DOI 10.1515/bot-2012-0226 — Botanica Marina 2013; 56(3): 261–273

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Variation in pigment content of *Thalassia testudinum* seedlings in response to changes in salinity and light

Abstract: Changes in pigment content allow seagrasses to photo-acclimate to dynamic light environments, but light- and salinity-induced stress may reduce this ability. In this study, *Thalassia testudinum* seedlings were exposed to two light treatments (50–70% reduction and full sun) and three salinity treatments (20, 35, and 50) in a 44-day mesocosm experiment. Variation in leaf pigment content (chlorophylls and carotenoids) was determined three times a day (0900, 1200, and 1800 h) during pretreatment, at treatment target conditions, and following a recovery period in the pretreatment conditions using reverse-phase high-performance liquid chromatography (HPLC). Seedlings in the salinity 50 treatment had reduced chlorophyll and carotenoid content after 6 days at their target salinity, a common stress response in plants. High salinity also induced increased nonphotochemical quenching (NPQ) during midday. The shaded seedlings had significantly lower chlorophyll *a*:*b* ratios, due largely to the increased chlorophyll *b* content. Epoxidation state (EPS) and NPQ significantly varied during the day among all the treatments, suggesting that the seedlings responded quickly to the diurnal fluctuations in light. No significant residual differences among the treatment seedlings were detected for chlorophylls or carotenoids from leaves sampled at the conclusion of the recovery period. The results indicate that hypersalinity caused increased sensitivity to high irradiances in *T. testudinum* seedlings, requiring an increased photoprotection.

Keywords: carotenoids; chlorophylls; diurnal variation; photoprotection; *Thalassia testudinum*.

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Introduction

Seagrasses have several mechanisms to acclimate their photosynthetic efficiency and capacity to changing environmental conditions (Dennison and Alberte 1986, Enríquez et al. 2002, Major and Dunton 2002). Changes in pigment pools allow plants to acclimate to longer-term changes in light regimes (Abal et al. 1994, Lee and Dunton 1997, Alcoverro et al. 2001), as the amount of light that a leaf is able to absorb for photosynthesis largely relies on its pigment content (Cummings and Zimmerman 2003). Near real-time changes in pigment contents and ratios also permit seagrasses to tolerate short-term light fluctuations by downregulating energy transduction (Dennison and Alberte 1986, Beer et al. 2000, Ralph et el. 2002, Belshe et al. 2007). Adjustments to chlorophyll concentrations can improve light absorption during periods of reduced light. For example, an increase in total chlorophyll content (Dennison and Alberte 1985, Longstaff and Dennison 1999, Cummings and Zimmerman 2003) and a reduction in the ratio of chlorophyll *a* to chlorophyll *b*, by increasing the chlorophyll *b* content relative to chlorophyll *a* (Ralph and Gademann 2005) can increase the amount of light absorbed for photosynthesis and converted to chemical energy. As a result, the total chlorophyll content of the leaves is usually higher, and the chlorophyll *a:b* ratio is usually lower in plants that are shade-acclimated (Geider et al. 1997, Matsubara et al. 2007).

Carotenoid pigments, including the xanthophyll cycle pigments, aid in light absorption as accessory pigments, acting in photoprotection and as structural units within the light-harvesting complexes (Paulsen 1999). The photoprotective mechanisms utilized by plants also include decreasing light absorption or providing alternative energy sinks (MacIntyre et al. 2000). When plants are exposed to increased light, the excess light energy may be diverted to the xanthophyll cycle in a process known as downregulation or nonphotochemical quenching (NPQ; Adams III et al. 1996). In the xanthophyll cycle, the carotenoid pigment violaxanthin becomes de-epoxidized

initially to antheraxanthin, then to zeaxanthin. This action of de-epoxidation safely de-excites chlorophyll pigments and converts the excess energy to heat, hence, contributing to NPQ (Demmig-Adams et al. 1996, Brown et al. 1999, Ralph et al. 2002). This process of photoprotection allows for carbon assimilation, while preventing photooxidative damage to photosystem II (PSII) reaction centers by oxidative free radicals (Demmig-Adams and Adams 1994, Ralph et al. 2002). The removal of the excess light energy from the photosystems also results in a reduction of the photosynthetic efficiency of PSII and, thus, lowered maximum photochemical efficiency of PSII (F_v/F_m). However, it prevents a prolonged depression in photosynthetic efficiency (photoinhibition) that would occur if damage to the photosystems was sustained (Ralph et al. 2002).

The de-epoxidation of violaxanthin to zeaxanthin is reversible overnight or under low light conditions; zeaxanthin is epoxidized back to violaxanthin, restoring efficient light absorption (Demmig-Adams and Adams 1994). Owing to the xanthophyll cycle's dependence on the prevailing light conditions, the changes in these pigment concentrations exhibit similar diurnal fluctuations to photosynthetic efficiency (Brown et al. 1999, Ralph et al. 2002). The size of the xanthophyll cycle pool tracks long-term changes in the light environment and photoprotective demand (Niinemets et al. 2003); seagrasses in reduced light conditions maintain lower concentrations of xanthophyll cycle pigments than their counterparts in higher light conditions (Collier et al. 2008).

β-carotene is another carotenoid accessory pigment that extends the absorption spectrum of plants to increase light absorption (Telfer 2002) and is a precursor to the xanthophyll pigments (Ralph et al. 2002). β-carotene also acts as an antioxidant in plants (Telfer 2002), scavenging reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) that have the potential to cause damage in plants (Elstner et al. 1988, Smirnoff 1993).

Salt stress may cause significant alterations in pigment contents and chloroplast structure in seagrasses (Parida et al. 2003, Trevathan et al. 2011). Increased salinity imposes ionic and oxidative stresses on chloroplasts, particularly by affecting the structural integrity of thylakoid membranes, causing a decreased efficiency in the ability to capture and convert photoenergy (Parida et al. 2003). As a result, seagrasses reduce the costly production of chlorophyll (Parida and Das 2005, Silva et al. 2010), perhaps relying on internal light scattering to increase light absorption (Cummings and Zimmerman 2003). For example, Parida et al. (2003) observed reduced chlorophyll *a*:*b* with an increased salt treatment (400 nM of NaCl) vs. the control (0 nM). In contrast, xanthophyll cycle activity and NPQ may increase under hypersaline conditions due to osmotic stress causing plants to be more sensitive to high irradiance (Masojídek et al. 2000, Qiu et al. 2003). Ralph et al. (2002) observed diurnal fluctuations of pigment content (particularly xanthophyll pigments) in the temperate seagrass *Zostera marina* in relation to light conditions, but information on diurnal variation in pigments in tropical seagrasses such as *Thalassia testudinum* is lacking.

Thalassia testudinum is the dominant seagrass in south Florida, a region that exhibits wide variations in salinity and water clarity (Zieman 1982). Recent widespread losses of *T. testudinum* have been attributed to hypersalinity, disease, anoxia, sulfide toxicity, and turbid water conditions created by algal blooms and resuspended sediments (Robblee et al. 1991, Durako et al. 2002, Fourqurean et al. 2003). Seedlings are an important source of recruitment in this species (Whitfield et al. 2004). Because *T. testudinum* exhibits continuous embryo development, and the seeds germinate as they are released from the parent plants (Kuo and den Hartog 2006), there is no seed bank. Thus, seedlings are produced as relatively uniform annual cohorts, which make them ideal as independent experimental units. Chlorophyll fluorescence and growth in *T. testudinum* seedlings exhibit significant changes in response to variations in light and salinity (Howarth and Durako 2013). The objective of this research was to compare leaf chlorophyll and carotenoid pigment levels of *T. testudinum* seedlings over diurnal cycles in response to changes in light and salinity. The associated null hypothesis was that the pigment levels will not change in response to irradiance and salinity variation. The changes in the pigment content were then compared to the diurnal trends observed in pulse amplitude modulated (PAM) fluorometry parameters (Howarth and Durako 2013).

Materials and methods

Collection of plant material

Seedlings of *Thalassia testudinum* Banks ex König collected from Key Biscayne, Florida (25.716°N 80.149°W), on August 14, 2010 were shipped overnight to the University of North Carolina Wilmington, Center for Marine Science

(UNCW/CMS), Wilmington, North Carolina. After the arrival at CMS, the seedlings were immediately planted in six-celled plastic nursery pots (each cell 5×5×7 cm) containing aragonite shell hash, and they were held in a holding vault (55×110×30 cm) with flow-through seawater (salinity 29–35). The Practical Salinity Scale (PSS) was used to determine salinity. Units are not assigned to salinity values because it is a ratio and has no units as defined by UNESCO (1985). The seedlings were allowed to grow for 4 weeks in the vault prior to their placement in the experimental aquaria.

Mesocosm experiment

At the start of the experiment, two six-celled pots were placed in each of four replicate 38-l treatment aquaria. The aquaria were placed outside within seawater-supplied fiberglass vaults (55×110×30 cm) located on the south side of CMS. The vaults acted as water baths to minimize daily water temperature fluctuations, and the aquaria were randomly arranged within the vaults (four aquaria per vault) to account for spatial differences. Water temperatures were monitored using Hobo temperature loggers (Onset®, Pocasset, MA, USA). During the 41-day experiment, water temperatures varied from 33.0°C during the day in September to 15.7°C during the night in October. The temperature variation was within the seasonal range known for south Florida (Fourqurean et al. 2003), and temperatures did not significantly vary among the treatment aquaria ($p>0.05$; data not shown). The experimental design consisted of two light treatments (full sun and 50–70% shade) and three salinity treatments (20, 35, and 50). The treatment salinities were representative of variations in salinity that are common in south Florida estuaries, due to interannual fluctuations in rainfall, freshwater runoff, and evaporation (Nuttle et al. 2000). Shorter-term variations in salinity can also occur due to tidal and wind advection (Wang et al. 2003). Both hypo- and hyper-salinity conditions are known to negatively affect *Thalassia testudinum* (Kahn and Durako 2006, Koch et al. 2007). Initially, all seedlings were acclimated to the control salinity (35), which was formulated with deionized (DI) water and Instant Ocean© salts, under full sun for 7 days (Pretreatment). Following the Pretreatment, neutral-density screens were placed over the shaded treatment seedlings and either deionized (DI) water or Instant Ocean© salts were added to the appropriate aquaria to decrease or increase the salinity by 2 per day until the target salinities were reached 8 days later (Changing). The salinity of the aquaria was checked daily for the duration of the experiment using a YSI Model 80 conductivity meter (Yellow Springs, OH, USA). The gradual change in salinity allowed the seedlings time to acclimate to the changes in salinity (Kahn and Durako 2006, Koch et al. 2007). The seedlings were kept at target salinities for 17 days (Target). Following the target period, the salinities were brought back to the control salinity over the course of 8 days by adding DI water or Instant Ocean© salt. At this time, shade cloths were removed, and the seedlings were kept at the control salinities for 6 days and monitored for recovery (Recovery). During the experimental period, seedling leaves were cleaned daily to remove settled particles and epiphytes.

Leaf samples for chlorophylls *a* and *b* and carotenoid analyses were taken for each light and salinity treatment combination three times a day (0900, 1200, and 1800 h) at the start of the experiment (day 0), at the end of Pretreatment (day 7), and 6 days after the seedlings had been at the Target salinities (day 22). Samples were also taken at 1200 h at the conclusion of the Recovery period (day 44), after the seedlings had been returned to the control salinity and full sun treatments for 6 days. Samples were only taken at the 1200-h sampling time on that day due to the limited number of seedlings remaining after our previous sample periods.

Pigment extraction and analysis

For each sampling, three replicate rank-2 leaves were collected, cleaned of any epiphytes, and patted dry. The leaves were weighed (fresh wt.) and then immediately ground using liquid nitrogen and a mortar and pestle under low light conditions. The mortar and pestle were kept in a -4°C freezer in order to minimize the damage to the chloroplasts. The ground leaf tissue was rinsed into centrifuge tubes wrapped in aluminum foil with 3 ml of 100% methanol (Fisher Scientific, Waltham, MA, USA) and stored in the dark at -4°C. The methanol leaf extracts were filtered under low light using 25-mm GF/F Whatman glass fiber filters and a vacuum pump, 500 µl of filtered extract, and 250 µl of 1 M ammonium acetate (Fisher Scientific, Waltham, MA, USA) was transferred to a glass test tube. The test tube was then covered and allowed to develop for 5 min before 200 µl of the extract was injected into a high-performance liquid chromatography (HPLC) Supelco (Sigma-Aldrich, St. Louis, MO, USA) MOS-2 HYPERSYL column (10 cm in length, 4.6 mm in diameter) with a flow rate of 0.01 ml min^{-1}. Pigments were separated via reversed-phase HPLC using a

Hewlett Packard (Palo Alto, CA, USA) HPLC model 1100, with techniques described by Vidussi et al. (1996). The concentrations in the extracts were calculated from the integrated peak area of the resulting chromatograms, based on retention times of the standards provided by the International Agency for ^{14}C Determination, VKI Water Quality Institute (Horsholm, Denmark). Chlorophyll *a*, chlorophyll *b*, violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), lutein, and β-carotene were the pigments included in the analysis. All chlorophyll contents are presented as mmol of chlorophyll $gram^{-1}$ of fresh weight, and all carotenoids are presented as mmol of pigment mol^{-1} of chlorophylls *a* and *b*. Owing to the low levels of antheraxanthin and zeaxanthin in the leaf samples, epoxidation state (EPS) was used to indicate xanthophyll cycle activity during the analysis. The EPS was calculated as (V+0.5A)/V+A+Z and is defined as the proportion of the xanthophyll cycle pigment pool that is in the de-epoxidized form in comparison to the proportion that is in the epoxidized form (Thayer and Björkman 1990, Ralph et al. 2002). Values closer to 1 are equivalent to more violaxanthin than antheraxanthin and zeaxanthin and indicate a low xanthophyll cycle activity. Non-photochemical quenching (NPQ) calculated as $F_m\text{-}F'_m/F'_m$, is also presented here as variation in these values reflects changes in xanthophyll cycle activity. The fluorescence values were determined from the measurements of rank 2 leaves taken at the 1200 h (F'_m) and 2100 h (F_m) sampling times with light (1200 h) and dark (2100 h) acclimated leaves, using a Mini-PAM (Walz GmbH, Effeltrich, Germany) fluorometer (Howarth and Durako, 2013). The NPQ values presented in the statistical analyses and figures are only from the days and times that the pigments were quantified.

Statistical analysis

The statistical analyses were completed using the JMP7® and SigmaStat® for Windows. The pigment content for each sampling date was tested for variance among light treatment, salinity treatment, and time of day using a three-way ANOVA with a Tukey test for pairwise comparisons when significant differences ($p<0.05$) were detected. The normality of the data was tested using the Shapiro-Wilk goodness of fit test. When the data failed this test, the nonparametric Kruskal Wallis (*H*, within and across the time periods) and the Wilcoxon signed rank (the treatment effects for the pooled data) tests were performed for each factor. All values are presented as means±standard error (s.e.).

Results

Chlorophyll

The leaf contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and the ratio of chlorophyll *a* to *b* were analyzed. For ease of comparison, the statistical results will be presented for comparisons among sampling times for the pooled treatment data (uppercase letters in the figures), among-salinity treatments within the specific sampling times for the shade and sun treatments (lowercase letters in figures), and for data pooled across sampling times when the levels among sampling times did not differ significantly (asterisks in figures). There were no significant among-aquaria or time of day variations detected for the leaf samples taken during the Pretreatment period (days 0 and 7; $p>0.05$ for all the chlorophyll data, data not shown). When the seedlings had been at the Target salinities for 6 days (day 22), the time of day was a significant source of variation only for the pooled chlorophyll *b* content, which was lowest at 1800 h in both the light treatments ($H=6.45$, $df=2$, $p=0.04$; uppercase letters in Figure 1C and D).

Significant treatment effects were detected in samples taken when the seedlings had been at the Target salinities for 6 days (day 22). At 0900 h, the high-salinity seedlings (50) had significantly lower pigment contents than the control (35) and the salinity 20 seedlings for chlorophyll *a* ($H=9.70$, $df=2$, $p=0.01$), chlorophyll *b* ($H=11.56$, $df=2$, $p=0.003$), and total chlorophyll ($H=9.70$, $df=2$, $p=0.01$; the lowercase letters in Figure 1A, C, and E, respectively). The salinity 20 and 35 seedlings were not significantly different from each other for all the three of these chlorophyll parameters. There was a significant light and salinity interaction effect for chlorophyll *b* ($H=12.98$, $df=5$, $p=0.02$); the shade 20 and 35 salinity seedlings had the highest pigment contents, and the shade 50 seedlings had the lowest, with the sun 20, 35, and 50 seedlings' pigment levels being intermediate.

Because of the relatively high variability among replicates during midday (1200 h), there were no significant differences detected in the chlorophyll contents among the treatments. At 1800 h, a significant salinity effect was detected for chlorophyll *a* ($H=12.43$, $df=2$, $p=0.002$), chlorophyll *b* ($H=7.30$, $df=2$, $p=0.03$), and total chlorophyll ($H=12.43$, $df=2$, $p=0.002$; lowercase letters in Figure 1A–F). Salinity and chlorophyll content were inversely related; the low (20) salinity seedlings had the highest contents, the salinity 50 seedlings had the lowest, with the control salinity seedlings being intermediate. Chlorophyll *a* and total chlorophyll also exhibited significant salinity and

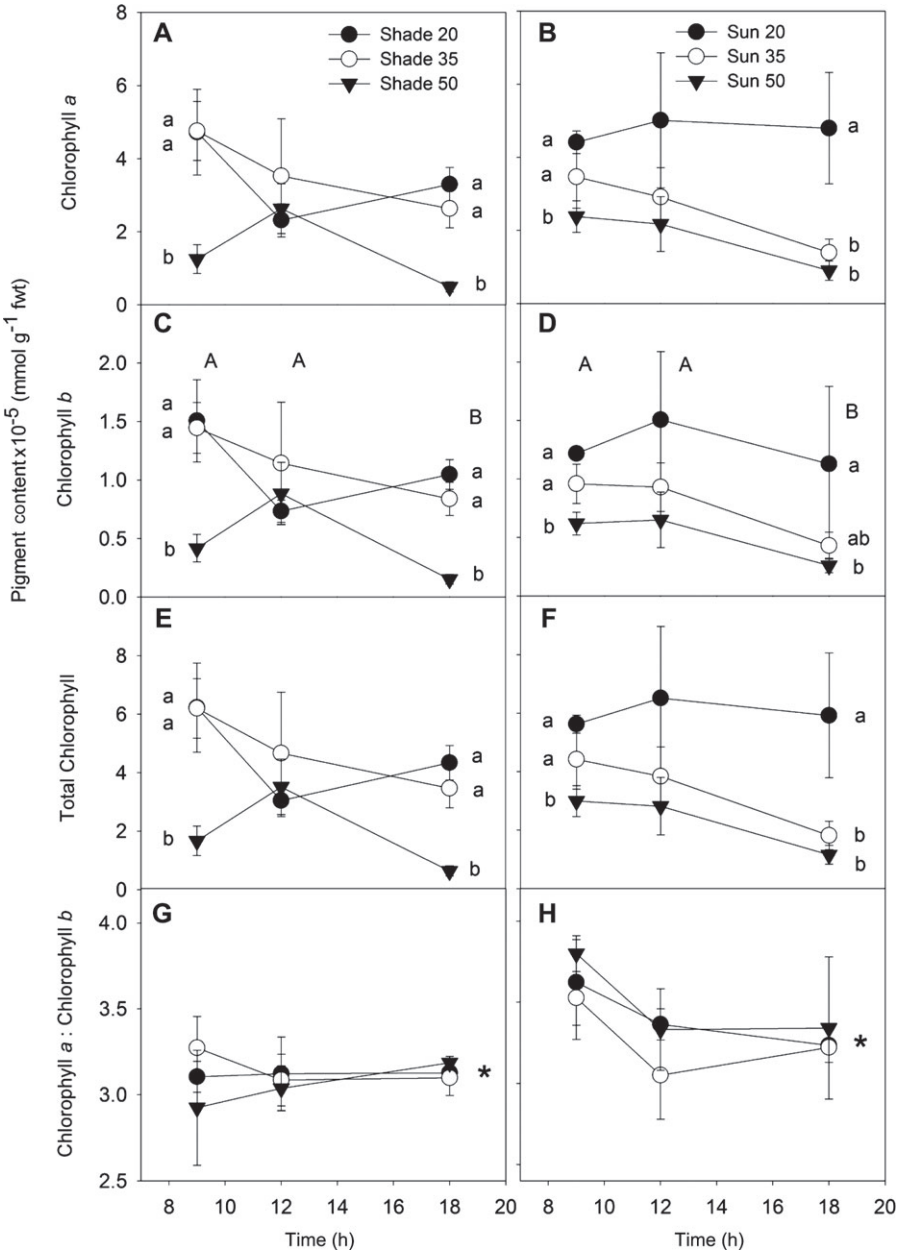

Figure 1 Mean chlorophyll *a* (A and B) and *b* (C and D), total chlorophyll (E and F) contents, and chlorophyll *a*:*b* ratios (G and H) for all the treatment combinations on day 6 of the target period (n=3). The different lowercase letters represent significant differences among the treatments within the specific sample times; the different uppercase letters represent significant differences among the sampling times for the pooled treatment data. The asterisks to the right of the graphs indicate a significant light-treatment effect.

light interactions (H=13.96, df=5, p=0.02; H=13.89, df=5, p=0.02, respectively). For both chlorophyll *a* and total chlorophyll, the sun 20 seedlings had the highest contents, and the sun 35 and 50 and the shade 50 seedlings had the lowest.

With the sampling times pooled, the salinity 50 seedlings had significantly less chlorophyll *a* and total chlorophyll than the salinity 35 and 20 seedlings, which did not differ from each other (H=18.02, df=2, p=0.0001; H=17.66, df=2, p=0.0001, respectively). The seedling chlorophyll *a* and total chlorophyll contents also exhibited significant salinity and light treatment interactions, with the sun 20 seedlings having the highest contents (H=20.37, df=5, p=0.001), the shade and sun 50 seedlings having the lowest (H=19.74, df=5, p=0.001). When the salinity treatments and sampling times were pooled, the sun treatment seedlings

had a significantly higher chlorophyll *a*:*b* ratio than the shade treatment seedlings (H=7.03, df=1, p=0.01, the asterisks in Figure 1G and H). After the treatment seedlings had been returned to the control salinities (35) and with full sun for 6 days (day 44, conclusion of the Recovery period), no significant residual differences among the treatments were detected for any chlorophyll measurements from the leaves sampled at midday (p>0.05 for all the chlorophyll data, data not shown).

Carotenoids and EPS

The variations in β-carotene, lutein, and the xanthophyll cycle pigments (violaxanthin, antheroxanthin, and zeaxanthin) and EPS were analyzed. No significant among-aquaria variation was detected for samples taken during the Pretreatment period (days 0 and 7; Figure 2A and C). There was a significant time of day effect for the EPS on day 0 ($F_{2,6}$=15.93, p=0.004) and day 7 ($F_{2,5}$=7.71, p=0.03), with 0900 h and 1200 h samples having lower values than 1800 h samples (capital letters in Figure 2B and D).

A significant difference in the carotenoid content and the EPS values among the salinity treatments was detected after the seedlings had been at the Target salinities for 6 days (day 22 of the experiment; Figures 3 and 4). At 0900 h, seedlings in the salinity 50 treatment had the lowest contents of lutein (H=6.63, df=2, p=0.036, the lowercase letters in Figure 3E and F). No treatment effects on the carotenoid contents were detected at noon. At 1800 h, significant treatment effects were detected for β-carotene and lutein. The salinity 20 seedlings had the highest β-carotene (H=8.05, df=2, p=0.02, lowercase letters in Figure 3A and B) and lutein (H=7.68, df=2, p=0.02, Figure 3E and F) contents. β-carotene and lutein contents also exhibited significant light and salinity interactions. The sun 20 seedlings had the highest amounts of β-carotene, and the shade and sun 50 and sun 35 seedlings had the lowest (H=11.34, df=5, p=0.04, Figure 3A and B). The sun salinity 20 seedlings also had the highest lutein contents, while the shade and sun salinity 50 seedlings had the lowest contents (H=11.32, df=5, p=0.046, Figure 3E and F). The EPS continued to vary significantly among the times of day during the Target period. The pooled treatment EPS values were highest at 0900 h, followed by 1800 h, with the 1200 h samples having the lowest values (H=7.91, df=2, p=0.02, uppercase letters in Figure 4C and D), indicating the highest xanthophyll cycle activity at noon.

Violaxanthin, lutein, or β-carotene contents did not significantly vary between the light treatments during the Target period, but a significant light effect was detected for the EPS; the shaded seedlings had significantly higher

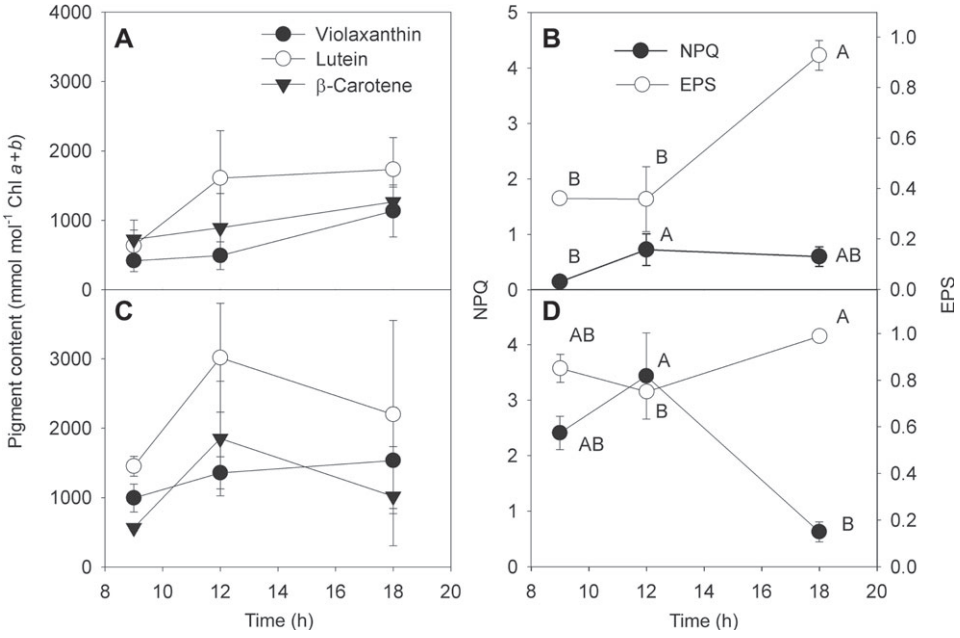

Figure 2 Mean carotenoid contents, EPS, and NPQ on day 0 (A and B) and day 7 (C and D) of the Pretreatment period. Light and salinities did not differ significantly, so the treatments have been pooled (n=18). The different uppercase letters represent significant differences among the sampling times.

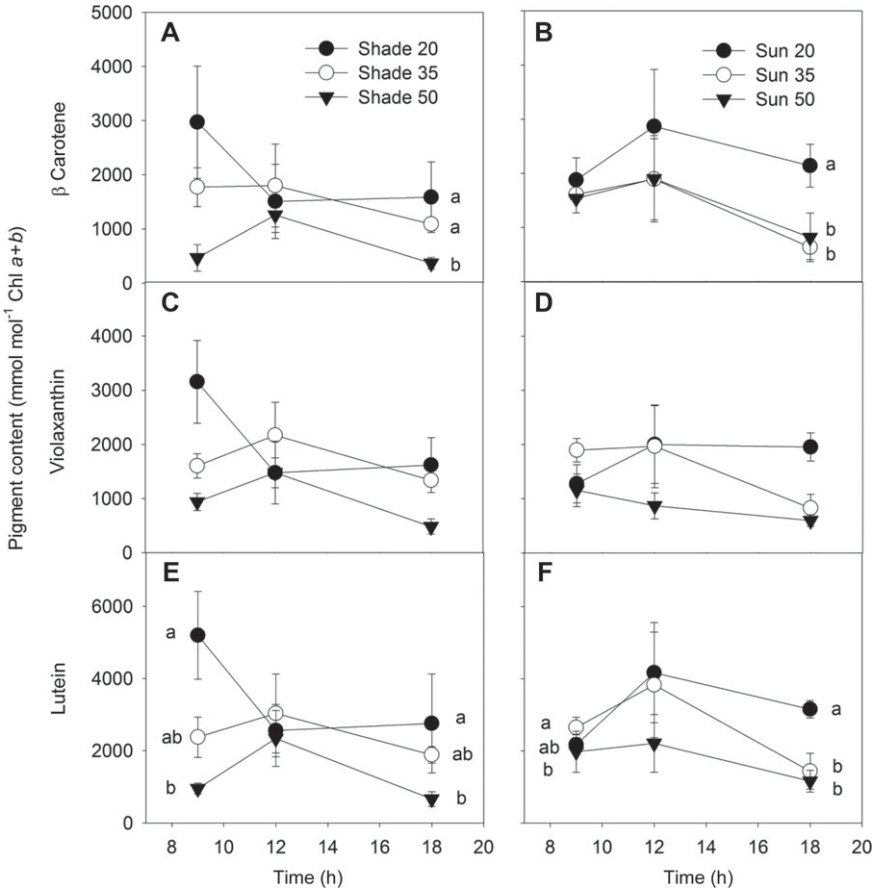

Figure 3 Mean β-carotene (A and B), violaxanthin (C and D), and lutein (E and F) contents for all the treatment combinations at day 6 of the target period (n=3). The different lowercase letters represent significant differences among the treatments within the specific sample times for the pooled treatment data.

EPS values than the full sun seedlings when pooled across time of day and salinity treatment, indicating a lower xanthophyll cycle activity with lower light (H=6.32, df=1, p=0.01; asterisks in Figure 4C and D). Comparing diurnal variation between the light treatments (salinity pooled), only at 1200 h were the EPS values for the shade treatment seedlings significantly higher than the sun treatment seedlings (H=10.34, df=1, p=0.001, Figure 4C and D). There was also a significant light and salinity interaction for the EPS values. The sun 50 seedlings had the lowest EPS values, the sun 35 and shade 20, 35, and 50 seedlings all had high values; the sun 20 seedlings had intermediate EPS values.

Significant salinity effects, as well as salinity and light interactions, were detected for β-carotene, violaxanthin, and lutein with the sampling times pooled. β-carotene and lutein and both showed the same significance pattern among the salinity treatments. The salinity 20 seedlings had the highest contents for both the pigments, the salinity 50 seedlings had the lowest, and the salinity 35 seedlings did not differ from either of the other two salinity treatments (H=13.48, df=2, p=0.001; H=8.76, df=2, p=0.01, respectively). For violaxanthin, the salinity 20 and 35 seedlings both had significantly higher contents than the salinity 50 seedlings (H=14.98, df=2, p=0.001). The significant light and salinity interaction for the β-carotene content indicated that the sun 20 seedlings had the highest content, the shade 50 seedlings had the lowest, and the other treatments were intermediate (H=12.78, df=5, p=0.03). The light and salinity interactions for lutein were only slightly different, with the shade 20 seedlings having the highest lutein content and the shade 50 seedlings the lowest, with the other treatments being intermediate (H=14.29, df=5, p=0.01). The shade 20 seedlings also had the highest violaxanthin content, the shade and sun 50 seedlings had the lowest contents, with the other treatments having intermediate levels of violaxanthin (H=15.70, df=5, p=0.01). The seedlings exhibited no residual treatment differences in the carotenoid pigment contents or the EPS at the conclusion of the Recovery

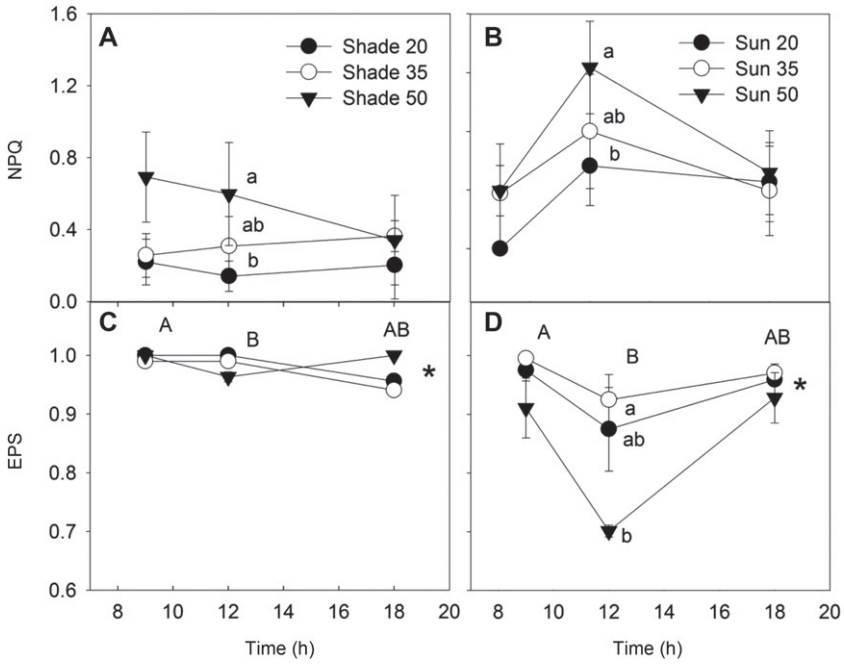

Figure 4 Mean nonphotochemical quenching (NPQ, A and B) and EPS (C and D) values for all the treatment combinations on day 6 of the target period (n=3). The different lowercase letters represent significant differences among the treatments within the specific sample times; the different uppercase letters represent significant differences among the sampling times for the pooled treatment data. The asterisks to the right of the graphs indicate a significant light-treatment effect.

period (day 44), after they had been back at the full-sun control treatment salinities for 6 days (p>0.05 for all the carotenoids and EPS, data not shown).

Nonphotochemical quenching

Time of day was a significant source of variability for the NPQ values on all the pigment sampling dates except for day 22 (p>0.05), which was very cloudy (maximum PAR=483 µmol quanta m^{-2} s^{-1} in our full sun treatment, vs. >900 µmol quanta m^{-2} s^{-1} during most other days of the experiment). When time of day was significant, the NPQ values were highest at 1200 h (H=17.98, df=2, p=0.0001; H=17.65, df=2, p=0.0001; and H=17.06, df=2, p=0.0002; for days 0, 7, and 44, respectively). During Pretreatment (days 0 and 7), there were no significant differences among the aquaria for NPQ values (Figure 2B and D). When the seedlings were at the Target salinities (day 22), significant treatment effects were only detected for the NPQ values at 1200 h (lowercase letters in Figure 4A and B). During this time, there was a significant salinity effect, with the salinity 50 seedlings having the highest NPQ values, the salinity 20 the lowest, and the 35 seedlings not significantly differing from either the salinity treatment (H=6.23, df=2, p=0.04). There was also a significant light and salinity interaction (H=11.43, df=5, p=0.04); the sun 50 salinity seedlings had the highest NPQ values, and the shade and sun 20 seedlings had the lowest. When the sampling times were pooled for day 22, NPQ varied significantly among the salinity treatments. The salinity 50 seedlings had significantly higher NPQ values than the salinity 35 and 20 seedlings, which did not differ from each other (H=7.84, df=2, p=0.02). At the conclusion of the Recovery period (day 44), there were no significant residual light or salinity treatment effects on NPQ detected (p>0.05, data not shown).

Discussion

Hypersalinity (50) significantly reduced the leaf chlorophyll and carotenoid pigment contents and induced increased nonphotochemical quenching (NPQ) relative to the control (35) and hyposaline (20) treatments in the *Thalassia testudinum* seedlings in this mesocosm experiment. The significant diurnal changes in the epoxidation state (EPS) in the sun treatments indicated that xanthophyll cycle pigments can respond rapidly to the changes in the ambient light conditions in this seagrass (Dennison

and Alberte 1986), but the xanthophyll cycle was also negatively affected by hypersalinity. These results indicate that hypersalinity increased the sensitivity to high irradiances of the *T. testudinum* seedlings relative to the control or hyposaline conditions, which required an increased photoprotection. The diurnal changes in the pigments correlated with the diurnal changes in effective photochemical efficiencies of PSII in response to the variations in salinity and light (Howarth and Durako 2013), which were inversely related to xanthophyll cycle activity. Diurnal fluctuations in xanthophyll cycle pigment content and photochemical efficiencies have also been observed in the temperate seagrass *Zostera marina* (Ralph et al. 2002) and in corals (Brown et al. 1999).

Chlorophylls

Chlorophyll *a*, *b*, and the total chlorophyll contents were all significantly lower in the high salinity treatment than the control and the low salinity treatments after the target salinities had been reached (day 22), suggesting that the salinity 50 treatment was the most stressful to the seedlings (Ralph 1999, Trevathan et al. 2011). Trevathan et al. (2011) observed reduced chlorophyll *a* and *b* contents due to pigment degradation and elevated oxidative stress under hypersalinity (45) compared to a control salinity of 30 in mature *Thalassia testudinum* short shoots. Similarly, Ralph (1999) observed a decline in photosynthetic capacity and decreased chlorophyll *a* and *b* in increased salinity treatments compared to control treatments in another tropical seagrass, *Halophila ovalis*. There were also significant differences in the chlorophyll *a*:*b* ratios of the seedlings between our light treatments during the Target period. The shaded seedlings had higher chlorophyll *a* and *b* contents than the full sun seedlings, but the difference in the contents between the treatments was not significant. However, the increased chlorophyll *b* content of the shaded seedlings relative to chlorophyll *a*, caused significantly decreased chlorophyll *a*:*b* ratios. The accumulation of light-harvesting complexes and, therefore, chlorophyll *a* and *b*, is a characteristic of shade-acclimated plants, so we can conclude that our shaded seedlings accumulated more chlorophyll *b* than our sun seedlings, which resulted in increased light absorption (Matsubara et al. 2007). Our results again concur with the findings of Ralph (1999), who detected increased chlorophyll *a* and *b* in decreased light treatments in *H. ovalis*. Similarly, Collier et al. (2008) observed that chlorophyll *a* and *b* increased with depth and, thus, reduced light availability, in the seagrass *Posidonia sinuosa*.

The daily mean PAR quantum flux for the shaded treatments was approximately 8 mol quanta m^{-2} day^{-1}, which exceeds the daily light requirements estimated for the other tropical seagrass species, ≈6 mol quanta m^{-2} day^{-1} (Chartrand et al. 2012). Perhaps no significant light treatment effect was detected in the individual chlorophyll contents because our shaded seedlings were still receiving irradiance levels exceeding their daily light requirements. Hence, the seedlings were not severely light stressed, but were simply becoming shade acclimated as indicated by their reduced chlorophyll *a*:*b*.

Chlorophyll *b* content exhibited significant diurnal variation during the Target period (day 22), with higher chlorophyll *b* occurring earlier in the day (0900 h and 1200 h) than in the evening (1800 h). Accessory pigments are known to respond quickly to a plant's light environment (Dennison and Alberte 1986), but we expected chlorophyll *b* to be highest in the evening, when light was the lowest. However, the PAR data show that the maximum irradiances on this cloudy day were much lower than the previous days (483 μmol quanta m^{-2} s^{-1} in our full sun treatment, vs. >900 μmol quanta m^{-2} s^{-1} on the previous days). It is possible that these seedlings increased the production of chlorophyll *b* in the earlier hours of the day due to this decline in irradiance, which would have increased the leaf light absorption (Dennison and Alberte 1985, Longstaff and Dennison 1999, Cummings and Zimmerman 2003).

It appeared that the seedlings had recovered from the salinity stress by the conclusion of the experiment, as no significant residual salinity effects were detected in the chlorophyll contents. We suggest that the *Thalassia testudinum* seedlings are able to recover from hypersaline stress once salinity conditions returned to within their salinity tolerance range of 20–40, as was also reported by Kahn and Durako (2006). No significant residual light treatment effects were detected either, suggesting that the shaded seedlings had acclimated to full sun by the end of the Recovery period, again reflecting that accessory pigments rapidly respond to light fluctuations (Dennison and Alberte 1986).

Carotenoids, EPS, and NPQ

The significant diurnal variation in the EPS values for the *Thalassia testudinum* seedlings was expected as xanthophyll cycle pigments usually exhibit near real-time responses to changing irradiance conditions (Dennison and Alberte 1986). The de-epoxidation of violaxanthin was occurring in these seedlings, which allowed them to

acclimate to light changes over relatively short periods of time (Demmig-Adams and Adams 1992). During the Target period, the EPS values were highest at 0900 h, showing that any of the antheraxanthin and zeaxanthin from the previous day was epoxidized overnight to restore the pool of violaxanthin (Demmig-Adams and Adams 1994). The EPS values dropped by 1200 h when the irradiance values were close to their maximum, indicating the de-epoxidation of violaxanthin. Later in the day, the EPS values began to approach 1 as zeaxanthin and antheraxanthin epoxidized back to violaxanthin as the light levels decreased (Thayer and Björkman 1990). The lack of a time of day effect for the violaxanthin content is most likely attributable to the large pools of violaxanthin in these seedlings relative to the antheraxanthin and zeaxanthin pools; the de-epoxidation of violaxanthin was occurring, as observed in our EPS values, but not enough to cause significant changes in the overall violaxanthin content.

The shaded seedlings had significantly higher EPS values than the full sun seedlings once the seedlings had been at the Target for 6 days. This suggests that the shaded seedlings were becoming low-light acclimated and had significantly less de-epoxidation of violaxanthin occurring because their light environment did not require as much photoprotection as the seedlings exposed to full sun.

The values of NPQ significantly varied diurnally during the pretreatment period and were inversely related to the EPS (i.e., the NPQ values were highest at the times of the highest irradiance). The only day that a significant time of day effect was not detected in the NPQ was on a cloudy day during the Target period (day 22). As stated before, the irradiance levels on day 22 were much lower than the previous sampling days. The NPQ values did vary during the day, but these changes were not significant. Ralph et al. (2002) observed increased NPQ with increased light in *Zostera marina*; they reported NPQ values around 0.2 at 350 μmol quanta m^{-2} s^{-1} (their moderate light treatment) and 0.6 at 530 μmol quanta m^{-2} s^{-1} (their high-light treatment). NPQ is related to a plant's xanthophyll cycle activity and, therefore, should change with diurnal light fluctuations (Demmig-Adams et al. 1996, Brown et al. 1999). Ralph et al. (2002) suggested that 800 μmol quanta m^{-2} s^{-1} PAR was needed to trigger a conversion of xanthophyll pigments for the temperate seagrass *Zostera marina*. *T. testudinum* is a tropical species with a much higher irradiance tolerance (Kalituho et al. 2007) so the PAR threshold for significant xanthophyll cycle activity would be expected to be higher for this species. We suggest that the maximum PAR values (483 μmol quanta m^{-2} s^{-1}) on this particular day did not reach the threshold of light required to trigger enough xanthophyll cycle activity to significantly affect NPQ.

In our study, NPQ was highest and the EPS was lowest in the salinity 50 seedlings, in both the shaded and full sun treatments, suggesting an increased sensitivity to high irradiance when these seedlings were salt-stressed. Trevathan et al. (2011) likewise observed an increased NPQ with increased salinity in adult *T. testudinum*, though this increase was not significant. NPQ and xanthophyll activity increases with the increased salinity have also been reported in green algae (Masojídek et al. 2000) and coastal plants (Qiu et al. 2003). Others have shown a poor correlation between zeaxanthin formation (EPS) and NPQ in higher plants and macroalgae (Jahns and Krause 1994, Niyogi et al. 1997). In the phytoplankton *Scenedesmus* and *Chlorella*, Masojídek et al. (2000) concluded that zeaxanthin formation made only limited contributions to NPQ. However, under full sun conditions, NPQ and EPS exhibited clear reciprocal diurnal behavior in our seedlings (Figure 4B and D) indicating that downregulation and xanthophyll cycle activity were correlated.

Lutein, the most abundant carotenoid pigment we measured in the *T. testudinum* seedling leaves and the most prominent xanthophyll pigment in higher plants (Matsubara et al. 2005, Esteban et al. 2007), is another pigment associated with the light-harvesting complexes (Ralph et al. 2002). This pigment may also play a role in photoprotection in some plants through a similar xanthophyll cycle called the lutein-epoxide cycle (Bungard et al. 1999, García-Plazaola et al., 2003, Matsubara et al. 2003, 2011, Förster et al. 2011). Lutein epoxide is de-epoxidized to lutein under high irradiance, and lutein is epoxidized back to lutein epoxide in the dark. However, the relaxation of the lutein epoxide cycle is much slower than that of the violaxanthin xanthophyll cycle (García-Plazaola et al. 2003). Owing to this slower relaxation, the de-epoxidation of lutein epoxide to lutein may "lock-in" photoprotection for longer periods of time rather than over diurnal periods (García-Plazaola et al. 2003, Matsubara et al. 2005, Förster et al. 2011). The lutein xanthophyll cycle may be a contributor to the NPQ in *T. testudinum*; however, the HPLC methods utilized here were unable to detect the lutein epoxide in our leaf material. In addition, the diurnal variations in lutein were not significant, so speculation on the possible photoprotective role of lutein in *T. testudinum* will not be attempted. Ralph et al. (2002) also detected no significant variations in the lutein content throughout the day in *Zostera marina*. However, because of its abundance, the role of lutein (and possible presence of lutein epoxide) in photosynthesis or photoprotection in seagrasses should be considered in future research.

β-carotene is a precursor to the xanthophyll cycle pigments, so we expected to see a lower β-carotene, as well as violaxanthin, content in the shaded seedlings compared to those in the full sun (Kalituho et al. 2007, Matsubara et al. 2007). The shaded seedlings did have less β-carotene and violaxanthin than the full sun seedlings, but these differences were not significant. Because hypersalinity induces excess ROS production in *T. testudinum* adults (Trevathan et al. 2011), the salinity 50 treatment seedlings were expected to increase their β-carotene production, which would potentially scavenge more of these harmful molecules. However, the high-salinity seedlings had the lowest β-carotene content. This may be because the production of antioxidants is metabolically expensive and requires carbon that may have been required for increased production of compatible solutes, which are necessary for the maintenance of a negative water potential and cell turgor in *T. testudinum* under high salinities (Kahn and Durako 2006).

Recovery of the *T. testudinum* seedlings at the conclusion of the experiment (day 44) was indicated by the lack of significant differences in the carotenoid contents for the seedlings from any of the light and salinity treatments. No time of day effect could be ascertained for the EPS on this sampling date because the samples were only taken at noon. However, NPQ significantly varied diurnally, being highest at noon suggesting that the downregulation was still occurring in all the treatment seedlings.

Conclusions

Our pigment results indicate that *Thalassia testudinum* seedlings exposed to a salinity of 50 were more stressed than the seedlings exposed to the salinities of 20 or 35. The salinity 50 seedlings had significantly lower chlorophyll, violaxanthin, lutein, and β-carotene contents, suggesting a decreased synthesis of these pigments under hypersaline conditions. Kahn and Durako (2006) reported that a salinity of 20 was within the physiological tolerance range for the *T. testudinum* seedlings, and they also observed significant increases in stress being exhibited in hypersaline (50 and 60) conditions. Higher pools of xanthophyll cycle pigments are typically seen in seagrasses that are exposed to high light conditions (Adams III et al. 1996). Here, the higher xanthophyll cycle pigment levels in the salinity 50 seedlings may be due to an increased sensitivity to higher irradiances brought on by salinity (and oxidative) stress. The pigment results presented here concur with diurnal PAM fluorescence data, which also indicated that the salinity 50 treatment was more stressful to the *T. testudinum* seedlings than the salinity 20 or 35 treatments (Howarth and Durako 2013). Significantly higher NPQ and lower EPS were observed in the salinity 50 seedlings, which correlates with decreased PSII photochemical efficiencies in response to hypersalinity (Kahn and Durako 2006, Howarth and Durako 2013). Increased downregulation typically results in decreased photosynthetic efficiency. The diurnal fluctuations in the xanthophyll cycle activity most likely contribute to the diurnal variation in the effective photochemical efficiencies and relative electron transport rates (rETR) observed in *T. testudinum* (Belshe et al. 2007, Durako 2012). While it is clear that changes in the PAM fluorometry parameters and plant pigment contents in response to environmental stressors are related, it would be useful to gain a better understanding of these relationships with respect to carbon allocation (antioxidants vs. compatible solutes) and the apparent need for increased photoprotection in *T. testudinum* seedlings under hypersaline conditions. Our results support the goals for water management and ecological restoration in south Florida, which are to increase the release of freshwater into Florida and Biscayne Bays in an effort to minimize the presence of the seasonally hypersaline conditions in these *T. testudinum*-dominated systems (Nuttle et al. 2000).

Acknowledgements: This research was supported by a grant from the Florida Fish and Wildlife Conservation Commission (Grant Nos. 509620 and 56990), which was funded by a cooperative agreement with the South Florida Water Management District (SFWMD #4600001348). The authors would like to thank Justin Campbell of Florida International University, Miami, Florida, for collecting and shipping the *Thalassia testudinum* seedlings.

Received 29 November, 2012; accepted 24 April, 2013; online first 21 May, 2013

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