortality to increasing salinity was dependent on plant species (significant species x salinity interaction, $p<0.0001$). The mortality of *Juncus* significantly increased at the 47 ppt salinity level, and became 100% at 59 to 71 ppt, while the mortality rate of *Avicennia* did not significantly increase until a salinity of 83 ppt; complete mortality occurred at 119 ppt. The mortality of Spartina was intermediate to that of *Juncus* and *Avicennia* with a significant increase at the 47 ppt salinity level, becoming 100% at 83 ppt. These results demonstrate that *Juncus* is more sensitive to salt stress than is *Spartina*, which is more sensitive than *Avicennia*.

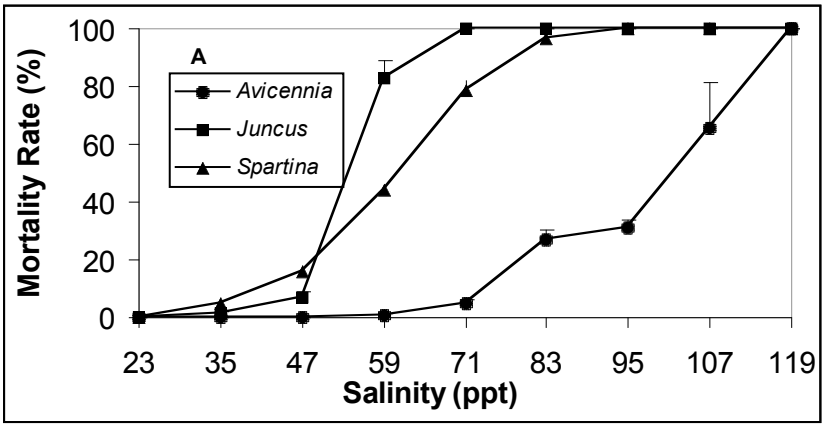


Fig. 1.6. Effects of increasing salinity (A) and the salinity held at 59 ppt (B) on percent mortality of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

For the treatment with salinity held at 59 ppt (Fig. 1.6B), the percent mortality of *Juncus* was significantly higher than that of *Spartina* and *Avicennia*. The mortality of *Spartina*, about 60% to 70%, was significantly higher than that of *Avicennia*, with less than 10%.

Discussion

Increasing salinity detrimentally affected marsh plant growth responses, with decreased photosynthetic rate and leaf expansion rate, fewer new shoots generated, and increased mortality. The effect of salinity was most severe on *Juncus* and least on *Avicennia*. *Spartina* tended to have an intermediate salinity tolerance.

In a salinity range from 35 to 47 ppt, growth of all three-marsh plants moderately decreased. However, plant mortality did not coincide with growth response, demonstrating that these species can survive at salinities < 47 ppt, but with reduced growth.

The salinity levels at which these species experience mortality has received little attention, although numerous studies have analyzed sub-lethal salt stress in these and other halophytes. We found that *Juncus* was the least tolerant of the three species to salinity stress. It was the species that had the greatest mortality both with increasing salinity and at the hold salinity of 59 ppt. *Juncus* was unable to survive at 59 ppt for four weeks. In contrast, *Avicennia* and *Spartina* had much greater salinity tolerances than *Juncus* and survived at 59 ppt for more than 10 weeks. Although the lethal salinity levels for *Juncus* and *Spartina* have not hitherto been

quantified, mangrove species have received some scrutiny. For example, along desert coast swamps of the United Arab Emirates (Tamaei, 2002), survival of *Avicennia marina* seedlings was negatively impacted at salinities of 60 ppt, and no survival occurred at 80 ppt due to dehydration through the roots. We found that one-year old *Avicennia germinans* plants had 30 % mortality at 83 ppt salinity and 100 % mortality at 119 ppt. The greater salt tolerance of A. germinans compared to A. marina may be a result of genetic differences between the species or to differences in the growth conditions when these experiments were conducted.

Most research has focused on plant growth responses to sublethal levels of salt (Cherian et al. 1999; Sobrado 1999; Noaman et al. 2000; Hester et al. 2001; Lewis et al. 2002). Concentrations of 7.8 g NaCl/L and 19.2 g NaCl/L reduced early seedling biomass of *Scirpus robustus* and *S. alterniflora*, respectively, when compared to plants grown in sediment containing no measurable salinity. Seedling survival was not affected at average concentrations ≤17.5 g NaCl/L for *S. robustus* and ≤22.3 g NaCl/L for *S. alterniflora* (Lewis et al. 2002). However, the study did not determine the lethal salinity level. Sublethal salinity levels (20 to 30 ppt) resulted in significant reductions in net CO_2 assimilation and leaf expansion of *Spartina alterniflora* (Hester et al. 2001). A common garden study indicated that halophyte species such as *Spartina alterniflora*, *Juncus roemerianus* and *Salicornia bigelovii* can grow with minimum reduction in aboveground biomass at soil solution salinities <20 ppt, however, the plants deteriorated at 40 ppt salinity in the irrigation water (El-Haddad et al, 2001). In contrast, CO_2 exchange and conductance of *Avicennia marina* were consistently higher at a high salinity site (35 ppt) compared to low salinity site (12 ppt) (Tuffers et al. 2001), indicating the high salt tolerance of this species. In the present study, *Avicennia germinans* survived at a salinity as high as 95 ppt. We increased the salinity by 12 ppt every 2-weeks to let the plants gradually acclimate to the increasing salinity levels. A gradual increase in salinity more likely simulates the increase in salinity and salt stress that might occur during a natural drought event, like the brown marsh phenomenon that occurred in Louisiana.

During the brown marsh event, dieback of *Spartina alterniflora* occurred, while *Avicennia germinans* and *Juncus roemerianus* survived (McKee et al., 2001). During the current salinity experiment, complete mortality occurred first for *Juncus roemerianus*, second for *Spartina alterniflora*, and last for *Avicennia germinans*. The species-specific mortality with increasing salinity was different from that during the brown marsh event. If salinity was primarily responsible for brown marsh event, *Spartina alterniflora* should have been the species first stressed by elevated salinities.

Conclusion

The three salt marsh plants, *Avicennia germinans*, *Juncus roemerianus* and *Spartina alterniflora*, have large differences in salt tolerance based on photosynthetic rate, leaf expansion rate, live stem density, cumulative live shoot height, and mortality rate. The maximum salt tolerance limit is < 59 ppt for *Juncus roemerianus*, and about 71 ppt for *Spartina alterniflora*, with reduction in growth rate at the high-end of the tolerance limit. *Avicennia germinans* has a tolerance limit > 95 ppt. It should be noted that long-term exposure to these salinities would likely reduce the salinity level at which 100 % mortality occurs. Because the less salt tolerant marsh plant, *Juncus roemerianus*, survived the brown marsh event while the more salt tolerant

species, *Spartina alterniflora*, did not, we conclude that elevated salinity, which may have occurred during the 2000 drought in coastal Louisiana, was not the primary cause of the sudden salt marsh dieback.

CHAPTER 2. DROUGHT TOLERANCE LIMITS OF THE SALT MARSH SPECIES *SPARTINA ALTERNIFLORA*, *JUNCUS ROEMERIANUS* AND *AVICENNIA GERMINANS*: AN ASSESSMENT OF PLANT WATER DEFICIT AS A CAUSE OF THE LOUISIANA BROWN MARSH EVENT

Extensive and sudden dieback of the perennial salt marsh grass *Spartina alterniflora* occurred in coastal Louisiana in 2000. However, sympatric species such as *Avicennia germinans* and *Juncus roemerianus* survived the event, termed brown marsh due to the brown coloration of the dead vegetation (McKee et al., 2001). Brown marsh coincided with extreme drought conditions and unusually low freshwater discharge from the Mississippi and Atchafalaya Rivers that prevailed during the 1999-2000 period (Swenson, 2001). Field surveys found that this severe drought caused the marsh surface to crack at some sites (McKee et al. 2001). Thus, a direct plant water deficit may have been a contributing cause of the brown marsh event.

The objectives of this experiment were to investigate the effect of increasing drought intensity (decreasing water potential) on plant growth responses to determine if drought could have been responsible for the brown marsh event.

Materials and Methods

Plant materials

The salt marsh plants, *Spartina alterniflora* (smooth cordgrass) and *Avicennia germinans* (black mangrove) were acquired from the Plant Materials Center of the National Resources Conservation Service (NRCS). *Spartina alterniflora* included populations E1-6, E2-3, E2-6 and E3-4. *Juncus roemerianus* (black needlerush) was collected from a salt marsh near Cocodrie, Louisiana. After acquired, all plants were planted into a substrate of commercial potting soil (Miracle Gro lawn, Inc.) and garden soil (Greenleaf Products, Inc.) (1:1) to grow to a satisfactory size before the initiation of the experiment. Because the mix of potting soil and garden soil can more readily be washed from plant roots than marsh sediment, the use of this rooting material reduced physical damage to the plants when they were transferred from the potting soil to sand, the substrate used in the experiment.

Experimental Procedures

Three weeks before the start of the experiment, the potting soil was carefully washed from the plant roots. The three marsh plant species (*Spartina, Juncus*, and *Avicennia*), free of the soil, were separately transplanted into sand filled plastic pots (20 cm height and 15 cm diameter) with 4 holes (2x2 cm) on the sidewall near the pot bottom. The holes were covered with a thin layer of sponge to let water drain freely, but to retain the sand inside the pots. Ten ramets (stems) of *Spartina* and *Juncus* and one individual of *Avicennia* were transplanted into each plastic pot. After transplantation, three experimental units (one pot of each species) were placed in a plastic tub (30x40x25 cm in depth, width and height). Thus, all three species were treated with the same salinity and other conditions, such as nutrient and pH levels. The experiment was conducted in the growth chamber with light intensity about 800 $\mu mol/m^2/s$ at the canopy level during the daytime and 0 $\mu mol/m^2/hr$ during the nighttime. The daytime temperature was 30 ºC

for 12 hours and nighttime temperature was 21 ºC for 12 hours in a 24-hour period. The tubs, containing the experimental units, were filled with half-strength Hoagland's nutrient solution to 0.5 cm above the soil surface. As soon as the plants recovered from initial transplant shock (about one to two weeks), salinity was increased to 7 ppt in the Hoagland's solution with sea salts (Forty Fathoms, MarineMix Corp.). Water was added to the tubs daily to maintain the 0.5 cm water level. The salinity was raised to 7 ppt one week after transplantation, 15 ppt after the second week, and 23 ppt after the third week. During this period, acidity of the nutrient solution was maintained at pH 6.5 with HCl. The experiment started with the plants acclimated to 23 ppt and pH 6.5.

Three water stress treatments were included in the experiment:

1. *Control*: No polyethylene glycol (PEG 8000) added to the nutrient solution during the experiment.
2. *Decreasing water potential*: The water potential of the nutrient solution was lowered from 0 to -11.0 MPa with different amounts of PEG at the rate of -0.15 to -3.0 MPa every two weeks.
3. *Water potential held at–1.1 MPa*: The water potential of the nutrient solution was lowered from 0 to - 1.1 MPa at the rate of 0.25 MPa every two weeks and then held at -1.1 MPa for 12 weeks.

Polyethylene glycol (PEG 8000) was used as an osmotic agent to precisely create a range of water availability for the plants. The water potential levels created by PEG were 0, -0.1, -0.25, -0.4, -0.6, -0.8, -1.1, -1.5, -1.9, -2.4, -3.0, -3.8, -4.7, -7.1, and -11 MPa. The salinity of the rooting solution was maintained at 23 ppt and the acidity at pH 6.5 throughout the experimental period for all treatments. In addition to the water potential created by PEG, the nutrient solution, which had a salt concentration of 23 ppt, created a water potential of about -1.8 MPa for all treatments.

When the treatment-levels were changed to the next lower water potential, the experimental units were flushed with the new nutrient solution at the next water potential level until the water potential of the drainage solution reached the new level. The drainage solution was discarded after flushing. Then the fresh nutrient solution at the new water potential was added to the pots and filled to a water level of 0.5 cm above the soil surface. The nutrient solution for the control (no PEG, pH 6.5, and 23 ppt salinity) was changed at the same intervals and with the same procedures as for the experimental treatments. The proper water potential of the nutrient solution was maintained during each two week interval by watering with tap water to replace the water lost by evapo-transpiration and by adding 1 N HCl daily to maintain the proper pH level.

Plant response to water potential was measured every two weeks (measurements started nine days after an increase in water potential). The plant response variables included: (1) leaf elongation rate, (2) photosynthetic rate, (3) stem density, (4) shoot height and (5) percent mortality

Methodologies

Leaf expansion rate. The difference in leaf length from a fixed point to the tip of a young leaf over a three-day period was measured to determine the leaf growth rate per day. The unit is cm/day.

Photosynthetic rate. Photosynthetic rate was measured with an infrared gas analyzer (Li-Cor 6400). A representative leaf was selected and enclosed in a leaf chamber to analyze net carbon dioxide exchange rate. Photosynthetic active radiation (PAR) of 2000 $\mu mol/m^2/s$ and CO_2 concentration of 350 ppm were used to measure plant photosynthetic rate. The unit is μmol $CO_2/m^2/s$.

Stem density (branch numbers for *Avicennia*). Live stem numbers of *Spartina* and *Juncus* were directly counted in each experimental unit. For *Avicennia*, instead of stem number, live branch number was measured in each experimental unit because there is only one stem of *Avicennia* in each pot. Thus, the live branch numbers of *Avicennia* were considered analogous to the live stem numbers of *Spartina* and *Juncus*. The unit is number/pot.

Total live shoot height. The height of each live shoot of *Spartina* and *Juncus* were measured with a meter stick. Total live shoot height was derived by summing the heights of all live shoots in each experimental unit. For *Avicennia*, instead of live shoot height, total live leaf number was measured. The number of live leaves served as a non-destructive surrogate for live leaf biomass similar to the use of live shoot heights of *Spartina* and *Juncus*.

Percent mortality. Percentage of dead tissue was estimated occularly every two weeks to determine mortality rate.

Statistics

Statistical analysis was conducted with the Mixed Model procedure of SAS (version 8.0) with repeated measurements. Least square means were used to compare between individual treatment-levels when interactions were significant. Saxton's macro was used for statistical separation of the means with letter groupings in Proc Mixed (SAS, 1998). The measured variables were converted to a percentage of the control for each species to account for the large differences in growth and physiology among the three plant species. The water potential-decrease experiment and water potential-hold experiment were statistically analyzed separately. Statistical significance was reported at a probability of 0.05 unless otherwise stated.

Results

Effect of increasing drought on plant condition

Overall, increasing the intensity of drought (lowering water potential) negatively affected plant condition (Figs. 2.1A-D). *Spartina alterniflora* was most sensitive to drought stress while *Avicennia* was most tolerant (Figs. 2.1A and 2.1F). *Juncus* tended to be intermediate in drought tolerance.

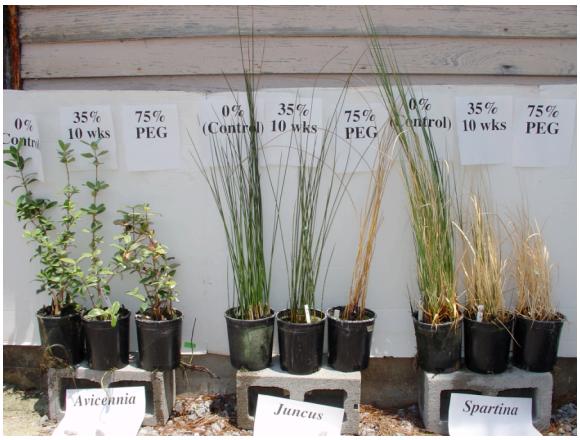
1A

1D

1B

1E

1C



1F

Fig. 2.1. Effect of increasing drought stress (decreasing water potential [WP] by adding PEG) on growth status of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*. 2.1A: WP at –0.6 and –0.8 MPa; 2.1B: WP at –0.8 and –1.5 MPa ; 2.1C: WP at –1.1 and –2.4 MPa; 2.1D: WP at -3.0 and at –1.1 MPa for 4 weeks; 2.1E: WP at -3.8 and –1.1 MPa for 6 weeks; 2.1F: WP at –7.1 and at –1.1 MPa for 10 weeks.

Effect of increasing drought stress on leaf expansion rate

Decreasing water potential significantly ($p<0.0001$) reduced leaf expansion rate, expressed as a percent of the control, of the three marsh plant species (Fig. 2.3A). However, there were no significant differences ($p=0.3504$) among the species nor was there a significant interaction ($p=0.9786$) between species and water potential.

For the treatment with water potential held at -1.1 MPa (Fig. 2.3B), the effect of stress duration on leaf expansion rate was significantly more severe on *Spartina* and *Juncus* than on *Avicennia* (Fig. 2.3B).

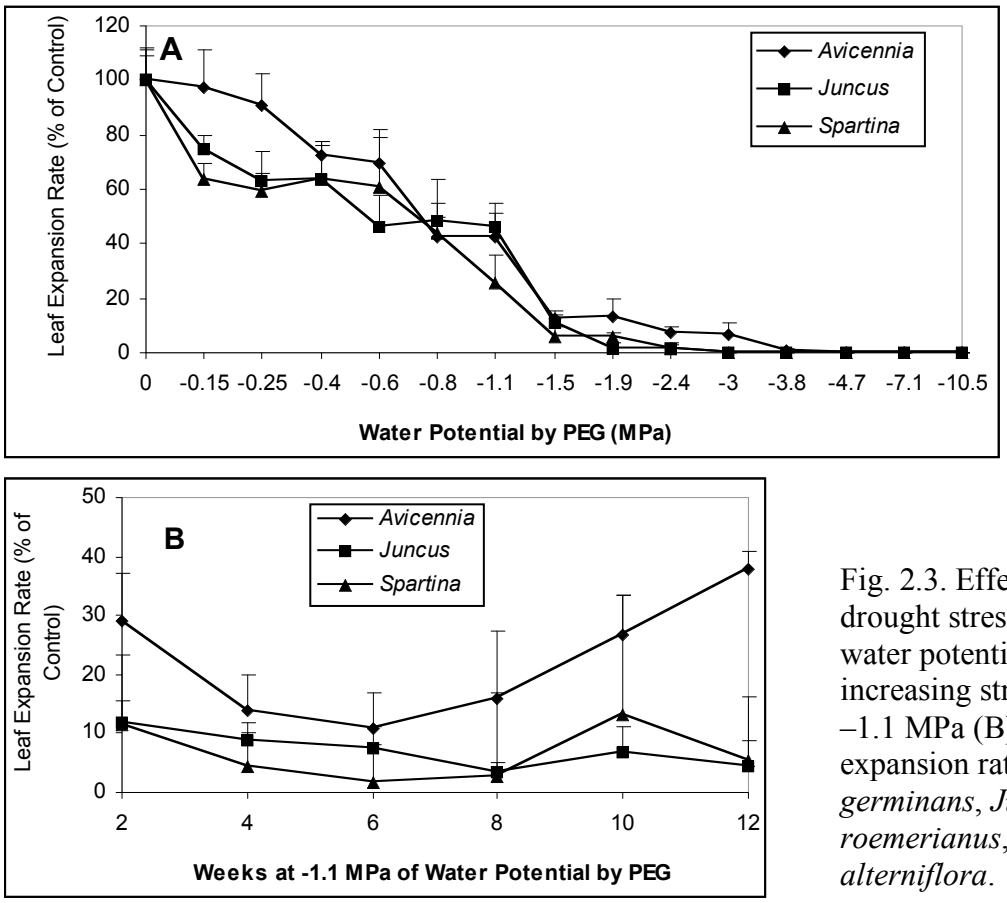
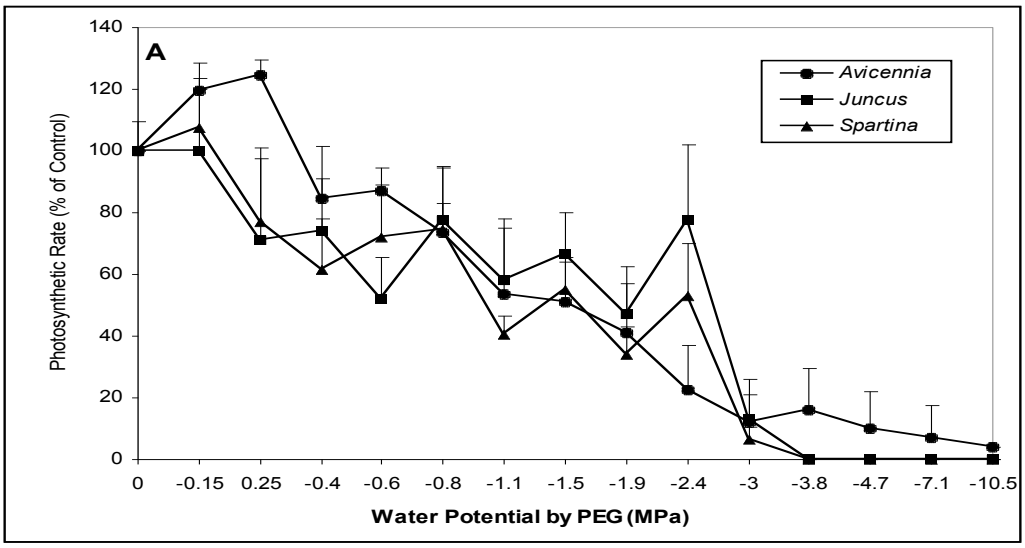


Fig. 2.3. Effect of increasing drought stress (decreasing water potential) (A) and increasing stress-duration at –1.1 MPa (B) on leaf expansion rates of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing drought stress on photosynthetic rate

The plant photosynthetic rate, expressed as a percent of the control, was significantly ($p<0.0001$) reduced with decreasing water potential (increasing drought) in the nutrient solution (Fig. 2.2A). However, the response of photosynthetic rate to decreasing water potential did not significantly differ with plant species ($p=0.26$) nor was there a significant interaction between species and water potential ($p=0.31$). This suggests that the photosynthetic rates of the three species respond similarly to decreasing water potential.

For the treatment with water potential held at -1.1 MPa (Fig. 2.2B), the photosynthetic response of the three species to decreasing water potential did not significantly differ ($p=0.1488$) nor was there a significant interaction between species and water potential ($p=0.2499$). Averaged over species, photosynthetic rate did show some significant differences with duration of stress, but these differences were not of ecological importance.



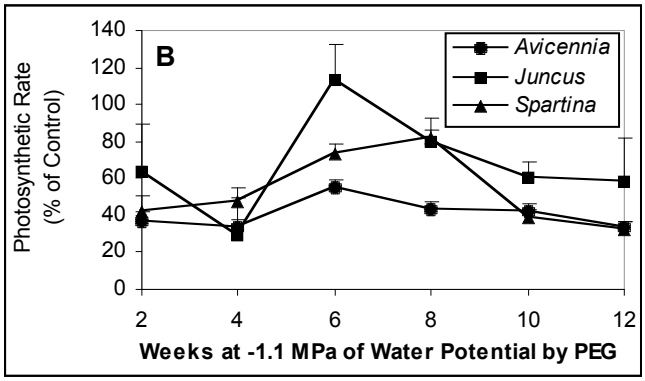
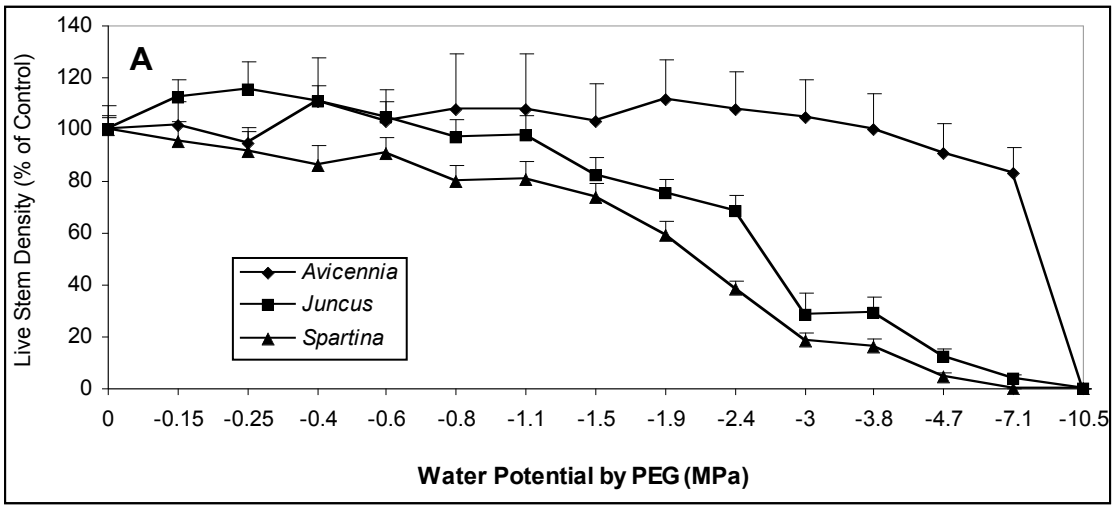


Fig. 2.2. Effect of increasing drought stress (decreasing water potential) (A) and increasing stress-duration at –1.1 MPa (B) on the photosynthetic rates of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing drought stress on the live stem density

Live stem density, expressed as a percent of the control, of the plant species significantly ($p<0.0001$) decreased with increasing drought stress (decreasing water potential) (Fig. 2.4A). The drought stress effect, however, was dependent on species (significant species x water potential interaction, $p<0.0001$). *Avicennia* was most resistant of the three species to water stress. The live stem density of *Avicennia* was not significantly affected by decreasing water potential until a water potential of -11.0 MPa. In contrast, live stem density of *Spartina* significantly decreased at a water potential of -0.4 MPa and continued to decrease as the water potential was lowered further. *Juncus* was generally intermediate in response to drought stress. Live stem density of *Juncus* significantly decreased at a water potential ≤ -0.8 MPa and continued to decrease in a similar fashion as did *Spartina*. Averaged over all water potentials, live stem density of *Avicennia* was significantly greater than that of *Spartina* with *Juncus* intermediate, but not significantly different from the other species.

For the treatment with water potential held at -1.1 MPa (Fig. 2.4B), increased duration of exposure to this water potential significantly ($p=0.0001$) affected live stem density. The main effect of plant species was also significant ($p=0.0204$); live stem density of *Avicennia* was significantly greater than that of *Spartina* (Fig. 2.4B). There were no significant differences in live stem density between *Avicennia* and *Juncus* or between *Juncus* and *Spartina*.



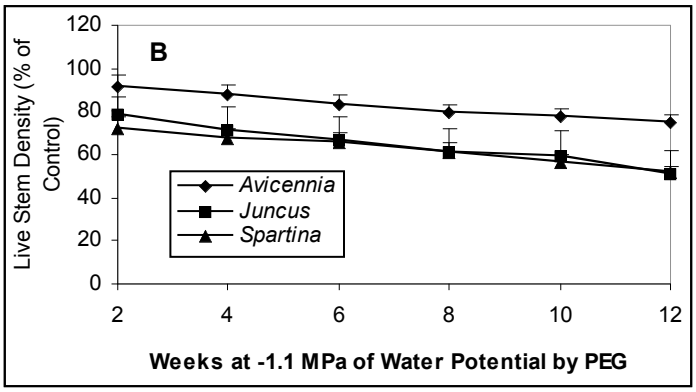
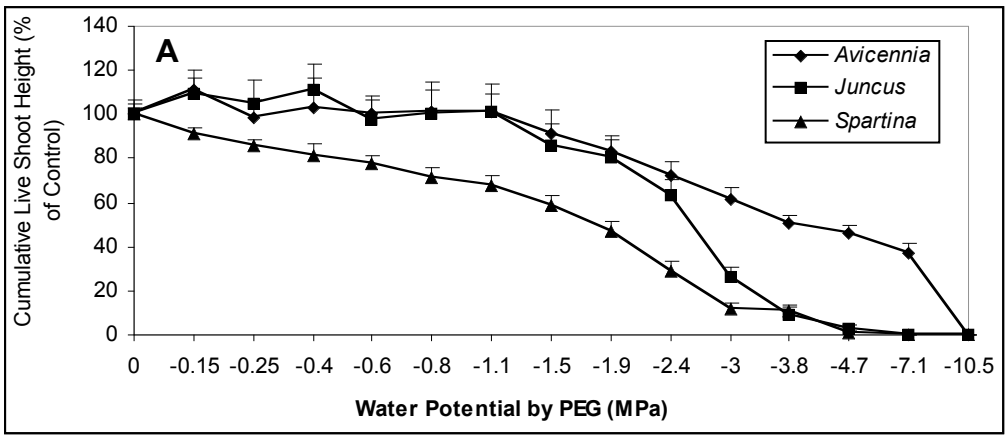


Fig. 2.4. Effects of decrease in water potential (A) and increasing stress duration at –1.1 MPa (B) on the live stem density of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing drought stress on cumulative live shoot height

Cumulative live shoot height, expressed as a percent of the control, of the three plant species significantly ($p<0.0001$) decreased with decreasing water potential (Fig. 2.5A). However, this drought effect differed with plant species (significant plant species x water potential interaction, $p<0.0001$). Cumulative live shoot height of *Avicennia* did not significantly decrease until water potentials ≥ -1.9MPa. In contrast, cumulative live shoot height of *Spartina* significantly decreased at water potentials ≤ -0.4 MPa. Cumulative live shoot height of *Juncus* significantly decreased at the water potential ≤ -1.5 MPa. Averaged over all water potentials, cumulative live shoot height was significantly different among the three species ($P<0.05$) with *Spartina* greater than *Avicennia* and *Juncus*.

For the treatment with water potential held at -1.1 MPa (Fig. 2.4B), the effect of stress duration on cumulative live shoot height was significant, i.e., this growth parameter decreased with stress duration. In addition, cumulative live shoot height was significantly greater for *Avicennia* than for *Spartina* (Fig. 2.5B), but no significant difference between *Avicennia* and *Juncus* or between *Juncus* and *Spartina* was detected.



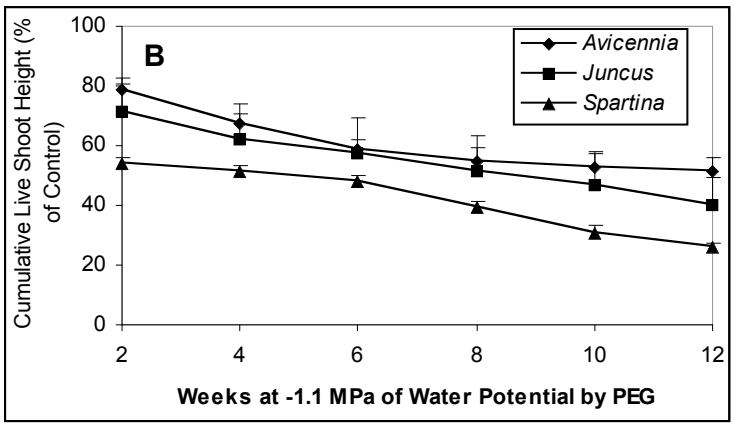


Fig. 2.5. Effects of increasing drought stress (decreasing water potential (A) and increasing stress duration at -1.1 MPa (B) on the cumulative live shoot height of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing drought stress on plant mortality

The percent mortality of the plant species significantly ($p<0.0001$) increased with decreasing water potential (Fig. 2.6A), and this increase in mortality was dependent on plant species (significant species x water potential interaction, $p<0.0001$). Percent mortality of *Avicennia* and *Juncus* did not significantly increase until water potentials ≥ -1.5MPa. In contrast, the percent mortality of *Spartina* significantly increased at water potentials ≥ -0.6 MPa. Averaged over all water potentials, the percent mortality was significantly greater for *Spartina* than *Juncus*, which had a percent mortality significantly greater than that for *Avicennia* (main effect of species significant at $p<0.0001$).

For the treatment with water potential held at -1.1 MPa (Fig. 2.6B), increasing stress duration significantly ($p<0.0001$) increased plant mortality, but this effect was dependent on plant species (significant stress duration x species interaction, $p<0.0001$). The effect of increasing stress duration was greater for *Spartina* than for the other species (Fig. 2.6B). Averaged over all time periods, the percent mortality for *Spartina* was significantly greater than for *Avicennia* and *Juncus*.

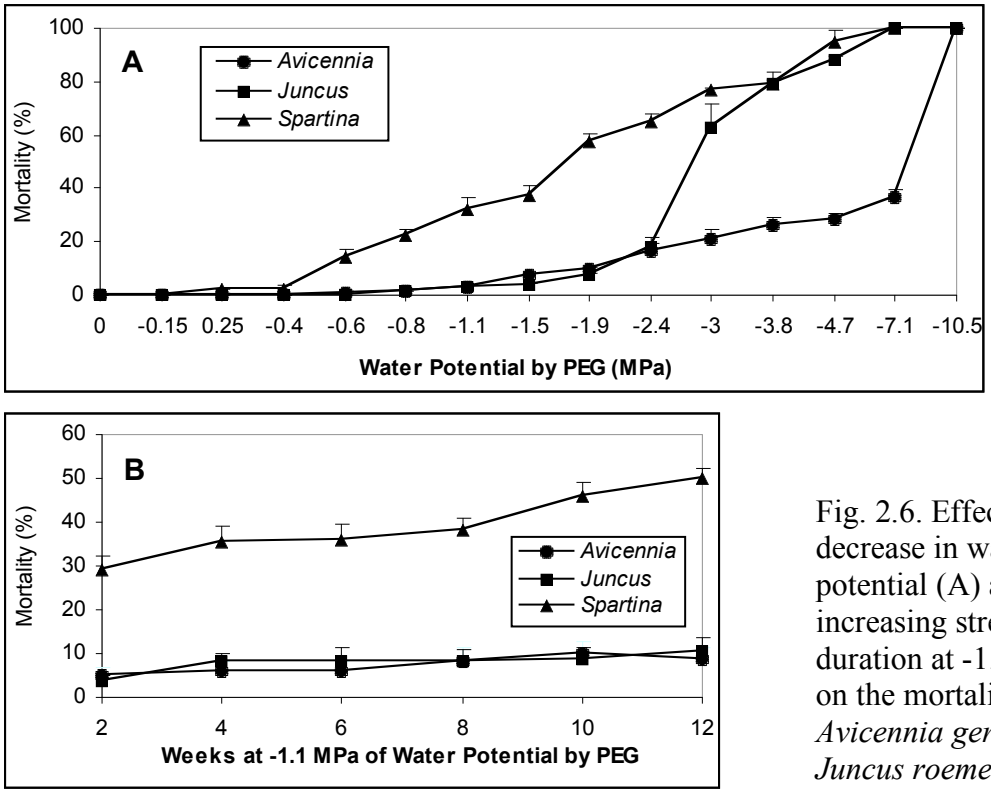


Fig. 2.6. Effects of decrease in water potential (A) and increasing stress duration at -1.1 MPa (B) on the mortality rate of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Discussion

Increasing drought stress (decreasing water potential) negatively affected marsh plant growth responses with decreased leaf expansion rate, photosynthetic rate, new shoot production (numbers), and live cumulative shoot height and increased percent mortality. However, the severity of the drought stress differed with species. The effect of water deficit stress was generally much more severe on *Spartina* than on *Avicennia* and *Juncus*.

In the present experiment, we use polyethylene glycol (PEG 8000) as an osmotic agent to reduce water availability and induce plant water deficits. PEG is one of the most used osmotic agents for water stress studies (Perez Alfocea et al. 1993, Zhao and Harris 1992, Kerepesi and Galiba 2000 and many others). The water potential of the plant nutrient solution decreases with increases in PEG concentration; the lower the water potential (more negative values) the more severe the simulated drought, and thus the less free water available for plant uptake. By using PEG as an osmotic agent, we were able to precisely control the drought intensity (water potential) in contrast to allowing the soil to dry over time, which allows other environmental factors like soil chemistry to confound inferences on drought stress, per se. Most water stress studies in the literature investigate sub-lethal effects on physiological response to drought. During drought, plants may accumulate organic solutes, such as sugars, amino acids and organic acids to decrease plant water potential (primarily solute potential) for water uptake (Perez Alfocea et al. 1993, Kerepesi and Galiba 2000), especially for drought tolerant plants.

Few studies in the literature have been conducted to precisely determine the drought tolerance limits of plants. Even fewer studies have evaluated water stress on wetland plants. Wetlands usually have an overabundance of water; thus, drought is not a primary driving force in most coastal wetlands. However, wetlands indeed periodically suffer drought stress. Wetland plant composition may change during dry periods because wetland plants have different drought tolerance limits. For example, vegetation dynamics of subalpine wetlands in Sierra Nevada, California, were studied from 1988 through 1996 (Rejmankova et al. 1999). During this period, the region experienced a drought lasting from 1988 to 1994 and reaching its extreme in 1992. Four plant communities, dominated respectively by *Carex rostrata*, *Juncus balticus*, *Scirpus acutus* and *Nuphar polysepalum*, were clearly discernible in the marsh along the water depth gradient. Dominance of *Carex* and *Juncus* has not changed over the years, however, *Scirpus* almost totally disappeared from the marsh, and the *Nuphar* zone became dominated by the rhizomatous perennial, *Hippuris vulgaris*, and terrestrial ruderals in dry years. In coastal Louisiana salt marshes, the sudden dieback of *Spartina alterniflora*, which occurred during an unprecedented drought in the year 2000, left sympatric species such as *Avicennia germinans* and *Juncus roemerianus* literally untouched (McKee et al., 2001). These observations suggest that large differences exist in the tolerance limits of these species to environmental factor(s) associated with the drought, such as water deficiency stress.

In the present study, *Spartina alterniflora* was most sensitive to drought stress. Percent mortality of *Spartina* significantly increased at a water potential of –0.6 MPa created by PEG. Live shoot cumulative height of *Spartina* significantly decreased at an even higher water potential of –0.4 MPa. When the additional water potential created by the 23 ppt salinity of the culture solution is taken into consideration, the combined water potential in the treatments were about –2.1 to -2.3 MPa, of which – 0.4 to –0.6 MPa were

created by PEG and -1.8 MPa created by the 23 ppt of salt. Unlike *Spartina*, percent mortality and cumulative live shoot height of *Avicennia* and *Juncus* were not significantly affected by drought until the water potential was reduced to - 1.5 to - 1.9 MPa created by PEG or - 3.3 to - 3.7 MPa, when the water potential of the saline culture solution is included. In our investigation of salinity tolerance of these same marsh species (see Chapter 1), *Spartina alterniflora* exhibited more tolerance to salinity stress than *Juncus roemerianus*; percent mortality and cumulative live shoot height of *S. alterniflora* first became significantly affected at a salinity of 59 ppt, equivalent to a water potential of about - 4.6 MPa. A comparison between water stress (drought) and salt stress indicates that drought impacted *Spartina alterniflora* more severely than salt stress at same water potential.

A handful of studies (Zhao and Harris. 1992, Perez Alfocea et al. 1993, Kerepesi and Galiba 2000) have indicated that certain plants are able to tolerate salinity stress but were unable to tolerate the same water potential from drought. For example, seedlings of the halophytes *Atriplex lentiformis* and *Suaeda salsa*, and the drought resistant *Prospis chilensis* were grown in liquid culture with their roots exposed to salt (NaCl) or water stress (PEG). *Atriplex lentiformis* and *S. salsa* were exposed to 0, -0.496 and -0.992 MPa osmotic potentials imposed by each treatment, and *P. chilensis* to 0, -0.248 and -0.496 MPa. Dry weight and moisture contents of *A. lentiformis* and *S. salsa* were reduced by PEG but were unaffected, enhanced, or only slightly reduced by NaCl (Zhao and Harris. 1992). Similarly, iso-osmotic stresses (-0.5 MPa) of NaCl (140 mM) and PEG 6000 (150 g l^{-1}) were applied to one-month old plants for 3 weeks. A similar decrease in leaf dry weight occurred *in L. pennellii* or *L. esculentum cv. P-73* and *Volgogradskij* under both stresses, while leaf dry weight of *Lycopersicon esculentum cv. Pera* decreased more under PEG stress than under NaCl stress (Perez Alfocea et al. 1993). In the present study, *S. alterniflora* was able to survive salinities as high as 59 ppt (-4.6 MPa of water potential), but was unable to tolerate - 2.9 MPa of drought (-1.1 MPa by PEG and –1.8 MPa by salt). In contrast, *J. roemerianus* tolerated drought better than salinity. It is well known that *S. alterniflora* excretes excess salt through salt glands, thus reducing ion toxicity. However, *J. roemerianus* does not have salt glands, thus cumulative ion toxicity probably results in the lower salt tolerance of *J. roemerianus* compared to *S. alterniflora*.

During the brown marsh event, dieback of *S. alterniflora* occurred, while *A. germinans* and *J. roemerianus* survived (McKee et al. 2001). In the current drought experiment, the performance of the selected salt marsh plants to drought was similar to their response during the brown marsh event. Significant mortality occurred for *Spartina* at a water potential as high as - 0.6 MPa, but little mortality for *Avicennia* and *Juncus* at water potentials as low as – 1.5 MPa. Also, for the treatment with the water potential held at -1.1 MPa, *Spartina* showed the greatest impact, with about 50% mortality. However, the other two species, *Juncus* and *Avicennia*, showed little stress, with less than 15 % mortality. The species-specific mortality was similar to that observed for these species during the brown marsh event.

Conclusion

Three salt marsh plants, *Avicennia germinans*, *Juncus roemerianus* and *Spartina alterniflora*, demonstrated large differences in drought tolerance based on their growth

responses to increasing drought. The drought tolerance limits are about - 0.8 to - 1.1 MPa for *Spartina alterniflora*, - 2.4 to - 3 MPa for *Juncus roemerianus*, and - 7 MPa for *Avicennia germinans* although long-term exposure to the drought may reduce their drought tolerances even further. The species-specific responses to decreasing water potential suggested that drought could be responsible for the brown marsh event in coastal Louisiana. Moreover, drought-induced soil acidification could result in additional stress to the vegetation. Furthermore, under field conditions, soil acidification may enhance the solubility and availability of metals, such as Al and Fe, adding even more stress to the plants. Consequently, higher water potentials (i.e., less severe drought) than those that initiated plant stress in the present experiment may cause mortality in the field.

Chapter 3. Acidity tolerance limits of the salt marsh species *Spartina alterniflora*, *Juncus roemerianus* and *Avicennia germinans*: An assessment of low pH as a cause of the Louisiana brown marsh event

Extensive and sudden dieback of the perennial salt marsh grass, *Spartina alterniflora*, occurred in coastal Louisiana in 2000. However, sympatric species such as *Avicennia germinans* and *Juncus roemerianus* survived the event, termed brown marsh due to the brown coloration of the dead vegetation (McKee et al., 2001). The brown marsh event coincided with extreme drought conditions in the north-central Gulf of Mexico and unusually low freshwater discharge from the Mississippi and Atchafalaya Rivers that prevailed during the 1999-2000 period (Swenson, 2001). Chemical changes may occur in sediments upon soil drying. When mineral sediments containing metal sulfides dry, oxidation of pyrite and other metal sulfides generates sulfuric acid, which leads to soil acidification (Nordstorm, 1982). Field monitoring indicated that some dieback sites showed evidence of soil desiccation in dead and transition zones with relatively low soil pH and high soil Eh, and root coatings of ferric iron, indicating oxidation (McKee et al. 2001). Although the causes of brown marsh event are unclear, soil oxidation-induced acidification may be a contributing factor.

The objectives of this experiment were to investigate the effects of increasing acidity (decreasing pH) on plant growth responses to determine if increased acidity. induced by soil oxidation, could have been responsible for the brown marsh event.

Materials and Methods

Plant materials

The salt marsh plants, *Spartina alterniflora* (smooth cordgrass) and *Avicennia germinans* (black mangrove) were acquired from the Plant Materials Center of the National Resources Conservation Service (NRCS). *Spartina alterniflora* included populations E1-6, E2-3, E2-6 and E3-4. *Juncus roemerianus* (black needlerush) was collected from a salt marsh near Cocodrie, Louisiana. After acquired, all plants were planted into a substrate of commercial potting soil (Miracle Gro lawn, Inc.) and garden soil (Greenleaf Products, Inc.) (1:1) to grow to a satisfactory size before the initiation of the experiment. Because the mix of potting soil and garden soil can more readily be washed from plant roots than marsh sediment, the use of this rooting material reduced physical damage to the plants when they were transferred from the potting soil to sand, the substrate used in the experiment.

Experimental Procedures

Three weeks before the start of the experiment, the potting soil was carefully washed from the plant roots. The three marsh plant species (*Spartina, Juncus*, and *Avicennia*), free of the soil, were separately transplanted into sand filled plastic pots (20 cm height and 15 cm diameter) with 4 holes (2x2 cm) on the sidewall near the pot

bottom. The holes were covered with a thin layer of sponge to let water drain freely, but to retain the sand inside the pots. Ten ramets (stems) of *Spartina* and *Juncus* and one individual of *Avicennia* were transplanted into each plastic pot. After transplantation, three experimental units (one pot of each species) were placed in a plastic tub (30x40x25 cm in depth, width and height). Thus, all three species were treated with the same salinity and other conditions, such as nutrient and pH levels. The experiment was conducted in the growth chamber with light intensity about 800 $\mu mol/m^2/s$ at the canopy level during the daytime and 0 $\mu mol/m^2/hr$ during the nighttime. The daytime temperature was 30 ºC for 12 hours and nighttime temperature was 21 ºC for 12 hours in a 24-hour period. The tubs, containing the experimental units, were filled with half-strength Hoagland's nutrient solution to 0.5 cm above the soil surface. As soon as the plants recovered from initial transplant shock (about one to two weeks), salinity was increased to 7 ppt in the Hoagland's solution with sea salts (Forty Fathoms, MarineMix Corp.). Water was added to the tubs daily to maintain the 0.5 cm water level. The salinity was raised to 7 ppt one week after transplantation, 15 ppt after the second week, and 23 ppt after the third week. During this period, acidity of the nutrient solution was maintained at pH 6.5 with HCl. The experiment started with the plants acclimated to 23 ppt and pH 6.5. Three treatments were included in the experiment:

4. Control: Acidity of the nutrient solution maintained at pH 6.5 throughout the experiment.
5. Increasing acidity: pH of the nutrient solution was decreased from pH 6.5 to pH 0.75, as described below, at the rate of 1 to 0.25 pH units every two weeks.
6. Acidity held at pH 2.5: pH of the nutrient solution was decreased from pH 6.5 to pH 2.5, as described below, and then held at pH 2.5 for 12 weeks.

The salinity was maintained at 23 ppt for all treatments throughout the experimental period. The pH was decreased at rate of 1 pH unit every two weeks from pH 6.5 to 4.5 and at the rate of 0.5 pH units every two weeks from pH 4.5 to 2.5. Below pH 2.5, the pH was decreased at rate of 0.25 pH units at two weeks interval. Thus, the pH levels were 6.5, 5.5, 4.5, 4, 3.5, 3, 2.5, 2.25, 2, 1.75, 1.5, 1.25, 1.0 and 0.75.

When the treatment solution was changed to the next pH level, the experimental units were flushed with fresh nutrient solution at the next pH level until the pH of the drainage water reached the appropriate pH. The drainage solution was discarded after flushing. Then the fresh nutrient solution at the new pH was added to the pots and filled to a water level of 0.5 cm above the soil surface. The nutrient solution for the control (pH 6.5) was changed at the same intervals and with the same procedures as for the experimental treatments. The proper pH of the nutrient solution was maintained by watering with tap water to replace the water lost by evapo-transpiration and by adding 1 N HCl daily to maintain the appropriate pH level.

Plant response to acidity was measured every two weeks (measurements started nine days after an increase in acidity). The plant response variables included: (1) leaf elongation rate, (2) photosynthetic rate, (3) stem density, (4) shoot height and (5) percent mortality

Methodologies

Leaf expansion rate. The difference in leaf length from a fixed point to the tip of a young leaf over a three-day period was measured to determine the leaf growth rate per day. The unit is cm/day.

Photosynthetic rate. Photosynthetic rate was measured with an infrared gas analyzer (Li-Cor 6400). A representative leaf was selected and enclosed in a leaf chamber to analyze net carbon dioxide exchange rate. Photosynthetic active radiation (PAR) of 2000 $\mu mol/m^2/s$ and CO_2 concentration of 350 ppm were used to measure plant photosynthetic rate. The unit is $\mu mol\ CO_2/m^2/s$.

Stem density (branch numbers for *Avicennia*). Live stem numbers of *Spartina* and *Juncus* were directly counted in each experimental unit. For *Avicennia*, instead of stem number, live branch number was measured in each experimental unit because there is only one stem of *Avicennia* in each pot. Thus, the live branch numbers of *Avicennia* were considered analogous to the live stem numbers of *Spartina* and *Juncus*. The unit is number/pot.

Total live shoot height. The height of each live shoot of *Spartina* and *Juncus* were measured with a meter stick. Total live shoot height was derived by summing the heights of all live shoots in each experimental unit. For *Avicennia*, instead of live shoot height, total live leaf number was measured. The number of live leaves served as a non-destructive surrogate for live leaf biomass similar to the use of live shoot heights of *Spartina* and *Juncus*.

Percent mortality. Percentage of dead tissue was estimated occularly every two weeks to determine mortality rate.

Statistics

Statistical analysis was conducted with the Mixed Model procedure of SAS (version 8.0) with repeated measurements. Least square means were used to compare between individual treatment-levels when interactions were significant. Saxton's macro was used for statistical separation of the means with letter groupings in Proc Mixed (SAS, 1998). The measured variables were converted to a percentage of the control for each species to account for the large differences in growth and physiology among the three plant species. The acidity-increase experiment and acidity-hold experiment were statistically analyzed separately. Statistical significance was reported at a probability of 0.05 unless otherwise stated.

Results

Effect of increasing acidity on plant condition

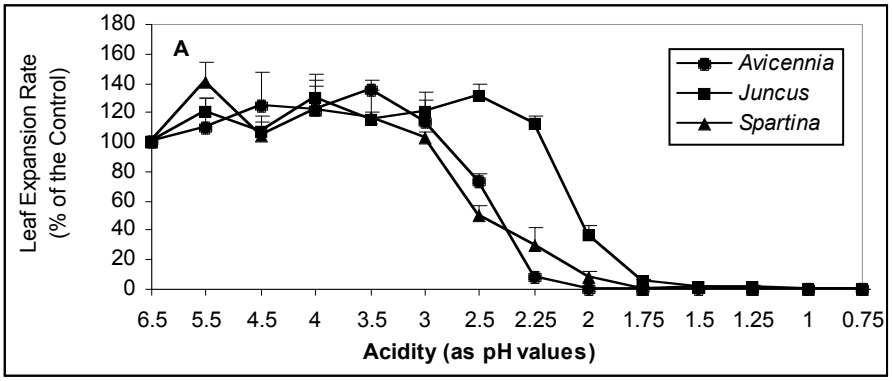
In general, increasing acidity (decreasing pH) negatively affected plant condition (Fig. 3.1A-D). *Spartina alterniflora* and *Avicennia germinans* were most sensitive to acidification and *Juncus roemerianus* was the most tolerant to acidity stress (Figs. 3.1B and 3.1C). Acidities ≥ pH 3 (pH 3 and above) did not adversely affect any of the plant species (Fig. 3.1A).

Fig. 3.1. Effect of increasing acidity (decreasing pH) on the growth status of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*. 3.1A: Acidity at pH 3.5 and 4; 3.1B: Acidity at pH 2.5 and 3; 3.1C: Acidity at pH 2.25 and 3; 3.1D: Acidity at pH 2.5 for 4 weeks and at pH 1.75.

Effect of increasing acidity on leaf expansion rate

Increasing acidity had a significant negative effect on leaf expansion rate, expressed as a percent of the control (pH=6.5), but the degree of impact was species-specific (significant species x acidity interaction, $p<0.0001$) (Fig. 3.3A). Generally, acidity levels ≥ pH 3 did not significantly affect the leaf expansion rate of any of the 3-plant species. However, at acidities between pH 2.5 and 2.0, leaf expansion rates of *Spartina* and *Avicennia* were significantly suppressed and significantly lower than that of *Juncus*. *Juncus* was very tolerant to high acidity and showed no acidity-effect even at a pH as low as 2.25. In contrast, the leaf expansion rates of *Spartina* and *Avicennia* were significantly suppressed at pH 2.5 and were near zero at pH 2.

For the treatment in which acidity was held at pH 2.5 (Fig. 3.3B), leaf expansion rate significantly decreased with duration of treatment from 2 to 12 weeks. However, the effect of stress-duration at pH 2.5 on leaf expansion rate was dependent on plant species (significant species x duration interaction, $p=0.0005$). *Juncus* had a relatively constant and high leaf expansion rate with increasing duration of acidity stress. In contrast, both *Avicennia* and *Spartina* had decreasing leaf expansion rates with increasing stress duration and their leaf expansion rates were significantly lower than that for *Juncus* (Fig. 3.3 B).



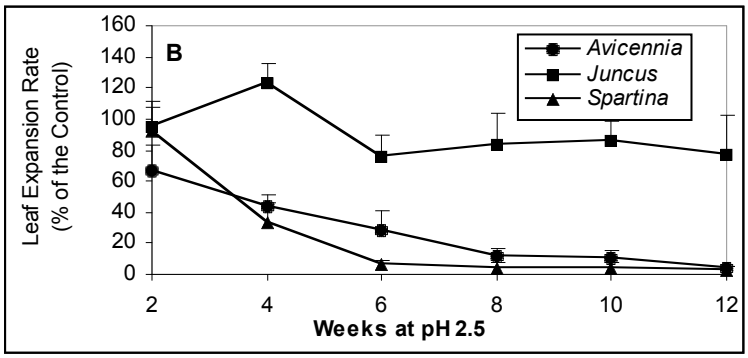
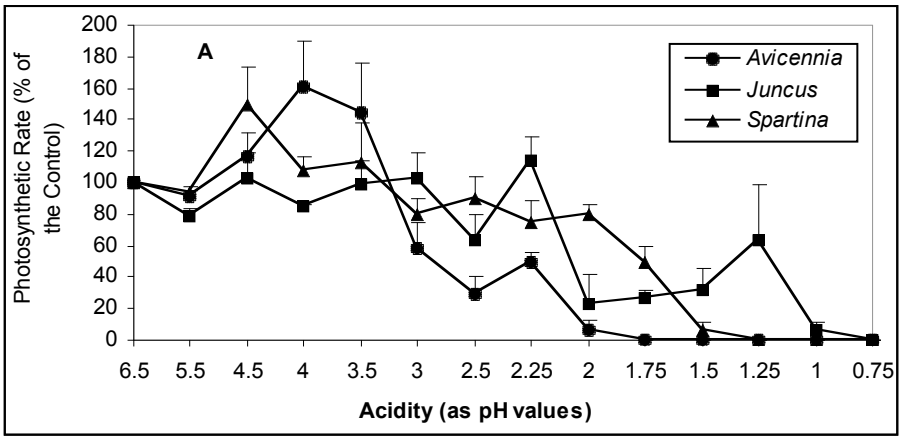


Fig. 3.3. Effect of increasing acidity (A) and increasing stress-duration at pH 2.5 (B) on the leaf expansion rates of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing acidity on photosynthetic rate

Increasing acidity (decreasing pH) significantly ($p<0.0001$) reduced plant photosynthetic rate, expressed as percent of the control (pH=6.5) (Fig. 3.2A); however, this effect was dependent on plant species (significant species x pH interaction, $p=0.0069$). At pH's ≤ 2.5, *Avicennia* was most affected of the three plant species. In contrast, Juncus was most tolerant of low pH, with photosynthetic rates still detectable at an acidity level of pH 1.25

For the treatment with acidity held at pH 2.5 (Fig. 3.2B), the photosynthetic rate of *Juncus* was significantly higher than that of *Avicennia* and *Spartina*. Photosynthetic rates of *Spartina* and *Avicennia* were not significantly different when acidity was held at pH 2.5. The main effect of acidity and the acidity x species interaction were not significant.



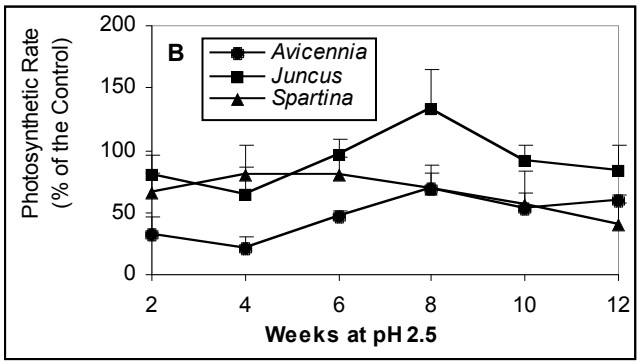
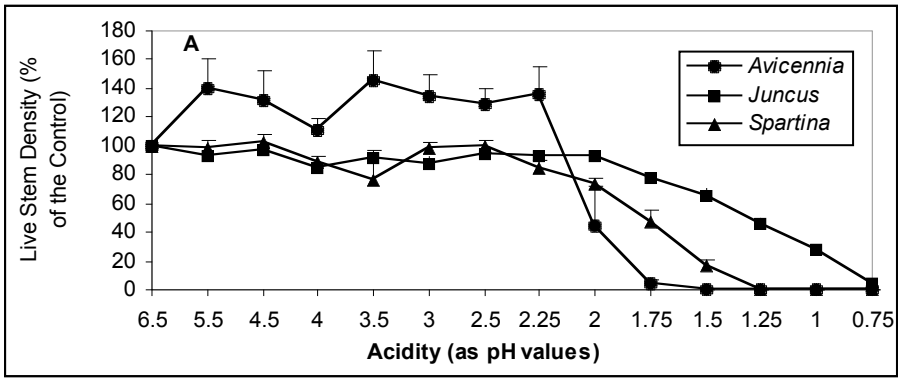


Fig. 3.2. Effect of increasing acidity (decreasing pH) (A) and increasing stress-duration at pH 2.5 (B) on the photosynthetic rates of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing acidity on the live stem density

Live stem density, expressed as a percent of the control, significantly ($p<0.0001$) decreased with increasing acidity (decreasing pH) (Fig. 3.4A). The acidity effect, however, was dependent on species (significant species x acidity interaction, $p<0.0001$). Generally, acidity levels ≥ pH 2.25 did not significantly impact live stem density of the three plant species. However, species responses differed when the acidity was < pH 2.25. Live stem density of *Avicennia* and *Spartina* was significantly lower than those of *Juncus* at pH values 2.00 and less. No live stems were recorded at pH's ≤1.75 and ≤1.25 for *Avicennia* and *Spartina*, respectively. However, live stems of *Juncus* existed even at acidity of pH 1.0.

For the treatment with acidity held at pH 2.5 (Fig. 3.4B), the effect of increasing acidity on live stem density was dependent on species ($p=0.0086$). This growth parameter significantly decreased with increasing acidity for *Avicennia* and *Spartina*, but was unaffected in *Juncus*.



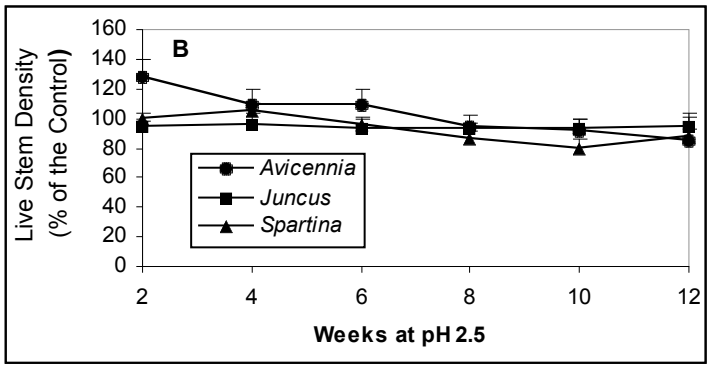
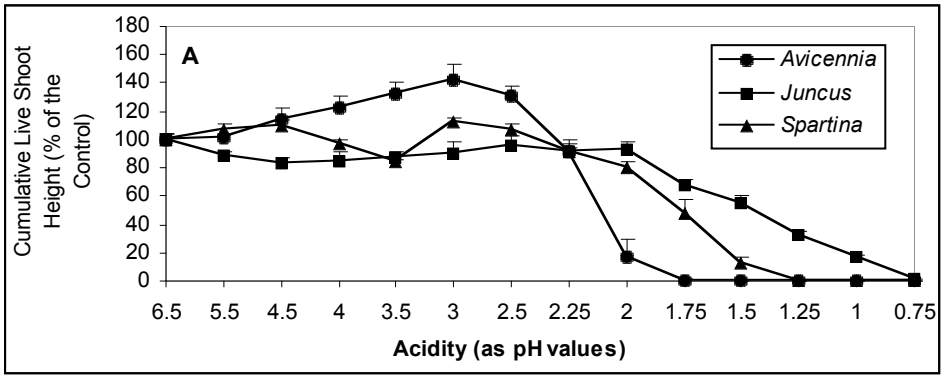


Fig. 3.4. Effect of increasing acidity (A) and increasing stress-duration at pH 2.5 (B) on live stem density of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Effect of increasing acidity on the cumulative live shoot height

Increasing acidity had a significant ($p<0.0001$) effect on cumulative live shoot height, expressed as a percent of the control. This effect, however, significantly differed with species (significant species x acidity interaction, $p<0.0001$). Generally, acidity levels ≥ pH 2.5 did not adversely affect cumulative live shoot height of the three species (Fig. 3.5A). Interestingly, cumulative live shoot height of *Avicennia* significantly increased as pH decreased from 6.5 to 2.5. However, a negative effect of acidity was apparent and differed among plant species at acidities < pH 2.5. Cumulative live shoot height of *Avicennia* and *Spartina* decreased steeply between pH 2.25 and 1.5, especially for *Avicennia*. Although the cumulative live shoot height of *Juncus* also decreased with decreasing pH, its rate of decrease was not as steep as for the other two species.

For the treatment with the acidity held at pH 2.5 (Fig. 3.5B), cumulative live shoot height of *Spartina* decreased steadily with time elapsed and was significantly lower than that of *Juncus*, which was constant, after 12 weeks. *Avicennia* again showed a positive response to moderately low pH as evidenced by a cumulative live shoot height that was greater at pH 2.5 than at the control pH of 6.5 (percentage of control ≥ 100%).



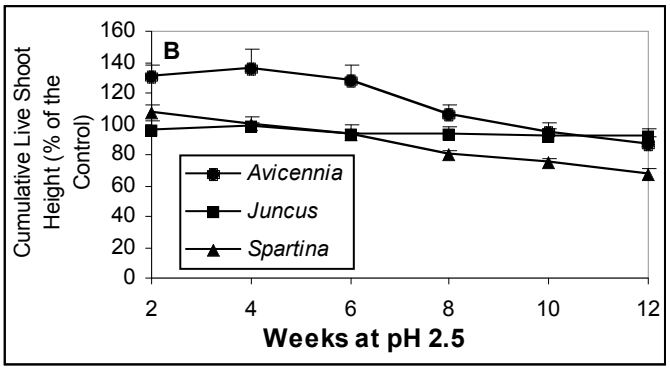
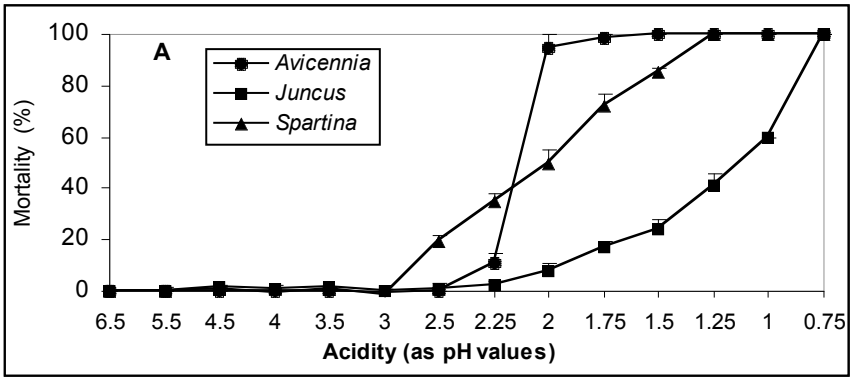


Fig. 3.5. Effect of increasing acidity (A) and increasing stress-duration at pH 2.5 (B) on the cumulative live shoot height of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina*

Effect of increasing acidity on percent mortality

The mortality of all three-plant species significantly increased with decreasing pH values ($p<0.0001$). This decrease, however, significantly differed with species (significant species x acidity interaction, $p<0.0001$). Percent mortality did not significantly differ among the three species at acidity levels ≥ pH 3 (Fig. 3.6A). However, at pH 2.5, the percentage of dead tissue significantly increased for *Spartina*, but not for *Juncus* and *Avicennia*. At pH 2.25, the percent mortality of *Avicennia* was significantly higher than for *Juncus*, but significantly lower than for *Spartina*. The percent mortality of *Avicennia* dramatically increased to near 100 % when the pH was lowered to 2.0 from a pH of 2.25. The percent mortality of *Spartina* increased steadily with decreasing pH from 3.0 to 1.5. Surprisingly, the percent mortality of *Juncus* was less than 50% even at pH 1.25. *Juncus* only reached 100 % mortality at a pH of 0.75.

For the treatment with the acidity held at pH 2.5 (Fig.3.6B), percent mortality was significantly higher for *Spartina* than *Juncus* and *Avicennia*, with about 60% dead tissues for *Spartina* and less than 10% for *Avicennia* and *Juncus*. Although some minor changes in percent mortality of *Juncus* and *Avicennia* occurred during the 12-week period at pH 2.5, *Spartina*, in contrast, had a linear increase in percent mortality with increasing stress-duration (significant species x duration interaction, $p<0.0001$)



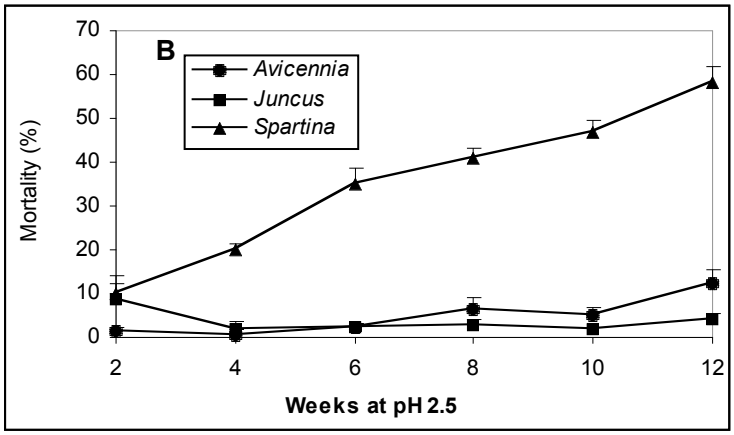


Fig. 3.6. Effect of increasing acidity (A) and increasing stress-duration at pH 2.5 (B) on percent mortality of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*.

Discussion

Acidification detrimentally affected marsh plant growth responses with decreased leaf expansion and photosynthetic rates, fewer new shoots generated, lower live cumulative standing shoot height, and increased percent mortality. However, these adverse effects did not occur until an acidity level < pH 3. In addition, the effect of acidity was generally more severe on *Spartina* and *Avicennia* than on *Juncus*.

During the 8-week period in which pH was lowered in the rooting medium from pH 6.5 to pH 3.0, growth of the three marsh plants was not detrimentally affected. In fact, for some plant growth variables, such as leaf expansion rate and photosynthetic rate, they increased with decreasing pH in these acidity ranges. The positive growth responses were probably due to the increased availability of mineral nutrients in the acidic solutions. A notable effect of low pH is an increase in the solubility of metal ions (Fyson 2000). The availability of micronutrients, such as Fe, Cu, Mn and Zn, as well as the macronutrient, P, to plants increases with lower pH in the rhizosphere soil (Chen et al. 2002). A handful of wetland plants, such as *Juncus bulbosus, Juncus effusus, Typha angustifolia, and Typha latifolia*, *Sparganium emersum*, *Corex rostrata*, *Eriphorum angustifolium*, *Phragmites australis*, and *Schoenoplectus lacustris*, are able to grow naturally in sediment with a pH around 3 or below (Fyson 2000, Pietch 1998, Sand-Jensen and Rasmussen 1978, Hargreaves et al. 1975, Whitton and Diaz 1981).

Plants responses to acidification greatly differed among the plant species when the acidity was less than pH 3.0. At acidity levels of pH 2.5 and pH 2.25, *Spartina alterniflora* was most stressed of the three species with decreased leaf expansion rate and obviously increased plant tissue mortality. *Avicennia germinans* was moderately stressed, and *Juncus roemerianus* was not stressed at these acidity levels. However, at a pH of 2.0, *Avicennia* was most impacted by the acidity with a dramatically increased mortality rate to more than 90% and photosynthetic rate and leaf expansion rate near zero. With further decreases in pH level, *Avicennia* completely died at pH 1.75, *Spartina* at pH 1.5 and *Juncus* at pH 0.75. The current study is the first research to demonstrate the effect of increasing acidity on these salt marsh plants. The dose-response relationship of plant growth to acidity provides quantitative information on the acidity tolerance limits of these salt marsh plant species.

The longer-term exposure to a relatively low, but non-lethal, pH level, such as pH 2.5 in the current study, is likely more applicable to the brown marsh event than exposure to the extremely low pH's as in the increasing acidity experiment. Acidity levels between a pH of 2 and 3 can occur naturally in the field (Fyson 2000; Feijtal et al. 1988), and some wetland plants, such as *Juncus bulbosus*, dominate in such low pH environments (Fyson 2000). In a mesocosm experiment for the brown marsh study that simulated drought on salt marshes (R. Twilley, personal communication), the drought induced a decrease in pH to below 2.5 in some marsh sods. In the current experiment in which acidity was held at pH 2.5, *Spartina alterniflora* showed the greatest impact, with more than a 60% mortality, and aboveground shoot and leaves turned to brown overall. However, the other two species, *Juncus* and *Avicennia*, showed little stress, with less than 15% mortality. In the present study, we decreased the pH level by 0.5-pH units every 2-weeks between pH 4.5 and pH 2.5 to let the plants gradually acclimate to the increased acidification. Gradually increasing acidity would likely better simulate the oxidation-

induced acidification that could have occurred during the brown marsh event. Thus, the growth response of the plant species when held at a relative low, but constant, pH may be more realistic with respect to the brown marsh event.

During the brown marsh event, *Spartina alterniflora* died-back, while *Avicennia germinans* and *Juncus roemerianus* survived (McKee et al. 2001). In the current acidity experiment, the performance of the selected salt marsh species was similar to their response during the brown marsh event. Between pH 2.5 and pH 2.25, considerable mortality occurred for *Spartina alterniflora*, but little for *Avicennia germinans* and *Juncus roemerianus*. In addition, for the treatment with acidity held at pH 2.5, *Spartina alterniflora* showed the greatest impact, with more than 60% mortality. However, the other two species, *Juncus* and *Avicennia*, showed little stress, with less than 15 % mortality. The species-specific mortality was similar to that during the brown marsh event, i.e., *Spartina* impacted and *Juncus* and *Avicennia* remaining unaffected..

Conclusion

Three salt marsh plants, *Avicennia germinans*, *Juncus roemerianus* and *Spartina alterniflora*, had large differences in acidity tolerance based on their growth responses to increasing acidity. The acidity tolerance limits are about pH 2.5 for *Spartina alterniflora*, pH 2.25 for *Avicennia germinans*, and pH 1.5 for *Juncus roemerianus*, although long-term exposure to these acidities may reduce their acidity tolerances even further. More importantly, longer-term exposure to pH 2.5 for almost 3 months revealed that *Spartina alterniflora* was stressed most at this acidity level while the other two species, *Avicennia germinans* and *Juncus roemerianus*, were little affected. The species-specific responses to decreasing pH suggest that increased acidification caused by drought-induced sediment oxidation could be partially responsible for the brown marsh event in coastal Louisiana. Furthermore, under field conditions, soil acidification may enhance the solubility and availability of potentially toxic metals, such as Al and Fe, adding additional stress to the salt marsh plants (see Chapter 4). Consequently, higher pH levels than those that initiated stress in this experiment could cause mortality in the field, where other stressors, such as toxic metals, elevated salinities and soil moisture deficiency, may add additional stress to the plants.

Chapter 4. Iron and Aluminum tolerance limits and effect on salt marsh plants *Spartina alterniflora*, *Juncus roemerianus*, and *Avicennia germinans* - brown marsh case studies on increasing ferrous iron and aluminum concentration

Extensive and sudden dieback of the perennial salt marsh grass, *Spartina alterniflora*, currently termed "brown marsh", occurred in coastal Louisiana in 2000. However, sympatric species such as *Avicennia germinans* and *Juncus roemerianus* survived the event (McKee et al., 2001). Brown marsh coincided with extreme drought conditions and unusually low freshwater discharge from the Mississippi and Atchafalaya Rivers that prevailed during the 1999-2000 period (Swenson, 2001). Chemical changes in sediments may occur upon soil drying. When some saline sediments are oxidized, microbial oxidation of pyrite and other metal sulfides generates sulfuric acid, which leads to soil acidification (Nordstorm, 1982). Increased soil acidity may lead to increased concentrations of potentially toxic metals such as iron and aluminum. Field monitoring indicated that some dieback sites showed evidence of soil desiccation in dead and transition zones with low soil pH and high soil Eh, and root coatings of ferric iron, indicating oxidation (McKee et al. 2001). Although the causes of brown marsh are unclear, soil acidification and increased metal toxicity may be a contributing cause of the brown marsh event.

The objectives of this experiment were to examine the effect of increasing soluble iron and aluminum concentrations on plant growth responses as a result of increased ferrous iron and aluminum availability.

Materials and Methods

Plant materials

The salt marsh plants, *Spartina alterniflora* Loisel. (smooth cordgrass) and *Avicennia germinans* L. (black mangrove) were provided by the Plant Materials Center of the National Resources conservation Service (NRCS). *Juncus roemerianus* Scheele. (black needlerush) was collected from a salt marsh near Cocodrie, Louisiana. After acquisition and collection in March 2002, the plants were established in commercial potting soil for propagation of sufficient experimental material.

Experimental Procedures

In June 2002, the plant material was divided into similar sized planting units (*Spartina* and *Juncus*: 10 – 20 stems; *Avicennia*: single, intact plant). Prior to transplanting, the plants were washed to remove potting soil from roots, and dead shoots were trimmed. Prepared planting units were transferred to plastic pots (16.5 cm diameter x 22.2 cm depth) containing acid-washed sand; mesh screen lined the pots to prevent sand leakage. Pots were submerged to just above the sand surface in larger plastic, acid-washed bins (Rubbermaid Roughneck; 61 cm length x 40.6 cm width x 22.2 cm depth; 20 L volume). The bins were filled with a half-strength nutrient solution at 0 ppt salinity

and pH of 6.5. One pot containing each species was placed in each of 30 bins (3 pots per bin). Plants were allowed to recover for one month, then salinity was increased over a 2-month period to 23 ppt where it was maintained for an additional month prior to initiation of experimental treatments. Bin solutions were changed on a biweekly basis, and algae were removed by scrubbing the surfaces of pots and bins. During the intervening week when solutions were not changed, water levels, salinity, and pH were checked and adjusted as needed, and nitrogen and phosphorus solutions were added to prevent deficiency. The experiment was conducted in a greenhouse with light intensity of 1200 to 1500 μmol m^{-2} s^{-1} at canopy level, humidity range of 75 to 90 %, and a temperature range of 25 to 32 ºC during the daytime.

Three metal treatments were applied by randomly assigning five bins to one of the following treatments:

1. Control: concentrations of Fe and Al were maintained at levels in the nutrient solution.
2. Ramp: increase concentration of Fe or Al in the nutrient solution until plant mortality occurs.
3. Hold: increase metal concentration until a negative growth response is observed in one of the plant species, then maintain that metal concentration

Metal treatments were initiated at 100 μM Fe ($Fe(II)SO_4$) or 0 μM Al ($AlCl_2$) in all bins. The pH of solutions was adjusted to 3.5-4.0 with 1 N HCl to maintain metals in solution. Controls were maintained at this pH and initial metal concentrations for the duration of the experiment. For the iron experiment, concentrations in treatment bins were raised according to the following sequence: 100, 400, 1000, 1600, 2200, 2800, 3200, 4000 μM Fe. For the aluminum experiment, concentrations were: 0, 300, 600, 900, 1200, 1500, 1800, 2100 μM Al. Metal concentrations were adjusted biweekly during the solution change. In the Fe experiment, the Hold concentration was 6,500 μM Fe for 16 weeks, and the Ramp concentration reached 14,000 μM Fe. In the Aluminum experiment, the Hold concentration was 13,500 μM Al for 16 weeks and the Ramp concentration reached 24,000 μM Al. Salinity was maintained at 23 ppt and pH at 3.7; nutrients were supplied as described above.

Plant response to metals was measured every 2 weeks (measurements started 9 days after an increase in concentration). The plant response variables included

Methodologies

Leaf expansion rate. A difference in distance from a fixed point to the tip of a young leaf between day 0 and day 3 was measured to determine the daily leaf growth rate. The unit is cm/day.

Photosynthetic rate. Photosynthetic rate was measures by infrared gas analyzer (Li-cor Li-6400). The representative leaf was selected and enclosed in a leaf chamber to analyze photosynthetic rate. The photosynthetic active radiation (PAR) of 2000 μmol/m^2/s and

CO_2 concentration of 350 ppm were used to measure plant photosynthetic rate. The unit is $\mu mol/m^2/s$.

Stem density (branch numbers for *A. germinans*). Live stem number of *Spartina* and *Juncus* were counted directly in each experimental unit. For *Avicennia*, instead of stem numbers, live branch number of was measured in each experimental unit because there is only one stem of *Avicennia* in each pot. So the live branch numbers of *Avicennia* measured were equivalent to the live stem numbers of *Spartina* and *Juncus*. The unit is number/pot.

Cumulative live shoot height. Each live shoot heights of *Spartina* and *Juncus* were measured by a meter ruler. The cumulative live shoot height was derived by summing all live shoot height in each experimental unit. For *Avicennia*, instead of live shoot height, cumulative live leaf numbers were measured to be equivalent to the live shoot heights of *Spartina* and *Juncus*. The unit is cm/pot.

Plant mortality rate. Percentage of dead tissues was estimated every two weeks as the mortality rate.

Response was expressed as percentage of the control for each species in order to standardize differences among the plant species for most variables. An overall estimate of plant mortality was based on the difference between control and treatment percent dead (a visual estimate of brown shoots or leaves).

Statistics

Statistic analysis was conducted by using SAS's (version 8.0) Mixed Model with repeated measurement. Ramp and Hold responses were analyzed separately. Least square means was used to compare individual treatments when the interaction was significant. A Saxton's macro for converting mean separation output to letter grouping in Proc Mixed was used (SAS, 1998). The significance was at $\alpha = 0.05$ unless otherwise stated. Biomass data in the Ramp and Hold treatments were analyzed separately with an ANOVA (JMP® Version 4.0.2; SAS, 2002). Plants with significant ANOVAs were compared with least squares means post-test comparison (Student's t).

Results

Effect of iron on plant health status

Increases in ferrous iron concentration resulted in visual signs of plant stress and decreased plant vigor (Fig. 4.1). Overall, plant vigor was affected at about 6-7 mM Fe with *Spartina* (browning and stunting) and *Juncus* (stunting) showing the first visual signs of stress (Fig. 4.1 A,B). By the end of the experiment, *Spartina* was the most

visibly affected by Fe treatment, whereas *Juncus* appeared to be the least affected (Fig. 4.1 C,D,E).

Figure 4.1. Effect of increased iron concentrations on visual status of *Spartina alterniflora*, *Avicennia germinans*, and *Juncus roemerianus* (A) Control – 0.1 mM Fe and (B) Iron - 7 mM Fe treatments. Effect of Control (0.1 mM Fe), Hold (6.5 mM Fe for 15 weeks), Ramp (14 mM Fe) treatments at the end of the experiment on (C) *Spartina*, (D) *Avicennia*, (E) *Juncus*.

Effect of iron on photosynthetic rate

Net CO_2 assimilation rate (percent of control) varied with increasing Fe concentration, but the pattern differed among species (Fig. 4.2A; Table 4.1). Excepting a spike in Avicennia at 6 mM Fe, net CO_2 assimilation rate appeared similar among species at all Fe concentrations up to 8 mM Fe. Above 8 mM Fe net CO_2 assimilation rates of *Avicennia* and *Spartina* continued to decline to 12 mM Fe, whereas that of Juncus show no further decline. From 12 to 14 mM Fe, both *Juncus* and *Avicennia* exhibited some recovery of photosynthesis.

When Fe concentration was maintained at 6.5 mM, the three species also showed different patterns of response over time (Fig. 4.2B; Table 4.1). At the inception of the Hold concentration, Avicennia and Spartina had lower relative net CO_2 assimilation rates than Juncus. Spartina relative net CO_2 assimilation rates became comparable to Juncus in weeks 5, 7, and 9, but dropped to the lowest of the three species by weeks 11 and 13 (<50%). Avicennia, which displayed the lowest relative net CO_2 assimilation rate in weeks 5, 7, and 9, increased to a level comparable to Juncus (100%) by the conclusion of the study.

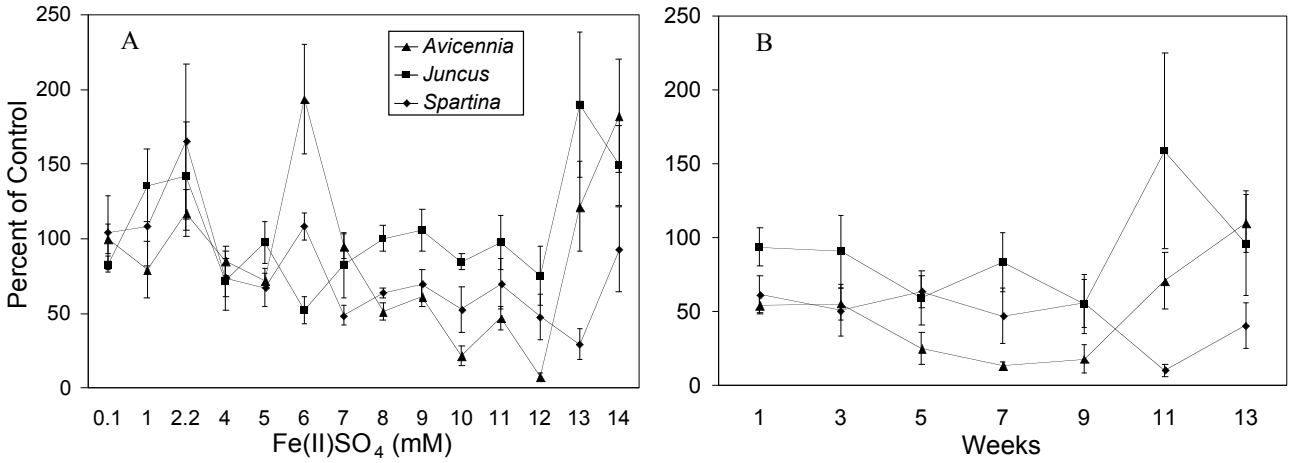


Figure 4.2. Effects of increasing iron concentrations (A) and iron concentration held at 6.5 mM Fe over time (B) on net CO_2 assimilation of *Avicennia*, *Juncus*, and *Spartina*. Data are presented as means ± SE (n=5).

Table 4.1. Results of a repeated measures ANOVA showing effects of iron on plant variables in (A) Ramp (repeated measure is concentration) and (B) Hold (repeated measure is time) treatment applications.

Source	A. Ramp (concentration)				B. Hold (time)			
	Num DF	Den DF	F	p	Num DF	Den DF	F	p
% Mortality								
Species (S)	2	5.68	20.84	0.0022	2	8.1	12.08	0.0037
Repeated Measure (R)	11	108	69.35	<.0001	6	68.4	12.27	<.0001
S x R	22	76.7	14.51	<.0001	12	44.7	2.24	0.0257
Leaf Elongation								
Species (S)	2	9.36	4.10	0.0527	2	8.91	2.48	0.1396
Repeated Measure (R)	13	156	27.16	<.0001	6	61.7	4.39	0.0009
S x R	26	102	2.71	0.0002	12	55.2	2.93	0.0033
Live Density								
Species (S)	2	4.97	0.71	0.5347	2	9.38	0.10	0.9092
Repeated Measure (R)	13	126	37.51	<.0001	6	63.3	19.33	<.0001
S x R	26	123	1.58	0.0525	12	43.4	1.62	0.1228
Height								
Species (S)	2	8.4	2.38	0.1519	2	6.33	3.67	0.0874
Repeated Measure (R)	13	144	46.42	<.0001	6	43.7	51.01	<.0001
S x R	26	95.9	10.10	<.0001	12	35.7	3.51	0.0017
Net CO_2 Assimilation								
Species (S)	2	7.96	3.91	0.0657	2	8.42	5.21	0.0336
Repeated Measure (R)	13	138	6.84	<.0001	6	39.3	1.90	0.1049
S x R	26	92.1	3.35	<.0001	12	45.9	3.15	0.0024
Stomatal Conductance								
Species (S)	2	5.52	1.2	0.3700	2	8.89	1.41	0.2933
Repeated Measure (R)	13	71.1	1.63	0.0981	6	62.2	2.44	0.0351
S x R	26	64.2	0.32	0.1828	12	53.8	0.51	0.8978

Effect of iron on stomatal conductance

Stomatal conductance relative to controls declined with increasing Fe concentration, but the trend was not significant. There were also no significant effects of species or an interaction effect on stomatal conductance (Fig. 4.3A; Table 4.1). When Fe concentrations were held at 6.5 mM , stomatal conductance of all three species declined significantly over time (Fig. 4.3B, Table 4.1). There were no significant effects of species or a species*time interaction.

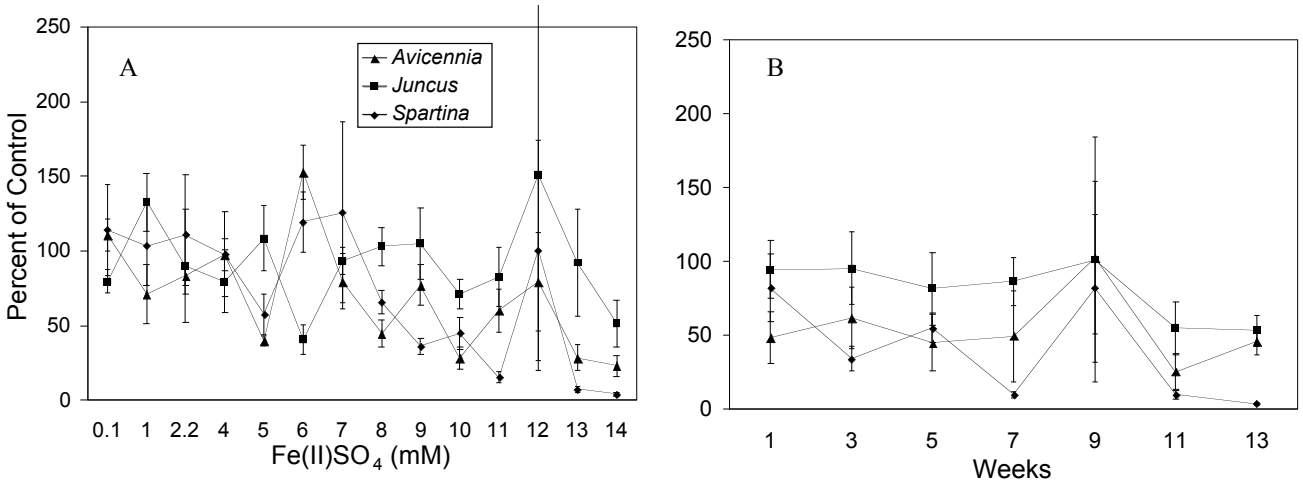


Figure 4.3. Effects of increasing iron concentrations (A) and iron concentration at 6.5 mM Fe over time (B) on stomatal conductance of *Avicennia*, *Juncus*, and *Spartina*. Data are presented as means ± SE (n=5).

Effect of iron on leaf expansion rate

Leaf elongation rates of all species decreased relative to controls as Fe concentration increased, but the individual patterns differed (Fig. 4.4A; Table 4.1A). Both *Avicennia* and *Spartina* showed greater fluctuations in leaf elongation than *Juncus*, which showed a steadier decline with increasing Fe concentration. When Fe concentrations were held at 6.5 mM, leaf elongation of both *Avicennia* and *Juncus* continued to decrease to 40 % of controls by week 13, whereas that of *Spartina* increased from 60 to near 80 % of controls (Fig. 4.4B; Table 4.1B). The increased elongation of *Spartina* was primarily attributable to inflorescence development.

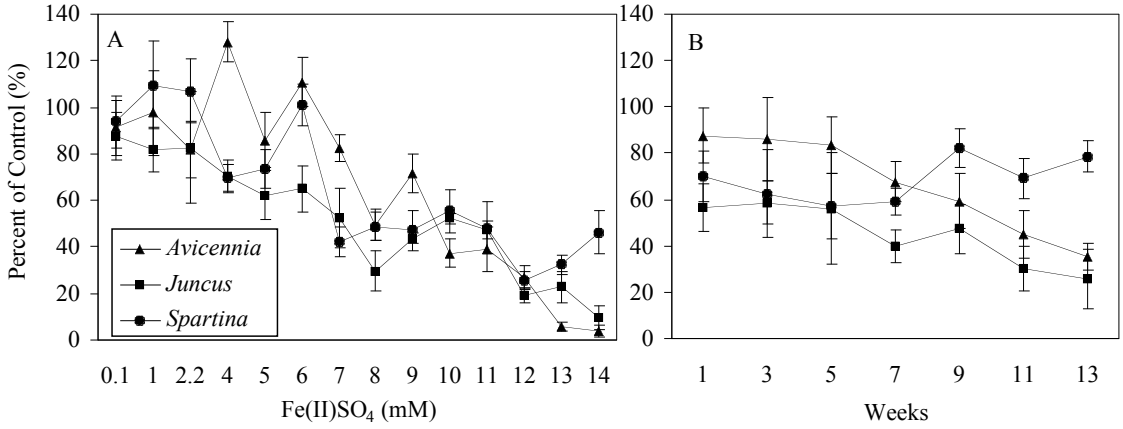


Figure 4.4. Leaf expansion of *Avicennia germinans*, *Juncus roemerianus*, *and Spartina alterniflora* (A) increasing iron concentrations and (B) over time at 6.5 mM Fe. Data are presented as means ± SE (n=5).

Effect of iron on live stem density

Increasing Fe concentration caused a decrease in numbers of live shoots (*Spartina* and *Juncus*) or leaves (*Avicennia*) relative to controls in all the species (Fig. 4.5A, Table 4.1A). However, the effect was least on *Juncus* compared to *Spartina* and *Avicennia*, which were similar. By the end of the experiment (14 mM Fe), live shoot number for *Juncus* was near 70 % of controls, whereas that of the other two species was near 40 % of controls. When Fe concentrations were held constant at 6.5 mM, live shoot (*Spartina* and *Juncus*) or leaf (*Avicennia*) numbers remained above 60 % and did not differ among species (Fig. 4.5B, Table 4.1B).

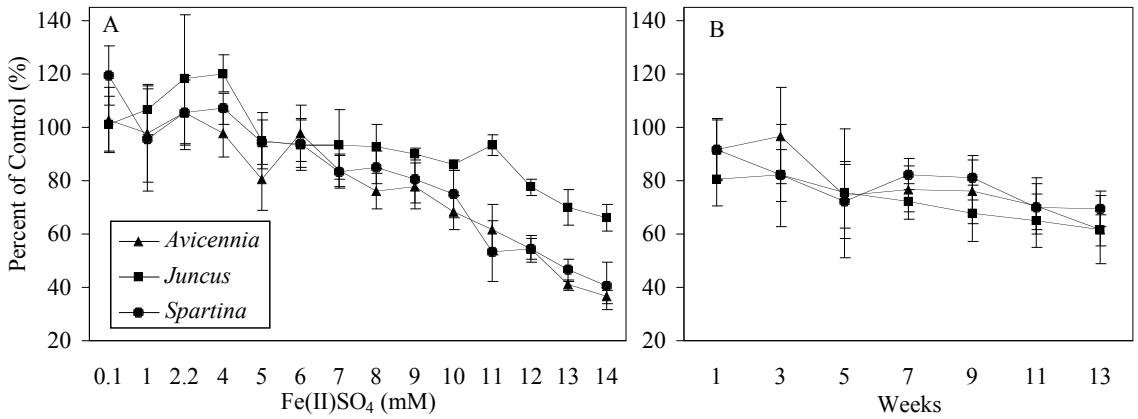


Figure 4.5. Effects of increased iron (A) and iron concentration held at 6.5 mM Fe over time (B) on the live stem/leaf density of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora*. Data are presented as means ± SE (n=5).

Effect of iron on live shoot height

Shoot height declined relative to controls with increasing Fe concentration, but the pattern differed among species (Fig. 4.6A, Table 4.1A). Height of Spartina shoots declined from 100 to 60 % of controls with increasing concentration to 14 mM Fe. Height of Juncus shoots also declined, but less sharply. *Avicennia* did not significantly change height in response to Fe treatment. When held at 6.5 mM, all plant species showed a decline in shoot height over time, but the rate of decline was greater for Avicennia (Fig. 4.6B, Table 4.1B).

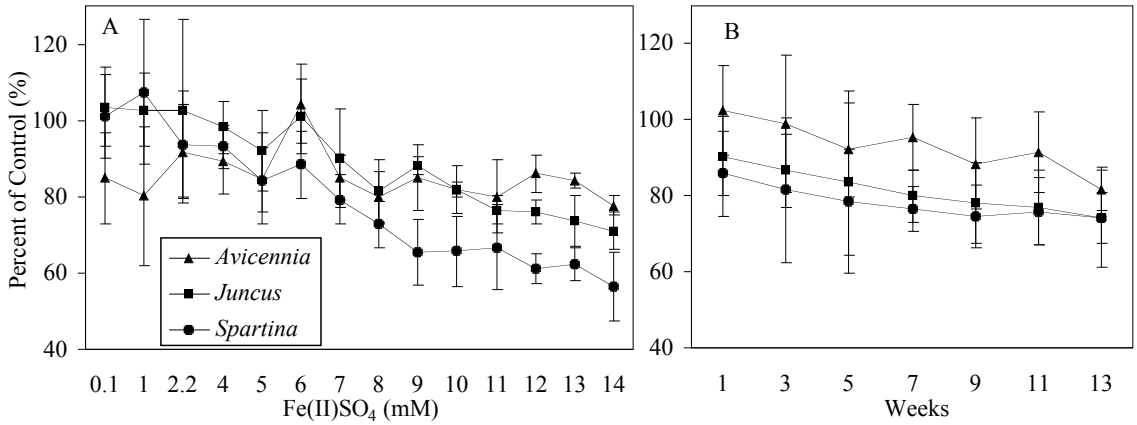


Figure 4.6. Effects of increasing iron concentrations (A) and iron concentration at 6.5 mM Fe over time (B) on height of *Avicennia*, *Juncus*, and *Spartina*. Means ± SE (n=5).

Effect of iron on mortality rate

The three species all showed increased mortality with increasing Fe concentration, but the patterns differed dramatically (Fig. 4.7A, Table 4.1A). Spartina mortality increased dramatically between 6 and 8 mM Fe, whereas that of the other two species remained low. Above 12 mM, mortality of Avicennia increased and reached almost 60% by 14 mM. Mortality of Juncus remained low (< 20%) up to the highest treatment level.

When Fe concentrations were held constant at 6.5 mM, *Spartina* mortality remained higher than that of the other two species, but did not increase much beyond 30 % (Fig. 4.7B; Table 4.1B). Mortality of *Avicennia* leaves gradually increased from near 0 to about 20 % over 13 weeks at 6.5 mM Fe (Fig. 4.7B; Table 4.1B). Browning of *Juncus* increased slightly over time when Fe concentrations were held at 6.5 mM, but remained low overall (less than 20 %) (Fig. 4.7B).

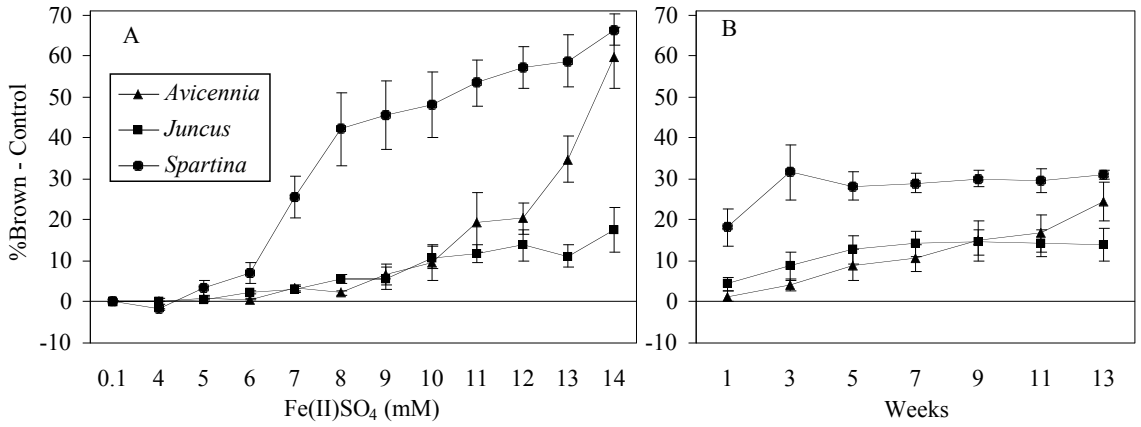


Figure 4.7. Browning of *Avicennia germinans*, *Juncus roemerianus*, *and Spartina alterniflora* (A) increasing iron concentrations and (B) over time with concentrations held at 6.5 mM Fe. Data are presented as means ± SE (n=5).

Effect of iron on final biomass

By the end of the experiment, the treatment plants all had 60% or less live biomass compared to their controls. *Spartina* had less live biomass and more dead biomass compared to *Avicennia* and *Juncus* (Fig. 4.8A and B, Table 4.2A). When the concentration of iron was held at 6.5 mM Fe, the salt marsh plants averaged 55% of the live biomass of their respective controls. Differences among species were similar to the ramp treatment except that dead biomass of Spartina was not significantly different from that of Avicennia (Fig. 4.8C and D, Table 4.2B).

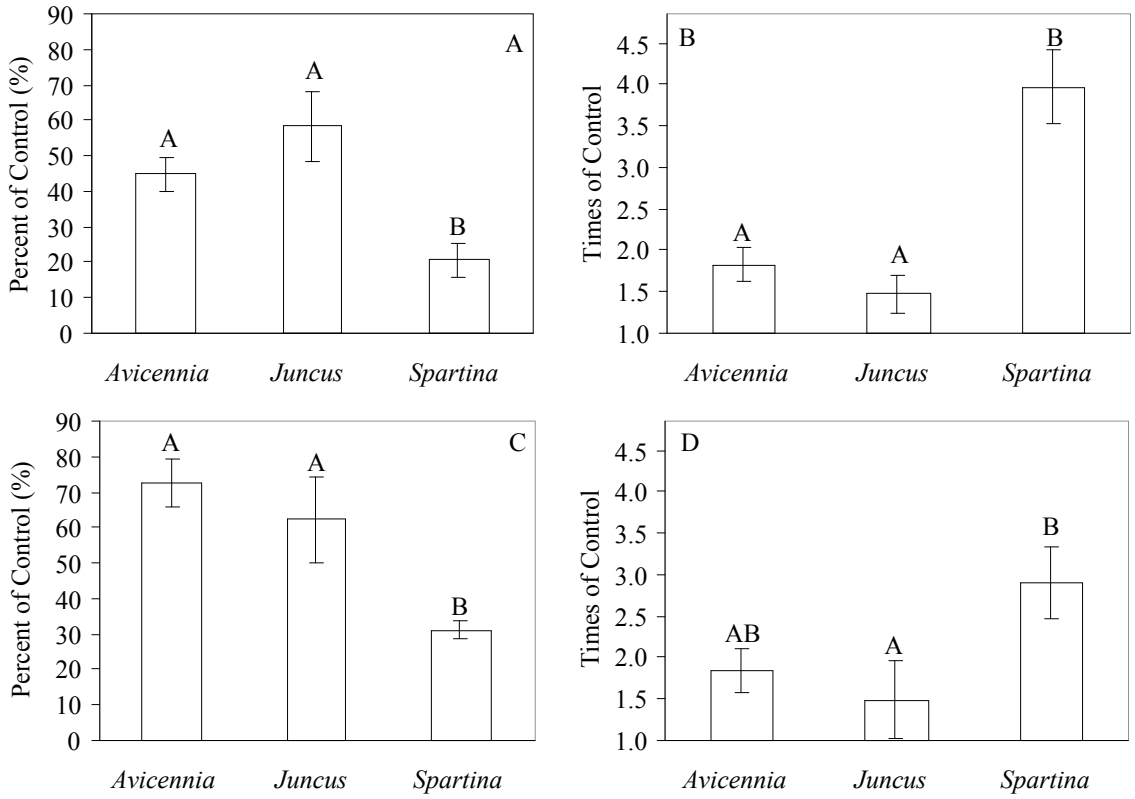


Figure 4.8. Effects of increasing concentration to 14 mM Fe on live (A) and dead (B) biomass; and, effects of iron concentration being held at 6.5 mM Fe for 13 weeks on live (C) and dead (D) biomass. Data are presented as means ± SE (n=5).

Table 4.2. ANOVA results for biomass of plants in (A) increasing concentration to 14 mM Fe and (B) held concentration at 6.5 mM Fe for 13 weeks (n=5).

Source	DF	F-ratio	p
A. Increasing Concentration			
Live Biomass	2	7.9451	0.0063
Dead Biomass	2	9.1804	0.0002
B. Hold Concentration			
Live Biomass	2	7.0759	0.0093
Dead Biomass	2	3.4394	0.0660

Effect of aluminum on plant health status

Increases in aluminum concentration caused visual changes in *Spartina* and *Avicennia* plants (Fig. 4.9). Visible symptoms of stress were most apparent in *Spartina*, followed by *Avicennia*, whereas *Juncus* exhibited no visual evidence of stress during the experiment.

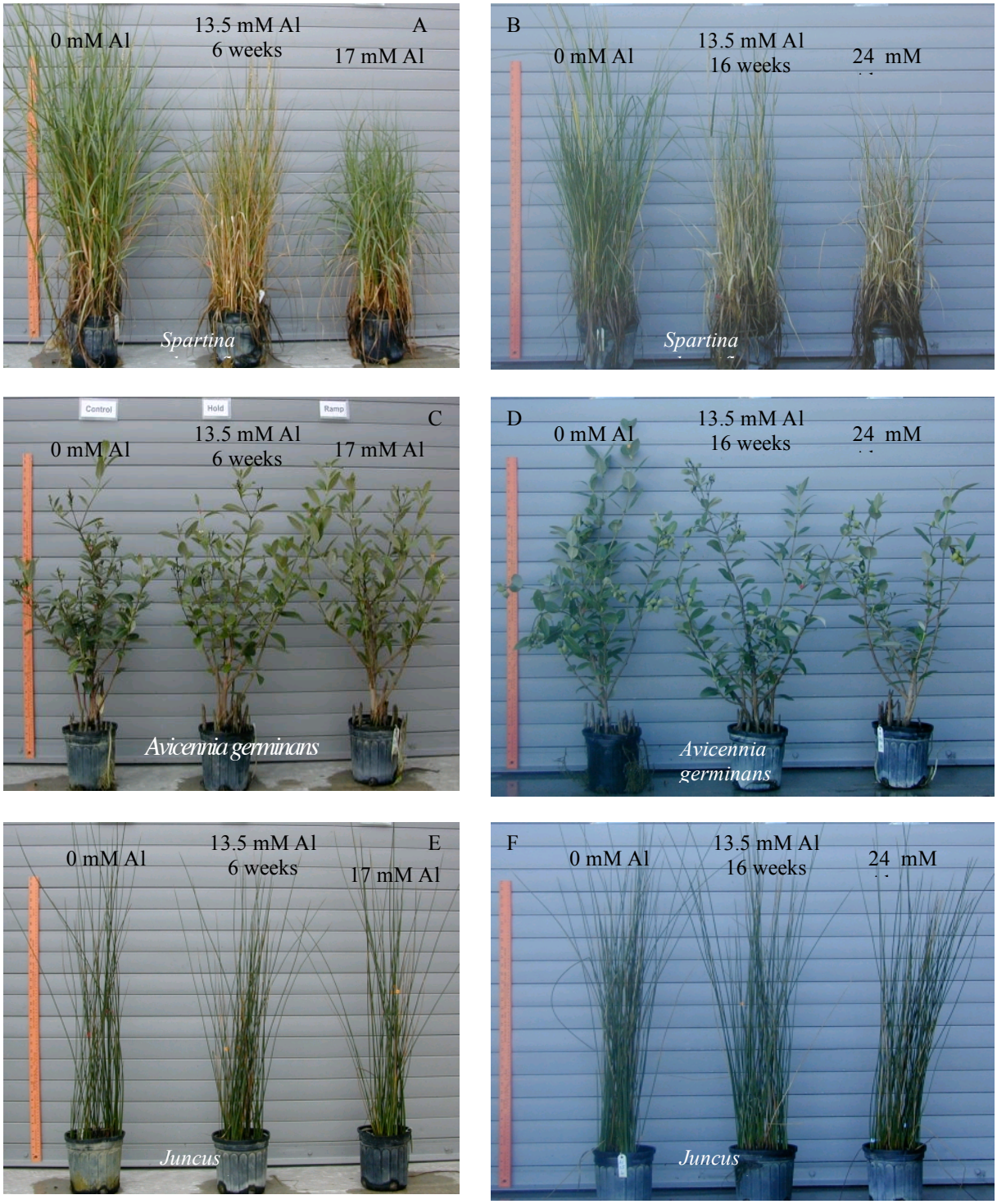


Figure 4.9. Effect of increased aluminum concentrations on growth status of saltmarsh plants. Effects of Control (0 mM Al), Hold (13.5 mM Al for 6 weeks), Ramp (17 mM Al) treatments and Control (0 mM Al), Hold (13.5 mM Al for 16 weeks), Ramp (24 mM Al) treatments on *Spartina alterniflora* (A and B, respectively), *Avicennia germinans* (C and D, respectively), and *Juncus roemerianus* (E and F, respectively).

Effect of aluminum on photosynthetic rate

Net CO_2 assimilation rates varied with increasing Al concentration, but the patterns differed among species (Fig. 4.10A; Table 4.3). Both Spartina and Avicennia showed declines in net photosynthesis in response to Al treatment, but Juncus did not. When Al concentrations were held at 13.5 mM, both Spartina and Avicennia photosynthetic rates declined over time, whereas that of Juncus increased relative to controls (Fig. 4.10B; Table 4.3). By the 15th week, Juncus photosynthetic rates were 150% of controls, but those of the other two species were less than 100% of controls.

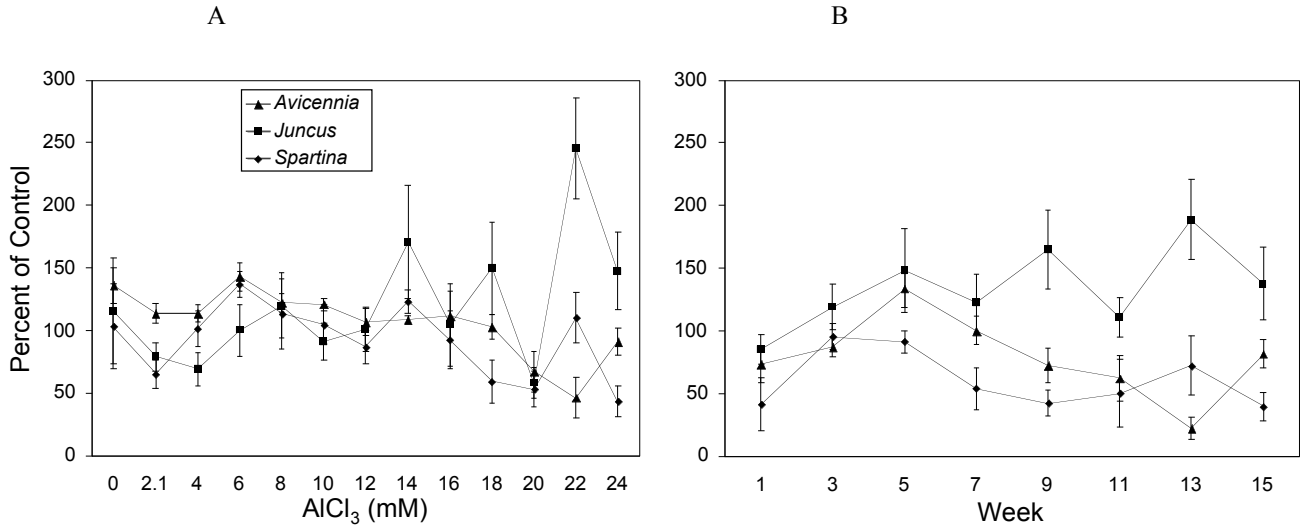


Figure 4.10. Effects of increasing aluminum concentrations (A) and aluminum concentration at 13.5 mM Al over time (B) on net CO_2 assimilation of *Avicennia*, *Juncus*, and *Spartina*. Data are presented as means ± SE (n=5).

Table 4.3. Results of a repeated measures ANOVA for effects of aluminum on plant variables in (A) Ramp (repeated measure is increasing concentration) and (B) Hold (repeated measure is time) treatment applications.

Source	A. Ramp (concentration)				B. Hold (time)			
	Num DF	Den DF	F	p	Num DF	Den DF	F	p
% Mortality								
Species (S)	2	5.81	62.26	0.0001	2	5.08	14.79	0.0076
Repeated Measure (R)	11	80.8	49.39	<.0001	7	71.8	4.88	0.0002
S x R	22	64.8	24.23	<.0001	14	47.5	2.36	0.0140
Leaf Elongation								
Species (S)	2	6.89	9.51	0.0104	2	8.31	14.15	0.0021
Repeated Measure (R)	12	130	7.74	<.0001	7	77.6	2.07	0.0565
S x R	24	100	1.41	0.1239	14	61.7	0.56	0.8847
Live Density								
Species (S)	2	7.21	2.07	0.1952	2	5.76	4.93	0.0564
Repeated Measure (R)	12	116	57.30	<.0001	7	59.1	13.79	<.0001
S x R	24	79.2	10.62	<.0001	14	43.9	2.13	0.0290
Height								
Species (S)	2	6.92	2.90	0.1213	2	6.69	44.37	0.0001
Repeated Measure (R)	12	140	16.05	<.0001	7	59.4	4.13	0.0009
S x R	24	91.9	3.04	<.0001	14	39.9	11.69	<.0001
Net CO_2 Assimilation								
Species (S)	2	9.35	7.65	0.0108	2	9.63	13.84	0.0015
Repeated Measure (R)	12	98.9	2.90	0.0018	7	66.1	2.64	0.0184
S x R	24	116	2.87	<.0001	14	66.9	2.62	0.0043
Stomatal Conductance								
Species (S)	2	4.52	5.39	0.0635	2	7.98	1.43	0.2950
Repeated Measure (R)	12	74.2	0.99	0.4644	7	51.4	2.26	0.0435
S x R	14	88.4	2.41	0.0016	14	64.5	0.97	0.4906

Effect of aluminum on stomatal conductance

Stomatal conductance varied with increasing Al concentration and the pattern among species differed (Fig. 4.11A; Table 4.3). Stomatal conductance by Avicennia declined as Al concentrations were increased; this decline was particularly steep initially (0 to 12 mM Al). Spartina showed a similar decline in stomatal conductance, but the change was less dramatic. No clear pattern emerged for Juncus due to large variation. When Al concentrations were held constant, stomatal conductance varied over time, but no clear patterns emerged (Fig. 4.11B; Table 4.3).

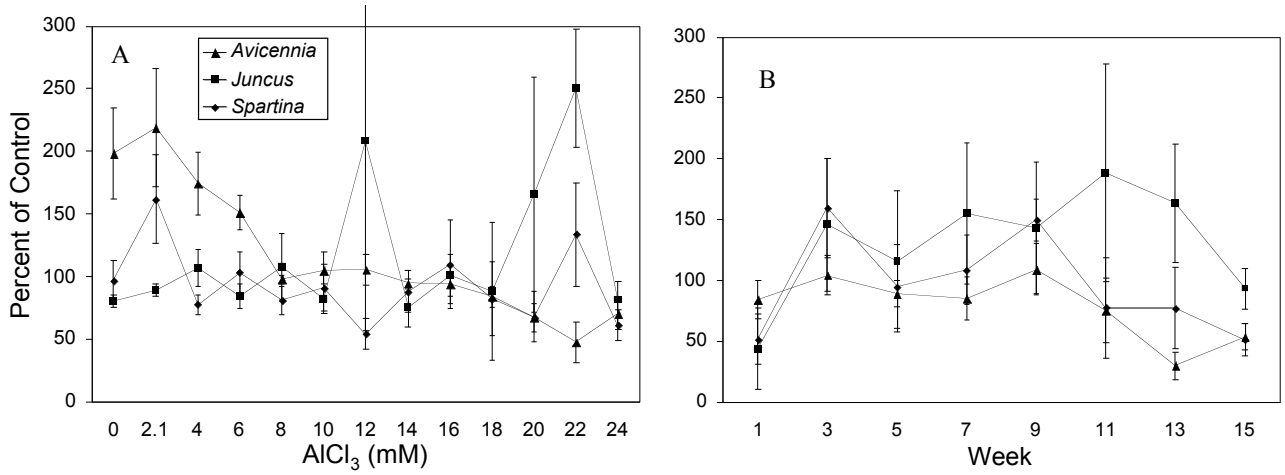


Figure 4.11. Effects of increasing aluminum concentrations (A) and aluminum concentration at 13.5 mM Al over time (B) on stomatal conductance of *Avicennia*, *Juncus*, and *Spartina*. Data are presented as means ± SE (n=5).

Effect of aluminum on leaf expansion rate

Leaf expansion rates decreased relative to the controls as aluminum concentration increased, and the pattern was similar across species (Fig. 4.12A; Table 4.3A). Leaf expansion was steady from 0 to 8 mM (116 %) then decreased thereafter, becoming significantly different at 20 mM Al. *Juncus* had the highest expansion rate overall (significant species effect).

Plants held at 13.5 mM Al for 15 weeks showed similar patterns of leaf expansion over time, although rates were significantly lower for *Spartina* overall (Fig. 4.12B; Table 4.3B).

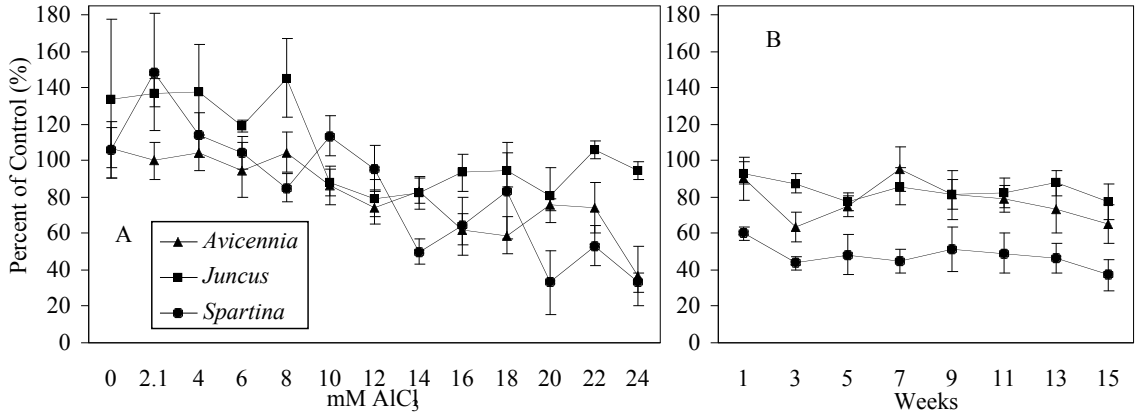


Figure 4.12. Effect of increasing aluminum concentration (A) and aluminum concentration at 13.5 mM Al over time (B) on leaf expansion.

Effect of aluminum on live stem density

Live stem density responded differently among species to increasing Al concentration (Fig. 4.13A; Table 4.3A). All three species showed little change in live density up to 16 mM Al. Thereafter, both Spartina and Avicennia showed sharp decreases to about 50% of controls, whereas Juncus showed no change in live stem density up to 24 mM Al.

Live density of the three species held at 13.5 mM Al decreased differently over time (Fig. 4.13B, Table 4.3B). Both Spartina and Avicennia showed declines in live stem density over 15 wks. *Juncus* exhibited a slight decline over time, but remained within 5% of its control.

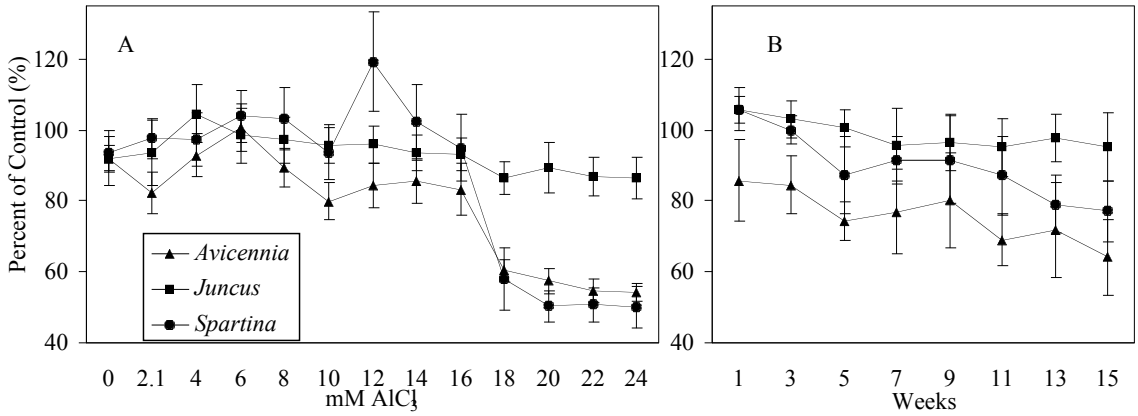


Figure 4.13. Effects of increasing aluminum concentrations (A) and aluminum concentration at 13.5 mM Al over time (B) on live density.

Effect of aluminum on shoot height

Although all species remained within 20 % of their controls throughout all concentrations (Fig. 4.14A), a significant interaction indicated different patterns across species (Table 4.3A). Shoot height of *Spartina* and *Avicennia* declined with increasing Al concentration, but Juncus height remained close to that of controls. Beyond 16 mM, *Spartina* height showed the greatest divergence from its control.

When Al concentrations were held constant, there was little further decline in shoot height of *Avicennia* or *Juncus*, but *Spartina* height continued to decline to about 70% of controls (Table 4.3B). Overall, Spartina height remained less than 80% of controls, whereas height of the other two species remained close to that of controls.

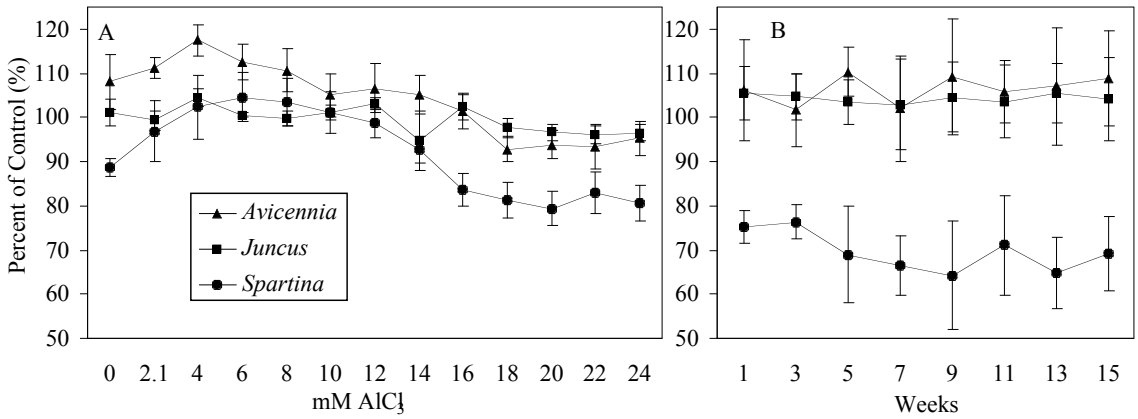


Figure 4.14. Effects of increasing aluminum concentrations (A) and aluminum concentration at 13.5 mM Al over time (B) on tree (*Avicennia*)/stand (*Juncus* and *Spartina*) height.

Effect of aluminum on mortality rate

Al treatment caused dramatic differences in mortality rate of the three species (Fig. 4.15A; Table 4.3A). Spartina mortality was consistently higher than the other two species from 8 to 24 mM Al. The rate of Spartina mortality increased at 18 mM and reached almost 75% of controls by 24 mM. *Avicennia* remained within 5% of its control through 18 mM, then mortality increased to 18 %. *Juncus* mortality remained less than 5 % of controls.

The plants held at 13.5 mM Al also showed different mortality patterns over time (Fig. 4.15B, Table 4.3B). Mortality of *Spartina* and *Avicennia* continued to increase over time, whereas *Juncus* showed less mortality than its control.

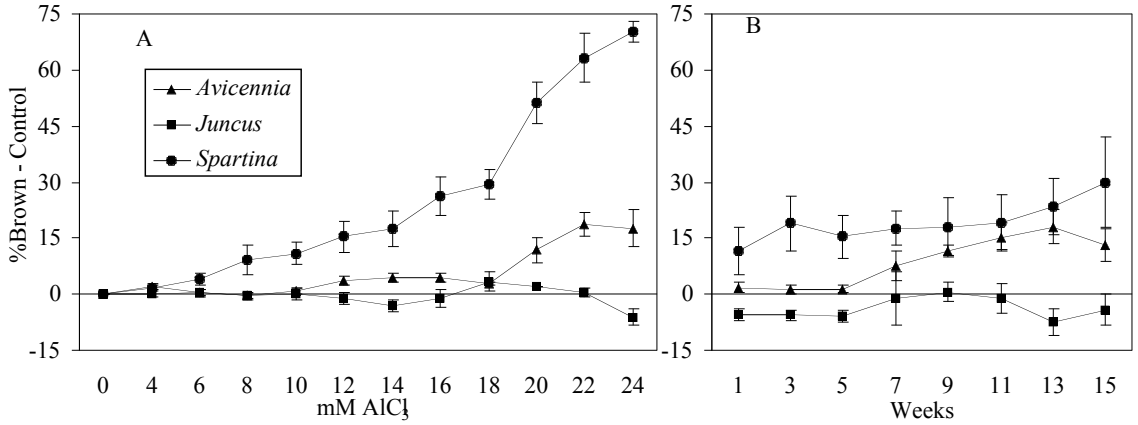


Figure 4.15. Browning of *Avicennia germinans*, *Juncus roemerianus*, *and Spartina alterniflora* from increased aluminum concentration(A) and over time at 13.5 mM Al(B).

Effect of aluminum on final biomass

Spartina was the most affected by increasing Al concentrations, with the least live biomass and the most dead biomass at the end of the experiment (Fig. 4.16A and B, Table 4.4 A). *Juncus* was least affected by Al, with 98% live biomass. *Avicennia* live biomass was 70 % of control, indicating an intermediate response to Al. The plants held at 13.5 mM Al also showed *Juncus* to be least sensitive to Al. However, final live biomass of *Spartina* and *Avicennia* were similar at the end of 15 wks.

Table 4.4. ANOVA results for biomass among plants in (A) increasing concentration to 24 mM Fe and (B) held concentration at 13.5 mM Fe for 15 weeks.

Source	DF	F-ratio	p
A. Increasing Concentration			
Live Biomass	2	31.15	<.0001
Dead Biomass	2	26.60	<.0001
B. Held Concentration			
Live Biomass	2	7.152	0.0315
Dead Biomass	2	16.18	0.0004

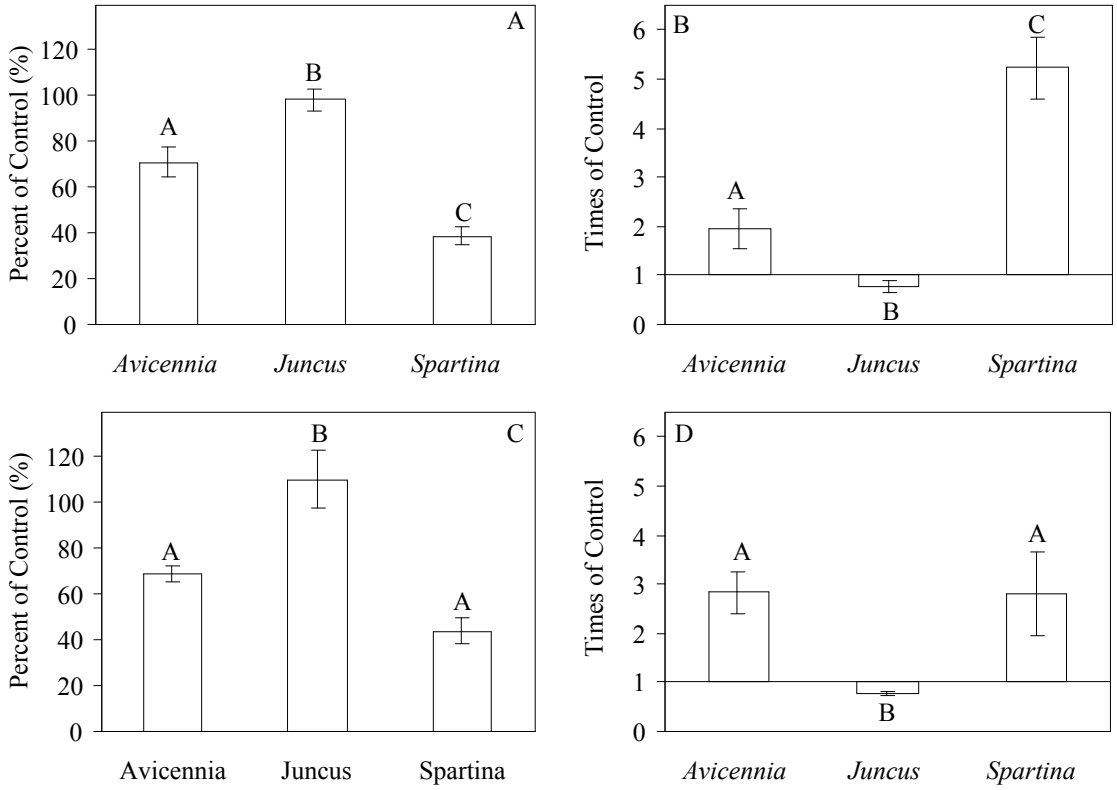


Figure 4.16. Effects of increasing aluminum concentration to 24 mM Al on live (A) and dead (B) biomass; and effects of Al concentration being held at 13.5 mM for 15 weeks on live (C) and dead (D) biomass. Data are presented as means and standard errors with different letters indicating statistical differences among plants within a frame.

Discussion

Salt marsh soils in coastal Louisiana are characterized by low redox potentials during summer and fall months, leading to formation of pyrite (FeS_2) (Feijtel et al. 1988). Sulfate, which is abundant in seawater, is reduced to sulfide in the anaerobic marsh soils and subsequently reacts with Fe(II) to form pyrite (Prasittikhet and Gambrell 1989). During early spring when water levels fall, oxidation of accumulated pyrite and other metal sulfides leads to extremely low pH (3-4) (Feijtel et al. 1988). Solubility of Fe, Mn, and Al increases several-fold with decreases in pH, and concentrations may ultimately reach toxic levels in the soil solution (Gambrell and Patrick 1978; Prasittikhet and Gambrell 1989). When pH decreases to 5 or lower, solubility of metals such as Al

increases substantially, and solution concentrations may reach phytotoxic levels (Foy 1974).

Low water levels are generated naturally along the Louisiana coast during the months of January and February due to the predominance of northwesterly winds that push water out of salt marshes. As a consequence of low water, intense oxidation of soils occurs in late winter and early spring and results in redox values above +300 mV (Smith et al. 1983; Gosselink 1984; Feijtel et al. 1988). Microbial oxidation of metal sulfides, including pyrite, generates sulfuric acid (H_2SO_4), which leads to soil acidification (Nordstrom 1982). With a decrease in pH, metals such as Al^{3+}, Fe^{2+}, and Mn^{2+} may become more mobile in the soil solution and reach levels toxic to plants. Field observations in Louisiana salt marshes show that porewater concentrations of Fe^{2+} increase from <25 to > 200 μM and Mn^{2+} from ~10 to 30 μM during the spring (Feijtel et al 1988). Together, these observations suggest that during early spring, soil acidification and release of potentially toxic metals is a natural phenomenon that does not normally damage the vegetation. However, a prolonged period of low water and/or drought could readily lead to greater oxidation and subsequent acidification and metal release upon reflooding.

Seasonal patterns of Fe^{2+} and Mn^{2+} fluctuation also occur in brackish marshes, but are mainly controlled by temporal pH changes independent of oxidation-reduction phenomena (Feijtel et al. 1988). Also, the pH decline (to about ~4) in brackish marshes occurs in fall and winter months, rather than in the spring when reduction of soils is maximal. Freshwater marshes in Louisiana show no consistent seasonal patterns in pH, although variability is high (Feijtel et al. 1988). These observations are consistent with the findings that the dieback phenomenon occurred mainly in salt marshes (McKee et al. 2004). In addition, the seasonal pattern of low pH in late winter and early spring in Louisiana salt marshes is consistent with the appearance of dieback in the spring of 2000 (G. Linscombe and R. Chabreck, public communication, www.brownmarsh.net). Consequently, the focus on potentially toxic concentrations of metals as a factor in dieback is justified by natural patterns of pyrite formation and dissolution, soil acidification, and porewater concentrations of Fe^{+2} and Mn^{+2} in Louisiana salt marshes.

In this study, we assessed the relative sensitivity of three salt marsh species to Fe and Al, which would have been increased by soil acidification. Fe and Al were selected since they were implicated by field observations as being greatly elevated in dieback plant tissues (McKee et al. 2004). Both Fe and Al are normally present in marsh soils, but not at toxic concentrations due to pH buffering near neutrality and redox reactions (Gambrell 1994). However, both metals can become more soluble under acidic conditions and readily taken up by plants. As discussed in the introduction, field observations indicated that dieback was limited to *Spartina* spp., and *Avicennia* and *Juncus* were unaffected (McKee et al. 2004). The experimental approach was designed to assess relative tolerance of Fe or Al, rather than simulating naturally occurring concentrations of these metals. In fact, the metal concentrations applied in our experiment greatly exceed those found in salt marsh porewater (Feijtel et al. 1988). It is not known what concentrations of metals occurred during the dieback event, and soil

analyses conducted after the event may not reveal this information. Consequently, subjecting the three species to incremental increases in metal concentrations would provide a way to identify the critical toxicity level without requiring knowledge of metal concentrations occurring during the dieback event.

Two types of tolerance tests were applied: 1) a ramp test in which concentration of the metal is raised at intervals until mortality and 2) a hold test in which metal concentration is raised until signs of stress and held at that level for 15 wk. Neither of these approaches subjects the test plants to the conditions that occurred naturally in the dieback phenomenon. Instead, these approaches allow the determination of the relative sensitivity of these three species to Fe or Al. With this information, we can then compare relative sensitivity to metals determined under controlled conditions with field observations of species dieback. To be consistent with field dieback patterns, *Spartina* would be most sensitive to metals, followed by *Juncus* and then *Avicennia*.

One limitation of the study was that metal concentrations in the bin solutions were difficult to control due to oxidation and precipitation, particularly of Fe. Although extreme measures were taken to maintain metals in solution (by adjusting pH and deoxygenating solutions), some precipitation occurred during the treatment intervals due to natural oxygenation and pH fluctuations. Thus, the actual concentrations of metals may have been less than the target concentrations. However, since plant responses continued to change with each incremental increase in metal concentration, it appears that the metal treatment did impose increasing amounts of stress on the sensitive species. Also, since all three species were subjected to exactly the same treatments, the relative plant responses to metals are valid.

Increasing the solution concentration of Fe or Al negatively affected growth of marsh plants with decreased photosynthetic rate and leaf expansion rate, lower shoot height and live biomass, and increased shoot mortality rate. Neither Fe nor Al treatment resulted in total mortality of any species. However, the relative sensitivity of the three species to these metals differed. *Spartina* was clearly the most affected by metal treatment overall with almost 70% mortality at highest concentrations of Fe or Al. *Avicennia* responded to Fe with mortality of 60% at highest concentrations, and to Al with decreased growth relative to controls. *Juncus* was very resistant to metal treatment and showed little or no or increased shoot mortality or decreased growth relative to controls.

Little previous work has documented the metal sensitivity of the three species examined in this study. Only one extensive study of Al toxicity in *S. alterniflora* has been conducted (Becker and Mendelssohn 2005). In that study, the critical toxicity level (determined based on stem elongation rate relative to controls) was reported to be between 5 and 11 mM Al. The critical toxicity level was defined as the concentration of metal ion that caused a growth indicator to drop below 30 % of the control (Lux and Cumming 1999). In our study, the critical toxicity level (as defined) was slightly higher (between 12 and 14 mM Al). However, leaf expansion did not distinguish among species sensitivity to metals well, whereas the percent mortality (% browning of shoots) provided

better separation of species responses to metals. Using 30 % mortality (relative to controls), the critical toxicity level for *Spartina* was 18 mM Al, but the critical level for *Juncus* and *Avicennia* was never reached (Fig. 4.15). Similar experiments to determine Fe toxicity levels for *S. alterniflora* or the other species have not been conducted previously. Using the 30% mortality criterion, the critical toxicity levels for *Spartina* and *Avicennia* were 7-8 and 13 mM Fe, respectively; the toxicity level for *Juncus* was never reached and presumably exceeded 14 mM Fe (Fig. 4.7).

Symptoms of Fe or Al toxicity include leaf bronzing and tissue necrosis (death). Bronzing is the result of loss of chlorophyll, leaving other pigments that impart a bronze, yellow, or orange coloration to the leaves (Van Breeman and Moorman 1978; Jugsujinda and Patrick 1993). The salt marsh plants involved in the 2000 dieback showed a distinct coloration (reddish brown to orange) of shoot tissues in some field sites (K. McKee and I. Mendelssohn, personal observation). In rice, tissue oranging was symptomatic of indirect toxicity of Fe, Mn, and Al affecting uptake of nutrients by plant roots; coatings of precipitated iron and manganese oxides limited absorption of nutrients from soils (Jugsujinda and Patrick 1993). During the metal experiments, heavy coatings of iron oxide (Fe experiment) were noted on the roots of test plants. Also, the shoots of *Spartina*, and to some extent *Juncus*, subjected to Fe treatment turned a reddish brown (Fig. 4.1), very reminiscent of the tissue coloration observed in the dieback areas during the 2000 event. This coloration was not apparent in the Al-treated plants (Fig. 4.9).

Although no previous work reports metal toxicity levels for *J. roemerianus*, the genus *Juncus* is well known for its resistance to low pH and metals. Species such as *J. bulbosus* and *J. effusus* are commonly found growing in mining lakes or acidic streams from lignite mining areas (Sand-Jensen and Rasmussen 1978; Pietsch 1998). In addition, wetland plants appear to be more resistant to metals than terrestrial (crop) species (Otte 2001). The current study supports this finding. Critical toxicity levels for tulip poplar (0.2 mM Al; Lux and Cumming 1999), water spinach (0 to 1.8 mM Al; Sun and Wu 1998), maize (~0.3 mM Al; Lidon and Barreriro 1998) and red spruce (~0.1 mM Al; Schier 1996) are all much lower than that found for the three salt marsh species examined in the current study (18-24 mM Al). Exposure of *Glyceria fluitans* to high groundwater concentrations of Fe (2.6 mM) caused necrotic spots on 67% of leaves (Lucassen et al. 2000). Thus, all three species appeared to be very resistant to Al and Fe, but *Juncus* and *Avicennia* were clearly more resistant than *Spartina* when concentrations became sufficiently elevated.

The exact reason for differential plant sensitivity to metals is not clear and beyond the scope of this investigation. However, other work has shown that species may exclude metals from roots, prevent translocation from roots to shoots, concentrate metals in older leaves or in stems, and/or modify metal solubility by altering pH in the rhizosphere (e.g., through selective uptake of anions and cations) (Foy 1978). One study has shown that *S. alterniflora* blocks root uptake of Al and Fe and also limits translocation to shoots (Alberts et al. 1990). Xeromorphic and/or halophytic features may also confer resistance or tolerance of Fe and Al since plant strategies to resist drought, salinity, and metal

toxicity have many similarities. Further work may reveal a suite of tolerance mechanisms that explain the relative sensitivities of these species to Fe and Al.

Conclusions

The metal tolerance experiments demonstrated that *Spartina* would have been more negatively affected than *Juncus* or *Avicennia* if concentrations of Fe or Al increased into the 7-8 mM range as a consequence of soil acidification. However, all three species showed high levels of tolerance to both Fe and Al, especially in comparison with terrestrial species. The greater tolerance of wetland plants to metals may reflect inherent plant strategies for dealing with the inimical conditions in waterlogged, saline soils. Since the concentrations of Fe or Al imposed in this study exceeded naturally occurring levels without causing total mortality of any species, it is unlikely that metal toxicity was the sole cause of marsh dieback. There is also no evidence that Fe or Al reached concentrations during the dieback that would have generated differential growth or mortality of *Spartina*, *Avicennia*, and *Juncus*. For these reasons, we conclude that metal toxicity alone did not cause *Spartina* dieback during the 2000 event. However, the greater sensitivity of *Spartina* compared to *Juncus* and *Avicennia* is consistent with field observations of the dieback pattern. Also, the known seasonal pattern of soil oxidation and acidification during the spring in Louisiana salt marshes matches the timing of the dieback event and provides a plausible mechanism for increased concentrations of potentially toxic metals in the soil solution. Further work to examine the interaction of metal toxicity with pH and drought conditions may reveal what combination of factors led to dieback of *Spartina*.

CHAPTER 5. INTERACTIVE EFFECTS OF DROUGHT, ACIDITY AND HEAVY METALS ON THE SALT MARSH SPECIES *SPARTINA ALTERNIFLORA*, *JUNCUS ROEMERIANUS* AND *AVICENNIA GERMINANS*

The extensive and sudden dieback of salt marshes dominated by the perennial grass *Spartina alterniflora* occurred in coastal Louisiana in 2000. Interestingly, sympatric species such as *Avicennia germinans* and *Juncus roemerianus* survived the incident, known as the brown marsh event (McKee et al., 2001). The occurrence of brown marsh coincided with extreme drought conditions and unusually low freshwater discharge from the Mississippi and Atchafalaya Rivers and low coastal and estuarine water levels, which prevailed during the 1999-2000 period (Swenson, 2001).

Species-specific differences in drought tolerance of *Spartina*, *Avicennia* and *Juncus* may have been the reason for the differential survival of these species observed during the brown marsh event. Our research investigating the drought tolerance of these plant species (see Chapter 2) demonstrated that *Spartina* was the least drought tolerant of the three species. This result was in agreement with observed differential field susceptibility of the species during the brown marsh event and suggested that drought could have been a primary cause of the dieback.

However, in addition to direct water deficits, chemical changes in sediments may occur upon soil drying, when the marsh water table was lower as a result of the drought. When saline sediments with high mineral content are excessively drained, oxidation of pyrite and other metal sulfides generates sulfuric acid, which may lead to soil acidification (Nordstorm, 1982). Field monitoring during the brown marsh event revealed that some dieback sites showed evidence of soil desiccation, low soil pH, high soil Eh, and root coatings of ferric iron, indicating oxidation (McKee et al. 2001). Our research investigating the acidity tolerance of the three marsh species (see Chapter 3) indicated that *Spartina alterniflora* was least tolerant of the three species to moderately acid conditions. This finding was in agreement with the differential survival of the three species during the brown marsh event. However, field surveys (McKee et al. 2004) during the brown marsh event did not quantify pH's low enough to cause appreciable mortality of *Spartina* (see Chapter 3). Nonetheless, pH's around 3 and below were generated when marsh sods were exposed to simulated drought conditions in the greenhouse (see R. Twilley, Task 2.2 Report). Thus, this degree of acidity could have occurred during the brown marsh event.

Further stressing the plants, a low soil pH during the drought could have increased the solubility and availability of some metals, such as Al and Fe, which may harm salt marsh plants. Our research investigating the metal tolerance of the three marsh plant species (Chapter 4) demonstrated that *Spartina alterniflora* was the most sensitive to elevated metal concentrations, although the metal concentrations that initiated plant impact were relatively high.

During the drought that generated the brown marsh event, it is likely that a number of the environmental stressors discussed above and investigated in this report occurred simultaneously. Hence, the interactive effect of multiple stressors, rather then any single stressor, could have been responsible for the plant dieback. This hypothesis becomes all the more plausible when one considers that, based on the species environmental tolerance limits determined in this report, individual stressor levels that

would have caused 100% mortality of *S. alterniflora* were never found in the field. However, the effect of these stressors acting together may have surpassed the tolerance threshold of the species, i.e., the stress threshold of the *Spartina* is likely much lower in response to a combination of stressors compared to one stressor acting alone. Because our results demonstrated that species susceptibility to three stressors, water deficit, acidity, and heavy metals, was in agreement with the species-specific impact during the brown marsh event, these factors were selected for further investigation.

Therefore, the objectives of this research were to (1) investigate the interactive effects of drought, acidity and heavy metals on the plant growth responses of the salt marsh species *Spartina alterniflora*, *Juncus roemerianus* and *Avicennia germinans* and (2) determine if the interaction of sublethal levels of these stressors, when acting together, could explain the mortality of *Spartina* but the survival of *Avicennia* and *Juncus* during the brown marsh event

Materials and Methods

Plant materials

The salt marsh plants, *Spartina alterniflora* (smooth cordgrass) and *Avicennia germinans* (black mangrove) were acquired from the Plant Materials Center of the National Resources Conservation Service (NRCS). *Spartina alterniflora* included populations E1-6, E2-3, E2-6 and E3-4. *Juncus roemerianus* (black needlerush) was collected from a salt marsh near Cocodrie, Louisiana. After acquired, all plants were planted into a substrate of commercial potting soil (Miracle Gro lawn, Inc.) and garden soil (Greenleaf Products, Inc.) (1:1) to grow to a satisfactory size before the initiation of the experiment. Because the mix of potting soil and garden soil can more readily be washed from plant roots than marsh sediment, the use of this rooting material reduced physical damage to the plants when they were transferred from the potting soil to sand, the substrate used in the experiment.

Experimental Procedures

One month before the start of the experiment, the potting soil was carefully washed from the plant roots. The three marsh plant species (*Spartina, Juncus*, and *Avicennia*), free of the soil, were separately transplanted into sand filled plastic pots (20 cm height and 15 cm diameter) with 4 holes (2x2 cm) on the sidewall near the pot bottom. The holes were covered with a thin layer of sponge to let water drain freely, but to retain the sand inside the pots. About 20 stems of *Spartina* and *Juncus* and one *Avicennia* plant were transplanted into each plastic pot. After the plants recovered from any initial transplant shock, 6 pots (experimental units), two of each species, were placed in one plastic tub (30x45x20 cm in depth, width and height). The experimental treatments, acidity, Al, Fe, and drought, were varied as described below to investigate the interactive effect of these stressors on each plant species. The experiment was conducted in the growth chamber with light intensity about 800 $\mu mol/m^2/s$ at the canopy level during the daytime and 0 $\mu mol/m^2/hr$ during the nighttime. The daytime temperature was

30 ºC for 12 hours and nighttime temperature was 21 ºC for 12 hours in a 24-hour period. The tubs containing the experimental units were filled the appropriate treatment combination in half-strength Hoagland's nutrient solution to 1 cm above the soil surface. The salinity was 23 ppt in the culture solution.

The treatment combinations included in the experiment were as follow:

- pH treatment: pH4, pH3 and pH2.5 These pH's in the single factor acidity experiment (Chapter 3) did not impact any of the three species.
- Metal treatment-combinations: low Fe (1 mM) and low Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM), high Al (20 mM). The Al and Fe concentrations used in these experiments were selected based on results from the single factor experiments (Chapter 4).
- Drought treatment: PEG at -1.1 MB versus no drought with no addition of PEG The -1.1 MPa treatment-level was selected because it only had sublethal effects in the single factor drought experiment (Chapter 2).
- Control: 1 mM of Al and Fe at pH 6.5, 4.0, 3.0 or 2.5, depending on experiment. I mM Al and Fe caused no negative impacts to any of the species in the single factor experiments (Chapter 4).

The salinity was maintained at 23 ppt for all treatments throughout the experimental period. The proper pH of the porewater nutrient solution was maintained by watering with tap water to replace the water loss by evapo-transpiration and adding the 1 N HCl or NaOH daily. The experimental units were flushed with the appropriate solution daily.

There were 3 experimental stages to this study:

1. The three plant species were treated with four metal concentrations at both pH 4 and pH 2.5 for 2 months: 1 mM Al and 1mM Fe, 1 mM Al and 10 mM Fe, 20 mM Al and 1 mM Fe, and 20 mM Al and 10 mM Fe. The treatment combinations were replicated 6 times. Plants in the treatments receiving pH 2.5 were harvested after 2 months because they were substantially stressed.
2. After the 2 months in the pH 4 treatment-level, the acidity of the treatment solution was lowered to pH 3 because pH 4 had little effect on the plants. Thus, the treatments during stage 2 were as follow: 1 mM Al and 1 mM Fe, 1 mM Al and 10 mM Fe, 20 mM Al and 1 mM Fe, and 20 mM Al and 10 mM Fe with the acidity at pH 3. These treatment-combinations were replicated 6 times.
3. After treatment at pH 3 for 2 months, half the experimental units were exposed to simulated drought by adding PEG (an osmotic drought agent) to the treatment solution to generate a water potential of -1.1 MP. The remaining experimental units did not receive PEG (control). All experimental units (drought and control) were maintained at pH 3 for 6 weeks. The treatments were as follow: 1 mM Al and Fe, 1 mM Al and 10 mM Fe, 20 mM Al and 1 mM Fe, and 20 mM Al and 10 mM Fe with or

without PEG. All treatments had an acidity of pH 3. These treatment-combinations were replicated 3 times.

Plant responses to the interactive stressors were determined by measuring the following parameters: (1) leaf elongation rate, (2) photosynthetic rate, (3) stem density, (4) shoot height and (5) percent mortality.

Methodologies

Leaf expansion rate. The difference in leaf length from a fixed point to the tip of a young leaf over a three-day period was measured to determine the leaf growth rate per day. The unit is cm/day.

Photosynthetic rate and Fluorescence rate Photosynthetic rate was measures with an infrared gas analyzer (Li-Cor 6400). A representative leaf was selected and enclosed in a leaf chamber to analyze net carbon dioxide exchange rate. Plant photosynthetic rate was determined at a photon flux density of 2000 $\mu mol/m^2/s$ and a CO_2 concentration of 350 ppm. The unit is $\mu mol\ CO_2/m^2/s$. Leaf fluorescence was measured after dark adaptation with the fluorescence chamber of the LiCor 6400 infrared gas analyzer. The variable fluorescence to maximum fluorescence ratio (Fv/Fm) was used to determine plant health status.

Stem density (branch numbers for *Avicennia*). Live stem numbers of *Spartina* and *Juncus* were directly counted in each experimental unit. For *Avicennia*, instead of stem number, live branch number was measured in each experimental unit because there is only one stem of *Avicennia* in each pot. Thus, the live branch numbers of *Avicennia* were considered analogous to the live stem numbers of *Spartina* and *Juncus*. The unit is number/pot.

Total live shoot height. The height of each live shoot of *Spartina* and *Juncus* were measured with a meter stick. Total live shoot height was derived by summing the heights of all live shoots in each experimental unit. For *Avicennia*, instead of live shoot height, total live leaf number was measured. The number of live leaves served as a non-destructive surrogate for live leaf biomass similar to the use of live shoot heights of *Spartina* and *Juncus*.

Plant mortality. Percentage of dead tissue was occularly measured to estimate mortality.

Statistics

Statistic analysis was conducted by using SAS's (version 8.2) General Linear Model. Duncan's test was used to determine significant differences between treatment levels when there was no significant interaction between main effects. Least square means were used to compare individual treatment-levels when the interaction of the main

effects was significant. Statistical significance was reported at $p \leq 0.05$ unless otherwise mentioned.

The plant mortality data are presented in this chapter because this parameter best integrated plant response to the interactive stressors. The response of the other growth parameters to the multiple stressors were variable and did not result in interpretable results.

Results

1. Interactive effects of Al and Fe at pH 2.5 and pH 4 on plant condition

In this experiment, plants were exposed to different metal concentrations at either pH 4 or pH 2.5 for 2 months to test the interactive effect of metals and acidity on plant growth responses. Generally, responses of the plants to the treatments differed with plants species; *Spartina alterniflora* was most impacted, while *Juncus* and *Avicennia* were relatively unaffected (Figs. 5.1 and 5.2).

Plant mortality significantly differed with different plants species ($p<0.0001$); percent mortality was highest for *Spartina* and lowest for *Avicennia,* averaged over all treatments (Fig. 5.3). Acidity also significantly affected plant mortality ($p<0.0001$), however, the acidity effect varied with plant species (significant plant species x acidity interaction, $p<0.0001$). While percent mortality of *Spartina* at the pH 2.5 was significantly greater than at the pH 4.0, *Juncus* was unaffected by a decrease in pH from 4.0 to 2.5. *Avicennia* had a small but significant increase in mortality with lower pH. A significant 3-way interaction ($p<0.005$) among Al addition, pH, and plant species was identified. Higher Al concentration and lower pH level significantly affected *Spartina alterniflora*, but not the other two species. The Fe addition also significantly increased percent mortality, but only for *Spartina* (significant plant species x Fe interaction, $p<0.0001$).

In addition to plant mortality, we also measured leaf expansion rate, leaf photosynthetic rate, and leaf fluorescence (Fv/Fm). These growth/plant health parameters, which were measured on single, young, live leaves, were unaffected by the treatments (data not shown) although the impacts of treatments were visually obvious. The reason for the absence of a significant effect was likely due to the fact that these measurements were conducted on individual leaves that were still live and growing. Young leaves were the last to be affected by the stressors and thus they stayed green even when older leaves had turned brown. These young, actively growing leaves were able to maintain their growth rates, even while older leaves died due to the stressors. We obviously did not select brown leaves for these measurements because they were no longer viable.

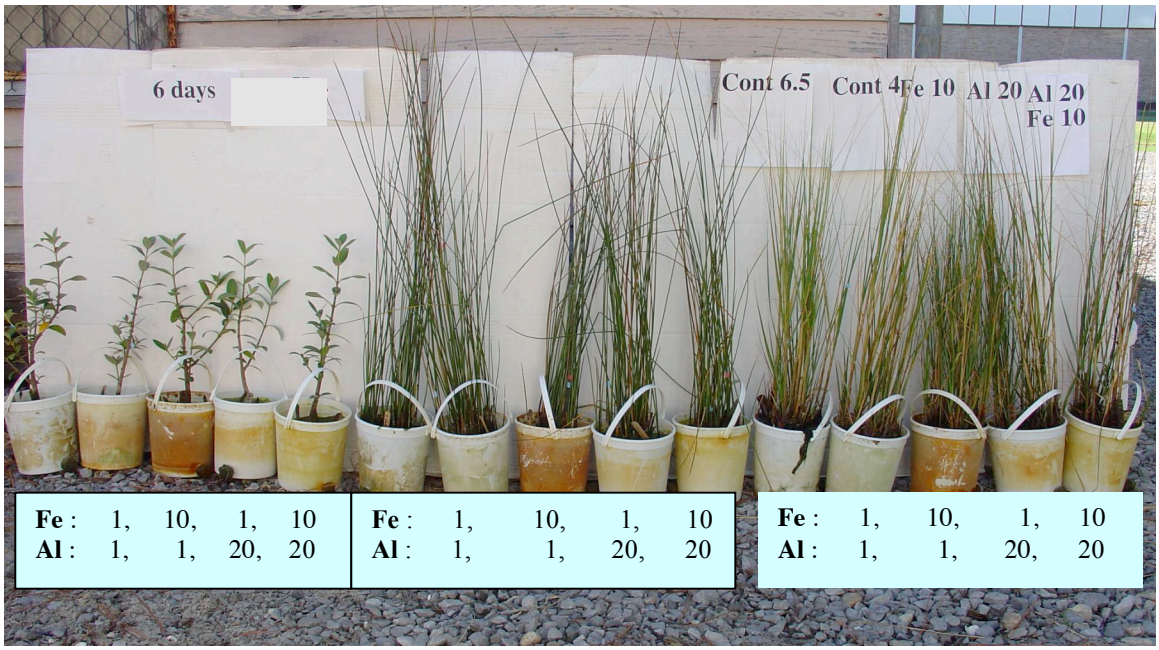


Fig. 5.1. Effect of the Fe and Al addition at pH 4 on the growth status of *Avicennia germinans* (left five pots), *Juncus roemerianus* (middle five pots), and *Spartina alterniflora* (right five pots) 8-weeks after treatment initiation. Four metal combinations: low Fe (1 mM) and low Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

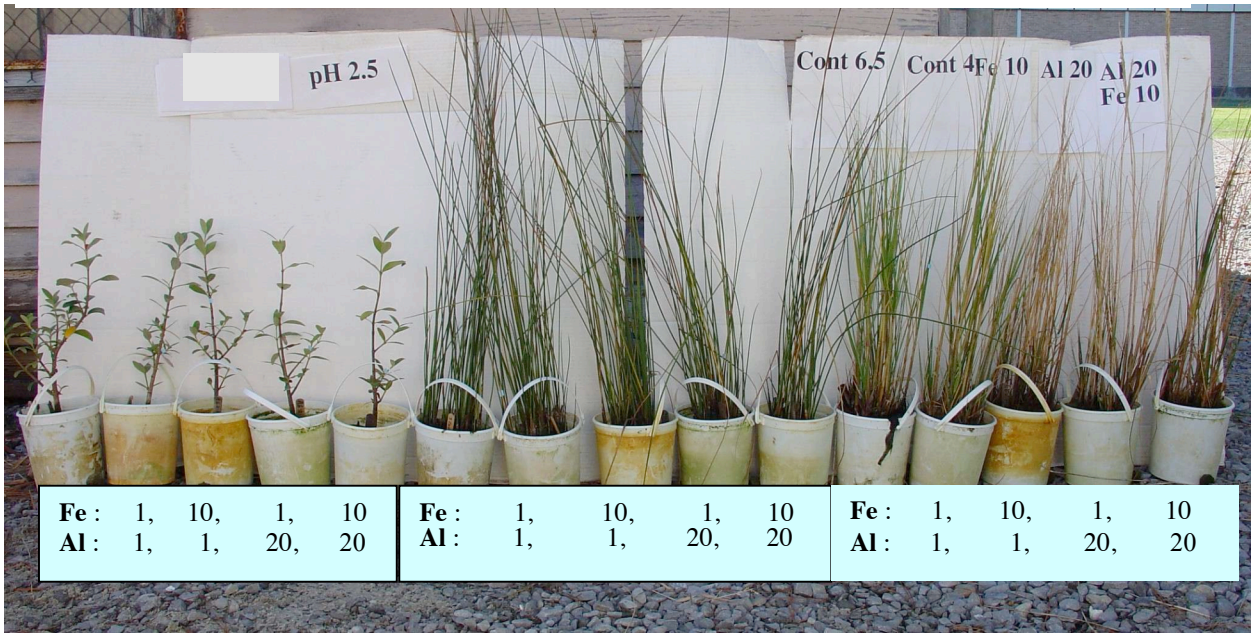


Fig. 5.2. Effect of the Fe and Al additions at pH 2.5 on the growth status of *Avicennia germinans* (left five pots), *Juncus roemerianus* (middle five pots), and *Spartina alterniflora* (right five pots) 8-weeks after treatment initiation. Four metal combinations: low Fe (1 mM) and low Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

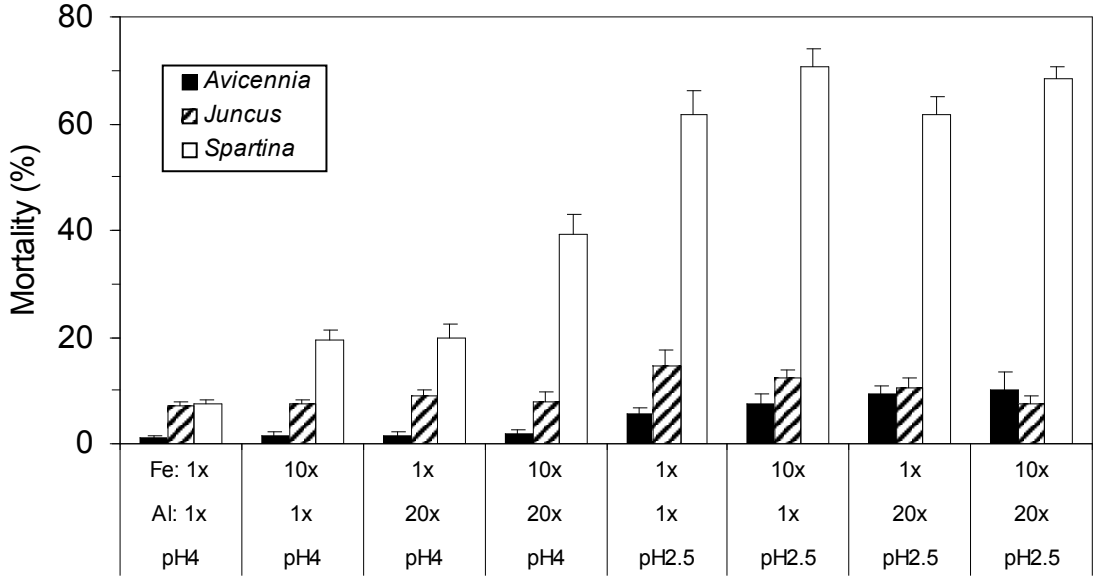


Fig. 5.3. Effect of Fe and Al additions at pH 4 and pH 2.5 on percent mortality of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora* 8-weeks after treatment initiation (n=6). Metal combinations: low Fe (1 mM) and low Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

2. Interactive effects of Al and Fe at pH 3 on plant health status

All plants in this experiment had been at pH 4 for two months, and then the pH level was lowered to pH 3 for another two months to evaluate the effect of heavy metals at a moderately low, but realistic, pH level. Generally, plant responses to the treatments differed with plant species, with the most severe impact on *Spartina*, but no obvious impact on *Juncus* or *Avicennia* (Fig. 5.4). An increase in Al concentration from 1 mM to 20 mM caused a significant increase in percent mortality of *Spartina*, but not of *Juncus* or *Avicennia* (significant species x Al interaction ($p<0.0001$). The effect of iron addition on plant mortality also significantly varied with plant species (significant species x Fe interaction ($p=0.0003$). Similar to the response to Al, increasing the Fe concentration from 1 mM to 10 mM significantly increased percent mortality of *Spartina*, but not *Juncus* or *Avicennia*.

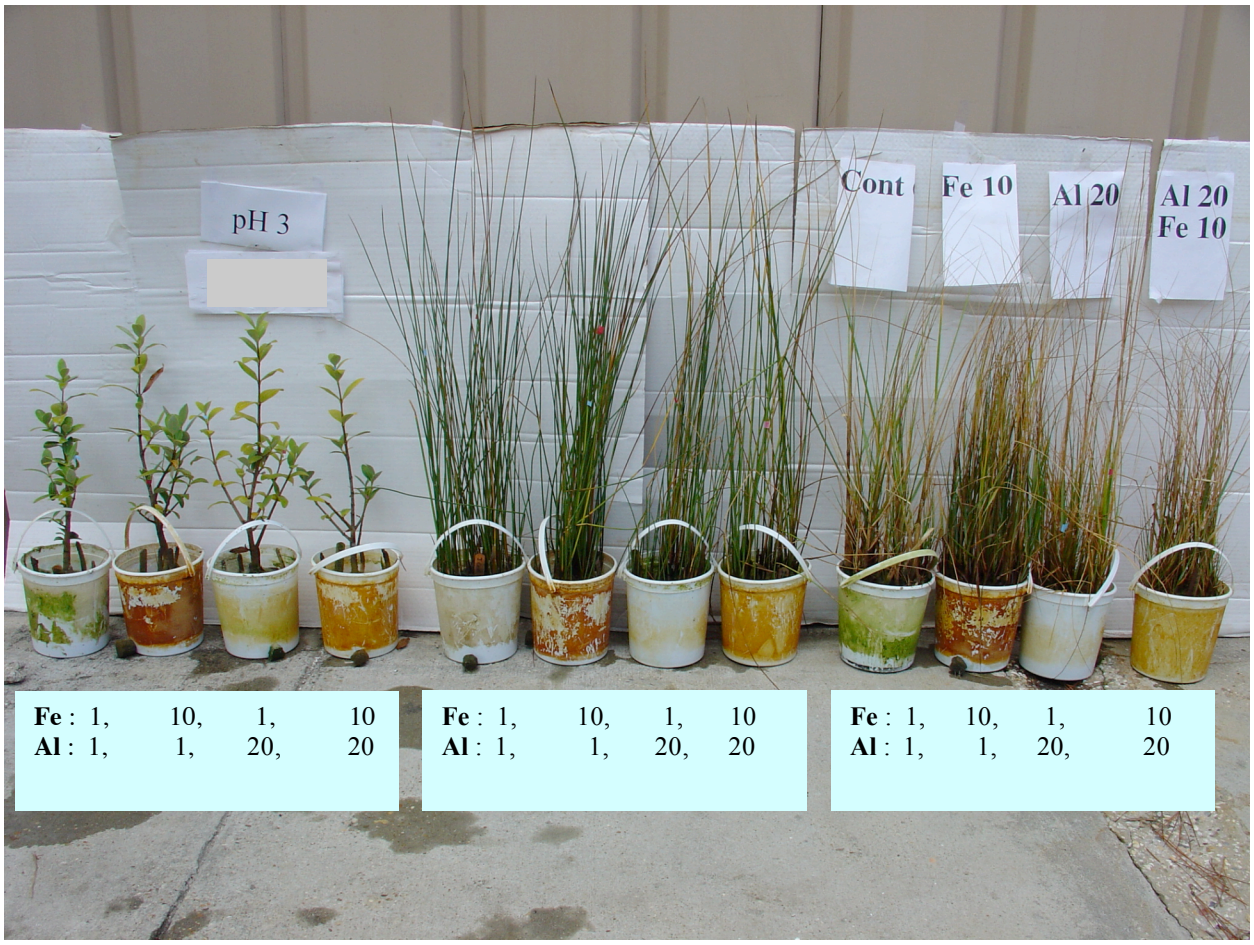


Fig. 5.4. Effect of Fe and Al additions at pH 3 on the growth status of *Avicennia germinans* (left four pots), *Juncus roemerianus* (middle four pots), and *Spartina alterniflora* (right four pots) 6-weeks after treatment initiation. Four metal combinations: low Fe (1 mM) and low Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

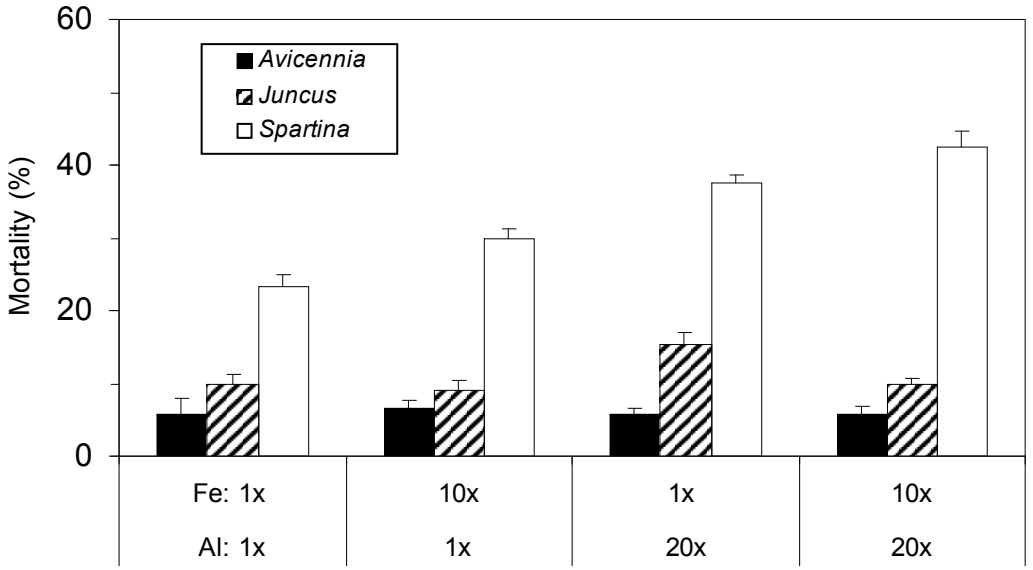


Fig. 5.5. Effect of Fe and Al additions at pH 3.0 on percent mortality of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora* 6-weeks after treatment initiation (n=6). Four metal combinations: low Fe (1 mM) and Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

3. Interactive effects of drought, Al, and Fe with the acidity of pH 3 on plant health status

Prior to the initiation of this experiment, the plants had been held at a pH of 3 with no added PEG (i.e., no simulated drought) for 2 months, and then half of the plants was treated with PEG for six weeks to induce a water potential of –1.1 MPa in the plant culture medium. This experiment was designed to evaluate the interactive effects of drought and heavy metals on plant response at moderately low pH. The percent mortality in response to the stressor treatments differed significantly among the three plant species ($p<0.0001$) with *Spartina* being the most severely impacted (Figs. 5.6 and 5.7). However, the species responses varied as a function of both drought and heavy metals (significant species x drought x Al interaction and significant species x drought x Fe interaction, $p=0.0185$ and $p=0.0474$, respectively) (Fig. 5.8). These interactions suggest that although *Spartina* is more sensitive to elevated metal levels (at pH 3.0) than are *Juncus* and *Avicennia*, drought exacerbates this negative effect such that mortality of *Spartina* approached 100% when all three stressors, drought, heavy metals and moderately acid conditions, were present.

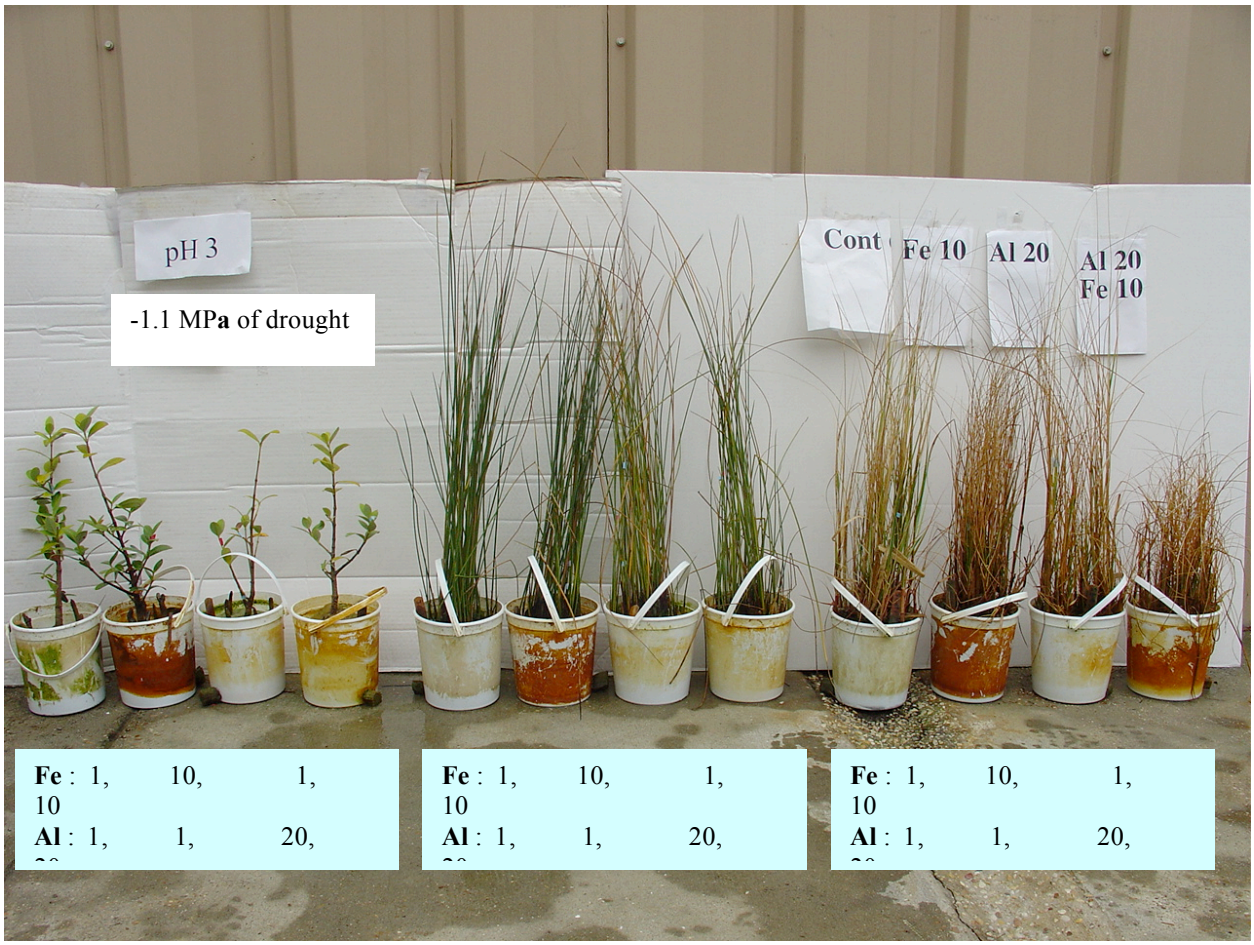


Fig. 5.6. Effect of water deficit (-1.1 MPa), Fe, and Al at pH 3.0 on growth status of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora* 6-weeks after treatment initiation. Four metal combinations: low Fe (1 mM) and Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

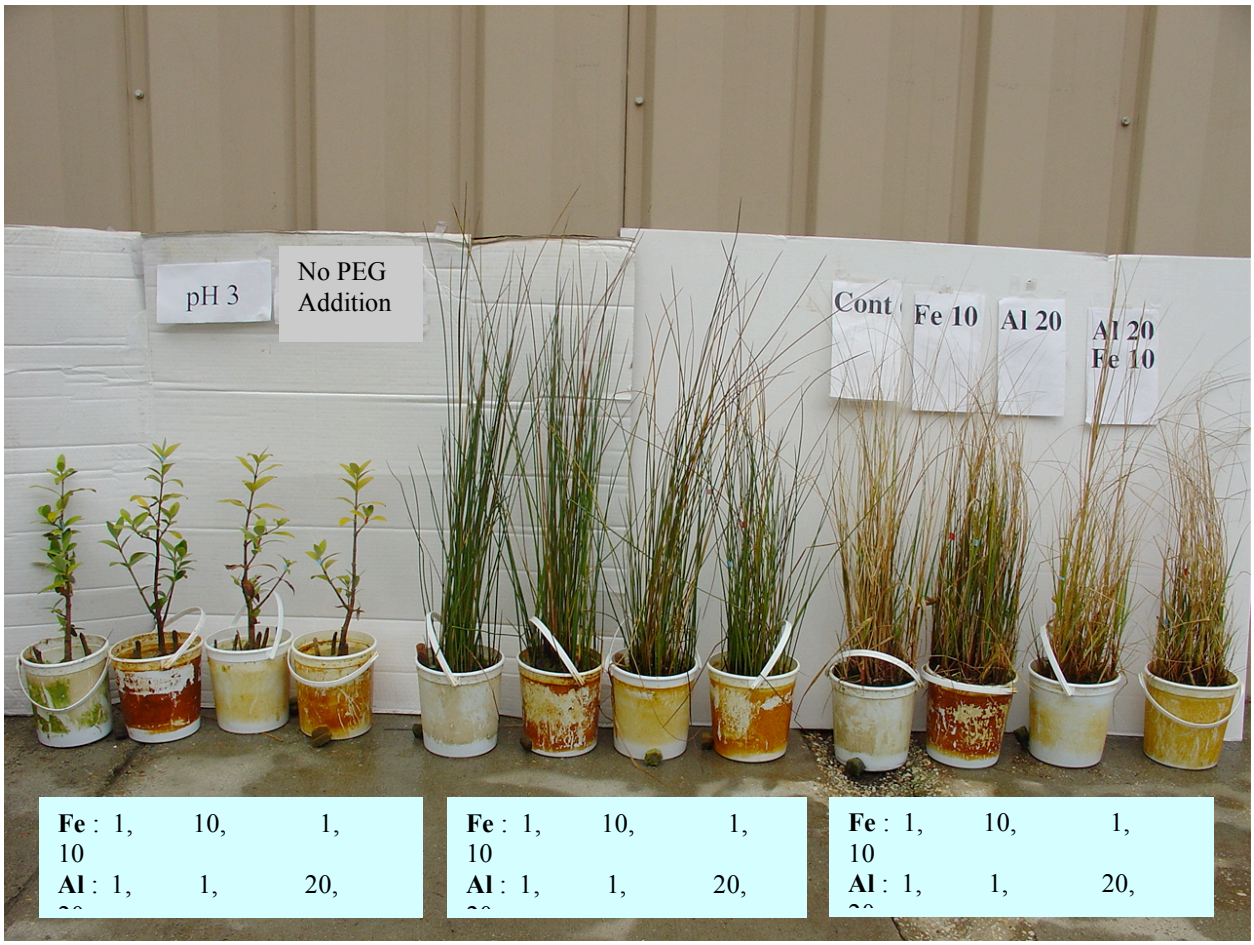


Fig. 5.7. Effect of Fe and Al additions without water deficit (no PEG added) at pH 3 on the growth status of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora* 6-weeks after treatment initiation. Four metal combinations: low Fe (1 mM) and Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

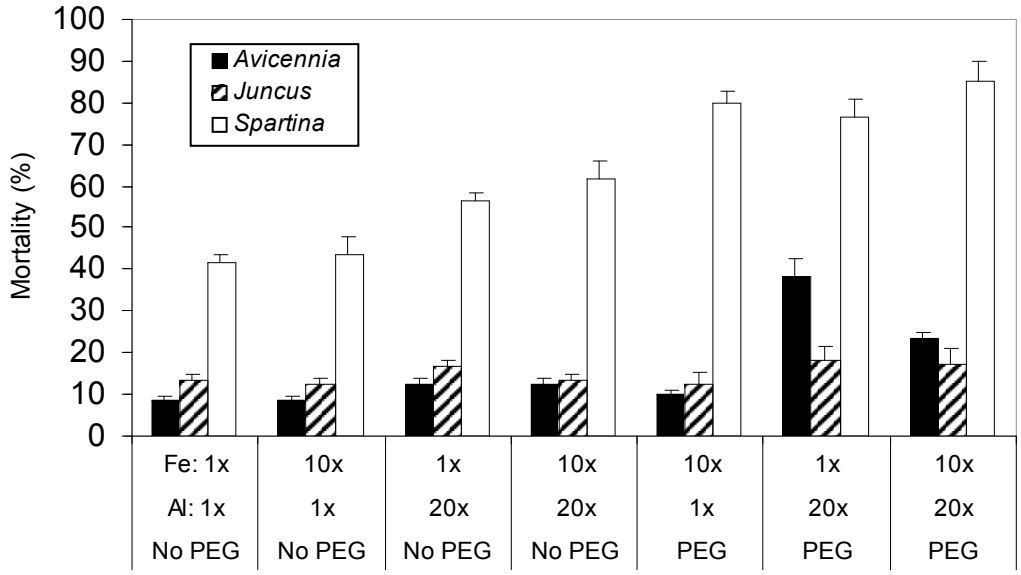


Fig. 5.8. Effects of water deficit (-1.1 MPa by PEG) and Fe and Al additions at pH 3 on percent mortality of *Avicennia germinans*, *Juncus roemerianus*, and *Spartina alterniflora* 6-weeks after treatment initiation (n=3). Four metal combinations: low Fe (1 mM) and Al (1 mM), high Fe (10 mM) and low Al (1 mM), low Fe (1 mM) and high Al (20 mM), and high Fe (10 mM) and high Al (20 mM).

Discussion

The series of experiments presented in this chapter demonstrate the importance of stressor-interactions in potentially causing the brown marsh event, in which *Spartina alterniflora* was impacted, but *Avicennia germinans* and *Juncus roemerianus* were not.

During the 8-week period in which all three-plant species were treated with pH 4 and pH 2.5 nutrient solutions with combinations of 1 and 20 mM Al and 1 and 10 mM Fe, percent mortality differed with plant species. At pH 4, 20 mM Al or 10 mM Fe significantly increased the percent mortality of *Spartina*. The combination of 20 mM Al and 10 mM Fe further increased the mortality of *Spartina* more than either metal individually. However, neither 10 mM Fe nor 20 mM Al, provided individually or in combination at this pH (4.0), significantly stressed *Juncus* or *Avicennia*. These results demonstrate that *Spartina* is more sensitive to metal toxicity than either of the sympatric species.

At pH 2.5, compared to pH 4.0, the percent mortality of *Spartina* increased 3-fold to approximately 70% while *Juncus* was unaffected; percent mortality of *Avicennia* only increased to ca. 8%. Thus, *Spartina* was obviously most sensitive of the three species to increases in acidity. At pH 2.5, neither 20 mM Al nor 10 mM Fe further increased the percent mortality of *Spartina* above that of the control (1 mM Al and Fe). The reason was most likely that the extremely low acidity at the pH 2.5 overwhelmed the effects of the metals. The mortality in all treatments at pH 2.5 was more than 65%. In addition,

this low pH might have affected plant root uptake of Al and Fe regardless of their high concentrations in the interstitial water. Yang et al. (2005) reported that root elongation in spinach was significantly inhibited at pH 4.5, and concluded that the sensitivity to acid stress in spinach could mask the potential role for oxalate to protect the plant roots from Al toxicity.

During the next 8-week period, the experimental plants, which had previously been exposed to pH 4, were treated with pH 3 nutrient solution at the same metal concentrations as used previously. Even at 1 mM concentrations of the metals, *Spartina* mortality was greater at pH 3 (Fig. 5.5) than at pH 4 (Fig. 5.3). At pH 3, elevated concentration of either Fe or Al increased percent mortality of *Spartina*. Furthermore, Al increased the percent mortality of *Spartina* more than Fe. Similar to the plant response at pH 4, the combination of 20 mM Al and 10 mM Fe increased the mortality of *Spartina* more than either metal individually. Impacts to *Spartina* were qualitatively greater at pH 3 than 4, but the extent of difference was dependent on metal concentrations.

During the 6-week period in which the three species were treated with and without the osmotic agent, PEG, at the same metal concentrations as previously, the mortality again differed among the three plant species. Of the three-species, *Spartina* was most affected after 12 weeks at pH 3.0. Elevated metal exposure, especially with Al, further increased percent mortality. However, when the plants were exposed to drought stress at these same metal concentrations and pH's, percent mortality of *Spartina* almost doubled. Thus, the combination of direct drought induced water stress, moderately low pH, and metal stress lowered the impact threshold of *Spartina* such that the combined effect of these stressors was greater than the effect of any of the factors acting alone. The combination of these stressors severely impacted *Spartina* with mortality as high as 90% in less than 2 months.. The combined effect of these stressors on *Avicennia* and *Juncus* was small (ca. 20 % mortality) in comparison to that for *Spartina* (ca. 90%).

During the brown marsh event, *Spartina alterniflora* died back, while *Avicennia germinans* and *Juncus roemerianus* survived (McKee et al. 2001). In the present experiment, the performance of the three plant species was similar to their response during the brown marsh event. *Avicennia* and *Juncus* survived the brown marsh incident, but *Spartina* did not. This experiment suggests that relatively low to non-lethal environmental stressors, if occurring simultaneously in the field, could result in the near complete dieback of *Spartina* while little affecting sympatric species.

Conclusion

Three salt marsh plants, *Avicennia germinans*, *Juncus roemerianus* and *Spartina alterniflora*, showed large differences in response to the combination of acidity, Al, Fe, and drought. Generally, *Spartina* was most sensitive to this combination of stressors; *Avicennia* and *Juncus*, in contrast, were little stressed by the same treatment-combinations. The stressors acting in combination appeared to lower the impact thresholds determined for each individual stressor. The species-specific responses to

drought, acidity, and elevated metal concentrations suggest that the brown marsh event in coastal Louisiana was a result of all these stressors acting in concert. The interactive effects of drought, lower pH induced by drought, and greater availability of potentially toxic metals in acid soils may have had lowered the impact threshold of *Spartina alterniflora* to these stressors, thus, enabling low-lethality stressors to cause near complete mortality.

SUMMARY AND CONCLUSION

This research determined the environmental tolerance limits of *Spartina alterniflora*, the species that died during the brown marsh event, and those of the sympatric species, *Juncus roemerianus* and *Avicennia germinans*, that survived. These three salt marsh species were exposed in 10 separate experiments to (1) increasing stressor levels of salinity, water deficiency, acidity, and heavy metals (Al and Fe) and (2) increasing stress duration at sublethal levels of these stressors. In addition, a series of three experiments were conducted to assess the interactive effects of these stressors on plant mortality. We hypothesized that the stressor or stressors most likely responsible for sudden salt marsh dieback would be one(s) that negatively impacted *Spartina*, while not appreciably affecting *Juncus* or *Avicennia*, the two species that survived the brown marsh event.

With respect to salinity, the experimental findings demonstrated that *Juncus roemerianus* was most sensitive to salinity stress, while *Avicennia* was most tolerant. *Spartina* had an intermediate response. Hence, if salinity was the primary factor driving the dieback, *Juncus* should have died with *Spartina*. Yet *Juncus* was not affected. Hence, salinity, per se, could not have been the primary cause of the dieback.

In contrast to the species-specific responses of these plants to salinity, *Spartina* was the most sensitive of the three species to the other stressors (water deficit, acidification, and heavy metals). Hence, any of these stressors or their interaction could have been responsible for the dieback. So which of these stressors could have most likely been responsible for *Spartina* mortality?

Although a water deficit has the potential to stress *Spartina*, it is unlikely that soil water potential would have become so negative as to cause, by itself, 100 % mortality of this species. Even though the marshes may not have flooded as frequently during the drought as normal, tidal inundation did periodically occur and the marsh substrate, even in marshes with cracked surface soils, was moist within the deeper rooting zone (personal observation, I. Mendelssohn). Relative to acidity, it is clear that *Spartina* is sensitive to low pH. However, the pH level that caused 100 % mortality of *Spartina* would have also resulted in 100% mortality of *Avicennia*. Yet, *Avicennia* was not affected by the brown marsh event. Hence, it is unlikely that acidity alone caused the dieback of *Spartina*. Increasing concentrations of the heavy metals, Al and Fe, affected *Spartina* much more than *Avicennia* and *Juncus*. The concentrations of these metals, however, that caused 100% mortality of *Spartina* were extremely high and probably did not occur, at least in the bulk soil, during the dieback. So our results strongly suggest that if the stressors were acting individually during the dieback event, none of them would have been stressful enough to have caused the dieback. Rather, we hypothesize that water deficiency, acidity and metal toxicity acted in tandem to cause a stress to *Spartina* much greater than would have resulted from any of these stressors individually. Our field and laboratory research during the dieback (McKee et al. 2004) provided the basis for proposing that reduced marsh inundation and lower precipitation during the drought dried the marsh soil enough to induce soil oxidation, which caused moderate soil acidification, and an

increase in availability of metals. The interaction of moderate soil acidification and metal toxicity in conjunction with decreased water availability, possibly exacerbated by somewhat higher soil salinities than normal, could have killed *Spartina* while leaving *Avicennia* and *Juncus* unaffected. What evidence do we have to support this hypothesis?

We tested the preceding hypothesis in a series of interaction experiments described in Chapter 5. These experiments showed that at a non-lethal pH of 3.0, a pH that could have occurred during the brown marsh event, the presence of Fe and Al, even at non-lethal concentrations (1 mM at pH 6.5, see Chapter 3), resulted in *Spartina* mortality ranging from 40 - 60 %. Further stress in the form of a sublethal water deficit resulted in *Spartina* mortality near 90 %, while having little impact on the sympatric species *Avicennia* and *Juncus*. Thus, the research presented in this report supports the hypothesis that low precipitation and reduced marsh flooding during the brown marsh event induced soil oxidation, soil acidification and metal toxicity, acting in conjunction with plant water stress, to cause *Spartina* mortality without an effect on co-occurring plant species. At this time, evidence from Task 2.1 - Fungal Pathogens, indicate that the pathogenic fungus *Fusarium* sp. could have played a role in the initial plant dieback. However, additional research is required to clarify their part, if any, in the dieback event. Also, although the marsh periwinkle, *Littoraria irrorata*, has been demonstrated to have enlarged the areas of initial dieback in Louisiana, there is no direct evidence that they caused the dieback (Silliman et al. 2005).

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