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Determining the physiological response of a subtropical seagrass, *Thalassia testudinum*, to salinity stress using pulse amplitude modulated (PAM) fluorometry

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

DETERMINING THE PHYSIOLOGICAL RESPONSE OF A
SUBTROPICAL SEAGRASS, *THALASSIA TESTUDINUM*, TO SALINITY STRESS
USING PULSE AMPLITUDE MODULATED (PAM) FLUOROMETRY

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

by

Dorothy A. Byron

2006

To: Interim Dean Mark Szuchman
College of Arts and Sciences

This thesis, written by Dorothy A. Byron, and entitled Determining the Physiological Response of a Subtropical Seagrass, *Thalassia testudinum*, to Salinity Stress using Pulse Amplitude Modulated (PAM) Fluorometry, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Kelsey Downum

Steven Oberbauer

James W. Fourqurean, Major Professor

Date of Defense: November 13, 2006

The thesis of Dorothy A. Byron is approved.

Interim Dean Mark Szuchman
College of Arts and Sciences

Dean George Walker
University Graduate School

Florida International University, 2006

DEDICATION

This thesis is dedicated to my parents, Verna and Charles W. Byron, Jr., and my eldest sister Suzanne Byron for their continual support during my educational career and for instilling in me a great love and appreciation of nature.

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There are many people in which I wish to thank for their help or support during this project. First, I would like to thank my committee, James W. Fourqurean, Kelsey Downum and Steven Oberbauer, for their patience and support. Second I would like to thank the Seagrass Rangers, past and present, in particular, Kevin Cunniff and Leanne Rutten for teaching me how to operate a boat and for keeping the good times plentiful, Sergio (SAS) Ruiz for field assistance and emotional support during the last phase of this degree and last but not least, Virginia (Ginny) Cornett for her field and laboratory assistance and for her friendship. Thank you for keeping me sane.

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This project would not have been possible without funding from the Florida Coastal Everglades Long Term Ecological Research (FCE-LTER) program and the FKNMS Water Quality Protection Program funded by the EPA from which funding was used to purchase the PAM fluorometer.

ABSTRACT OF THE THESIS

DETERMINING THE PHYSIOLOGICAL RESPONSE OF A SUBTROPICAL SEAGRASS, *THALASSIA TESTUDINUM*, TO SALINITY STRESS USING PULSE AMPLITUDE MODULATED (PAM) FLUOROMETRY

by

Dorothy A. Byron

Florida International University, 2006

Miami, Florida

Professor James W. Fourqurean, Major Professor

I investigated how photosynthetic performance of *Thalassia testudinum* changed along a naturally occurring salinity gradient in Florida Bay, and to laboratory controlled hyper and hypo-osmotic stress. I found significant differences between sites in Florida Bay for yield ratios (Y and F_v/F_m); however, this difference does not seem to be based on the salinity regime, since sites with the greatest salinity range were not significantly different from the site with the lowest salinity range. Laboratory results showed declines in the minimum and maximum fluorescence values after a gradual ramping-up of salinity and after long-term exposure to a sustained drop in salinity, but these declines were not seen with the Y and F_v/F_m ratios.

Caution should be used when drawing conclusions about physiological stress from results obtained by PAM fluorometry, as acclimation may play a large role in the fluorescence response, limiting the use of this technique.

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CHAPTER I: Chlorophyll *a* fluorescence as an indicator of the stress response of *Thalassia testudinum* in Florida Bay across a salinity gradient using

A: Introduction

Seagrasses world-wide are in decline, mainly from anthropogenic influences, as coastal development increases. Increased turbidity from poor land use techniques, eutrophication, dredging and increased recreational use have reduced the quantity of light available to seagrasses, and poor watershed management has led to increased pollutants, and altered freshwater inputs to estuaries. Global environmental changes may also add to changes in seagrass distribution (see review by Short, et al., 1999; Orth et al., 2006). When changes in the nearshore environment become extreme from anthropogenic or natural sources, they can have negative consequences for seagrasses as the plants are stressed beyond the limits of their tolerance. The use of physiological parameters to detect environmental stress in seagrasses would be a valuable tool for management of coastal areas, as physiological changes occur before morphological changes are apparent, and observing negative effects early on could allow coastal managers to initiate action plans before large-scale seagrass die-offs occurred.

Various stressors (light, temperature and salinity) may affect seagrass in multiple physiological ways with the same end result: reduced net carbon fixation and eventually reduced growth. Light is required for photosynthesis; however, excessive irradiance can result in a breakdown of the photosystems from oxidative damage if photoprotective pathways, such as the xanthophyll cycle and D1 protein turnover seen with photoinhibition, are exceeded and overwhelmed (Demmig-Adams, et al., 1992; Thiele, et al., 1996). Low light levels can be stressful to seagrass depending on the duration of reduced light availability, as respiration becomes greater than photosynthesis and the net

carbon balance of the plant becomes negative (Fourqurean, et al., 1991; Longstaff, et al., 1999b). Large deviations from optimal temperatures can cause reductions in photosynthesis as many of the biochemical reactions that occur during photosynthesis and respiration are temperature dependent (Bulthuis, 1987). Temperature deviations can also influence light requirements needed to maintain a positive carbon balance, and affect nutrient availability and uptake, and protein synthesis (Bulthuis, 1987). Salinities outside of tolerance ranges can cause a variety of effects on seagrasses, including amino acid content changes (Pulich, 1986), changes in cell and chloroplast morphology (Iyer, et al., 1993), decreases in photosynthetic rate (Biebl, et al., 1971; Hellblom, et al., 1999), and decreases in above and below-ground biomass (Zieman, 1975; Kamermans, et al., 1999).

In addition to light, temperature and salinity stress, many seagrasses grow in severely nutrient limited environments (Fourqurean, et al., 1992a; Short, 1987; see review by Duarte, 1995). Lack of sufficient supplies of nitrogen or phosphorus to allow for optimal performance of cells lead to deviations of elemental ratios away from stoichiometric balance. Such deviations are associated with reduced plant size, abundance and vigor (Fourqurean, et al., 1992a; Fourqurean, et al., 1992b; Short, 1987), and it is likely that severe shortages of essential elements are stressful to the photosynthetic performance of seagrasses like that seen with terrestrial plants (Conroy, et al., 1986; Demmig-Adams, et al., 1992; Huang, et al., 2004; Lima, et al., 1999).

Chlorophyll content and chlorophyll *a* to *b* ratios have previously been measured in relation to light and temperature gradients with various species having similar responses. As with terrestrial plants, several species of submerged marine and freshwater macrophytes increase their total chlorophyll content in response to reduced light levels (Barko, et al., 1983; Longstaff, et al., 1999a; Longstaff, et al., 1999b; McMillan, et al.,

1979; Wiginton, et al., 1979), yet the relationship between light and chlorophyll *a:b* is variable and inconsistent between species or even within species growing in different locations (Barko, et al., 1983; Wiginton, et al., 1979). Both Winginton and McMillan (1979) and Macauley et al. (1988) found no relationship between chlorophyll *a:b* ratios and light availability for the seagrass *Thalassia testudinum* but, Macauley et al. (1988) did find a significant negative correlation between chlorophyll *a:b* ratios and temperature.

Few studies have investigated the relationship between salinity and chlorophyll content and chlorophyll *a:b* ratios. McMillan and Moseley (1967) found that the chlorophyll content of *T. testudinum* kept at higher salinities had a lower chlorophyll content than those kept at constant salinity (28-31 ppt). Ralph (1998) found that chlorophyll *a* content decreased at both low and high salinity extremes for *Halophila ovalis*, and this reduction caused the decline in the chlorophyll *a:b* ratio as the chlorophyll *b* content remain relatively unchanged across treatments (Ralph, 1998a). No significant correlations were found between chlorophyll and salinity for *T. testudinum* by Macauley (1988), however, the range of salinities studied (24 -35 ppt) were within the range of tolerance for *T. testudinum*. It has been shown, however, that photosynthetic activity of *Zostera marina* (Biebl, et al., 1971; Hellblom, et al., 1999) and *T. testudinum* (Hammer, 1968 as cited in Zieman, 1975) were inhibited by decreases in salinity to near freshwater conditions, and that growth of *T. testudinum* was reduced when salinity was low (between 13-15ppt, Zieman, 1975) or high (greater than 60 ppt, McMillan, et al., 1967).

Recent laboratory and field research has turned to pulse amplitude modulated (PAM) fluorometry to detect deleterious conditions on seagrass physiology that would occur before morphological changes could be detected. The amount and timing of

fluorescence can be used as an indicator of the function of Photosystem II because as light energy is harvested by chlorophyll it can follow five possible pathways to return to the stable ground state: 1) it can be used to drive photochemical work; 2) it can be given off as heat (thermal deactivation); 3) it can be emitted as a photon of light (fluorescence); 4) it can be transfer between chlorophyll molecules (resonance); and 5) it can fall from the second excited singlet state to a excited triplet state via thermal deactivation which can then fall to the ground state resulting in phosphorescence (Luttge, 1997; Nobel, 1999). Each pathway has a characteristic time in which it will occur. Within 10^{-15} to 10^{-9} seconds after a photon of light is absorbed, its energy is passed to a reaction center, with resonance transfers taking about 1 or 2×10^{-12} seconds. From the reaction center, it takes 10^{-7} to 10^{-4} seconds for photochemical pathways to be started (Nobel, 1999). In comparison, fluorescence of chlorophyll *a* takes approximately 10^{-9} to 10^{-6} seconds (Nobel, 1999). Therefore, for photochemical work to be done, it has to occur before the energy can be dissipated by fluorescence. If the photochemical pathways are obstructed or damaged due to stress causing a delay in the processing of the energy, any further energy harvested by the excitation of chlorophyll molecules will be dissipated through one of the other pathways, mainly heat or fluorescence. Larger measures of fluorescence would indicate that the photosynthetic apparatus was experiencing stress.

PAM fluorometry measures the fluorescence of the sample immediately before and after a saturating pulse of light is delivered to the sample giving minimum and maximum fluorescence values, respectively (Ralph, et al., 2005; Schreiber, et al., 1993). Minimum and maximum values may vary depending on preliminary treatment of the sample. If the measurements are taken after a period of dark-adjustment, which allows the reaction center chlorophyll molecules to be oxidized (i.e. the chlorophyll *a* reaction

centers are open and available to harvest light energy), the minimum fluorescence values are termed F_o and the maximum fluorescence values are termed F_m . These values are then used to calculate photosystem II (PSII) photochemical efficiency (F_v/F_m), where $F_v/F_m = (F_m - F_o) / F_m$, which provides insight to the potential photosynthetic capacity of the leaf sample. If the sample has been previously illuminated for a period of time and non-radiative dissipation processes (e.g. thermal deactivation) may be occurring, the maximum fluorescence values, now termed F_m' , may be lower than from the dark-adjusted samples, since another energy dissipation process is active. With previously illuminated samples, the minimum fluorescence value, now termed F , may be higher than the dark-adjusted sample as some chlorophyll reaction centers are reduced and some are oxidized. These values are used to calculate quantum yield (Y), where $Y = (F_m' - F) / F_m'$. Quantum yield measurements can be equated to the percentage of light that is being used to do photochemical work given the previous light history (in units of minutes to hours) of the leaf. Thus, it is expected that Y will be less than F_v/F_m in normal conditions, but that both measurements will be lower for stressed versus unstressed plants, with the Y values of stressed plants being very low as the plant attempts to dissipate as much excessive energy as possible before severe damage occurs to the photosynthetic apparatus.

Peter Ralph's extensive work on the seagrass *Halophila ovalis* has shown negative quantum yield and PSII photochemical efficiency responses to high light (Ralph, 1999b; Ralph, et al., 1995) but no significant response to low light (Ralph, 1999b). Thermal stress (heating and chilling) (Ralph, 1998b) and hyper-osmotic and hypo-osmotic conditions (Ralph, 1998a) have also showed negative Y and F_v/F_m responses. The interaction between the degree of intensity and the length of exposure was significant in

all of the aforementioned experiments, except the low light experiment (Ralph, 1999b), and greater decreases were seen in the fluorescence response with longer exposure times. The combination of light, temperature and salinity stresses resulted in declines in Y and F_v/F_m for all stress combinations, with thermal stress appearing to cause the most damage, followed by hyper-osmotic and then hypo-osmotic, and finally light stress; however, the combination of the stresses was additive in that they caused greater decline than each individual stress alone (Ralph, 1999a). It is important to note that the exposures times used in the aforementioned experiments lasted at most 96 hrs, and this can be considered a short time span as many stresses can last days to weeks to months.

While laboratory work has focused on the response of fluorescence to stressful conditions, most field work on the influence of stress on fluorescence in seagrasses has focused on methodological considerations such as within shoot and within leaf variability (Durako, et al., 2002; Enriquez, et al., 2002). In shallow marine environments, temperature, salinity, and water clarity leading to reduced light level may all interact in additive ways to intensify a stressful condition (Ralph, 1999a). Embayments that have salinity or water quality gradients can provide natural treatments, but there have been only a limited number of field studies looking at changes in chlorophyll fluorescence across environmental gradients (e.g. Campbell, et al., 2003). This may be due to the difficulties in teasing apart environmental variables that may be dependent on one another, making results difficult to interpret.

Florida Bay is a shallow estuarine system located at the southern tip of Florida, bounded to the east and south by the Florida Keys and to the west by the Gulf of Mexico. Divided into numerous basins due to the criss-cross carbonate mud bank system which restricts circulation, Florida Bay has numerous water quality gradients (salinity, nutrients,

light availability/chlorophyll, etc) which provide natural treatments for study on different response parameters (Fourqurean, et al., 1993). On average, the waters of Florida Bay are about 1 m in depth. This results in the water temperatures being more susceptible to atmospheric temperature changes than deeper offshore waters. During winter months as cold fronts become more frequent and severe, water temperature may drop rapidly in these shallow waters compared to the deeper coastal waters along the Florida Keys Reef tract. These larger temperature fluctuations may be stressful for the marine plants that reside there. The heterogeneity of the water quality parameters, the shallow nature of Florida Bay and the large variation seen in salinity both across time and space (Nuttle, et al., 2000), may result in many areas being stressful for submerged marine macrophytes (i.e. seagrass).

Florida Bay can be divided into three to four zones based on similar water quality parameters: the eastern region with mean salinity values lower than oceanic values, but large salinity variations (both hyper and hypo) on a temporal scale; the central zone which experiences periodic and persistent hypersaline conditions and also sees large temporal variation; and the south and west zones which have salinities similar to that found along the coastal waters and experience less extreme salinity variation due to direct contact with the Gulf of Mexico (McIvor, et al., 1994; Fourqurean, et al., 1999; Nuttle, et al., 2000). The east and central zones with their lower and higher mean salinity values as well as their large salinity variations may, at times, be stressful to the benthic plant communities that occur there. In addition to this northeast to southwest salinity gradient there is also a nutrient gradient, with phosphorus availability decreasing towards the north and east (Fourqurean, et al., 1992a). The focus of this study is to observe how seagrass fluorescence responses may change across a natural gradient of salinity stress.

Consequently, due to the length of the study, the seasonal temperature responses of fluorescence measurements were also explored.

To determine the feasibility of using PAM fluorometry to detect stressful conditions in Florida Bay, I measured the chlorophyll *a* fluorescence of *Thalassia testudinum* at fixed sites located across a salinity gradient (mean and standard deviation) and a temperature gradient, with the following expectations: higher fluorescence values (thus lower Y and F_v/F_m values) should be measured at sites with lower mean salinities and at times with the greatest fluctuations in salinity; all sites would show a seasonal shift in fluorescence during the colder months; although there is also a phosphorus gradient across Florida Bay, four of the five sites have similar nitrogen to phosphorus ratios (Fourqurean, et al., 1992a), thus, the P deficiency should be an additive but equivalent stress on those sites and would not interfere with the salinity gradient response.

B: Materials and Methods:

Site characteristics:

Five sites throughout Florida Bay were chosen using the established Florida Coastal Everglades Long Term Ecological Research (FCE- LTER) sites. The five sites, Little Madiera Bay (near TS/Ph-7), Trout Cove (near TS/PH-8), Duck Key(TS/PH-9), Bob Allen Keys (TS/PH-10) and Sprigger Bank (TS/PH-11) were chosen based on their salinity history and distance from Taylor slough (Figure 1), the only freshwater input into Northeastern Florida Bay other than rainfall. Extreme salinity variations have been measured across the interior of Florida Bay, with fluctuations of salinity above 50 ppt occurring across much of the bay about every 10 years from the 1950s to the present (Fourqurean, et al., 1999). During the five year period immediately preceding our study, salinity was variable across Florida Bay, but the bay as a whole did not experience

historically high salinities. Salinity records obtained from Everglades National Park, dating back to 1998, indicate that Little Madiera Bay and Trout Cove are similar with regards to salinity with the range varying between 0 and 35 ppt. Duck Key and Bob Allen Keys had salinity values ranging between 15 and 36 ppt and 25 and 44 ppt, respectively. Sprigger Bank was chosen due to its relative stability, with salinity values only ranging between 30 and 40 ppt.

On each sampling date, 10 *T. testudinum* shoots were randomly chosen for chlorophyll fluorescence measurements, so that in theory, no shoot was measured twice. Biweekly measurements were taken to increase the chance of observing a quick salinity change; however, when biweekly measurements were not possible, each site was visited at least once a month. Measurements were taken for approximately one year so that any seasonal effects, if any, on chlorophyll fluorescence could be observed. The leaves used for the first five PAM measurements were clipped at the base and brought back to the laboratory for analysis of photosynthetic pigments (chlorophyll *a* and *b*). *Thalassia testudinum* above-ground tissue samples were also collected for nutrient analysis (carbon, nitrogen, and phosphorus) as part of regular monitoring for the FCE-LTER monitoring program and to further distinguish any differences between sites. Measurements of salinity, temperature, and light (PAR and turbidity) were also taken at each site.

Chlorophyll Fluorescence Measurements:

Chlorophyll fluorescence parameters were measured with a portable underwater Pulse Amplitude Modulated (PAM) fluorometer (Walz, Germany) using SCUBA. Dark leaf clips were used to keep the distance between the sample and the fiber optics at a uniform distance (10mm). All measurements were taken on the adaxial surface of the second youngest leaf at the middle portion of the leaf. Quantum yield measurements and

F_v/F_m measurements were taken on the same leaf in the same position, with F_v/F_m measurements occurring after a 10 minute dark adaption period determined adequate for relaxation of the chlorophyll *a* reaction center based on preliminary work (Byron, unpublished data). Minimum and maximum fluorescence values are measured as volts, however, as Y and F_v/F_m are ratio values, there are no units associated with these measures.

Photosynthetic pigment analysis:

At each site, the first five leaves utilized for fluorescence measurements were removed at the base of the leaf, placed in a dark plastic bag and returned to the laboratory for chlorophyll analysis. Within 24 hours of collection, leaves were scraped free of epiphytes, cut with a razor blade to a standard length of 40 mm, and rinsed with deionized water. Each leaf segment was then wrapped in an aluminum foil packet and freeze dried for a minimum of 12 hours. The samples were then ground with a mortar and pestle to increase surface area during chlorophyll extraction, and placed in pre-weighed 7 mL scintillation vials. The vials and sample were weighed so that the chlorophyll values could be standardized to weight (g) as well as area (cm²). Following a modification of the technique utilized by Dennison (1990), chlorophyll *a* and chlorophyll *b* were extracted by adding 5mL of 90% acetone to the samples, and placing the vials in a -20°C freezer to keep the samples cold and dark during the extraction. After 24 hours, the extracts were analyzed using a Shimadzu RF-Mini 150 filter fluorometer using a 440 nm excitation filter and 670 nm emission filter for chlorophyll *a* and 460 nm excitation filter and 650 nm emission filter for chlorophyll *b* (see Welschmeyer, 1994). The relative fluorescence measurements for each pigment were then compared to a standard curve produced from pure chlorophyll *a* and chlorophyll *b* spinach extracts following the

methodology of Jeffrey and Humphrey (1975) and chlorophyll *a* and chlorophyll *b* concentrations (μM) were calculated.

Nutrient analysis:

Seven *Thalassia testudinum* shoots were collected at each site during each date for nutrient analysis. Photosynthetic tissues were scraped free of epiphytes, dried at 70°C , and ground to a fine powder using a mortar and pestle and placed in a 7mL scintillation vial for storage. Using 4-7 mg of sample, duplicated C and N content analysis (wt/wt) was conducted using a Fison NA 1500 elemental analyzer. Phosphorus content (wt/wt) was determined colorimetrically after acid-hydrolysis following the methods of Fourqurean et al. (1992) using 17 – 21 mg of dried and ground sample. Nutrient ratios were also calculated on a mol:mol basis for C, N and P.

Statistics:

To test for intersite differences in temperature, turbidity, amount of PAR reaching the canopy level, and nutrient concentrations the data were pooled across date and compared with a one-way ANOVA. Post hoc analyses were conducted using Tukey's Multiple Comparison test on significant tests to determine which sites differed. The fluorescence data (Y , F_v/F_m , minimum and maximum fluorescence) were also pooled across date and one-way ANOVAs were used to determine if there were significant differences along the mean salinity gradient. Significant tests were further analyzed with Tukey's Multiple Comparison test to determine if the sites grouped in accordance with the mean salinity gradient. In order to avoid confusion between the reported fluorescence parameters (F_o , F_m , F , and F_m') and the values of the F statistic associated with ANOVA, all reported F statistics will be represented by an uppercase italics "*F*".

Correlation matrices were analyzed to determine if and how the physical data (salinity, salinity deviation from the 5 year (1998-2002) site-specific salinity mean, temperature, turbidity, and PAR) related to the fluorescence data. The same statistical analysis that was carried out for the fluorescence data was also conducted for the chlorophyll pigment (chlorophyll *a*, chlorophyll *b*, total chlorophyll, and chlorophyll *a:b*) data.

All data were tested for normality and homogeneity of variances. If the raw data failed one or both these tests, transformations were performed. If the transformed data violated one of the assumptions, parametric statistical analyses were performed on the raw data, as ANOVA tests on large samples sizes are robust to violations of the assumptions. However, if the transformation failed both normality and homogeneity of variance, the equivalent nonparametric statistical analysis was used. All statistical analysis was done using the SAS 9.1 statistical package.

C: Results:

Site characteristics:

Temperature varied seasonally but was not significantly different among sites, with the lowest temperatures recorded from December 2002 through February 2003 (Figure 2). Neither turbidity nor the fraction of incoming PAR reaching the canopy were significantly different among the five sites, and no seasonal pattern was detected. Turbidity ranged from 0.07 to 11.55 NTU, but most of the measured values were between 0 and 4 NTU (Figure 3). PAR measured reaching the canopy ranged from 53 $\mu\text{E m}^{-2} \text{s}^{-1}$ to over 2400 $\mu\text{E m}^{-2} \text{s}^{-1}$ (Figure 4), with the minimum values of PAR most likely the result of overcast days rather than high attenuation in the water column; however, turbidity and

PAR reaching the bottom were significantly, negatively correlated (Spearman correlation coefficient = -0.30260, $p = 0.0128$).

Mean salinity during the study period was significantly different among sites and varied from 16 ppt to 35 ppt, with the lowest salinity occurring in the northeastern portion of the bay at Little Madiera and Trout Cove, followed by Duck Key and then Bob Allen Keys and Sprigger Bank (Figure 5a). These values were similar to the mean salinity calculated from a five year average from 1998 – 2002 (Table 1). Little Madiera Bay and Trout Cove saw the greatest range of salinities with deviations from the 5 year mean of approximately -15 ppt during the end of the wet season from July to November 2002 (Figure 5b). During the dry season, from December to May, these sites showed salinity deviations of +5 ppt to +10 ppt (Little Madiera Bay and Trout Cove, respectively). Duck Key, Bob Allen and Sprigger Bank showed smaller deviations, with salinities ranging approximately ± 5 ppt from the five year mean.

The carbon content (%C) of *Thalassia testudinum* had similar values at all five sites (Table 2), but both %N and %P were significantly different among sites. There was not an obvious gradient with the nitrogen content (%N), but the phosphorus content (%P) grouped out along a northeast to southwest gradient. Unexpectedly, Little Madiera and Trout Cove had higher concentrations of phosphorus than did Duck Key and Bob Allen Keys (Figure 6). C:P and N:P ratios followed the same grouping trend as the %P, with Duck Key and Bob Allen Keys having similar ranges, Little Madiera Bay and Trout Cove having similar ranges and Sprigger Bank separated from both groups (Figure 7). Carbon content, for all sites, ranged from 32.2% to 42.2%, N content ranged from 1.8% to 2.9%, and P content ranged from 0.038 to 0.191%, equaling a five fold difference between minimum and maximum recorded values for %P.

Neither %C, %N or %P were significantly correlated to the site salinities measured during this study, and %C and %P were not significantly related to the deviation of salinity from the site-specific 5 year mean. However, nitrogen content was significantly, positively correlated with the deviation of salinity from the site-specific 5 year mean (Spearman correlation coefficient = 0.25144, $p = 0.0264$)(Table 3). Both %N and %P were significantly, negatively correlated with temperature ($p = 0.0031$ and $p = 0.0208$, respectively), while %C was less strongly negatively correlated with temperature ($p = 0.0613$).

Chlorophyll Fluorescence Measurements:

Across all sites Y varied between a minimum of 0.238 and a maximum of 0.817 and F_v/F_m varied between 0.267 and 0.819, with both showing the lowest values at the end of January 2003. This corresponds with the lowest temperatures recorded during the study period (Figure 8). Y and F_v/F_m , when averaged across date, were both significantly different among sites ($F_{4, 825} = 30.11$, $p < 0.0001$; $F_{4, 823} = 4.17$, $p = 0.0024$, respectively); however, the lowest mean fluorescence values did not follow the salinity gradient. Post Hoc analysis showed that the effective quantum yield at Bob Allen Keys was significantly lower than at the other sites, with a mean value of 0.548 (Figure 9). There were less obvious significant differences among sites for the F_v/F_m ratio; however, Bob Allen Keys again had a slightly lower value. The F_v/F_m ratio at Bob Allen Keys was 0.681, which was significantly lower than the other sites, except at Duck Key and Trout Cove whose mean F_v/F_m ratios were 0.712 and 0.723, respectively (Figure 9). Most importantly, the sites with the lowest mean salinities and the greatest salinity fluctuations (Little Madiera Bay and Trout Cove) were not significantly different from the site that

had near optimum salinity known for *T. testudinum*, and the least fluctuation from that mean (Sprigger Bank).

Correlation analysis indicated that temperature, measure site salinity, deviation of site salinity from the site-specific 5 year mean, and PAR reaching the canopy were all significantly correlated with both Y and F_v/F_m (Table 4). All salinity variables were significantly, negatively correlated to the yield ratios, as was the amount of PAR reaching the canopy. Temperature was significantly, positively correlated to both response variables.

Further examination of the fluorescence parameters revealed that there were significant site differences for minimum fluorescence for both the illuminated (F) and dark-adjusted leaves (F_o) when these data were pooled across date; however, again the sites did not group along the mean salinity gradient (Figure 10). F was significantly higher at Duck Key ($F = 195.66$ V) than at the other sites, while F_o was significantly higher at Bob Allen Keys ($F_o = 227.31$ V). Maximum fluorescence, when pooled across date, was significantly different for the illuminated samples ($F_{4,575} = 18.52$, $p < 0.0001$), while the dark adjusted samples were not significantly different ($F_{4,573} = 2.09$, $p = 0.0809$). Bob Allen Keys had the lowest F_m' value of 411.67 V, which explains the lower Y ratio was due to the lower F_m' value and not a higher F . However, the opposite occurred for the F_v/F_m ratio, as a higher F_o value was the cause for the lower F_v/F_m ratio as F_m was not significantly different across sites.

Correlation analysis indicated that temperature was positively correlated with F , F_m' , F_o and F_m (Table 5), with lower fluorescence values observed during times of lower temperature (December to February), and maximum fluorescence (F_m' and F_m) having a more obvious response than the respective minimum fluorescence values (Figure 11).

Turbidity and PAR reaching the canopy were both negatively correlated with the minimum and maximum fluorescence values. Unlike the Y and F_v/F_m ratios, site salinity and the deviation of site salinity from the site-specific 5 year mean did not exhibit the same response across all fluorescence variables. The deviation of site salinity from the site-specific 5 year mean was not significantly correlated to F_o , but was significantly, positively correlated to F . F_m was not significantly correlated to the deviation of site salinity from the site-specific 5 year mean, while F_m' was significantly, negatively correlated ($p = 0.09$). The salinity measured at during the study was significantly, positively correlated to both F and F_o , but significantly negatively correlated to F_m' (Table 5).

Photosynthetic pigments:

Total chlorophyll content ranged across sites from 0.83 to 1.24 $\mu\text{g}/\text{cm}^2$, with chlorophyll *a* constituting approximately 70 to 80 % of the total chlorophyll content. Chlorophyll *a:b* ratios did not vary as greatly, with the sites ranging from 2.48 to 2.66. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll were significantly different among sites and were grouped in accordance with the mean salinity gradient, while chlorophyll *a:b* was not significantly different between sites (Table 6; Figure 12). Over time, measurements generally were divided into two groups: December to February/March and March/April to June (Figure 13). The December to February/March group had higher chlorophyll *a* and chlorophyll *b*, and a lower chlorophyll *a:b* ratio than the March/April to June group. Chlorophyll *b* concentration was always lower than chlorophyll *a* but the increase in the chlorophyll *a:b* ratio that occurred in April 2003 was due to a larger decline of chlorophyll *a* than chlorophyll *b* (Figure 13).

Correlation analysis confirmed our general grouping as chlorophyll *a* and chlorophyll *b* were both significantly, negatively correlated with temperature, whereas the chlorophyll *a:b* ratio was significantly, positively correlated (Table 7). Chlorophyll content (chlorophyll *a*, chlorophyll *b*, and total chlorophyll) was significantly, positively correlated to the salinity measured during the study, but the chlorophyll *a:b* ratio remained unchanged with increasing salinity. Chlorophyll content increased with increasing turbidity, but chlorophyll *b* increased at a greater rate due to the weak negative correlation between chlorophyll *a:b* ratio and turbidity.

D: Discussion:

Variations in salinity away from marine conditions have often been cited as stressors for seagrasses (e.g. Biebl, et al., 1971; Hellblom, et al., 1999; Iyer, et al., 1993; Kamermans, et al., 1999; Ralph, 1998a; Short, et al., 1999; Zieman, 1975). In particular, *Thalassia testudinum* is considered a relatively stenohaline plant that displays reductions in vigor and growth when salinity varies from constant marine conditions (Zieman, 1975). Although PAM fluorometry has been shown to be a sensitive indicator of stress in vascular plants in general, and seagrasses in particular (Campbell, et al., 2003; Dawson, et al., 1996; Kamermans, et al., 1999; Longstaff, et al., 1999b; Ralph, 1998a; b; 1999a; Ralph, 1999b; Ralph, 2000; Ralph, et al., 1995; Ralph, et al., 1998; Schwarz, et al., 2000), I found only weak relationships between salinity and fluorescence of *T. testudinum* across a marked salinity gradient in Florida Bay. Despite very large differences in mean salinity across sites, and also in the deviation in salinity from the site-specific mean across sites, especially at the beginning of our observations, I did not see large differences in the fluorescence measurements. Even if the plants had acclimated to the salinity regime at each site, I would have expected to see some reduction in

fluorescence measurements at Little Madiera Bay and Trout Cove locations during times when the deviation from the mean salinity was negative and large (over 15 ppt difference); however, a reduction was not evident.

In contrast to the weak effects of salinity on fluorescence, Y and F_v/F_m values declined when the temperature dropped below 20°C, indicating a strong temperature dependent seasonal response. Light availability may play a role in the decreased values as all sites were visited during the course of one day, yet the order and thus the time of visit was not consistent between visits and I did not find significant difference in the average light received at each site across the whole of the observational period.

There was a correlation between salinity measured during the study and the deviation of site salinity from the site-specific 5 year mean with the Y and F_v/F_m values; however, caution must be exercised in drawing conclusions about these relationships as they may be artifacts from the negative correlation of both salinity measures with temperature. Further analysis of the individual fluorescence parameters response indicated that the negative response in Y and F_v/F_m to increasing mean salinity were most likely due to the maximum fluorescence values, as both F_m' and F_m were also negatively correlated with mean salinity, and F and F_o were not. This agrees with Ralph's (1998) findings that maximum fluorescence values for *Halophila ovalis* in both hypo and hyper osmotic treatments were the cause of the reduction in Y and F_v/F_m measurements. The decrease in F_m' and F_m after the application of a stressful condition may be attributed to closing of chlorophyll *a* reaction centers as a photoprotection mechanism, whereas the increase in minimum fluorescence values may indicate photodamage (Campbell, et al., 2003; Ralph, et al., 1995). Perhaps the closure of the PSII reaction centers was related to structural changes of the chloroplast in response to a stressful salinity condition.

However, further conclusions regarding large salinity fluctuations on the individual parameters cannot be made because technical problems with the fiber optics of the fluorometer only allow for comparisons of data after October 2002, after the greatest salinity fluctuations occurred at Little Madiera Bay and Trout Cove.

The lower temperatures observed during this study had a significant negative impact on all of the fluorescence measurements. Previous research has demonstrated that *T. testudinum* experiences lower production in winter months (Fourqurean, et al., 2001; Zieman, 1975), so some degree of seasonality was expected, as growth is dependent on carbon fixation which is dependent on ATP and NADPH formation from the light reactions of photosynthesis. Since the enzymes and proteins used to transfer energy along the electron transport chain to make ATP and NADPH during photosynthesis have optimal temperatures ranges in which they function, large deviations from these ranges can result in suboptimal function or deactivation due to conformational changes. Ralph (1998) found similar results with *Halophila ovalis* in response to heating and chilling conditions, concluding again that the decline in the fluorescence parameters is related to the closure of PSII reaction centers.

As PAM fluorometry measures the fluorescence of chlorophyll *a*, it would be expected that the values of minimum and maximum fluorescence would be related to the chlorophyll *a* content the plant. Previous studies have shown a complementary relationship between fluorescence responses and chlorophyll responses (Ralph, 1998a; 1999a; Ralph, 1999b; Ralph, 2000), yet such a relationship was not seen in this study. Significant differences in chlorophyll *a* content among sites were seen across the salinity gradient in Florida Bay; however, the fluorescence responses (minimum and maximum fluorescences values) did not mimic this response. Our findings also suggest that even

though Little Madiera Bay and Trout Cove had the lowest chlorophyll *a* concentrations, the remaining chlorophyll *a* reaction centers at those sites were still as efficient in processing light energy as those found in the leaves of *T. testudinum* at Sprigger Bank, which had the highest chlorophyll *a* concentration. Further research is required to determine if plants with lower, but efficient, chlorophyll *a* concentrations can maintain the positive carbon balance that is required for growth.

Caution is required when drawing conclusions from studies that use natural environmental gradients as treatments, as one or more variables may be necessary to explain changes in photosynthetic parameters (e.g., light availability affecting chlorophyll ratios; nutrient availability affecting protein and enzyme synthesis needed for photosystem repair). In addition, consideration must be given to the time span at which changes occur (minutes to hours or day to weeks and months), as it has been shown that the interaction between the intensity of the stress and the duration of the stress is a significant factor in explaining magnitude of fluorescence changes (Ralph, 1998a; Ralph, et al., 1995). Fourqurean et al. (2003) provided a statistical model based on water quality and seagrass monitoring data, which predicts that as the salinity regime changes as more freshwater enters the system and salinities remain at lower levels for a longer time, the areas currently dominated by *Thalassia testudinum* would shift to *Ruppia maritima* and *Halodule wrightii* dominated habitats, since these two species have greater salinity tolerances (Fourqurean, et al., 2003). The question then becomes “How intense and for what duration would the salinity change have to be for the species shift to occur?”

Even though PAM fluorometry allows for a quick, nondestructive, *in situ* way to measure the photosynthetic efficiency of marine plants, caution should be employed when drawing conclusions from this method when observations are complicated by one or

more environmental variables that interact. More laboratory investigations are needed to determine each variable's individual effect on fluorescence responses, as well as experiments that investigate the importance of combined effects before this method would be appropriate for use as a monitoring tool.

Table 1: Temperature and salinity averaged over a 5 year period (1998-2002) for each of the study site locations. Minimum, maximum and median values are also presented. Data courtesy of Everglades National Park.

	Little Madiera Bay	Trout Cove	Duck Key	Bob Allen Keys	Peterson Keys (for Sprigger Bank)
Temperature (°C):					
Mean of 5 years	26.35	26.46	26.20	26.13	26.17
Minimum	17.81	18.09	20.96	10.64	17.62
Maximum	32.22	31.63	32.20	31.84	32.15
Median	26.37	26.63	26.13	26.42	26.47
Salinity (ppt):					
Mean of 5 years	20.80	16.85	27.03	33.50	34.98
Minimum	8.06	1.12	8.59	23.7	28.58
Maximum	37.73	36.3	37.21	43.63	40.89
Median	20.19	16.13	27.09	32.60	35.49

Table 2: Mean nutrient content (wt/wt) and ratios (mol:mol) \pm 1 SD for *Thalassia testudinum* collected at the five study sites with ANOVA results. Lettering indicate sites that are significantly different as determined by Tukey's Multiple Comparison analysis. * indicates that non-parametric equivalent test (Kruskal-Wallis) was used for statistical analysis.

Site	%C	%N	%P	C:N	C:P	N:P
Little Madiera Bay	40.02 \pm 1.36	2.29 \pm 0.21 abc	0.073 \pm 0.009 a	20.52 \pm 1.36 ac	1444.18 \pm 0200.06 a	70.62 \pm 10.09 a
Trout Cove	40.23 \pm 1.39	2.46 \pm 0.21 c	0.095 \pm 0.028 a	19.23 \pm 1.64 bc	1194.09 \pm 372.75 a	61.92 \pm 17.94 a
Duck Key	38.92 \pm 2.18	2.20 \pm 0.20 ab	0.053 \pm 0.007 b	20.71 \pm 1.40 ab	1940.95 \pm 306.06 b	93.65 \pm 12.38 b
Bob Allen Keys	38.80 \pm 2.49	2.38 \pm 0.24 bc	0.056 \pm 0.011 b	19.19 \pm 1.68 bc	1859.27 \pm 323.73 b	96.69 \pm 12.90 b
Sprigger Bank	39.23 \pm 2.28	2.09 \pm 0.20 a	0.154 \pm 0.020 c	22.06 \pm 2.14 a	671.02 \pm 106.69 c	30.41 \pm 3.57 c
ANOVA results						
F or χ^2	1.46	7.39	62.91*	8.41	60.51*	62.15*
df	4, 75	4, 75	4	4, 75	4	4
p	0.2224	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 3: Spearman's correlation matrix for nutrient content and environmental variables. Salinity deviation equals the deviation of the site salinity from the 5 year (1998–2002) salinity mean. Significant correlations are indicated by bold text. Values above the diagonal represent N values for pairwise comparisons.

	Salinity	Salinity deviation	Temperature	Turbidity	PAR	C	N	P	CN	CP	NP
Salinity											
Salinity deviation	78										
Temperature	0.26107 0.0210	73									
Turbidity	0.04010 0.7362	0.06234 0.6003									
PAR	-0.09084 0.4321	0.02558 0.8253	-0.32561 0.0049								
C	0.06284 0.6134	-0.03420 0.7835	0.01683 0.8967	-0.30260 0.0128							
N	-0.04798 0.6766	0.17189 0.1324	-0.21860 0.0613	0.05975 0.6009	-0.28634 0.0179						
P	-0.15178 0.1847	0.25144 0.0264	-0.33967 0.0031	-0.04582 0.6885	0.01825 0.8826	0.48701 <.0001					
CN	0.18701 0.1011	0.09953 0.3859	-0.26841 0.0208	0.47185 <.0001	-0.06302 0.6097	0.09495 0.4022	-0.02996 0.7919				

Table 3: continued.

	Salinity	Salinity deviation	Temperature	Turbidity	PAR	C	N	P	CN	CP	NP
CN	0.10153 0.3764	-0.21161 0.0629	0.19006 0.1048	0.10024 0.3794	-0.18508 0.1308	-0.04816 0.6714	-0.84865 0.09824	0.11620 0.3047		80	80
CP	-0.16976 0.1373	-0.06510 0.5712	0.22245 0.0568	-0.46705 <.0001	0.03649 0.7677	0.03583 0.7524	0.3860	-0.98170 <.0001	-0.10146 0.3705		80
NP	-0.12643 0.2700	-0.01190 0.9176	0.18101 0.1227	-0.47411 <.0001	0.05974 0.6284	0.05328 0.6388	0.25495 0.0225	-0.95818 <.0001	-0.29586 0.0077	0.96828 <.0001	

Table 4: Spearman's correlation matrix for fluorescence yield measurements (Y and F_v/F_m) and environmental variables. Significant correlations are indicated by bold text. Significant correlations are indicated by bold text. Values above the diagonal represent N values for pairwise comparisons.

	Salinity	Salinity Deviation	Temperature	Turbidity	PAR	Y	F_v/F_m
Salinity		820	770	820	691	820	818
Salinity Deviation	0.43680 <.0001		770	820	691	820	818
Temperature	-0.20616 <.0001	-0.25616 <.0001		780	641	780	778
Turbidity	-0.14892 <.0001	-0.08955 0.0103	-0.09691 0.0068		691	830	828
PAR	0.03698 0.3317	-0.05229 0.1698	0.08634 0.0288	-0.30907 <.0001		691	689
Y	-0.26115 <.0001	-0.13618 <.0001	0.47749 <.0001	-0.02647 0.4464	-0.10819 0.0044		828
F_v/F_m	-0.14639 <.0001	-0.09659 0.0057	0.32915 <.0001	0.03043 0.3819	-0.09322 0.0144	0.76172 <.0001	

Table 5: Spearman's correlation matrix for individual fluorescence parameters, minimum and maximum fluorescence values of illuminated and dark-adjusted leaf samples, and environmental variables. Significant correlations are indicated by bold text. Values above the diagonal represent N values for pairwise comparisons.

	Salinity	Salinity Deviation	Temperature	Turbidity	PAR	F	F _m '	F _o	F _m
Salinity		570	520	570	500	570	570	568	568
Salinity Deviation	-0.00095 0.9820		520	570	500	570	570	568	568
Temperature	-0.02439 0.5789	0.32103 <.0001		530	450	530	530	528	528
Turbidity	-0.02308 0.5824	0.07851 0.0611	-0.31750 <.0001		500	580	580	578	578
PAR	0.08054 0.0720	0.01750 0.6962	-0.19636 <.0001	-0.28878 <.0001		500	500	498	498
F	0.10133 0.0155	0.10339 0.0135	0.39218 <.0001	-0.14917 0.0003	-0.21497 <.0001		580	578	578
F _m '	-0.13790 0.0010	0.07098 0.0905	0.48942 <.0001	-0.18571 <.0001	-0.28100 <.0001	0.79399 <.0001		578	578
F _o	0.21040 <.0001	-0.03059 0.4669	0.36986 <.0001	-0.14346 0.0005	-0.11504 0.0102	0.56151 <.0001	0.33908 <.0001		578
F _m	-0.04937 0.2401	-0.03869 0.3574	0.49637 <.0001	-0.10798 0.0094	-0.25355 <.0001	0.66128 <.0001	0.73190 <.0001	0.64442 <.0001	1.000

Table 6: Chlorophyll concentration ($\mu\text{g cm}^{-2}$) \pm 1 SD for *T. testudinum* collected at the five study sites with ANOVA results. Lettering indicate sites that are significantly different as determined by Tukey's Multiple Comparison analysis.

Site	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Chlorophyll a:b
Little Madiera Bay	0.59 \pm 0.19 a	0.23 \pm 0.07 a	0.83 \pm 0.26 a	2.57 \pm 0.43
Trout Cove	0.70 \pm 0.31 ab	0.27 \pm 0.13 ab	0.97 \pm 0.43 ab	2.66 \pm 0.46
Duck Key	0.80 \pm 0.26 bc	0.32 \pm 0.10 bc	1.12 \pm 0.35 bc	2.51 \pm 0.39
Bob Allen Keys	0.88 \pm 0.26 c	0.36 \pm 0.12 c	1.24 \pm 0.37 c	2.48 \pm 0.29
Sprigger Bank	0.85 \pm 0.31 c	0.35 \pm 0.16 c	1.20 \pm 0.47 c	2.62 \pm 0.40
ANOVA results				
<i>F</i>	10.21	9.55	10.29	1.90
df	4, 265	4, 265	4, 265	4, 265
p	<0.001	<0.001	<0.0001	0.1108

Table 7: Spearman's correlation matrix for photosynthetic pigment content (chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), total chlorophyll (total chl), and chlorophyll *a:b* ratio (chl *a:b*)) and environmental variables. Significant correlations are indicated by bold text. Values above the diagonal represent *N* values for pairwise comparisons.

	Salinity	Salinity Deviation	Temperature	Turbidity	PAR	chl <i>a</i>	chl <i>b</i>	total chl	chl <i>a:b</i>
Salinity		265	265	260	230	265	265	265	265
Salinity Deviation	-0.20660 0.0007		265	260	230	265	265	265	265
Temperature	0.00492 0.9365	0.25646 <.0001		265	230	270	270	270	270
Turbidity	0.00613 0.9217	0.06810 0.2740	-0.35574 <.0001		225	265	265	265	265
PAR	0.12719 0.0541	0.11428 0.0838	-0.26669 <.0001	-0.19955 0.0026		230	230	230	230
chl <i>a</i>	0.28418 <.0001	-0.16381 0.0075	-0.13071 0.0318	0.16199 0.0082	0.02659 0.6884		270	270	270
chl <i>b</i>	0.25399 <.0001	-0.22528 0.0002	-0.31009 <.0001	0.18667 0.0023	0.12868 0.0513	0.92934 <.0001		270	270
total chl	0.27895 <.0001	-0.18574 0.0024	-0.19008 0.0017	0.17438 0.0044	0.06169 0.3517	0.99238 <.0001	0.96624 <.0001		270
chl <i>a:b</i>	-0.01672 0.7865	0.25467 <.0001	0.52585 <.0001	-0.10671 0.0830	-0.26918 <.0001	-0.21935 0.0003	-0.53677 <.0001	-0.32222 <.0001	

Figure 1: Map of the southern Everglades and Florida Bay. Sites used in the Florida Coastal Everglades- Long Term Ecological Research study are indicated by a (●). Site located in the boundaries of Florida Bay were used for this study.

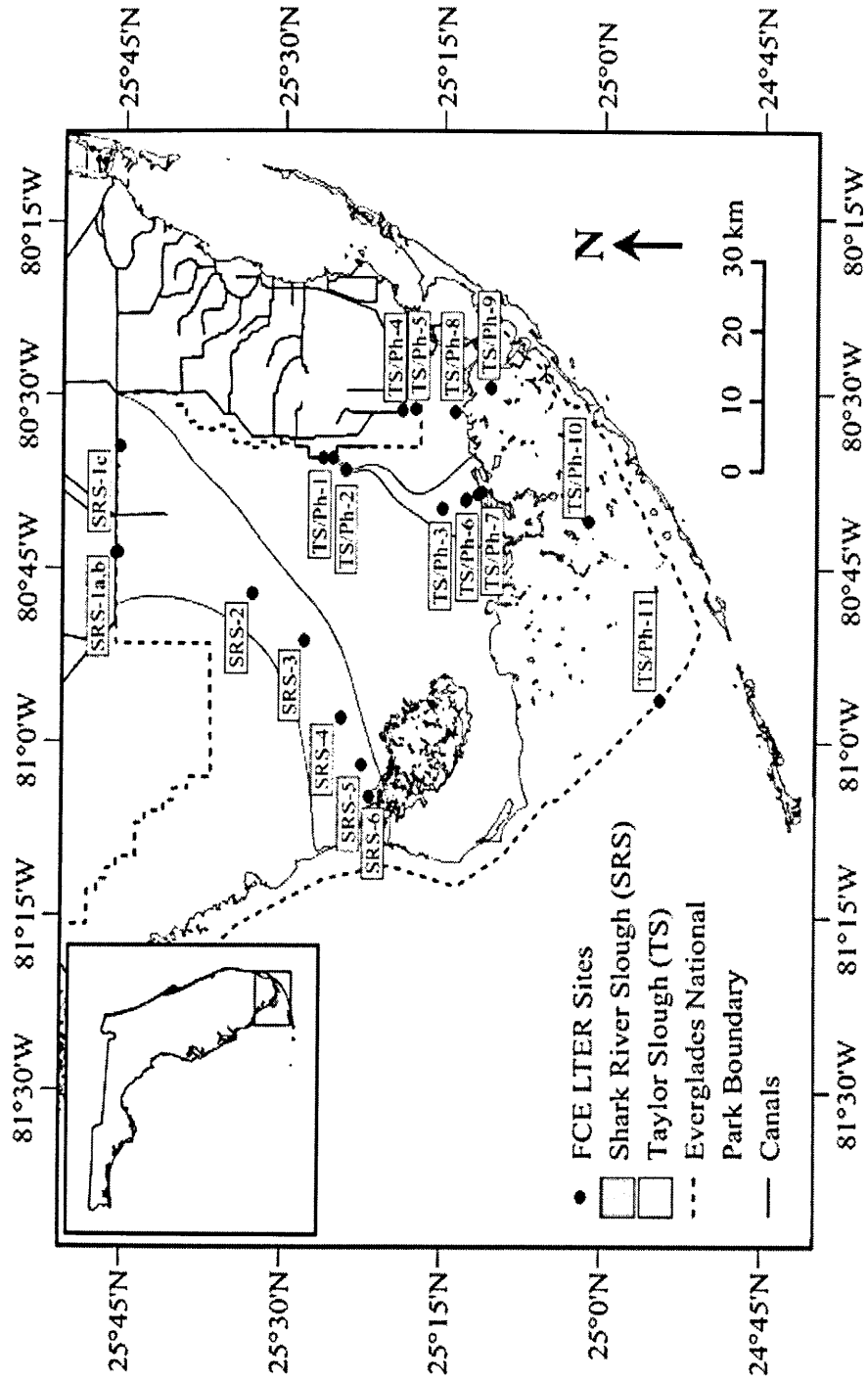


Figure 2: Temperature ($^{\circ}\text{C}$) values recorded during the study period at the five study sites. Symbols are as follows: Little Madiera (\bullet), Trout Cove (\circ), Duck Key (\blacksquare), Bob Allen Keys (\square), and Sprigger Bank (\blacktriangle).

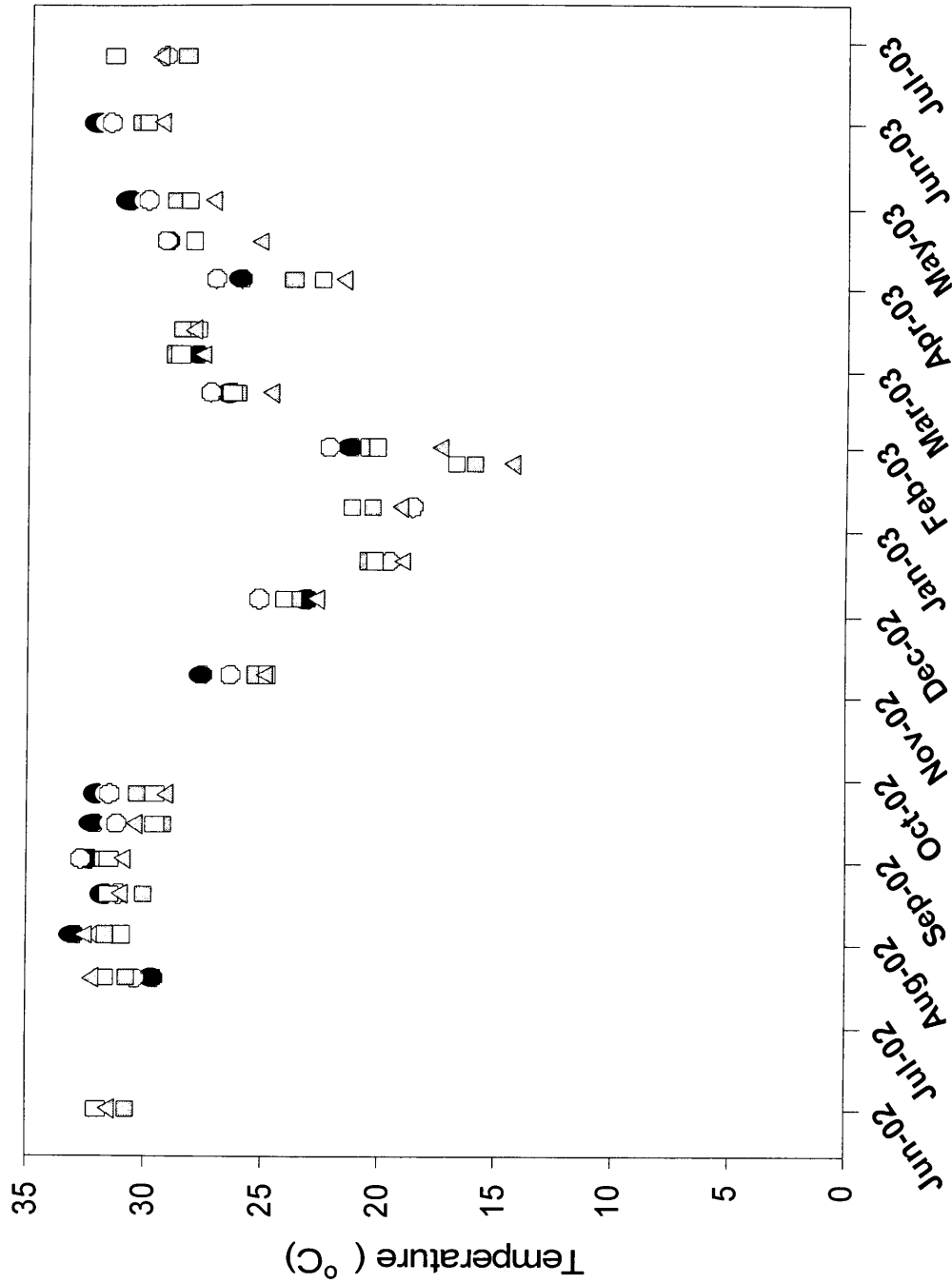


Figure 3: Turbidity (NTU) values measured during the study period at the five study sites. Symbols are as follows: Little Madiera (●), Trout Cove (○), Duck Key (■), Bob Allen Keys (□), and Sprigger Bank (▲).

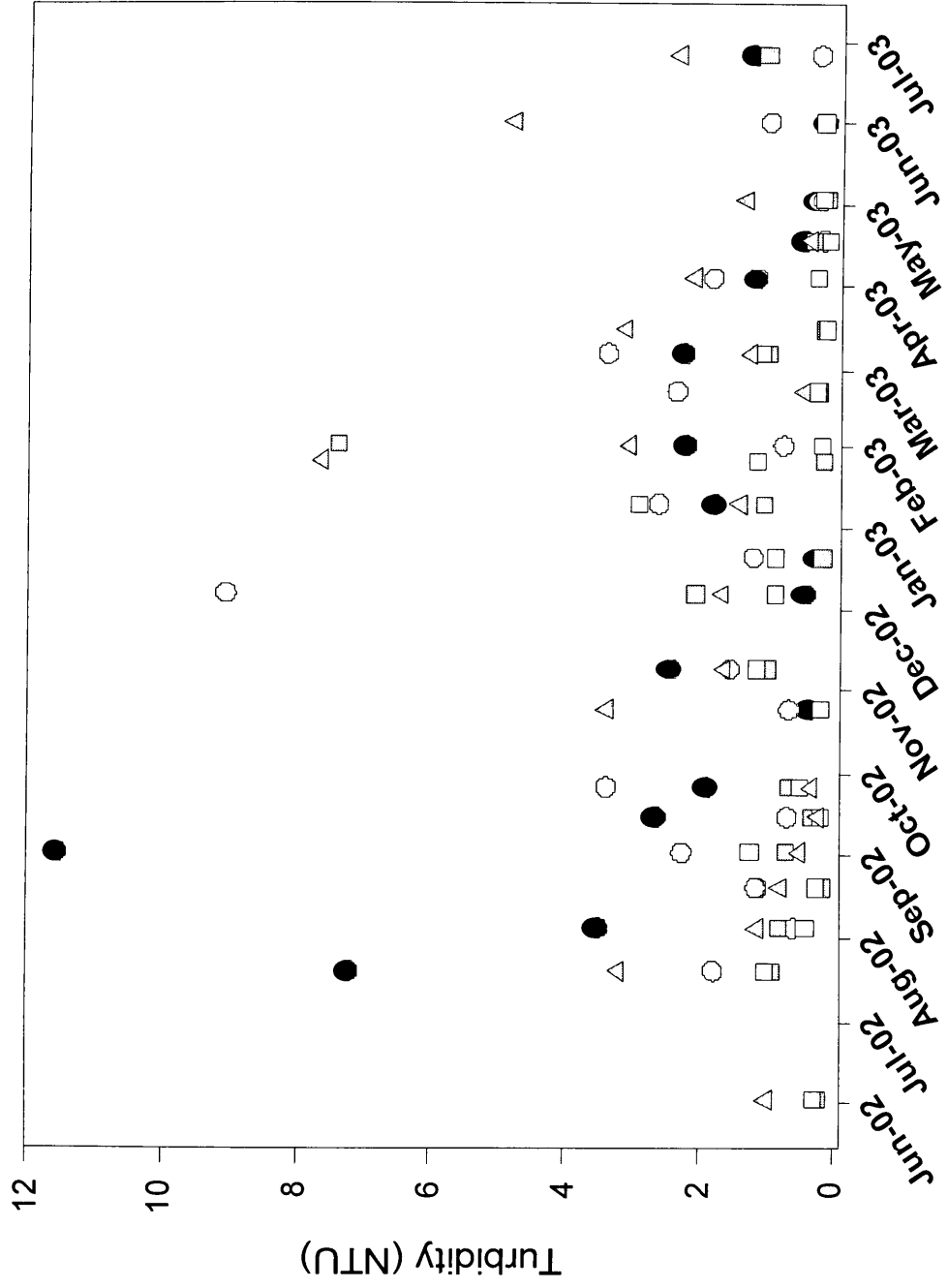


Figure 4: Photosynthetically active radiation ($\mu\text{E m}^{-2} \text{s}^{-1}$) reaching the canopy at each of the five sites during each of the sampling activities. Symbols are as follows: Little Madiera (●), Trout Cove (○), Duck Key (■), Bob Allen Keys (□), and Sprigger Bank (▲).

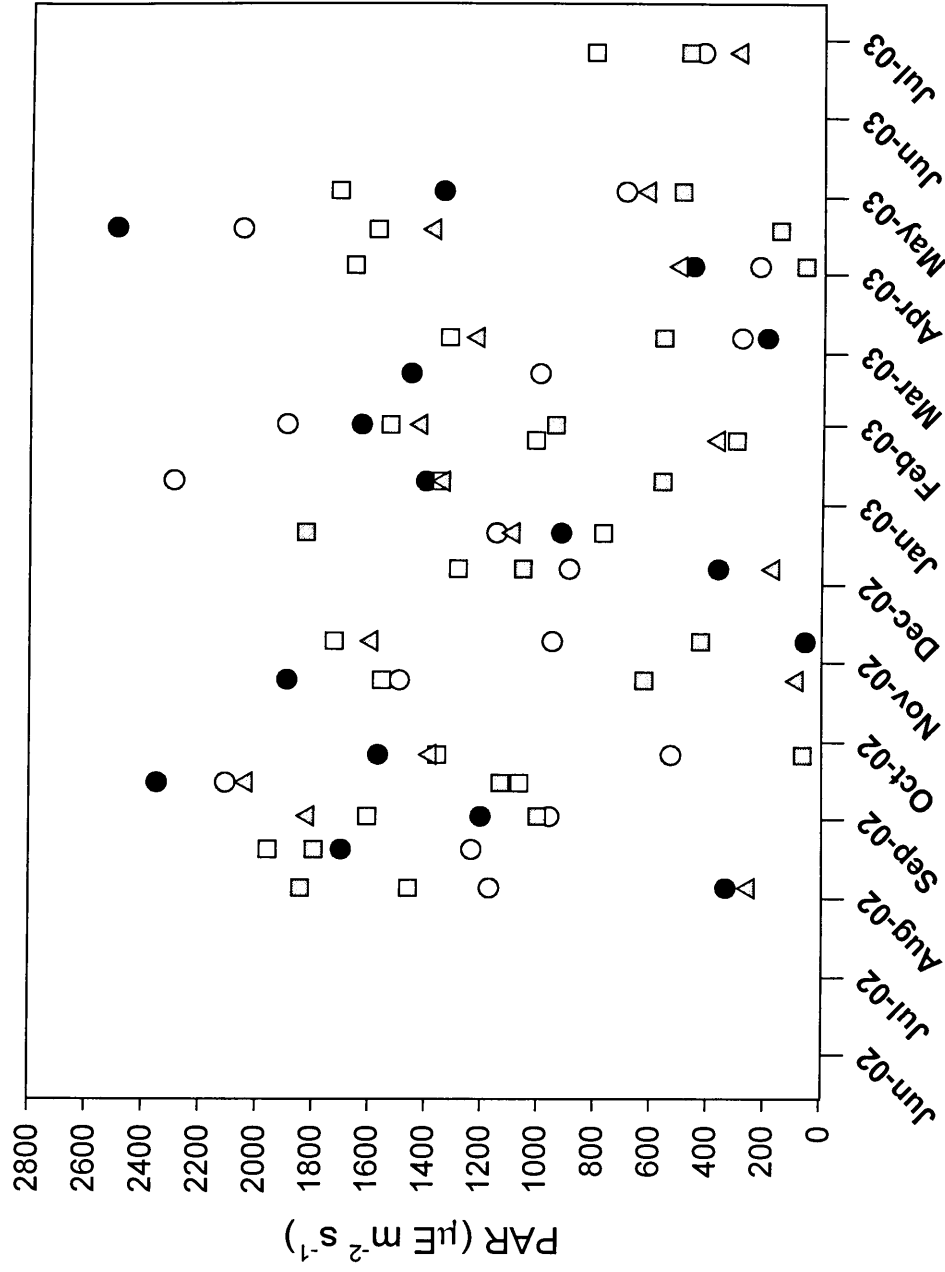


Figure 5a: Mean salinity at each site averaged across the duration of the observations. Error bars are ± 1 SD.

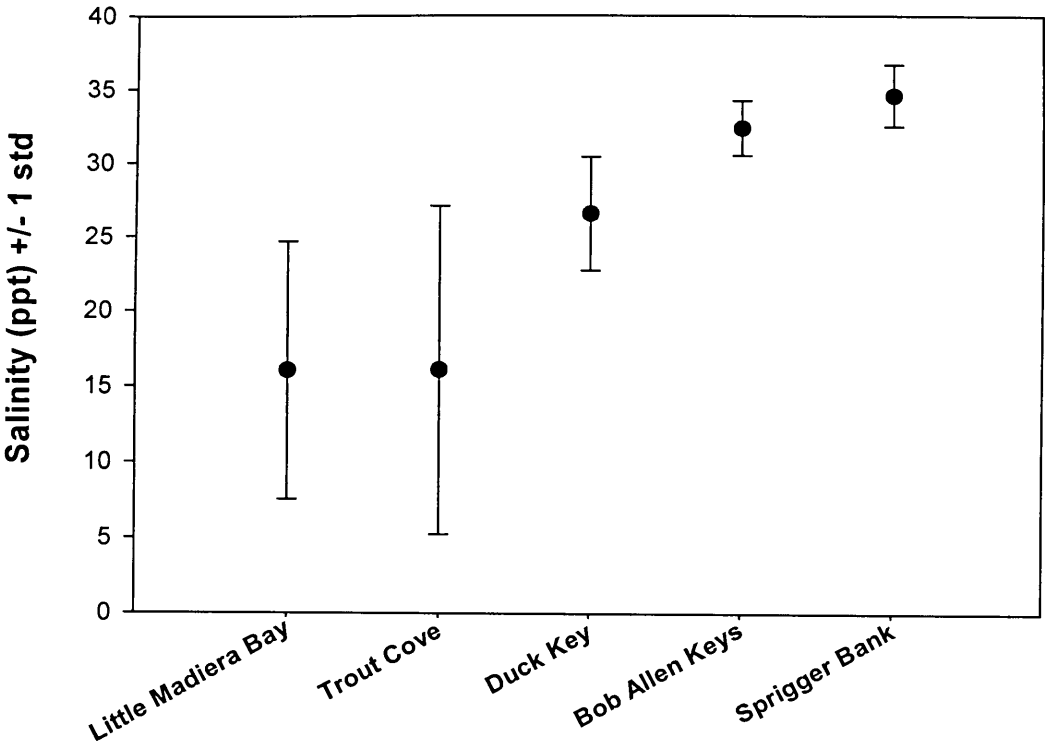


Figure 5b: Salinity deviation from the five year mean over the course of the study. Symbols are as follows: Little Madiera (●), Trout Cove (○), Duck Key (■), Bob Allen Keys (□), and Sprigger Bank (▲).

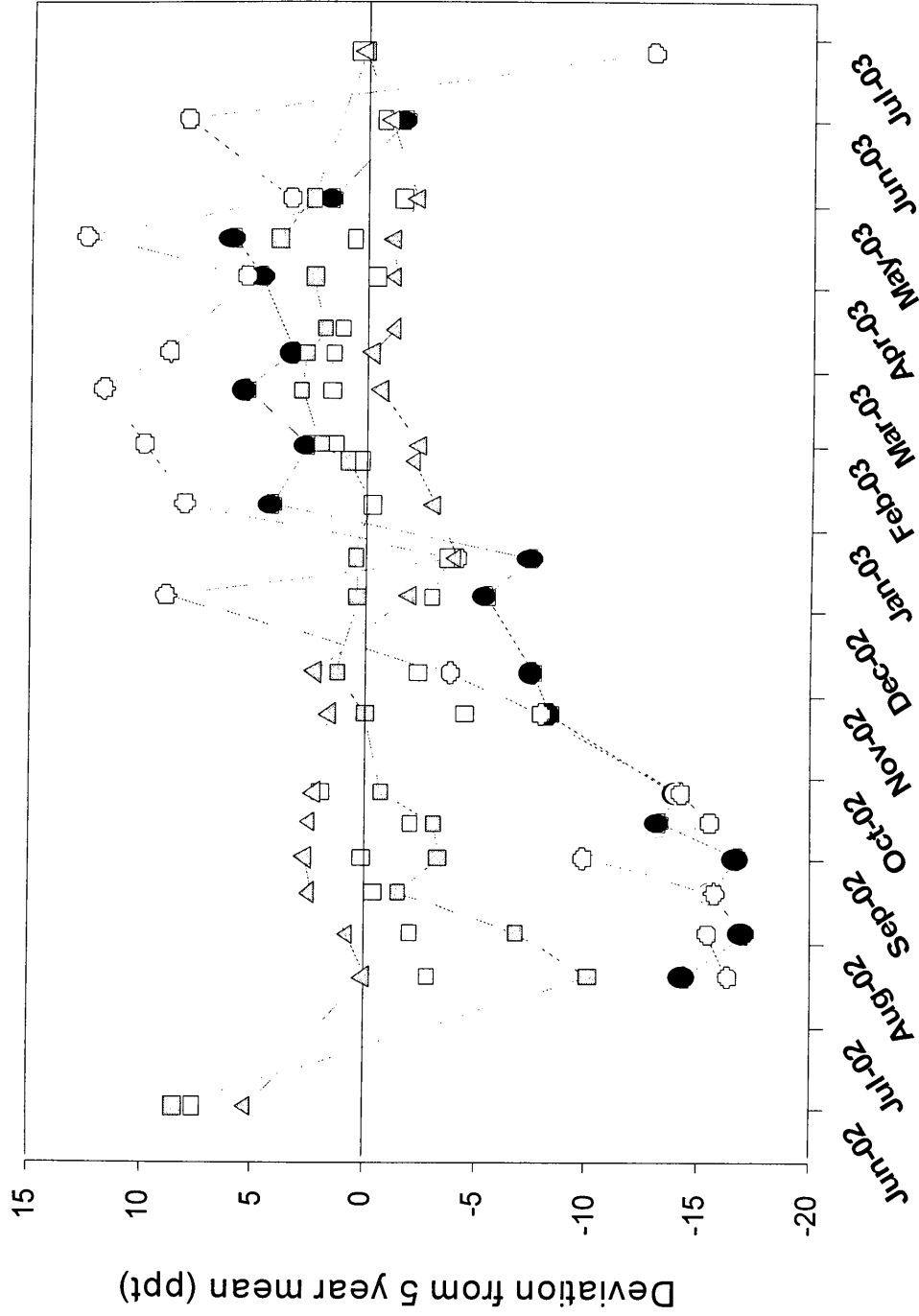


Figure 6: Mean phosphorus content (wt/wt) at each site. Error bars are ± 1 SE (n=13 for Little Madiera Bay and Trout Cove; n=18 for Duck Key, Bob Allen Keys and Sprigger Bank).

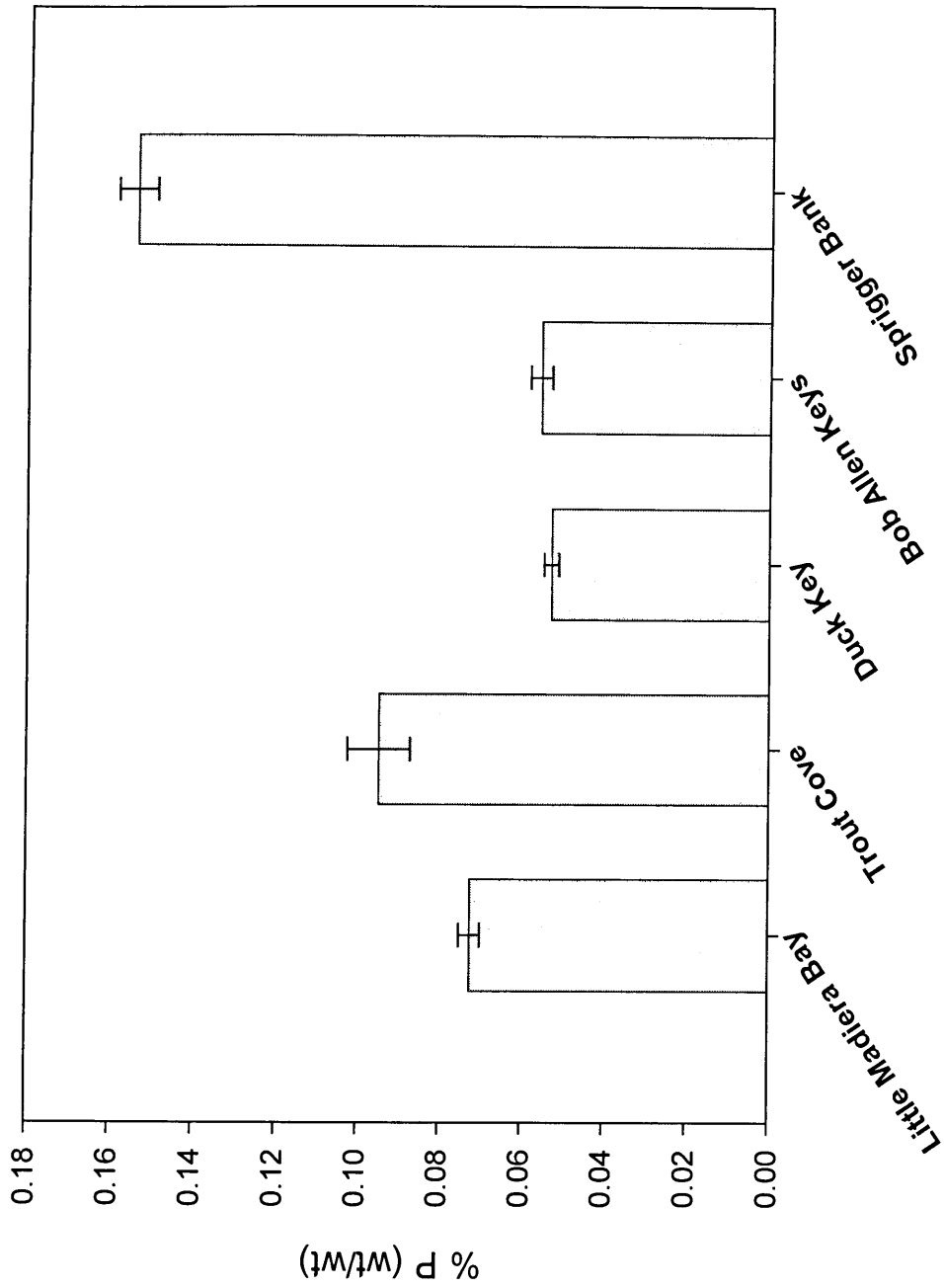


Figure 7: Mean nutrient ratios (mol:mol) at each site. Error bars are ± 1 SE (n=13 for Little Madiera Bay and Trout Cove; n=18 for Duck Key, Bob Allen Keys and Sprigger Bank).

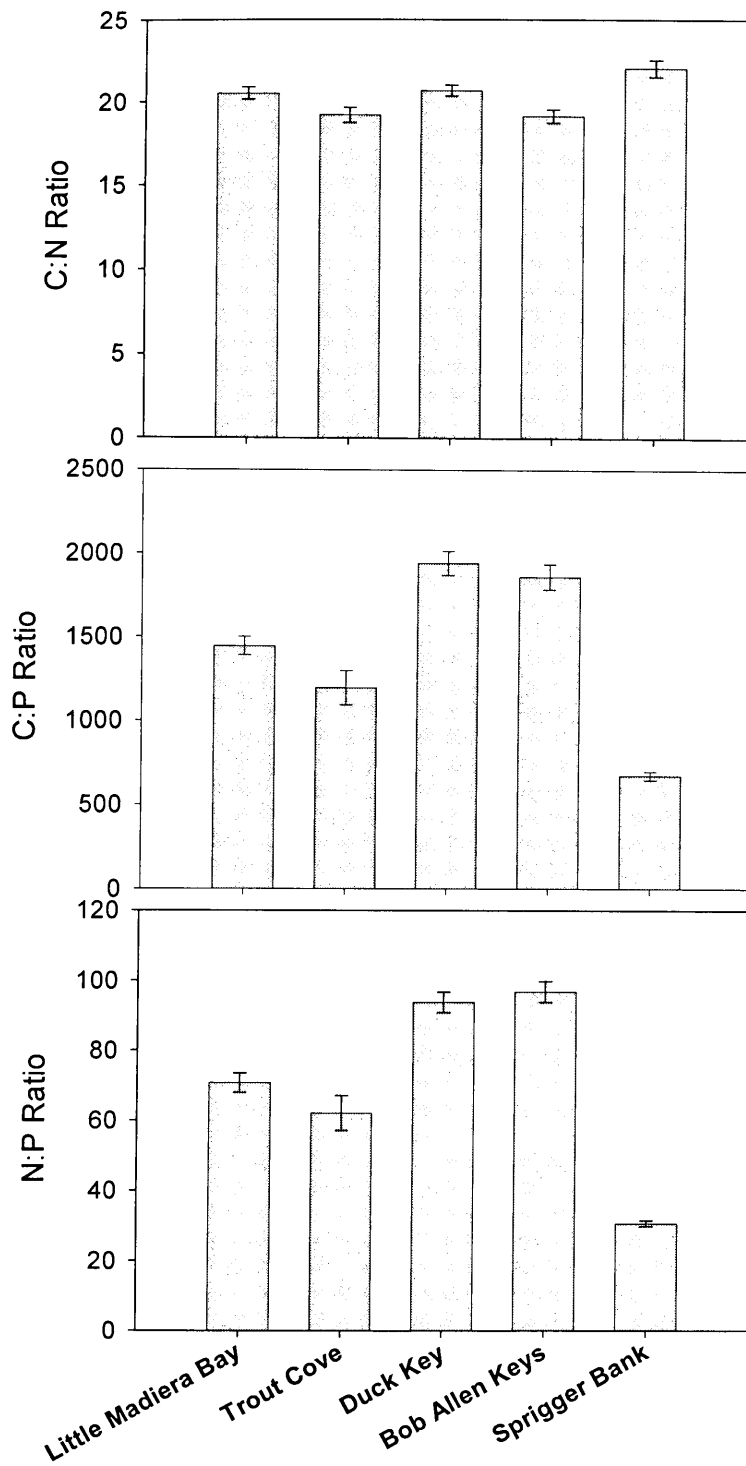


Figure 8: Quantum yield and PSII Photochemical Efficiency at each site at each sampling date. Error bars are ± 1 SE (n=10). Symbols are as follows: Little Madiera (●), Trout Cove (○), Duck Key (■), Bob Allen Keys (□), and Sprigger Bank (▲).

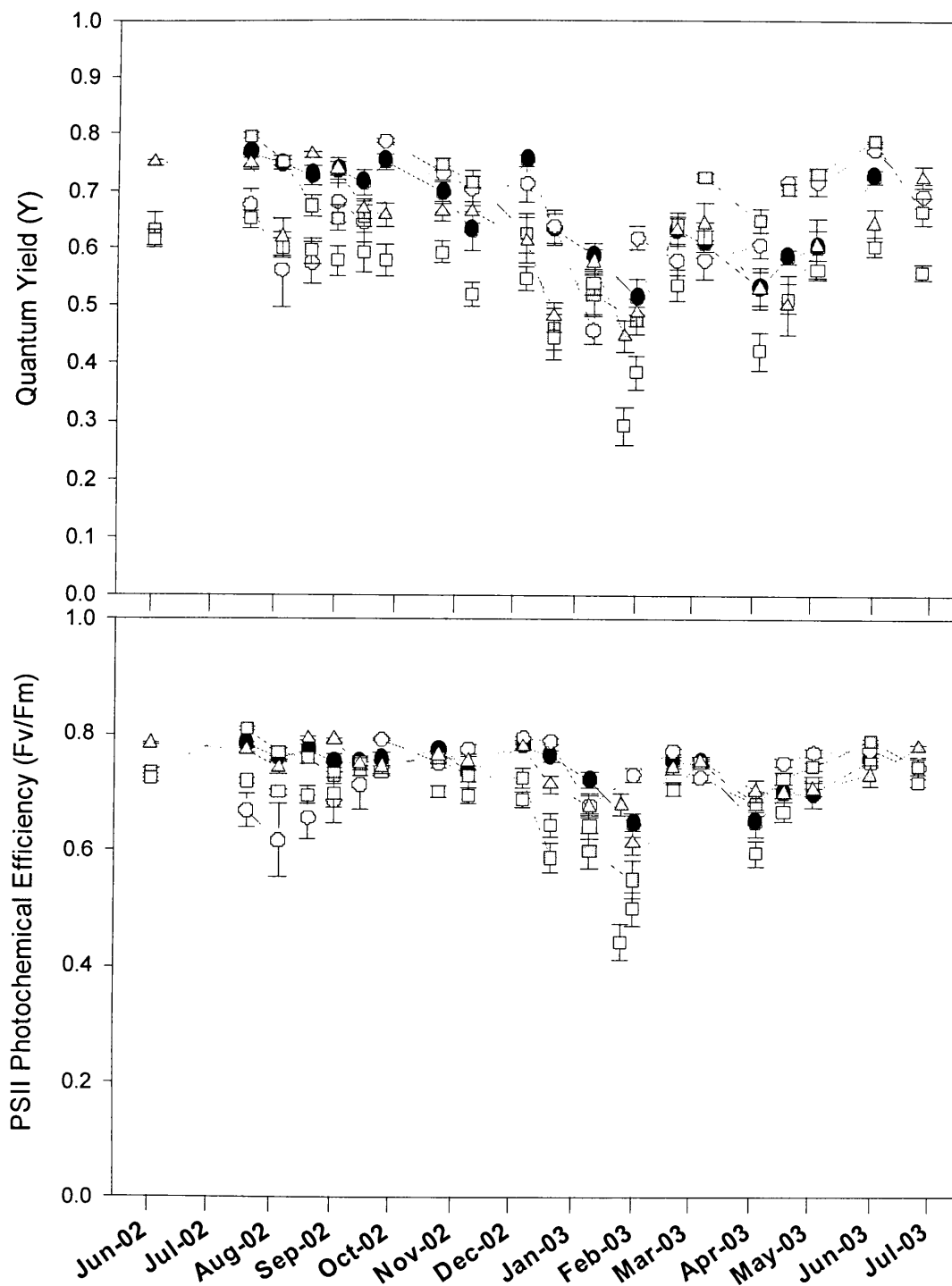


Figure 9: Quantum yield and PSII Photochemical Efficiency averaged by sampling location. Error bars are ± 1 SE (n=166). Letters indicated statistical differences between sites as determined by Tukey's Multiple Comparison Tests.

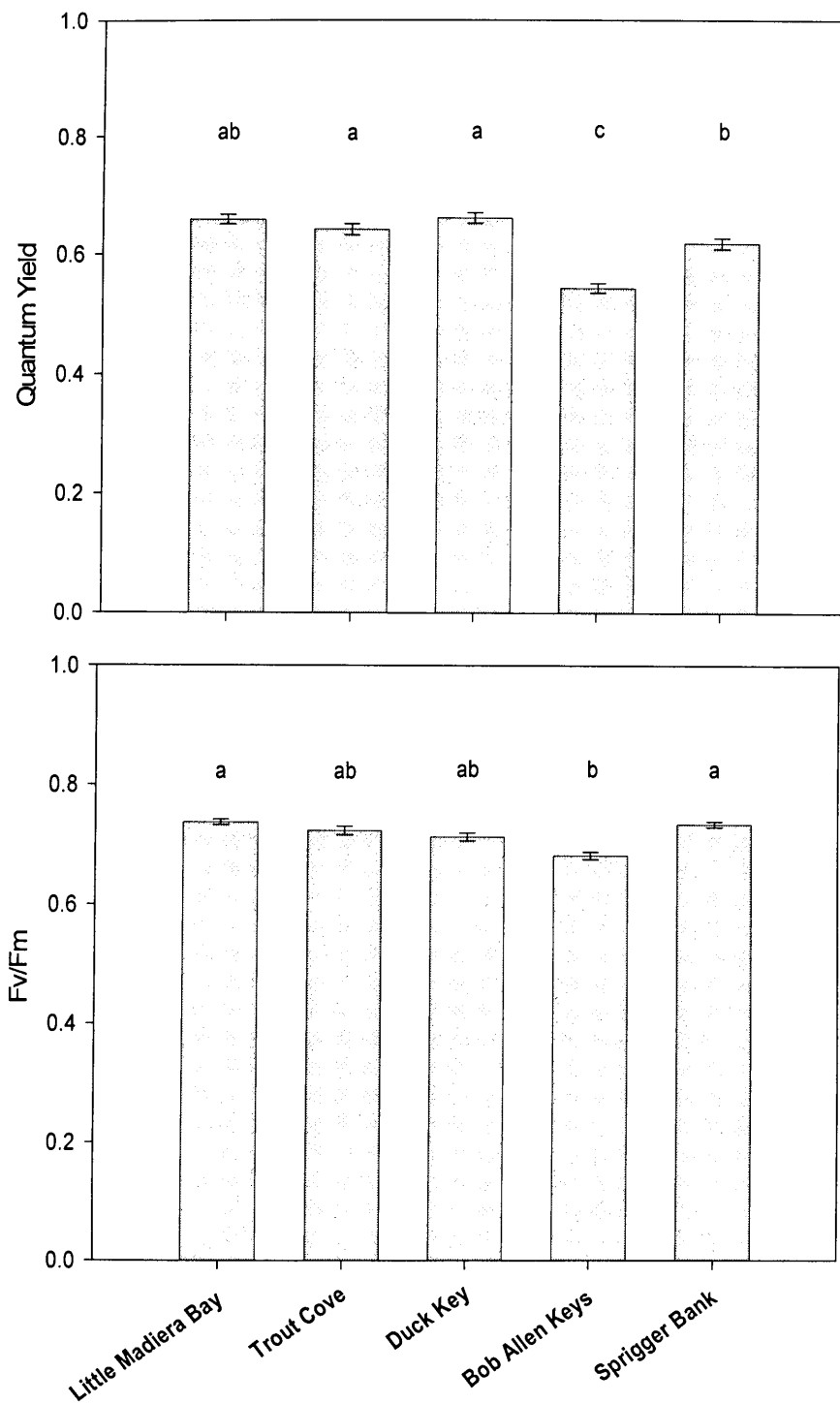


Figure 10: Minimum and maximum fluorescence measurements for both illuminated and dark adjusted leaf samples averaged across the study period by site. Error bars are ± 1 SE (n=116). Letters indicated statistical differences between sites as determined by Tukey's Multiple Comparison Tests.

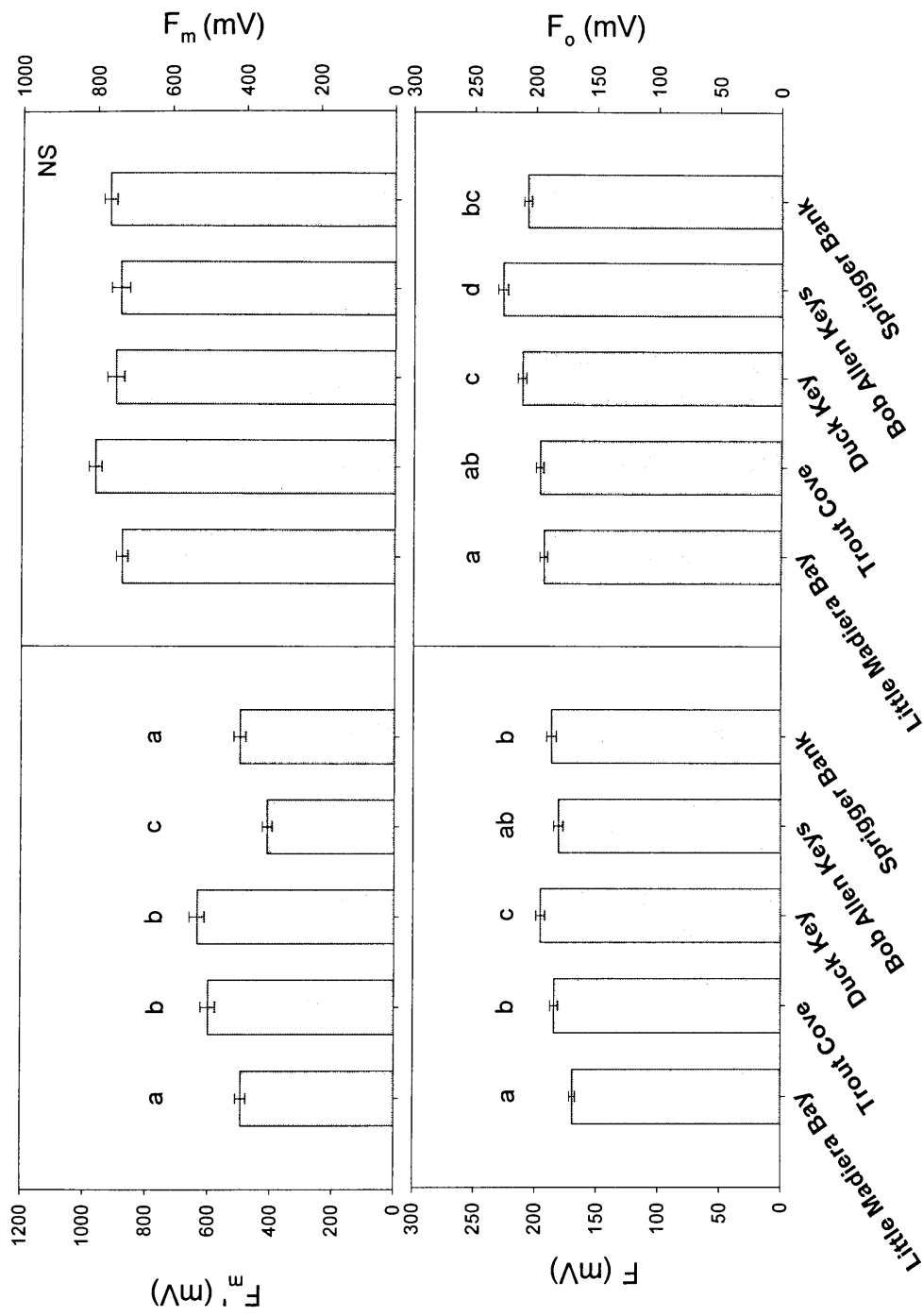


Figure 11: Minimum and maximum fluorescence measurements for both illuminated and dark adjusted leaf samples at each site at each sampling date. Error bars are ± 1 SE (n=10). Symbols are as follows: Little Madiera (●), Trout Cove (○), Duck Key (■), Bob Allen Keys (□), and Sprigger Bank (▲).

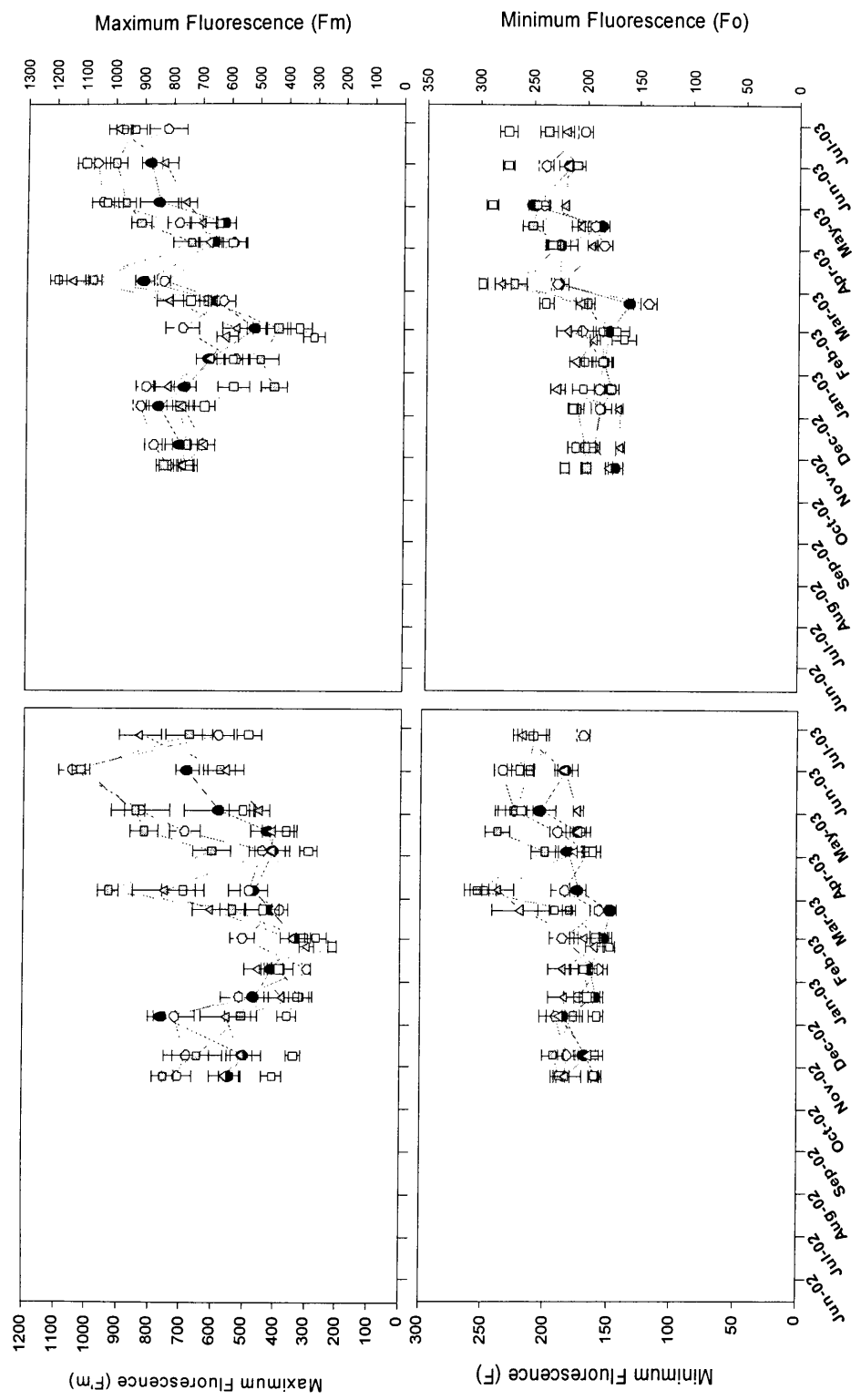


Figure 12: Photosynthetic pigment concentration averaged across the study period by site. Error bars are ± 1 SE (n=55).

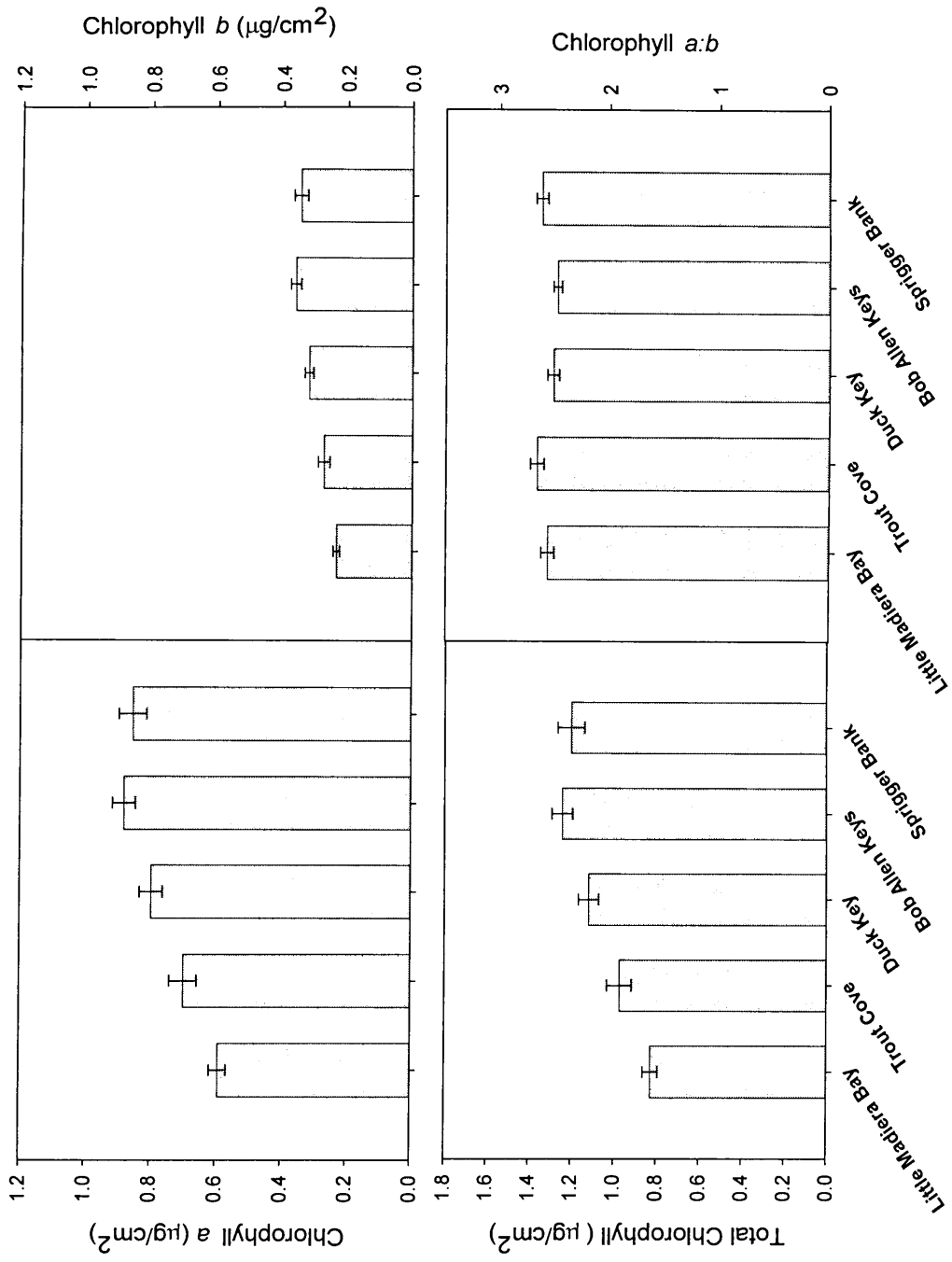
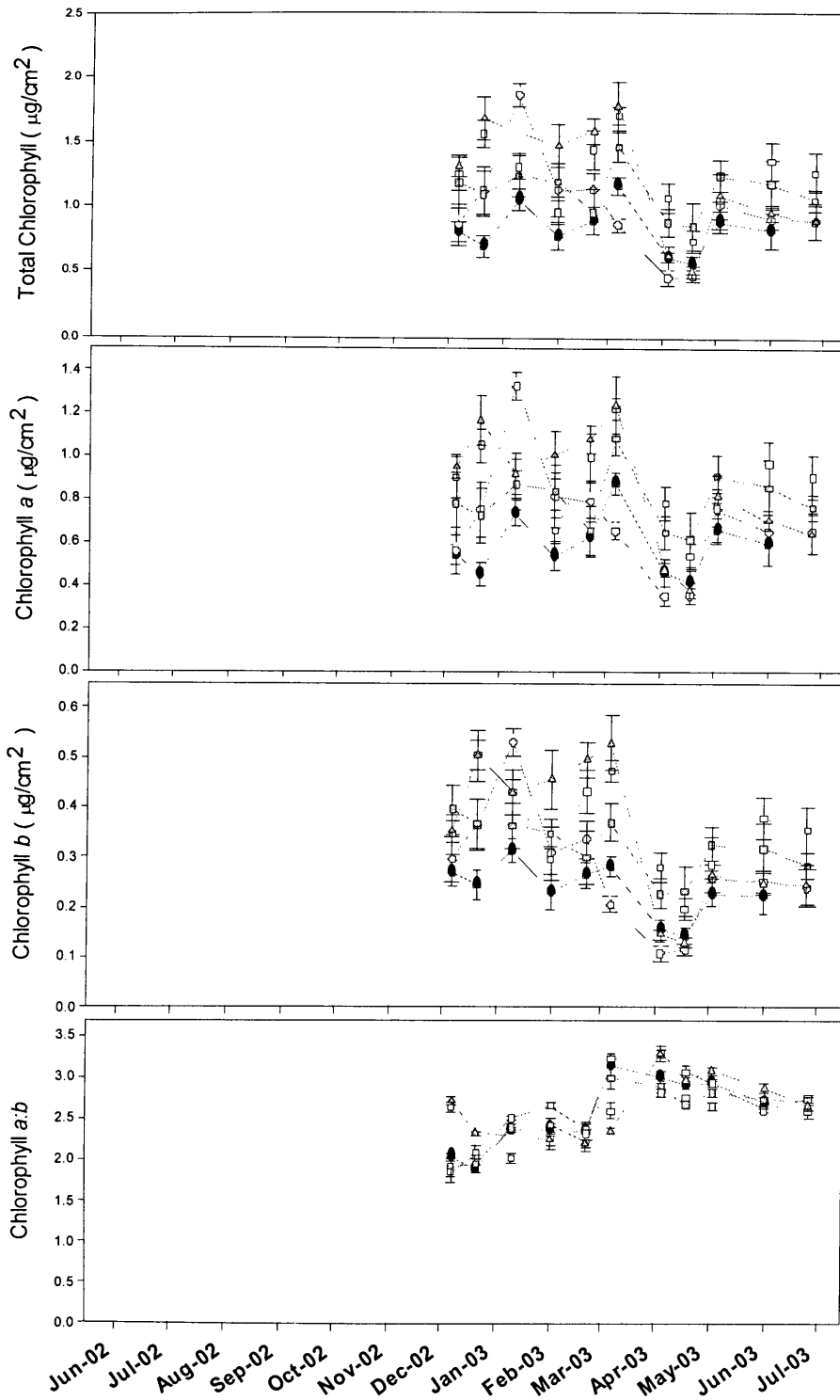


Figure 13: Photosynthetic pigment concentration at each site at each sampling date. Error bars are ± 1 SE (n=10). Symbols are as follows: Little Madiera (●), Trout Cove (○), Duck Key (■), Bob Allen Keys (□), and Sprigger Bank (▲).



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CHAPTER II: Chlorophyll *a* fluorescence response of *Thalassia testudinum* to hyper and hypo-osmotic conditions

A: Introduction:

In shallow estuaries, hyper-saline (i.e. > 35 ppt) and hypo-saline (i.e. < 35 ppt) conditions may be detrimental to the submerged aquatic vegetation. Hyper-saline conditions can occur naturally in long water residence time estuaries when evaporation exceeds freshwater input from either rainfall or river discharge (Nuttle, et al., 2000). Hyper-saline conditions can be induced by limiting inflow from damed rivers, outflow from desalinization plants and poor watershed management practices (Adams, et al., 1994; Kamermans, et al., 1999; Ralph, 1998a; Zieman, et al., 1999). Hypo-saline events can also occur from excessive rainfall, flood runoff, and watershed management practices (Nuttle, et al., 2000; Thorhaug, et al., 2006). Both hypersaline and hyposaline conditions exist at times in Florida Bay, a shallow water estuary on the southern tip of Florida, and these conditions may be stressful for the dominant benthic macrophyte, the seagrass *Thalassia testudinum*.

Hyper-saline and hypo-saline events may be stressful and can cause a variety of negative effects on seagrasses, including amino acid content changes (Pulich, 1986), changes in cell and chloroplast morphology (Iyer, et al., 1993), decreases in photosynthetic rate (Hammer, 1968 as cited in Biebl, et al., 1971; Hellblom, et al., 1999; Zieman, 1975), and decreases in above and below-ground biomass (McMillan, et al., 1967; Zieman, 1975; Kamermans, et al., 1999). Studies on chlorophyll content and salinities outside of tolerance ranges found that chlorophyll content of *T. testudinum* at higher salinities had a lower chlorophyll content than those kept at constant seawater salinity (28-31 ppt, McMillan, et al., 1967), and that chlorophyll *a* content decreased at

salinity extremes (hypo and hyper) for *Halophila ovalis* (Ralph, 1998a). Byron (Chapter 1) found that as mean salinity increased from 16 ppt to 35 ppt the chlorophyll *a* content of *T. testudinum* also increased.

Research on stress responses of marine plants within the past 10 years has begun to focus on chlorophyll *a* fluorescence. Enzymes and proteins have optimal conditions in which they function and large deviations from these conditions can result in suboptimal function or deactivation due to conformational changes. Since photosynthesis is dependent on membrane-embedded proteins and enzymes to pass energy along to make ATP and NADPH, when conditions vary outside of tolerance ranges, normal processes may be disrupted. Fluorescence is the process by which excessive light energy is dissipated before cellular damage occurs if the energy is unable to be used for photochemical work (i.e. photosynthesis). Pulse amplitude modulated (PAM) fluorometry can be used to detect deleterious conditions on seagrass physiology because the amount and timing of fluorescence can be used as an indicator of the function of the photosynthetic processes.

PAM fluorometry has been used to determine the effects of a variety of stresses on seagrass. Peter Ralph's extensive work on the seagrass *Halophila ovalis* has shown negative quantum yield and PSII photochemical efficiency responses to high light (Ralph, 1999b; Ralph, et al., 1995), but no significant response to low light (Ralph, 1999b). Thermal stress (heating and chilling, Ralph, 1998b) and hyper-osmotic and hypo-osmotic conditions (Ralph, 1998a) have also shown negative Y and F_v/F_m responses. The interaction between the degree of intensity and the length of exposure was significant in all of the aforementioned experiments, except the low light experiment (Ralph, 1999b), with greater decreases seen in the fluorescence response with longer exposure times. The

combination of light, temperature and salinity stresses resulted in declines in Y and F_v/F_m for all stress combinations, with thermal stress appearing to cause the most damage, followed by hyper-osmotic and then hypo-osmotic, and finally light stress; however, the combination of the stresses was additive in that they caused greater decline than each individual stress alone (Ralph, 1999a). Dessication stress on *Ruppia cirrhosa* and *Zostera capensis* resulted in lower F_v/F_m ratios after exposure, with the decline increasing with increasing exposure time for *R. cirrhosa* (Adams, et al., 1994). It is important to note that the exposures times used in the aforementioned experiments lasted at most 96 hrs, which can be considered a short time span as many stresses can last days to weeks to months.

In the previous chapter, I investigated the fluorescence response across a natural salinity gradient and determined that there were only weak correlations between fluorescence and mean salinity across Florida Bay, and that firm conclusions regarding salinity and *in situ* fluorescence measurement were difficult to draw because of other interacting environmental variables. It was the goal of this study to determine if the fluorescence measurements of *T. testudinum* would change as salinity conditions changed from normal marine salinities in a controlled setting so that one environmental variable would be influencing the fluorescence response. I was also interested in how the fluorescence measurements changed with length of exposure, as longer exposure times (i.e. > 96 hr) may result in acclimation by *T. testudinum* to salinities outside of the optimal range of 24 -34 ppt (Zieman, 1975). To try and understand why the fluorescence parameters changed, I was also interested in how the photosynthetic pigment concentrations changed in response to hyper and hypo-osmotic stresses.

B. Materials and Methods:

Hyper and Hypo-osmotic stress experiments:

In order to study the effects of osmotic stress on *Thalassia testudinum*, 35- 5 gal. aquaria were set up at Florida International University's outdoor aquaculture facility. Intact *T. testudinum* rhizomes with at least three short shoots were collected from The Boggies, Key Largo, Florida (25° 10.524' N, 80° 27.251' W), placed in coolers and transported to the aquaculture facility on November 8th, 2003. During the collection, chlorophyll fluorescence measurements were taken to establish a 'baseline' value of fluorescence for each individual. The intact shoots were transplanted into aquariums (one per aquarium) on November 11th, 2003 and allowed to acclimate under laboratory conditions. Salinity in each aquarium was maintained at 35ppt; however, since the facility was located outside, light and temperature could not be controlled.

Photosynthetically active radiation measured during mid-day (between 1100 and 1300 h) averaged $300 \mu\text{E m}^{-2} \text{s}^{-1}$ at the tops of the 5 gal aquaria, with a photoperiod of 11:13 h. Temperatures varied between 14.5 and 29.2°C; however, all fluorescence measurements were taken when the temperature was between 21 and 26°C (Figure 14). Acclimation time varied depending on treatment due to the techniques used to achieve the desired salinity within the aquaria. The hyper-osmotic treatments were started 22 days after transplanting and the hypo-osmotic treatments were started 42 days after transplanting.

Three hyper-osmotic treatments, three hypo-osmotic treatments and one control treatment were randomized across the 35 aquaria (Figure 15). Each treatment had five replicates. The hyper-osmotic treatments consisted of 150%, 200% and 250% seawater, which equal 52.5, 70, and 87.5 ppt, respectively. The hypo-osmotic treatments consisted of 0%, 25%, 50% seawater, which equal 0, 8.75, and 17.5 ppt, respectively. Hyper-

osmotic conditions were achieved by periodically adding a brine solution to each aquarium. Brine additions to the 150% treatment were made every two days; to the 200% treatment they were every other day; and to the 250% treatment, they were made every day. The desired hyper-osmotic conditions were achieved after 67 days, with rates of salinity increase of 0.6 ppt d⁻¹, 1 ppt d⁻¹ and 1.2 ppt d⁻¹ for the 150%, 200% and 250% treatments respectively (Figure 16). The brine solution was made by natural evaporation from two large tanks filled with 35 ppt seawater that was also set up at the outdoor aquaculture facility. Hypo-osmotic conditions were created using a pulse technique where the salinity was quickly dropped and then kept constant at the lower value. This was achieved by adding deionized water to each tank until the desired salinity was attained. The same control tanks (salinity equal to 35ppt) were used for both experiments. These treatments were used to measure the effects of evaporative ramping up of salinity and a sudden and sustained drop in salinity, mimicking salinity changes within Florida Bay, on chlorophyll *a* fluorescence.

Chlorophyll Fluorescence Measurements:

Chlorophyll fluorescence parameters were measured using a portable underwater Pulse Amplitude Modulated (PAM) fluorometer (Walz, Germany). Dark clips were used to keep the distance between the sample and the fiber optics constant at a uniform distance (10mm), and all measurements were taken on the adaxial surface of the second youngest leaf at the middle portion of the leaf. Fluorescence measurements were obtained immediately before and after a saturating pulse of light was provided to the leaf via a bulbed fiber optic probe. Providing saturating pulses of light to a leaf which was previously illuminated yields the following parameters: minimum fluorescence (F_m) and maximum fluorescence (F_m'). These values were then used to calculate quantum yield

(Y), where $Y = (F_m' - F) / F_m'$. Quantum yield measurements can be equated to the percentage of absorbed light being used to do photochemical work, given the previous light history (in units of minutes to hours) of the leaf. The dark clips were then closed for a 10 minute dark adaption period, allowing for adequate relaxation of the chlorophyll *a* reaction centers (Byron unpublished data). Once the dark adaption period was over, fluorescence measurements were again taken, yielding minimum and maximum fluorescence values now termed F_o and F_m , respectively. These values were then used to calculate photosystem II (PSII) photochemical efficiency (F_v/F_m), where $F_v/F_m = (F_m - F_o) / F_m$, which provides insight to the potential photosynthetic capacity of the leaf sample. It was expected that Y would be less than F_v/F_m in normal conditions, but that both measurements would be lower for stressed versus unstressed plants, with the Y values of stressed plants being very low as the plant attempted to dissipate as much excessive energy as possible before severe damage occurred to the photosynthetic apparatus.

To study the effect of hyper-osmotic conditions on fluorescence, measurements were taken prior to additions of brine to the treatment aquariums and then once the desired salinity was obtained, 67 days after the initial addition of brine. Measurements were also taken periodically (5, 15, 45, and 62 days) during the ramping-up phase of the hyper-osmotic experiment to determine whether fluorescence changed as the salinity was slowly raised.

To study the effect of a sudden and sustained drop in salinity, fluorescence measurements were taken immediately after the stress was applied (0 h), 2 hours after, 6 hours after and then daily for 6 days afterward. Fluorescence measurements were also taken 19 days and 33 days after the salinity was lowered to observe how prolonged exposure would affect the measurements.

Photosynthetic pigment analysis:

At the conclusion of the experiments, leaves used for fluorescence measurements were taken from each tank to determine how the chlorophyll pigments might have changed in response to hyper and hypo osmotic stress. Within 24 hours of collection, leaves were scraped with a razor blade to remove epiphytes, cut to a standard length of 30 mm, and rinsed with deionized water. Each leaf segment was then wrapped in an aluminum foil packet and freeze dried for a minimum of 12 hours. The samples were then ground using a mortar and pestle to increase surface area during chlorophyll extraction and placed in pre-weighed 7 mL scintillation vials. The vials and sample were weighed so that the chlorophyll values could be standardized to weight (g) as well as area (cm²). Following a modification of the technique utilized by Dennison (1990), chlorophyll *a* and chlorophyll *b* were extracted by adding 5mL of 90% acetone to the samples, and placing the vials in a -20°C freezer to keep the samples cold and dark during the extraction. After 24 hours, the extracts were analyzed using a Shimadzu RF-Mini 150 filter fluorometer using a 440 nm excitation filter and 670 nm emission filter for chlorophyll *a* and 460 nm excitation filter and 650 nm emission filter for chlorophyll *b* (see Welschmeyer, 1994). The relative fluorescence measurements for each pigment were then compared to a standard curve produced from pure chlorophyll *a* and chlorophyll *b* spinach extracts following the methodology of Jeffrey and Humphrey (1975) and chlorophyll *a* and chlorophyll *b* concentrations (μM) were calculated.

Statistics:

Fluorescence measurements made the day prior to the start of either treatment set (hyper or hypo-osmotic) were analyzed using a One-way Analysis of Variance (ANOVA) to ensure that no pre-treatment differences existed among groups due to

random differences in the aquaria at the initial application of any stress. I approached the analysis of each experiment in two ways. First, to determine if there were overall effects of the different treatments (hyper- and hypo-osmotic) on the fluorescence measurements, one-way ANOVAs were conducted on the difference between the initial and final fluorescence measurements. Significant ANOVA results ($p < 0.05$) were further analyzed with Tukey's Multiple Comparison tests to determine which treatments were significantly different. Then, as I was also interested in how the fluorescence measurements changed over time in the slow ramped-up hyper-osmotic and the quick pulsed-down hypo-osmotic experiments, repeated measures ANOVAs were conducted to determine if the fluorescence measurements differed across treatments, across exposure times and if the pattern of change over time for each treatment was different. Although many results for the hyper and hypo-osmotic experiments were plotted together to facilitate synthesis of the results, statistical analyses were conducted on each experiment separately. In order to avoid confusion between the reported fluorescence parameters (F_o , F_m , F , and F_m') and the values of the F statistic associated with ANOVA, all reported F statistics will be represented by an uppercase italics "*F*".

To test for differences in photosynthetic pigment measurements between treatments, one way ANOVAs were conducted. Significant ANOVA results were analyzed with Tukey's Multiple Comparison test to determine which treatments were significantly different.

Transformations were done on any data set that did not pass either normality or homogeneity of variances tests. Squaring or cubing any non-normal raw data most often resulted in a normal distribution. All statistical analysis was done using the SAS 9.1 statistical package.

C. Results:

Chlorophyll Fluorescence Measurements:

Analysis of the treatment tanks 24 hours prior to the application of the stress showed no significant differences in the fluorescence measurements among the groups of tanks (Table 8). Initial minimum (F_o) fluorescence values were between 207 and 218 V and final F_o values declined to between 63 and 153 V, with the higher salinity treatments having the lower values after 67 days of hypersalinity conditions (Figure 17). Maximum fluorescence (F_m) measurements also declined from an initial range of 805 to 888V to a final range of 237 to 671 V (Figure 17). The percent decline in F_o , normalized to the control treatment, was 15% in the 150% seawater treatment, 39% in the 200% seawater treatment, and 58% in the 250% seawater treatment. Similar declines were seen for F_m , with the 250% seawater treatment having the greatest decline between initial and final measurements (72%). The differences between the initial and final minimum and maximum fluorescence measurements across treatments were both significantly different ($p < 0.05$). Tukey's multiple comparison test revealed that the control treatment measurements were not significantly different than the 150% seawater or 200% seawater treatments, but were significantly different than the 250% seawater treatment. The 150%, 200% and 250% seawater treatments were not significantly different from one another (Figure 18).

As both F_o and F_m declined at similar rates, the decline in F_v/F_m ratios was minimal, and increases were actually seen, with the final measurements being higher for the control, the 150% seawater and the 200% seawater treatments (Figure 19). Initial F_v/F_m ratios ranged from 0.713 to 0.758 and final ratios ranged from 0.707 to 0.765, both of which are in the range of unstressed plants of 0.7 to 0.8 (see review by Touchette, et

al., 2000). The differences between the initial and final F_v/F_m ratios across treatments was not significantly different ($F_{3,16} = 1.08$, $p = 0.3841$). Similar results were found for the minimum and maximum fluorescence values and quantum yield (Y) ratios from measurements taken without the period of dark-adjustment (data not shown).

Repeated measures analysis conducted on the minimum and maximum fluorescence measurements from dark-adjusted leaves taken during the 'ramping up' procedure revealed that the treatments were significantly different (salinity main effect, $F_{3,15} = 3.77$, $p = 0.0338$; and $F_{3,15} = 4.63$, $p = 0.0175$ for F_o and F_m , respectively), and that F_o and F_m were significantly different at different times (time main effect, $F_{3,45} = 11.12$, $p < 0.0001$; and $F_{3,45} = 41.94$, $p < 0.0001$ for F_o and F_m , respectively). Salinity treatment also significantly influenced the way in which these measurements changed over time for both F_o and F_m (salinity x time interaction, $F_{9,45} = 3.50$, $p = 0.0024$; $F_{9,45} = 2.44$, $p = 0.0238$ for F_o and F_m , respectively)(Figure 20). Repeated measures analysis on the F_v/F_m values, revealed that both salinity and time were significant ($F_{3,15} = 8.22$, $p = 0.0018$; $F_{3,45} = 189.33$, $p < 0.0001$, respectively); however there was no significant salinity x time interaction. There was a decline in Y and F_v/F_m on day 15 (Figure 20); however, this also corresponded to a significant decrease in average tank temperature to 16.4°C (see Figure 14). The decline in the yield measurements (both Y and F_v/F_m) was mainly due to a decline in the maximum fluorescence values, as minimum fluorescence values remained relatively stable (Figure 20). It is important to note that the same results were seen when day 15 was removed from the statistical analysis, so that even though temperature is an important factor, there was a significant effect of salinity change on the fluorescence measurements.

Analysis of the hypo-osmotic treatments 24 hours prior to the application of the stress revealed no pre-treatment differences in fluorescence response variables among treatment groups (see Table 9). Initial minimum (F_o) fluorescence values were between 200 and 240 V and final F_o values declined to between 63 and 182 V after 33 days of exposure to hypo-osmotic conditions, with the lowest salinity treatment having the lowest final values (Figure 21). Maximum fluorescence (F_m) measurements also declined from an initial range of 512 to 730 V to a final range of 158 to 653 V (Figure 21). The differences between the initial and final minimum and maximum fluorescence measurements across treatments were both significantly different (F_o : $F_{3,16} = 4.57$, $p = 0.017$; F_m : $F_{3,16} = 6.95$, $p = 0.0033$). Tukey's multiple comparison test revealed that the differences between minimum fluorescence (F_o) values pre and post application of the stress of the control treatment were significantly less than the differences seen in the 0% seawater treatment, but not significantly different than the 50% seawater or 25% seawater treatments. Maximum fluorescence (F_m) values of the control treatment were not significantly different from the 50% seawater treatment, but was significantly lower than both the 25% and 0%. For both F_o and F_m the 0%, 25% and 50% seawater treatments were not significantly different from one another (Figure 18).

As in the hyper-osmotic experiment, since both F_o and F_m declined at similar rates, the percent of decline in F_o/F_m ratios was minimal (Figure 19). Initial F_o/F_m ratios ranged from 0.582 to 0.669 and final ratios ranged from 0.550 to 0.716. Although there were greater differences between pre and post stress across the treatments, these differences were not significant. As also seen in the hyper-osmotic experiment, similar results were found for the minimum and maximum fluorescence values and quantum

yield (Y) ratios from measurements taken without the period of dark-adjustment (data not shown).

Repeated measures analysis of the F_v/F_m ratios, concluded that both salinity and time were significant (salinity main effect, $F_{3,15} = 5.21$, $p = 0.011$; time main effect, $F_{10,150} = 4.00$, $p = 0.0001$); however their interaction was not (salinity x time interaction, $F_{30,150} = 0.95$, $p = 0.5466$). Similar results were seen for the Y ratio; however, the pattern of change over time for each treatment was more pronounced (Figure 22). Further analysis of the individual fluorescence measurements showed that both time and the salinity x time interaction were significantly different for F_o (time main effect, $F_{10,150} = 14.63$, $p = 0.0001$; salinity x time interaction, $F_{30,150} = 2.48$, $p = 0.0002$) and F_m (time main effect, $F_{10,150} = 33.46$, $p < 0.0001$; salinity x time interaction, $F_{30,150} = 3.01$, $p < 0.0001$), but only in the F_m measurements was the between subject effects of the treatments significant (salinity main effect, $F_{3,15} = 5.11$, $p = 0.0124$). Again, the same types of results that existed with the quantum yield ratios were also seen with the individual fluorescence measurements of non-dark adjusted leaves, but the pattern of change over time was more pronounced (Figure 23).

Photosynthetic pigments:

Total chlorophyll content across all treatments ranged from 0.17 to 0.55 $\mu\text{g}/\text{cm}^2$, with chlorophyll *a* constituting approximately 59 to 77 % of the total chlorophyll content (Figure 24). One-way ANOVA and post hoc analysis showed that chlorophyll *a* and total chlorophyll in the 200% and 250% seawater treatments had significantly lower concentrations than the control and 150% seawater treatments across the hypersaline treatments ($F_{3,34} = 10.53$ and 11.62, respectively, $p < 0.0001$), but none of the treatments were significantly different in the hyposaline treatments ($F_{3,38} = 2.29$, $p > 0.05$). When

both treatments were analyzed together, Tukey's multiple comparison test revealed that the chlorophyll *a* and total chlorophyll content at extreme salinity values (e.g. the 0%, 25%, 200% and 250% seawater treatments) were not significantly different from each other. Chlorophyll *b* concentrations were not significantly different across any treatment in either experiment (hyper-osmotic: $F_{3,34} = 1.12$ $p > 0.05$; hypo-osmotic: $F_{3,38} = 0.28$, $p > 0.05$), and although chlorophyll *a:b* ratios appeared to vary (Figure 24), with the sites ranging from 3.8 to 10.3, there were no significant differences between treatments in either the hyper or hypo-osmotic experiments ($F_{3,34} = 0.71$, $p = 0.5505$; $F_{3,38} = 0.26$, $p = 0.8572$, respectively).

D. Discussion:

Although seagrasses have evolved in the marine medium, both high and low salinities can be stressful for these marine vascular macrophytes, and large variations in salinity has often been cited as a stress for seagrasses (Biebl, et al., 1971; Hellblom, et al., 1999; Iyer, et al., 1993; Kamermans, et al., 1999; Ralph, 1998a; Short, et al., 1999; Zieman, 1975). *Thalassia testudinum*, in particular, is considered a relatively stenohaline plant that exhibits reductions in vigor and growth when salinity varies from constant marine conditions (Zieman, 1975). PAM fluorometry has been shown to be a sensitive indicator of stress in vascular plants in general and seagrasses in particular (Campbell, et al., 2003; Dawson, et al., 1996; Kamermans, et al., 1999; Longstaff, et al., 1999; Ralph, 1998a; b; 1999a; Ralph, 1999b; Ralph, 2000; Ralph, et al., 1995; Ralph, et al., 1998; Schwarz, et al., 2000), but only weak relationships between salinity and fluorescence were seen in *T. testudinum* across a marked salinity gradient in Florida Bay, as observations were complicated by the interaction of other environmental variables (Byron, Chapter 1).

In this investigation of slow ramping up and quick pulsing down of salinity, when Y and F_v/F_m were studied over time, there were significant differences among the salinity treatments and over time. In 1973, Selye (1973) introduced the biological stress concept, which has since been applied to plants (Levitt 1980; Luttge, 1997). This concept states that as stress is applied and then removed, the first response of the organism is the 'alarm phase'. This will result in a negative departure from the current or 'normal' state of the organism. During this phase, biochemical and morphological changes may occur to protect the organism from the stress. While the stress is still present the 'recovery phase' begins, followed by a 'hardening' and 'resistance phase'. These later phases represent acclimation. If during this process, the stress is increased, the stress is too intense upon application, or the exposure period is too great, the organism may undergo an 'exhaustion phase' which can lead to chronic or acute damage and death (Selye, 1973; Luttge, 1997).

During the hyper-osmotic experiment, I did not notice these phases; however, this may be due to the method used to achieve the desired salinity. The gradual change in salinity may have allowed some degree of acclimation as the fluorescence measurements remained relatively close together up to 45 days after the start of the salinity increase. After this time both minimum and maximum fluorescence values showed declines in the highest salinity (250% seawater) treatment. Again, because both measurements declined there were relatively small changes in the yield ratios, suggesting that the decreases were due to a loss of chlorophyll *a*. During the hypo-osmotic treatment, we saw that the Y response, and to a lesser degree the F_v/F_m response, followed the pattern described by the biological stress concept. There was an initial decline in fluorescence values after 24 h, after which the plants then entered into a recovery phase and fluorescence measurements were higher than those of the controls; however, as the salinity stress persisted, both

treatments declined to values below those measured in the controls. The greatest hypo-osmotic stress (0% seawater treatment) showed a decline in fluorescence immediately after the salinity was lowered and this decline increased with prolonged exposure. Peter Ralph's work on *Halophila ovalis* (Ralph, et.al, 1995; Ralph, et al., 1998, Ralph, 1998a; Ralph, 1998b; Ralph, 1999a; Ralph, 1999b; Ralph, 2000) may have also seen this pattern in the fluorescence measurements if the length of stress exposure was extended beyond 96hrs. Because of the reduced exposure times used in those experiments, the measured fluorescence response may only represent the response seen in during the 'alarm phase'. If the stress persisted, the plants, in this case *H. ovalis*, may have been able to recover and acclimate like what was seen for *T. testudinum* in this investigation.

Salinity deviations from marine conditions in either a positive or negative direction produced marked departures in both the final minimum and maximum fluorescence measurements. However, since both these measurements declined, the final yield ratios (Y and F_v/F_m) did not show great deviations from the control treatment. This decrease in F_m' and F_m after the application of the stress may be attributed to closing of chlorophyll *a* reaction centers as a photoprotection mechanism (Campbell, et al., 2003; Ralph, et al., 1995). The similar decrease in F and F_0 further indicate a complete loss of chlorophyll *a*. Measurements of chlorophyll *a* at the completion of each experiment support this notion since chlorophyll *a* values decreased as salinities varied further from marine conditions. However, the Y and F_v/F_m ratios were not different across salinity treatments, suggesting that those chlorophyll reaction centers still remaining are capable of transferring light energy just as efficient as the unstressed plants.

This study demonstrates that hyper and hypo-osmotic stress do affect fluorescence measurements, but that the way in which the stress is applied is important in determining

how fluorescence measurements respond. Gradual changes in salinity may not produce fluorescence responses until days to months, after there has already been a reduction in chlorophyll content, or a morphological change, whereas sudden changes induce fluorescence changes within hours to days. This study provides only the first steps in investigating how fluorescence measurements change in response to salinity stress, and further experimentation is required for a complete understanding of how the fluorescence measurements of *Thalassia testudinum* are affected by hyper and hypo-osmotic stress.

Table 8: ANOVA results for hyper-osmotic treatments 24 hours prior to the application of the stress.

	100% (control)	150% seawater	200% seawater	250% seawater	$F_{3,16}$	p-value
F	204	205.4	196.6	216.6	0.37	0.7733
F_m'	707.2	824.2	716.2	785.8	1.05	0.3971
Y	0.693	0.751	0.724	0.724	1.37	0.2867
F_o	218.6	215	207.6	215.8	0.08	0.9722
F_m	822.6	888	805.4	837.8	0.23	0.8754
$F_{\sqrt{F_m}}$	0.713	0.758	0.741	0.741	0.75	0.5371

Table 9: ANOVA results for hypo-osmotic treatments 24 hours prior to the application of the stress.

	100% (control)	50% seawater	25% seawater	0% seawater	$F_{3,16}$	p-value
F	182.2	197.2	215.6	184.8	0.71	0.5602
F_m'	439.4	560.4	616.6	484.8	0.99	0.4213
Y	0.559	0.645	0.649	0.570	2.06	0.1454
F_o	200.6	222.6	240.0	203.8	0.92	0.4534
F_m	511.8	680.8	730.4	596.2	1.58	0.2334
F_v/F_m	0.582	0.673	0.669	0.635	2.53	0.0943

Figure 14: Average temperatures ($^{\circ}\text{C}$) recorded within the tanks throughout the entire experiment. Circled points correspond to dates when fluorescence measurements were taken. Error bars are ± 1 SD.

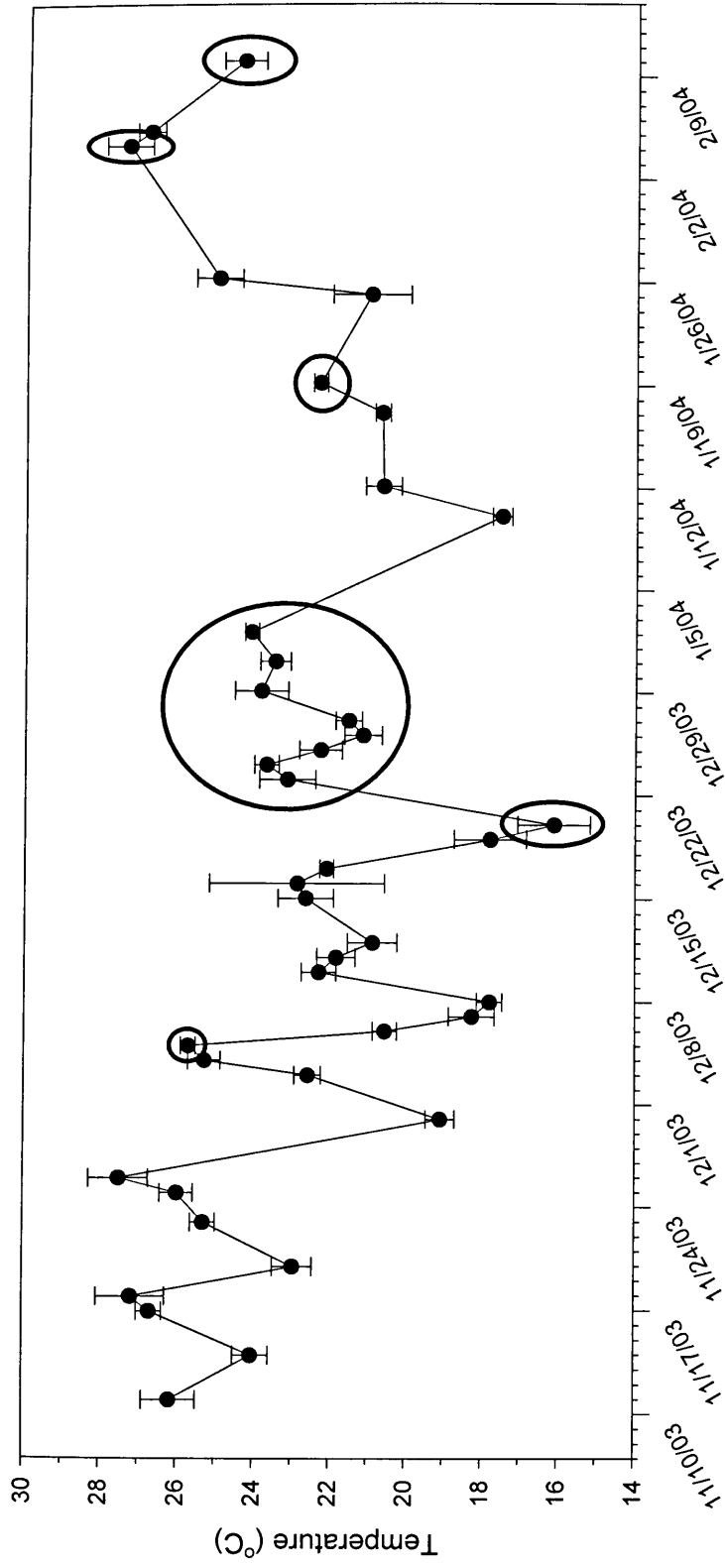


Figure 15: Randomization of treatments, experimental design

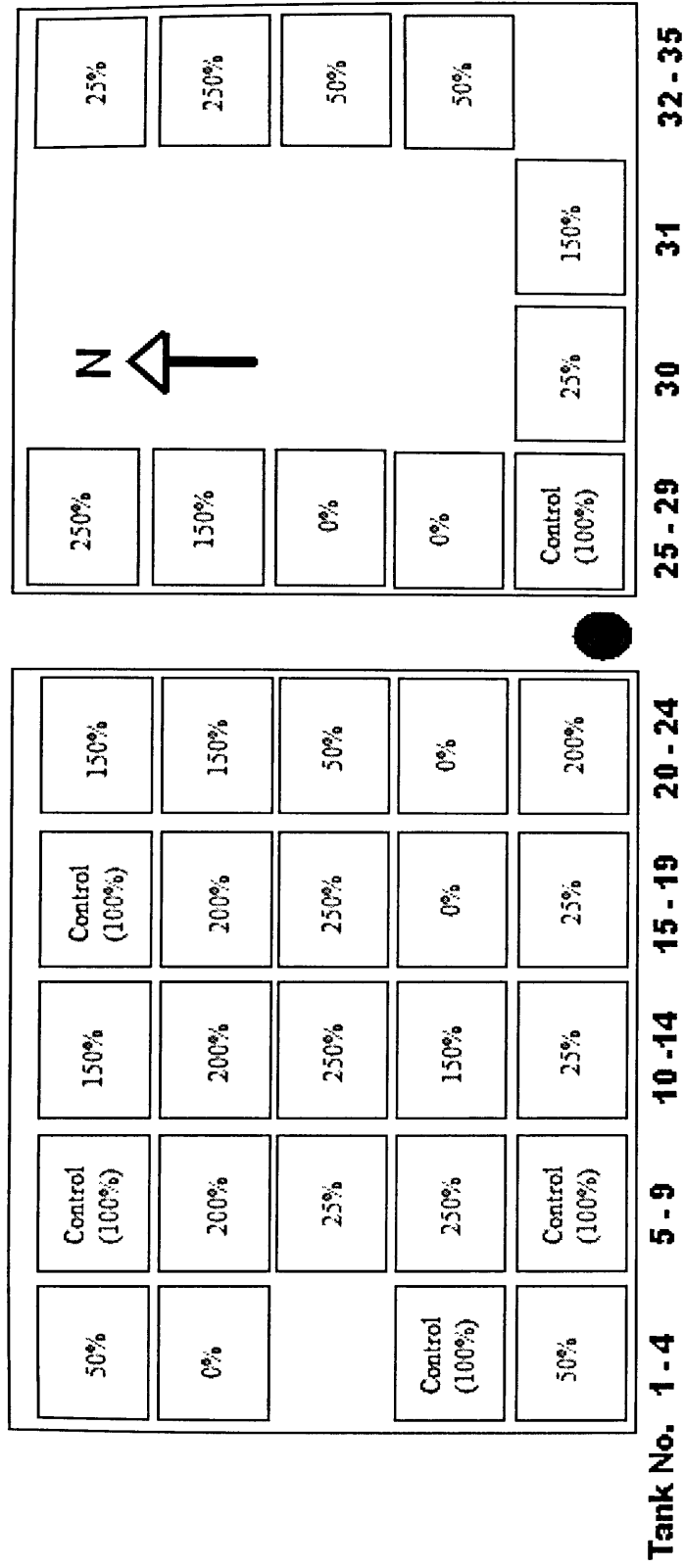


Figure 16: Salinity of tanks throughout entire experiment.

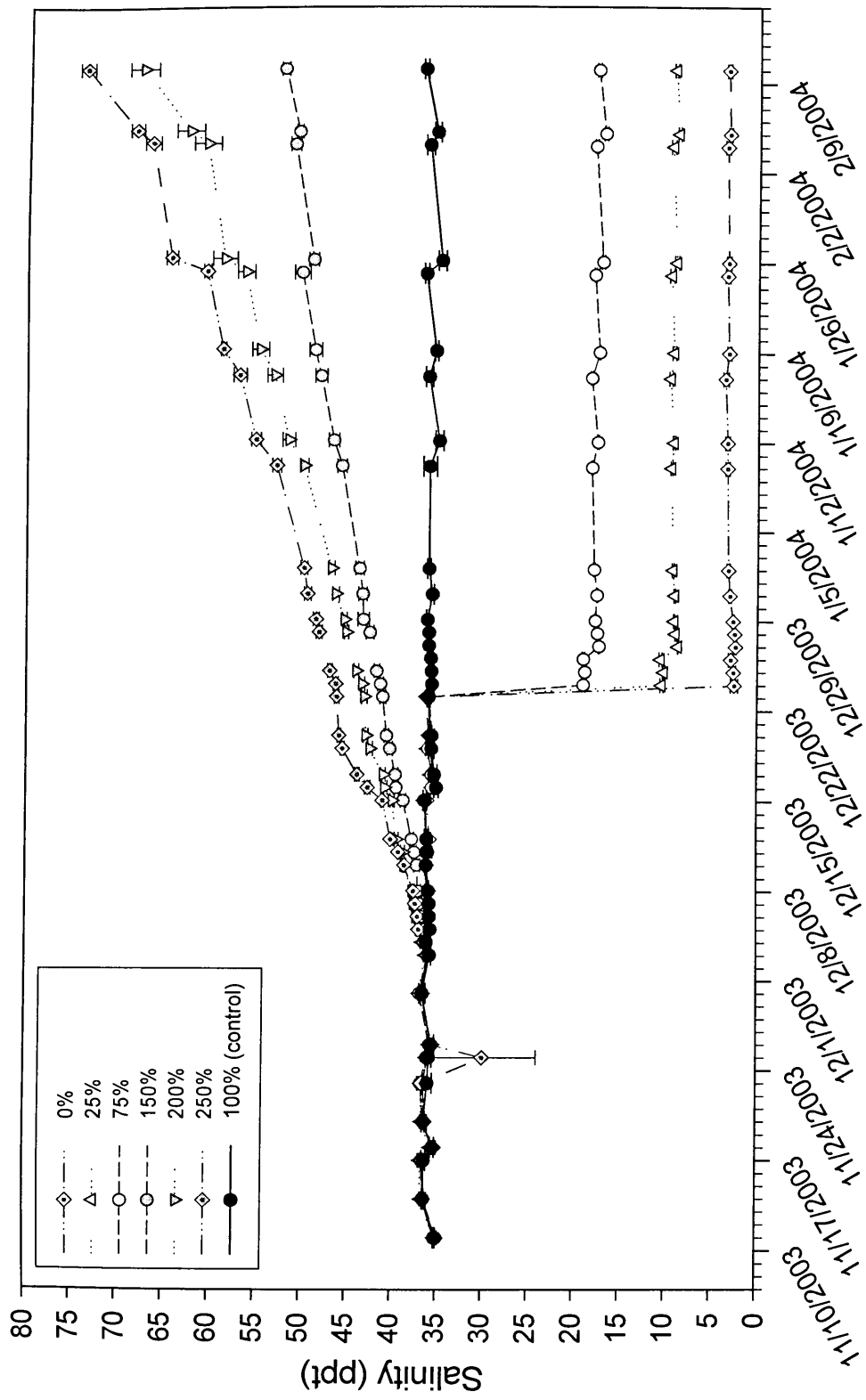


Figure 17: Minimum and maximum fluorescence values of dark-adjusted samples in hyper-osmotic treatments, pre and post salinity change. Error bars are ± 1 SE ($n=5$).

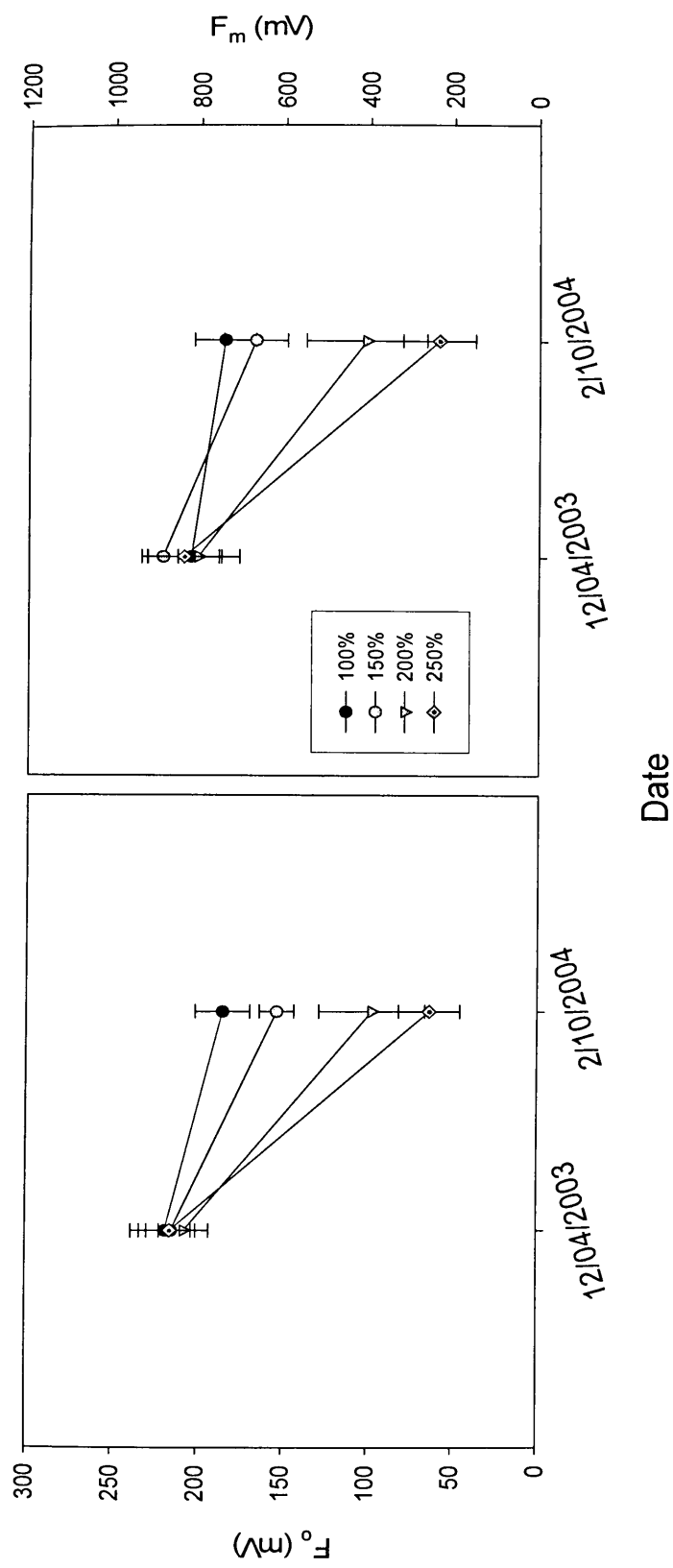


Figure 18: Differences between initial and final minimum and maximum fluorescence values of both the hyper and hypo-osmotic experiments. Error bars are ± 1 SE ($n=5$).

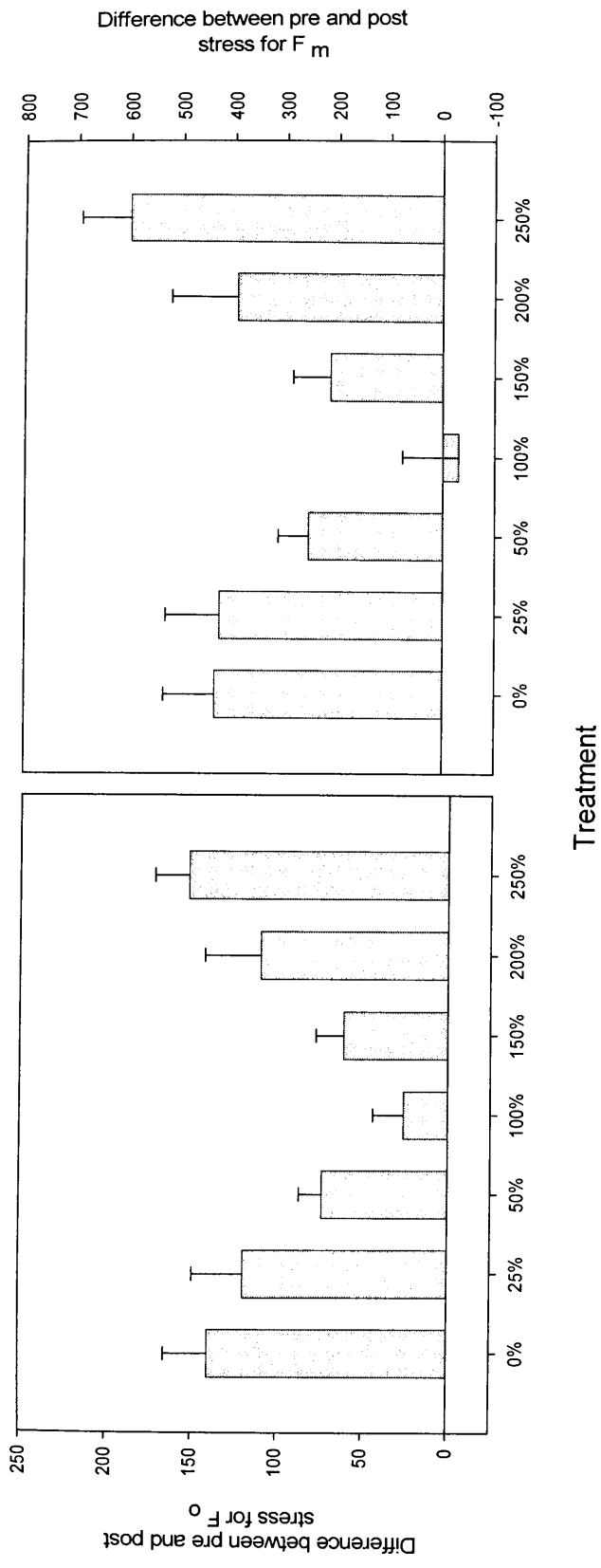


Figure 19: Difference between initial and final F_v/F_m measurements for both the hyper and hypo-osmotic experiments. Error bars are ± 1 SE (n=5).

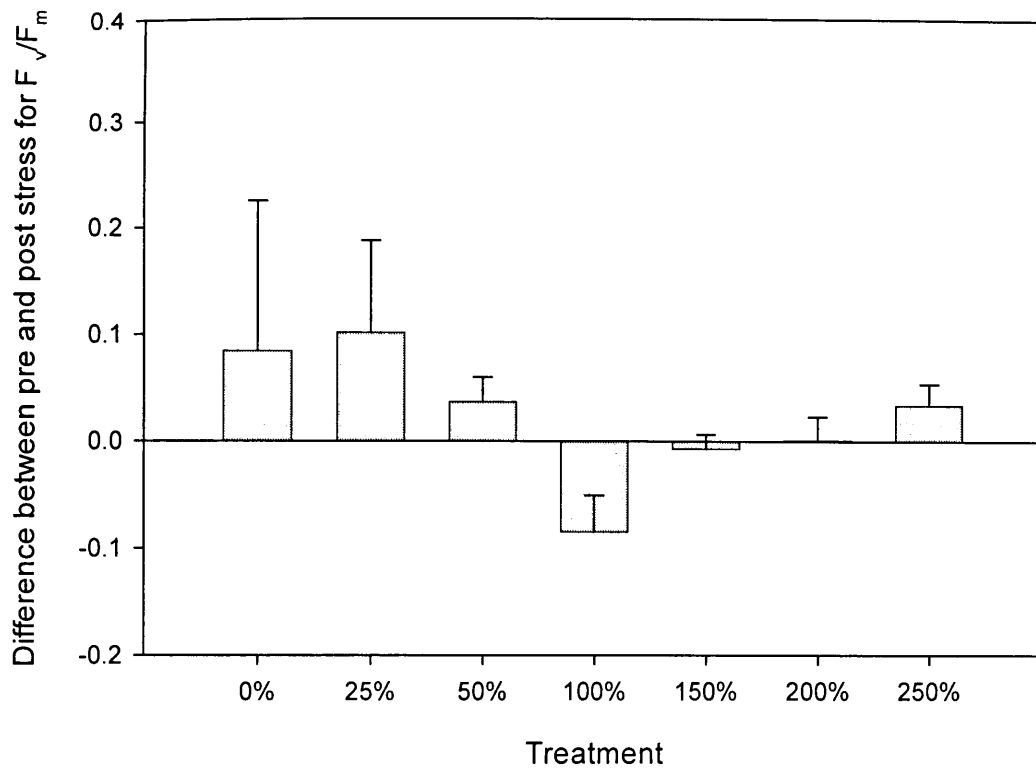


Figure 20: Fluorescence measurements during the 'ramping-up' phase of the hyperosmotic experiment taken on days 5, 15, 45, and 62. Error bars are ± 1 SE (n=5).

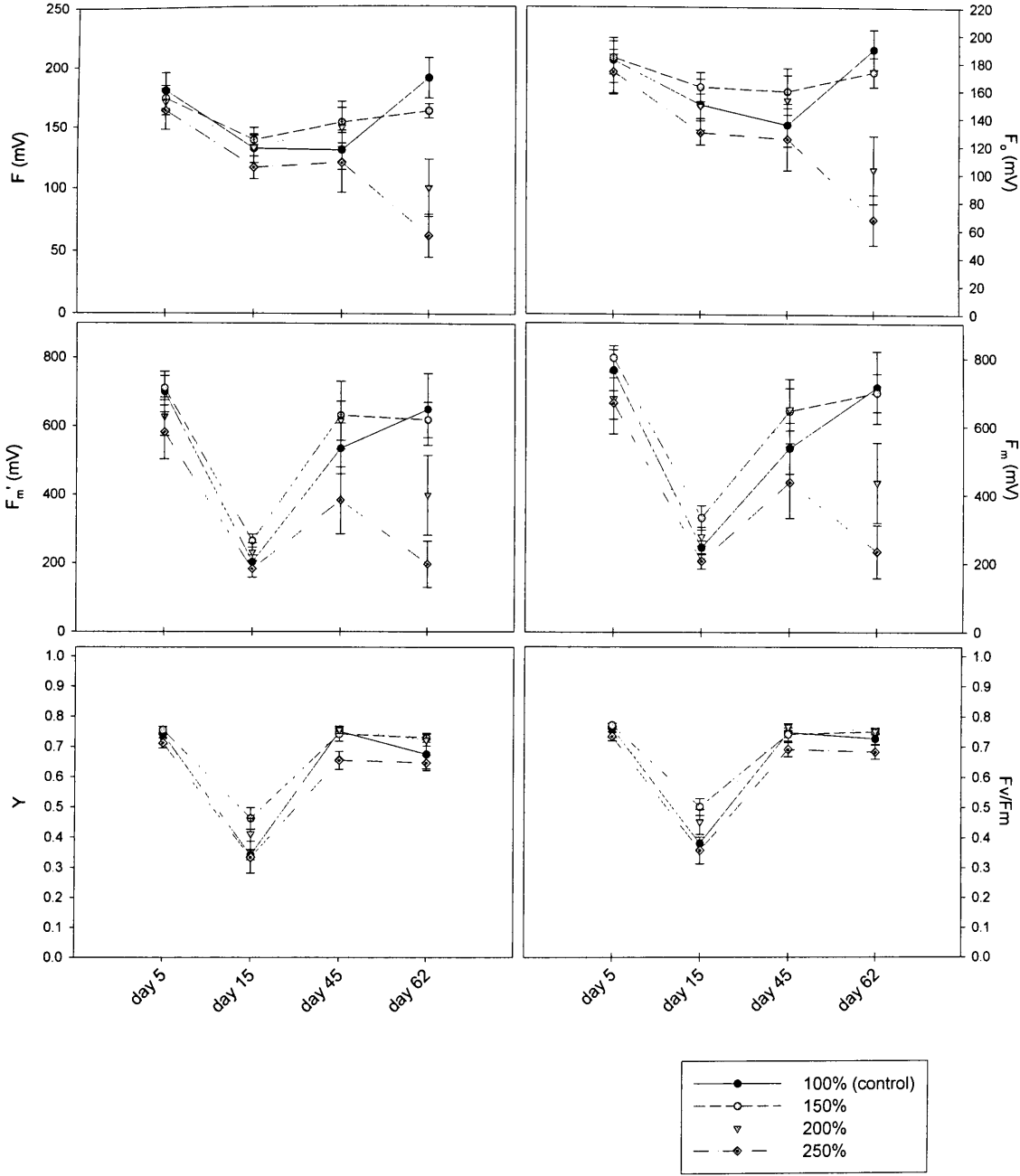


Figure 21: Minimum and maximum fluorescence values of dark-adjusted samples in hypo-osmotic treatments, pre and post salinity change. Error bars are ± 1 SE (n=5).

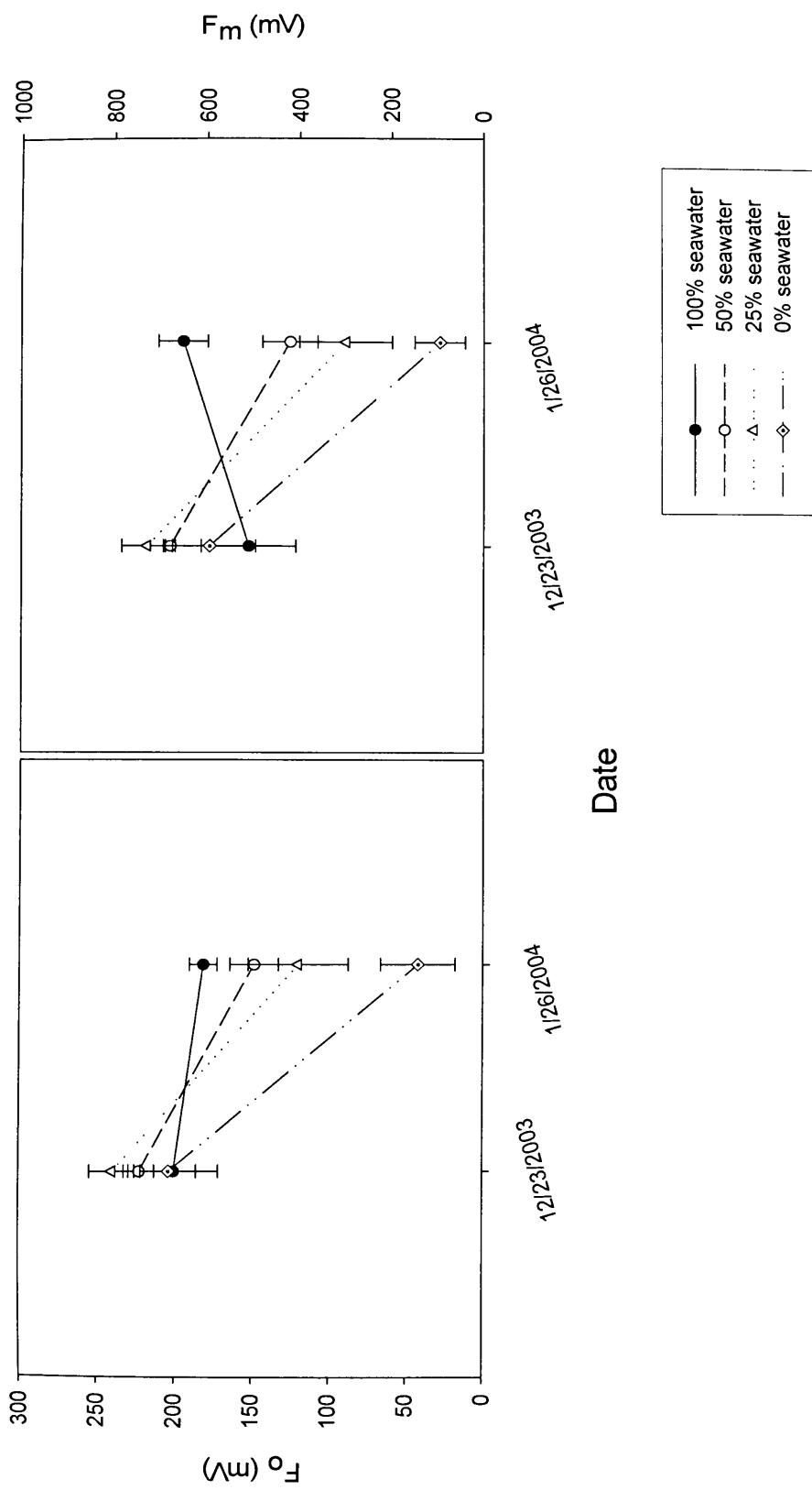


Figure 22: Minimum and maximum fluorescence measurements taken across time after the application of the hypo-osmotic stress. Error bars are ± 1 SE (n=5).

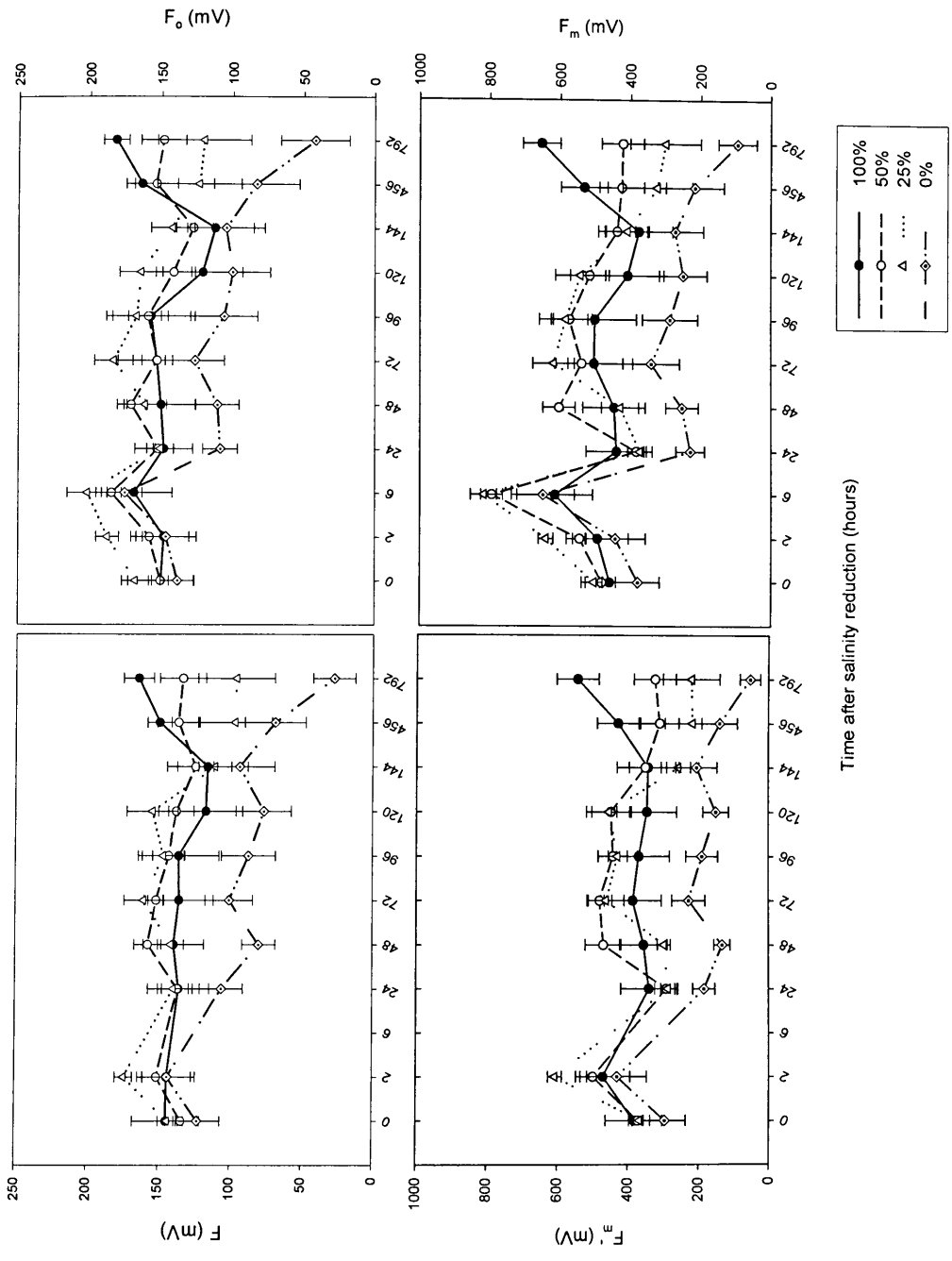


Figure 23: F_v/F_m and Y ratios across time after the application of the hypo-osmotic stress. Error bars are ± 1 SE ($n=5$).

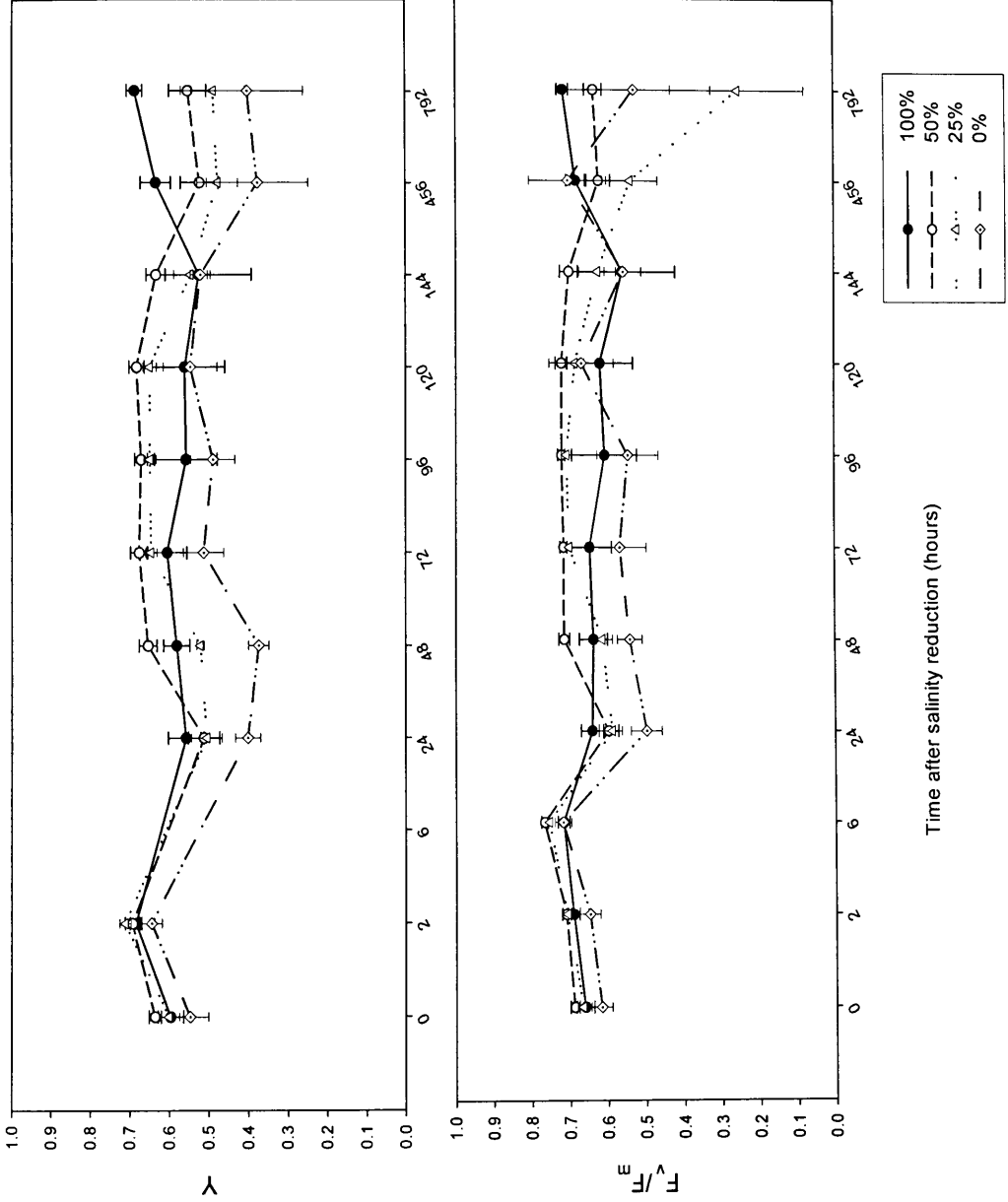
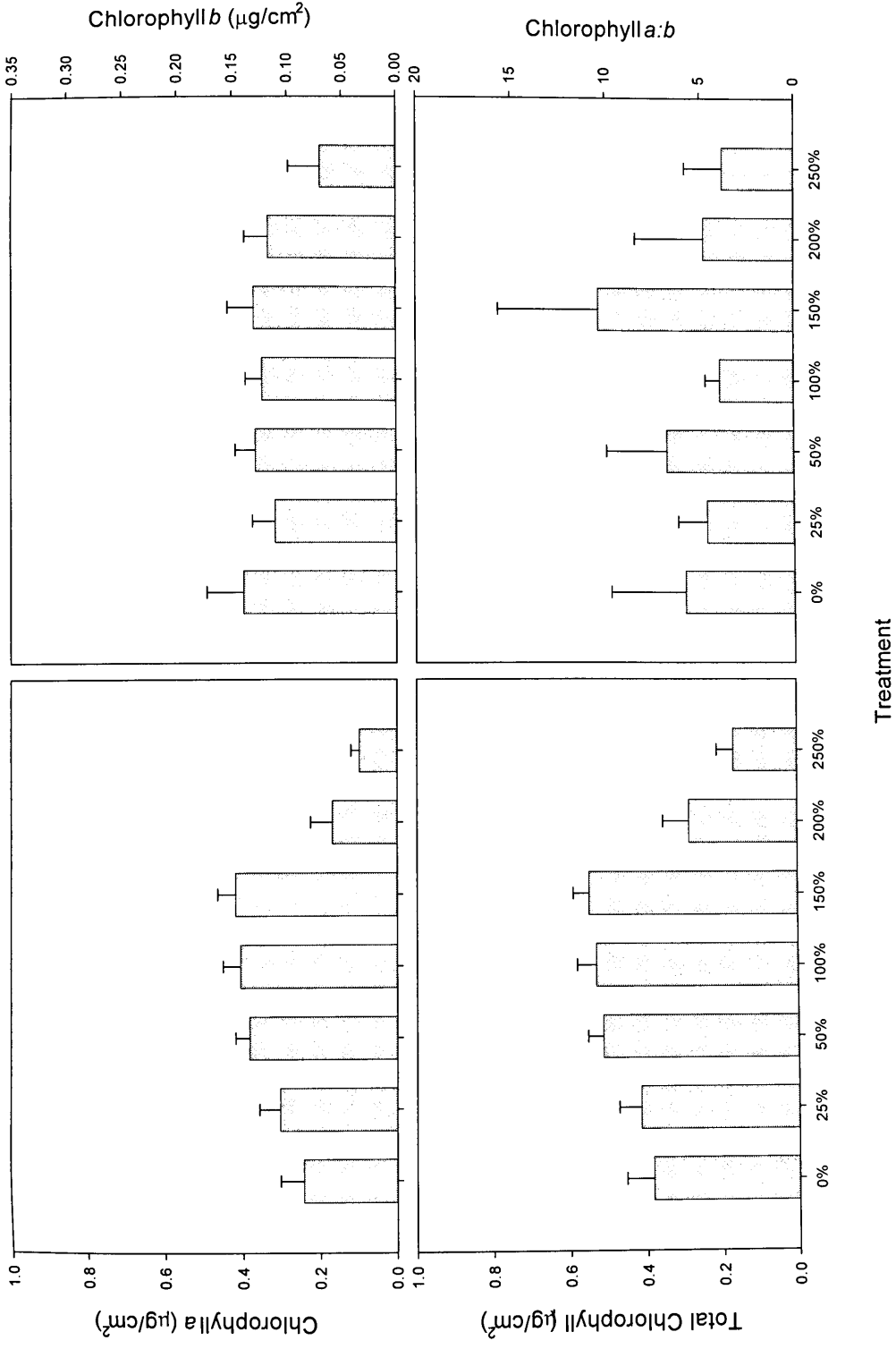


Figure 24: Photosynthetic pigment concentrations of both the hyper and hypo-osmotic experiments. Error bars are ± 1 SE (n=5).



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