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DETERMINING SALINITY-TOLERANCE OF GIANT SALVINIA USING CHLOROPHYLL FLUORESCENCE

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ABSTRACT: *Salvinia molesta* Mitchell, a floating invasive aquatic plant, is one of the top 10 worst invasive aquatic weeds in the world. It was discovered in the lower Pascagoula River in 2005 and evidence suggests that this non-native species is spreading along the northern Gulf of Mexico. These plants exhibit rapid growth and nutrient uptake rates, allowing them to out compete other plants in similar habitats. Distributional observations suggest that non-native *S. molesta* is able to survive in salinities of up to 7 ppt in the lower Pascagoula River. The response of *S. molesta* to three salinity levels (0, 5, 10 ppt) was tested using chlorophyll fluorescence. The health of the plants was measured over a period of one month, using a log scale series of observation intensities (hourly, daily, weekly). Plant responses indicated an acute salinity effect after about 4-6 hrs and then a gradual chronic decline. Compared to initial measurements, the final actual quantum yield ($\Delta F/F_m'$) dropped by 5%, 6% and 29%, while the final potential quantum yield (F_v/F_m) dropped 6%, 27% and 39% in the 0, 5, and 10 ppt treatments, respectively. Only plants in the 0 ppt treatment showed significant new growth. Plants in 5 ppt appeared to maintain themselves, but plants at 10 ppt all exhibited signs of severe stress and loss of color, turgor, and tissue viability after 10 d. Tolerance to brackish salinities has been reported in the past, and has implications for the use of the biological control agent, the weevil *Cyrtobagous salviniae*, that can only tolerate freshwater conditions.

INTRODUCTION

Aquatic plants can be grouped into three types: emergent, floating, and submerged (Pieterse and Murphy 1993), with some of the most successful invasive aquatic plants being in the floating group (e.g., *Eichhornia crassipes* and *Salvinia molesta*). These plants exhibit rapid growth rates, rapid nutrient uptake rates, are aggressive, and are competitive species that can impact aquatic environments, local economies, and human health (Holm et al. 1977). The impact of these species on a freshwater body is dramatically illustrated by *S. molesta*, one of the top 10 worst non-native invasive aquatic weeds in the world (Room and Julien 1995, Carley and Brown 2006). *Salvinia molesta* has become a world-wide problem, with invasions into freshwater bodies in most tropical countries, and was introduced into the United States in 1995 (Julien and Tipping 2002, USGS 2005).

Salvinia molesta has a doubling time of 4-6 d (Mitchell and Tur 1975) and was found in the lower Pascagoula River in 2005 (MS DMR 2005). Evidence reported in McFarland et al. (2004) suggests that this non-native species is spreading into the northern Gulf of Mexico (GOM). In Alabama and Mississippi, there are many suitable habitats for native and non-native invasive aquatic plants; there are four river drainage systems along the 121 km (75 mile) coastline of the state of Mississippi alone. The largest of these is the Pascagoula River, which holds the distinction of being the longest un-dammed, natural river remaining in the continental USA and provides habitat for numerous important and endangered salt marsh species (Schueler 2002). Much of this river system remains rela-

tively unimpacted by development, except for the very lower reaches between the towns of Gautier and Pascagoula, MS.

Distributional observations during an outbreak in 2005 by personnel with the Mississippi Department of Marine Resources (DMR) suggest that non-native *S. molesta* was able to survive in salinities of up to 7 parts per thousand (ppt) in the lower Pascagoula River; a similar tolerance has been reported earlier by Divakaran et al. (1980) from growth tests conducted on salinities of 0 to 11 ppt. This has implications for the use of the biological control agent *Cyrtobagous salviniae* on this infestation, as this weevil can only tolerate freshwater conditions (Thomas and Room 1986, Julien et al. 2002). This observation is distressing in two respects: (1) potential for a portion of the non-native *S. molesta* population in the Pascagoula River to escape biological control; and (2) a more salinity-tolerant variety of this species could easily spread into similar habitats that abound along the GOM and elsewhere.

Pulse amplitude modulated (PAM) fluorescence is a tool to measure photophysiological processes *in vivo*. While it cannot be used to directly measure the mechanisms of osmoregulation, it has been used successfully to demonstrate the physiological stress resulting from salinity change in a number of aquatic plant species (Ralph 1998, Kamermans et al. 1999, Murphy et al. 2003, Biber 2006). PAM fluorescence has been used in submerged aquatic plants to measure acute stress, such as desiccation (Adams and Bates 1994, Bjork et al. 1999), temperature or salinity shifts (Ralph et al. 1998, Ralph 1999), and even changes in ambient light over short

time durations (Beer and Bjork 2000, Major and Dunton 2002). However, this technique has not been evaluated in floating aquatic plants subject to salinity stress, and at the time of the early *S. molesta* research in the 1970-80s this tool was not available. This study aims to determine both the efficacy of using a PAM fluorometer on this invasive aquatic plant, as well as to confirm earlier work of Divakaran et al. (1980) on the salinity tolerance of this species. Specifically, the aim of this study was to test the ability of giant salvinia (*S. molesta*) to tolerate salinities of 5 and 10 ppt using the PAM fluorescence technique.

MATERIALS AND METHODS

The responses of non-native *S. molesta* plants (about 7 g dry weight) collected from the Pascagoula River delta (30°25.523' N, 88°34.640' W) were tested in three salinity levels (0, 5, 10 ppt). Plants recovered from field collecting and transport for 10 d prior to starting the experiment by being held in freshwater with adequate light; by this time plants were producing new leaves.

For each salinity level, a glass aquarium was placed under a 60W grow-light and a bank of fluorescent lamps (min 250 $\mu\text{E m}^{-2} \text{s}^{-1}$ PFFD) on a 12:12 hour cycle, in a growth chamber. Temperature was recorded in the 0 ppt and 10 ppt treatments every 15 min for the duration of the experiment by Hobo Tidbit recorders. Salinities were achieved by mixing de-ionized (DI) water with GF/F filtered (0.7 μm) estuarine water of 20 ppt salinity. Water was charcoal filtered and circulated using aquarium filters (Whisper 10i) and allowed to condition for 10 d prior to plants being introduced into the three salinity treatments. Salinities were tested twice weekly using a refractometer and adjusted with DI water to replenish evaporative losses.

Chlorophyll fluorescence of photosystem II (PSII) was measured by the PAM technique. This provides an instantaneous measure of the effective quantum yield ($\Delta\text{F}/\text{F}_m'$) of PS II under prevailing ambient light conditions. In addition, photo-inhibition or quenching was also determined by measuring the potential quantum yield (F_v/F_m) of dark-adapted samples (Genty et al. 1989). Samples can be dark adapted with leaf-clips that are supplied with the PAM instrument. These are attached to a plant leaf and serve to occlude a small area of the leaf and then a shutter built into the clip is opened, exposing the leaf area under the clip to very low intensity red light transmitted through fiber-optics. The chlorophyll in the dark-adapted area of the leaf fluoresces and the initial fluorescence (F_0) is recorded. Upon illumination with a high intensity burst of saturating light through the fiber-optics, the pigments associated with PSII become overwhelmed and the maximal fluorescence (F_m) is recorded. The difference between the maximal and initial fluorescence levels ($\text{F}_m - \text{F}_0$) is called the variable fluorescence (F_v) and from this the ratio F_v/F_m , or potential quantum yield is calculated. In an analogous fashion the

effective quantum yield $\Delta\text{F}/\text{F}_m'$ can be determined on leaf samples that are not dark adapted and are exposed to ambient light.

On the day prior to the salinity stress experiment (day 0), 30 leaves were haphazardly sampled, one per plant, for actual quantum yield ($\Delta\text{F}/\text{F}_m'$) and 6 leaves were sampled for potential quantum yield (F_v/F_m) after a minimum of 5 min dark adaptation time using the leaf clips and fiber optic supplied with the mini-PAM (Walz, Germany). Default instrument settings were used and measurement intensity (gain) was set to 1; these settings were maintained for the duration of the experiment. The population was then separated into three equal portions, determined by blotted wet weight (g).

On the morning of day 1, plants were sequentially introduced into the three experimental salinity treatments and one leaf per plant was selected for analysis. Immediately on immersion into the salinity treatment, 6 leaves were dark-adapted using the clips for F_v/F_m , then $\Delta\text{F}/\text{F}_m'$ measurements were taken on 30 leaves that were not dark adapted. When the measurements on the 30 leaves were complete, the 6 clipped leaves had dark-adapted and were also measured. All 36 measurements typically took less than 15 min to complete.

Every hour after the initial introduction, the cycle of 30 $\Delta\text{F}/\text{F}_m'$ and 6 F_v/F_m measurements were repeated for each of the three salinities on haphazardly selected plants. Hourly PAM measurements were continued for 12 hrs (8 am to 8 pm). The following day, and every day for the next 7 d, PAM measurements were repeated between 11-12 am. After the first week, any plants marked for new leaf growth were checked to count new leaf production. Over the next three weeks, plant fluorescence was measured at least once per week at the same time of day, and weekly observations on new-leaf production were recorded.

Chlorophyll fluorescence variation due to leaf age was tested in the control (0 ppt) mid-way through the experiment (day 16). PAM measurements were made in duplicate on each leaf pair on 15 individuals. Additionally, leaf size, shape, and color for each leaf pair was noted. Leaf age was denoted as immature (small green leaves formed apically), mature (large green leaves distal to the new leaves) and senescent (large leaves with discoloration and loss of turgor). From these data, mean fluorescent yield values by leaf age were determined to better understand within-plant variation.

At the end of the experiment, all plant material was removed from each tank and sorted into newly formed green leaves and original leaves. Five representative leaves were saved from each of the three salinity treatments for chlorophyll analysis using standard methods (Arar 1997). Wet weight (g) of the remaining biomass was recorded after blotting the sample dry. Samples were dried at 60-70 °C and reweighed to determine dry weight (g).

Statistical analysis was done in JMP (SAS Institute, Cary,

N.C.) using either a T-test or ANOVA as appropriate. Prior to the test, data were determined to satisfy the assumptions of normality and homoscedasticity; data transformation was not necessary. Significant results ($p < 0.05$) were followed by a Tukey's honestly significant difference posthoc test, and significantly different means are grouped by superscripts on the figures.

RESULTS

Some variation occurred in the treatment salinities because of evaporation in the growth chamber. The 0 ppt treatment remained in the optimal range of <1 ppt throughout the one month duration of the experiment. The salinity in the 5 ppt treatment ranged from 5-7 ppt and there was 30% less growth compared to the 0 ppt treatment. The salinity of the 10 ppt treatment ranged from 9-12 ppt, representing the upper lethal tolerance.

During day 1, the light adapted yield ($\Delta F/F_m'$) exhibited a slight, but nonsignificant, decline during the first 4 h in the 0 ppt and 5 ppt treatments, with mean fluorescence dropping from 0.736 at the first reading, down to 0.690 and 0.705, respectively, before recovering again (Figure 1a). There was a significant (T-test: $t_{1,8} = 1.86$, $p = 0.005$) drop

in $\Delta F/F_m'$ in the 10 ppt treatment after 4 h, from 0.716 to 0.518, with an increase back to fluorescence values similar to other salinities for the remainder of day 1 (Figure 1a). In contrast, the dark-adapted fluorescence yield, F_v/F_m , ranged between 0.770 and 0.820 for all three salinity treatments with very little change over the 12 h (Figure 1b).

During the remaining four weeks of the experiment, both $\Delta F/F_m'$ and F_v/F_m measurements showed similar responses by salinity treatment, although $\Delta F/F_m'$ was more variable. After day 5, there was a decline in chlorophyll fluorescence across all treatments with F_v/F_m dropping slightly from 0.813 to 0.778 by the end of week 1, and $\Delta F/F_m'$ declining from a mean of 0.738 to 0.712 (0 ppt), 0.732 (5 ppt), and 0.680 (10 ppt). Chlorophyll fluorescence was lower during week 2 than week 1, with $\Delta F/F_m'$ showing greater day-to-day fluctuations than F_v/F_m . At the end of the second week, $\Delta F/F_m'$ in the 10 ppt treatment was lower (0.663) than the other two treatments (Figure 1c). The greatest difference among salinity treatments became evident in the last two weeks of the experiment. In the 10 ppt treatment, both $\Delta F/F_m'$ and F_v/F_m declined substantially to ending values of 0.517 (Figure 1c) and 0.498 (Figure 1d), respectively. In

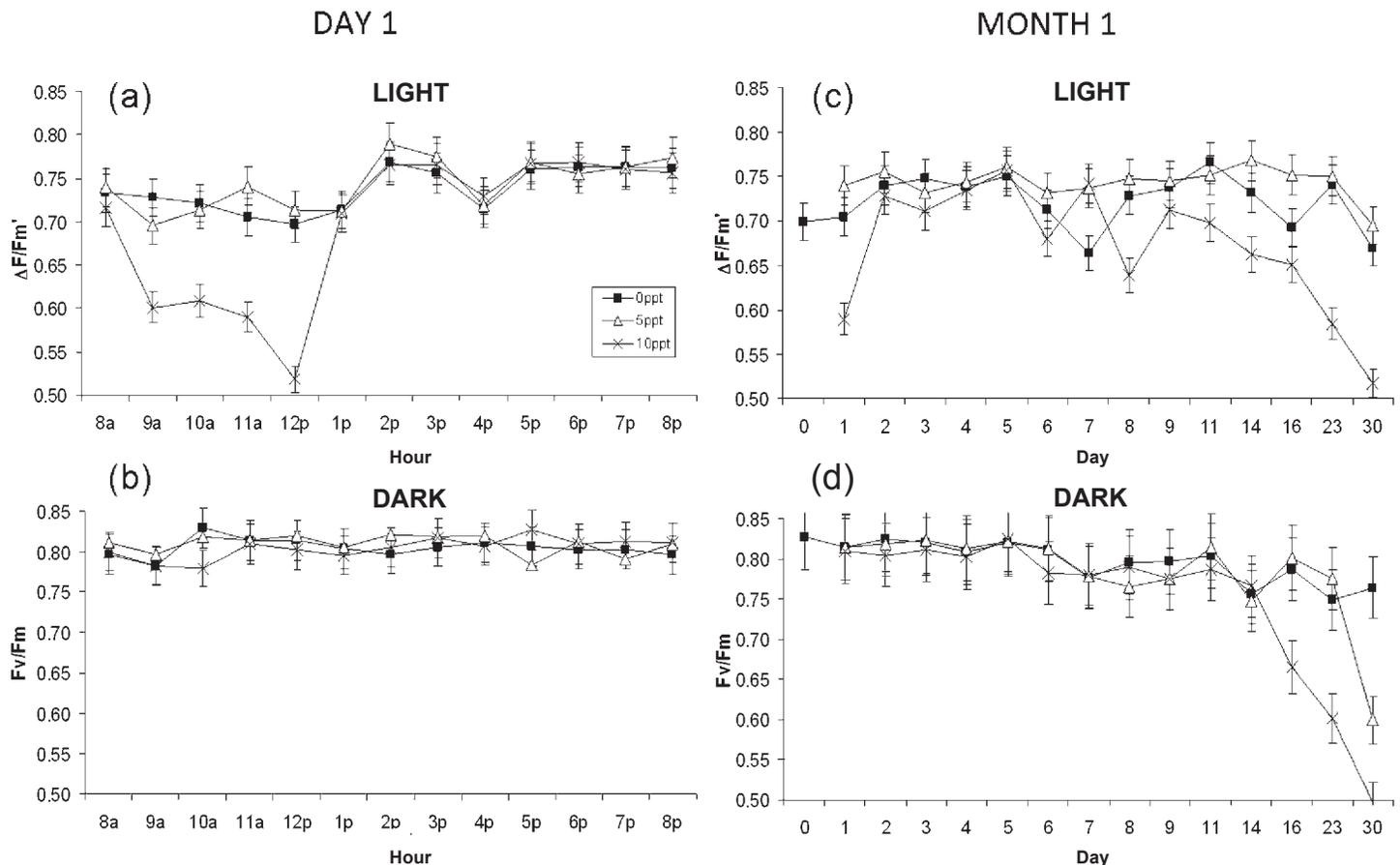


Figure 1. Mean (± 1 se) chlorophyll fluorescence over time in *S. molesta* plants exposed to three different salinities (0, 5, and 10 ppt). (a) Hourly effective quantum yield ($\Delta F/F_m'$) on 30 leaves on day 1 (b) Hourly potential quantum yield (F_v/F_m) from 6 dark-adapted leaves on day 1 (c) Noon-time effective quantum yield ($\Delta F/F_m'$) on 30 leaves from day 1 to day 30 (d) Noon-time potential quantum yield (F_v/F_m) from 6 dark-adapted leaves from day 1 to day 30.

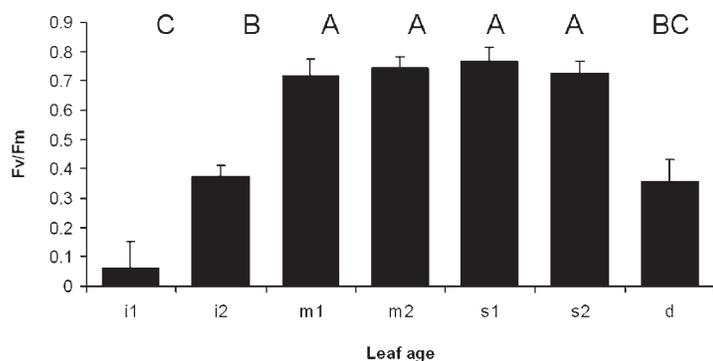


Figure 2. Mean (± 1 se) dark-adapted yields (Fv/Fm) measured on day 16, for leaves of different ages in 0 ppt. For each age, leaves were grouped into a smaller and a larger size class (1 = 0.3-1.0 cm, 2 = 1-3 cm mid-rib length). i1 and i2—immature green leaves, m1 and m2—large mature green leaves, s1 and s2—senescing brown leaves, d—black dead leaves. Superscript letters indicate significantly different means from Tukey's HSD posthoc test.

the 5 ppt plants, Fv/Fm also dropped to 0.599 in the last week of the experiment (Figure 1d). Compared to initial measurements, the final light-adapted yields were 95%, 94% and 71%, while the final dark-adapted yields were 94%, 73% and 61% in the 0, 5, and 10 ppt treatments, respectively.

PAM fluorescence was found to vary significantly by leaf age (one-way ANOVA: $F_{6,47} = 20.21$, $p < 0.001$). Immature and dead leaves had significantly lower Fv/Fm values compared to mature green and senescing leaves; Fv/Fm was ≥ 0.700 in mature and senescing leaves, and < 0.500 in all others (Figure 2). Immature leaves were smaller (< 1 cm) in size than mature leaves (> 1 cm mid-rib length).

Initial biomass in the three treatments was less than 1.63% different, with 1.73 g total dry weight in the 0 ppt, 1.71 g in 5 ppt and 1.74 g in the 10 ppt treatments, respectively (Figure 3a). Final biomass decreased with increasing salinity, and a 40.64% decline was noted in the 10 ppt compared to the 0 ppt treatment. There was 1.78 g total dry weight in the 0 ppt, 1.22 g in 5 ppt and 1.06 g in the 10 ppt treatments, respectively. New leaf growth contributed 0.235, 0.031, and 0 g dry weight in the 3 salinity treatments (Figure 3a). The plants exhibited some withering and decrease in leaf production at 5 ppt, and a gradual but incomplete senescence with no new leaf production at 10 ppt.

Leaf chlorophyll concentration was significantly different among treatments (one-way ANOVA: $F_{3,20} = 49.11$, $p < 0.001$). Plants in the 0 ppt treatment had significantly higher chlorophyll concentrations with 9.69 ± 3.97 $\mu\text{g}/\text{mg}$ in new leaves and 2.83 ± 1.31 $\mu\text{g}/\text{mg}$ in old leaves, compared to only 1.51 ± 0.22 $\mu\text{g}/\text{mg}$ in senescent leaves in the 10 ppt treatment (Figure 3b). The chlorophyll concentrations corresponded with observations of leaf color and tissue integrity among the three treatments.

DISCUSSION

Observations made on the non-native *S. molesta* plants over the course of the experiment indicated that higher salinity caused loss of turgor, pigments, and a reduction in new leaf formation. At the end of the first week, the plants in the 5 ppt treatment showed signs of browning, especially in the older leaves, and had fewer new leaves. In the 10 ppt treatment, the leaves had folded together, most of the green color was lost, and no new leaves were produced. At the end of the experiment, the 0 ppt treatment had substantial new leaf growth with 1-2 new leaf pairs per individual; all leaves were at least partly green in color. The 5 ppt treatment had minimal new leaf growth and the leaves were a darker green with some brown. In the 10 ppt treatment there was no new leaf growth and the remaining leaves were brown to black and had lost turgor.

Health of *S. molesta* has been determined in previous studies by assessing the general appearance of the individuals, pigment content and changes in texture of the leaves, the rate of decay and disintegration, as well as the production of new growth (Divakaren et al. 1980, Finlayson 1984). Since those studies, the technique of chlorophyll fluorescence has become widely adopted as a robust and reliable technique, easy to carry out, non-destructive and rapid (Maxwell and Johnson 2000, Ralph et al. 2007). In general, the greater

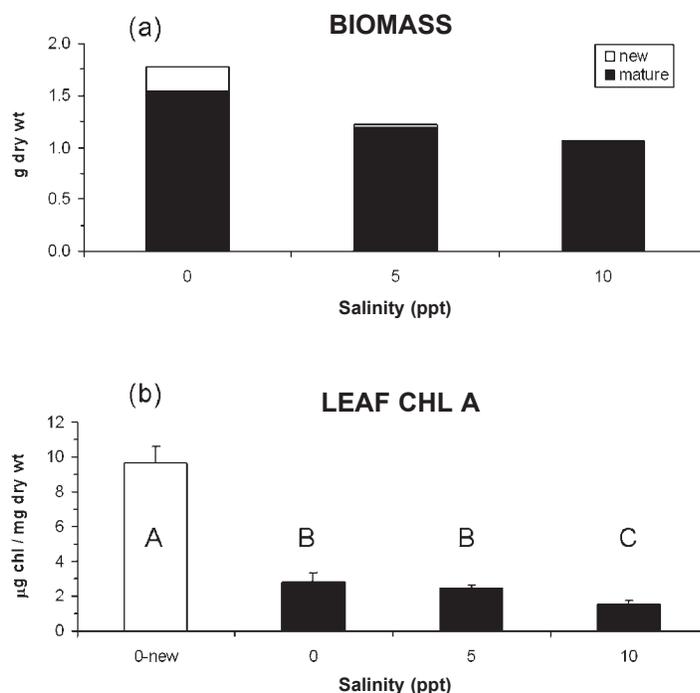


Figure 3. Biomass and chlorophyll content of new (immature) and mature leaves in 3 salinity treatments. (a) Final dry weight of all plant material. Biomass was separated into new immature leaves produced during the experiment, and mature leaves that were present at the beginning. (b) Mean (± 1 se) leaf chlorophyll content of immature leaves (0 ppt only) and mature leaves. Superscript letters indicate significantly different means from Tukey's HSD posthoc test.

the quantum yield of chlorophyll fluorescence as measured using PAM Fv/Fm, the higher the efficiency of the light reactions in photosynthesis, which equates to a plant under low physiological stress. Generally, the maximum possible proportion of the solar energy absorbed into photosynthesis is around 83% (Maxwell and Johnson 2000). Any decline in this ratio (either $\Delta F/F_m'$ or Fv/Fm) indicates a reduction in the efficiency with which light is converted to photosynthetic product and subsequently, growth or reproductive output, and such a decline is often seen when a plant becomes stressed (Krause and Weis 1991, Rohacek and Bartak 1999).

The stressor that was tested in this study was hyperosmotic stress in the non-native aquatic invasive *S. molesta*, which grows optimally in freshwater (Mitchell et al. 1980, Room and Gill 1985). In a previous study (Divakaran et al. 1980), the growth of this species was reduced by 25% at 3.5 ppt and growth was "very slow" at 7 ppt. Salinities above 7 ppt were reported to be unfavorable, with total withering taking place at salinities of 11 ppt and above (Divakaren et al. 1980). At lethal salinities of 11 ppt and above, *S. molesta* becomes more and more spongy and soft in texture. The stem and "roots" shrink and the color of the leaves turns from green to brown (also noted in Divakaren et al. 1980). In this experiment, new leaves grew only in the 0 and 5 ppt treatments, suggesting that one of the responses to increased salinity stress by *S. molesta* is a reduced ability to produce new leaf segments. The implications of this are a reduction in ramet production causing a reduction in the potential number of clonal fragments, leading to reduced population growth over time.

The chlorophyll fluorescence data indicates that there was a decrease in "plant health" in all 3 salinity treatments over the course of the 1 mo trial, but that the decline was more pronounced at 10 ppt than in the other 2 treatments.

Similar sub-lethal responses to salinity have been measured using chlorophyll fluorescence in submerged aquatic (Ralph 1998) and emergent wetland plants (Biber 2006). *Salvinia molesta* may be able to tolerate salinities of 5 ppt and even produce new leaves, but salinities of 10 ppt greatly stress the plants and they are unable to survive after prolonged exposure of more than 3 weeks. This coincides with previous data based on physical appearance and growth rate. These results suggest that *S. molesta* is not able to tolerate elevated salinities > 10 ppt for prolonged periods of time (> 1 mo). However, at salinities around 5 ppt, typical of the lower Pascagoula River where this invasive non-native aquatic was found, these plants did demonstrate the ability to maintain photosynthesis and new leaf growth for at least one month. Additional studies on the long term persistence of this species at salinities present in the lower Pascagoula are warranted to better understand the ability of *S. molesta* to persist in the absence of specific management actions.

These findings are cause for concern, as they indicate that *S. molesta* may be able to persist in the low salinity conditions typical of the upper reaches in many northern GOM estuaries. Further, it could be possible for this invasive non-native aquatic to establish populations in higher salinity conditions than the non-native biological control agent, *Cyrtobagous salviniae*, a freshwater weevil (Thomas and Room 1986, Julien et al. 2002), exacerbating difficulties in controlling *S. molesta* outbreaks. Other control options include herbicide applications, which can also affect native plants, or manual control, which is generally not cost-effective (Pieterse and Murphy 1993). For this reason, the Mississippi DMR commenced a comprehensive eradication program using herbicides, which appears to have been successful at controlling *S. molesta* in this outbreak (Dale Diaz, pers. comm., Mississippi Department of Marine Resources, Biloxi).

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