Physiological and Photosynthesis-related Gene Expression Analysis of Seagrass *Enhalus acoroides* (L. f.) Royle under Salt Stress

Pimpanit Kongrueang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Botany

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Thesis Title	Physiological and photosynthesis-related gene expression analysis of seagrass *Enhalus acoroides* (L. f.) Royle under salt stress
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Candidate

ชื่อวิทยานิพนธ์ การวิเคราะห์ทางสรีรวิทยาและการแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์ด้วยแสงของหญ้าทะเลชนิด *Enhalus acoroides* (L. f.) Royle ภายใต้สภาวะความเครียดเค็ม

ผู้เขียน นางสาวพิมพนิต คงเรือง

สาขาวิชา พฤกษศาสตร์

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บทคัดย่อ

การศึกษาครั้งนี้มีจุดมุ่งหมายเพื่อตรวจสอบการตอบสนองทางสรีรวิทยา (การตอบสนองทางสรีรวิทยาที่เกี่ยวข้องกับแสงและออสโมติก) และการแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์ด้วยแสงในหญ้าทะเลชนิด *Enhalus acoroides* หลังได้รับความเค็มแตกต่างกันคือ 10, 20 (ความเค็มต่ำ), 30 (ควบคุม), 40 และ 50 (ความเค็มสูง) เป็นระยะเวลา 20 วัน

จากการศึกษาครั้งนี้พบว่าสภาวะความเค็มต่ำและความเค็มสูงส่งผลต่อสรีรวิทยาที่เกี่ยวข้องกับแสงของต้นกล้า *E. acoroides* โดยประสิทธิภาพการใช้แสงสูงสุดของระบบแสงสอง (F_v/F_m) และปริมาณของคลอโรฟิลล์ในใบหญ้าทะเล *E. acoroides* ลดลง และพารามิเตอร์ดังกล่าวแสดงให้เห็นว่าการสังเคราะห์ด้วยแสงมีความไวต่อสภาวะความเค็มต่ำมากกว่าความเค็มสูง สภาวะความเค็มสูงส่งผลให้ปริมาณน้ำของรากเพิ่มสูงขึ้นและยังส่งผลให้ปริมาณโซเดียมไอออนในเนื้อเยื่อเพิ่มขึ้นแต่ไม่ส่งผลกระทบต่อการสังเคราะห์ด้วยแสง สรุปได้ว่าการรักษาสมดุลไอออนของต้นกล้า *E. acoroides* จะได้รับผลกระทบจากสภาวะความเค็มสูงน้อยกว่าความเค็มต่ำซึ่งสังเกตได้จากอัตราส่วน K^+/Na^+ ในใบที่ลดลงในวันที่ 20 ของการทดลอง นอกจากนี้พบว่าประสิทธิภาพการสังเคราะห์ด้วยแสง (F_v/F_m และปริมาณของคลอโรฟิลล์) ไวต่อการเปลี่ยนแปลงของความเค็มจึงสามารถนำมาใช้เป็นตัวบ่งชี้ของความเครียดเค็มในหญ้าทะเลชนิดนี้ได้

การแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์ด้วยแสงแสดงให้เห็นว่าสภาวะความเค็มต่ำและความเค็มสูงส่งผลให้การแสดงออกของยีน *LHCB* ในใบ *E. acoroides* มีแนวโน้มเปลี่ยนแปลงแต่ยังไม่ชัดเจน แนวโน้มการแสดงออกของยีน *LHCB* ที่ลดลงอาจส่งผลให้ปริมาณคลอโรฟิลล์และกระบวนการสังเคราะห์ด้วยแสงลดลงดังที่ปรากฏในการศึกษาทางสรีรวิทยา ในระยะท้ายของการศึกษาพบว่าการแสดงออกของยีน *RCA*, *psbA* และ *psbD* แสดงออกเพิ่มมากขึ้นซึ่งอาจเกี่ยวข้องกับการซ่อมแซมความเสียหายที่เกิดกับระบบแสงสอง

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Abstract

This study aims to examine physiological responses (photophysiological and osmotic responses) and photosynthesis-related gene expression in seedlings of the seagrass *Enhalus acoroides* after exposure to different salinity levels. Seagrass seedlings were grown for 20 days in control (salinity 30), hyposaline (salinity 10 and 20) and hypersaline (salinity 40 and 50) conditions.

The present study showed that both hypo- and hypersaline conditions affected the photophysiology of *E. acoroides* seedlings, reducing the maximum quantum yield of photosystem II (F_v/F_m) and total chlorophyll content. The photosynthetic system appeared to be more sensitive to hyposaline than to hypersaline conditions as shown by immediate declines in F_v/F_m and total chlorophyll content. Hyposaline conditions increased the water content in roots. The increase in tissue Na^+ content induced by hypersalinity did not affect photosynthetic integrity and was more pronounced in leaves than in roots. It is concluded that the ionic homeostasis of *E. acoroides* seedlings is less affected by shortterm hypersalinity than by hyposalinity. The K^+/Na^+ ratios in leaves with hypersalinity decreased by 20 days after treatment. Additionally, the photosynthetic efficiency (F_v/F_m and total chlorophyll content) is highly sensitive to salinity shifts and can be used as a marker for short-term acclimation to salinity stress in this seagrass species.

The photosynthesis-related gene expression showed that hypo- and hypersalinity conditions unclearly changed *LHCB* gene expression in *E. acoroides* leaves. The decline trend of *LHCB* transcript might correspond to the chlorophyll content and photosynthesis decreases in the physiological study. At the late

experiment, *RCA*, *psbA* and *psbD* gene were up-regulated which are possibly related to the repair of occurred photodamage in photosystem II.

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Pimpanit Kongrueang

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List of abbreviations and symbols

°C	= degree Celsius
µg ml^{-1}	= microgram per milliter
µl	= microliter
A_{470}	= absorbances at 470 nanometer wavelengths
A_{646}	= absorbances at 646 nanometer wavelengths
A_{663}	= absorbances at 663 nanometer wavelengths
Ca^{2+}	= calcium ion
cDNA	= complementary deoxyribonucleic acid
Chl *a*	= Chlorophyll *a*
Chl *b*	= Chlorophyll *b*
Cl^-	= chloride ion
cm	= centimeter
C_T	= the threshold cycle for reference or target amplification
DAT	= days after treatment
DW	= sample dry weight
F_m	= maximum value for chlorophyll fluorescence in the dark state
F_o	= minimum value for chlorophyll fluorescence in the dark state
F_v	= maximum variable chlorophyll fluorescence
F_v/F_m	= maximum quantum yield
FW	= sample fresh weight
h	= hour
HNO_3	= nitric acid
I	= intensity of the transmitted light.
i.e.	= id est (that is)
I_0	= intensity of the incident light
K^+	= potassium ion
KH_2PO_4	= potassium phosphate
LHCB	= light-harvesting chlorophyll *a*/*b* binding
LSD	= least significant difference

List of abbreviations and symbols (continued)

mg g^{-1}	= microgram per gram
mg l^{-1}	= milligram per liter
min	= minute
ml	= milliter
n	= number of sample
Na^{+}	= sodium ion
$NaNO_3$	= sodium nitrate
nm	= nanometer
PAM	= pulse amplitude modulated
PAR	= Photosynthetically Active Radiation
PPFD	= photon flux density
PSI	= photosystem I
PSII	= photosystem II
psbA	= photosystem II reaction center D1
psbD	= photosystem II reaction center D2
qPCR	= quantitative polymerase chain reaction
RCA	= rubisco activase
RNA	= ribonucleic acid
ROS	= reactive oxygen species
rpm	= revolutions per minute
s	= second
S.E.	= standard error
TW	= sample turgid weight

List of publications

Paper: **Kongrueang, P.,** Buapet, P. and Roongsattham, P. 2018. Physiological responses of *Enhalus acoroides* to osmotic stress. *Botanica marina*. 61(3): 257–267.

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Thank you very much

Yours sincerely

Pimpanit Kongrueang

SUMMARY OF CONTENT

CHAPTER 1

PHYSIOLOGICAL RESPONSES OF *ENHALUS ACOROIDES* TO OSMOTIC STRESS

1.1 Introduction

The coastal areas are dynamic environments with frequent shifts in light intensity, salinity and temperature which disturb seagrass growth (Vergeer et al., 1995; Blakesley et al., 2002; Trevathan et al., 2011). Natural phenomena and anthropogenic disturbances, such as heavy rainfall, fresh water inflows, storms, changes in watersheds or wastewater disposal, and decline of freshwater input due to consumption by agriculture, can lead to dramatic salinity changes in some coastal areas and estuaries, especially in areas adjacent to the shores (Adams and Bate, 1994; Tomasko and Hall, 1999; Fernandez-Torquemada and Sanchez-Lizaso, 2005; Thorhaug et al., 2006; Chollett et al., 2007; Touchette, 2007). For example, wastewater from desalination plants increased salinity of some Mediterranean coastal areas (from salinity of 37 to up to 44, even 90) (Fernandez-Torquemada and Sanchez-Lizaso, 2005).

Each seagrass species has different optimal salinity ranging from salinity of 20 to 42 (Les and Cleland, 1997; Collier et al., 2014). Nevertheless, rapid changes in salinity result in stress in this group of plants (Tyerman, 1982; Tyerman et al., 1984). Salinity stress alters seagrass biochemical and physiological processes which may subsequently affect their growth, reproduction and survival (Touchette, 2007). Hyposaline and hypersaline conditions have been shown to negatively affect photosynthetic activity of *Halophila johnsonii* at medium-term series (15 days) (Fernandez-Torquemada et al., 2005). Studies on *Cymodocea nodosa* under high salinity conditions at long-term (47 days) showed minor reduced photosynthetic rate that indicated *C. nodosa* can tolerate to hypersaline more than *Posidonia oceanica* which prefers stable salinity (Sandoval-Gil et al., 2012). Additionally, a prolonged

exposure to salinity stress may dramatically increase the mortality rate (Kahn and Durako, 2008; Griffin and Durako, 2012).

High salinity affects plant homeostasis by two means: 1. osmotic stress by removing water from plant tissues and 2. ionic toxicity by altering ion concentrations and metabolic processes, especially those of growth and photosynthesis (Munns and Tester, 2008; Cambridge et al., 2017). In contrast, hyposaline condition leads to hypo-osmotic stress in plants resulting from ion efflux from vacuoles and compatible solute (osmoprotectant) degradation (Bisson and Kirst, 1995; Griffin and Durako, 2012). Sudden hypo-osmotic conditions also increase turgor pressure, and consequently trigger hypo-osmotic shock (Takahashi et al., 1997; Walley et al., 2007; Beauzamy et al., 2014) by a steady decrease in plant cell osmolarity (Felix et al., 2000). The osmotic responses to unfavorable salinity are energy-demanding processes and may increase total energy requirements of the plants, thus decreasing growth and fitness (Fernandez-Torquemada and Sanchez-Lizaso, 2005; Touchette, 2007; Griffin and Durako, 2012). Short-term high salinity pulses have been shown to increase Cl^- and Na^+ ion concentrations and deplete K^+ and Ca^{2+} ions from the leaves and rhizomes of seagrass species. Many K^+ transporters have high affinities to Na^+, thus they serve as Na^+/K^+ symporters. Therefore, relatively high Na^+ levels in the environment can affect K^+ influx efficiencies in marine plants (Touchette, 2007; Garrote-Moreno et al., 2014). K^+ is necessary for managing the osmotic balance, as an auxiliary participating in biological reactions, and as a co-factor of enzymatic reactions (Touchette, 2007). Thus, a decline of K^+ uptake negatively affects plant growth (Touchette, 2007). The K^+/Na^+ ratio in plants has been proposed as a proxy for salinity tolerance (Lopez and Satti, 1997). Seagrass species that are tolerant to hypersaline conditions have been shown to be able to maintain their K^+/Na^+ ratio (Garrote-Moreno et al., 2014).

Enhalus acoroides (L.f.) Royle is distributed along the coastal areas and in estuaries in the tropical Indo-Pacific regions that have salinity fluctuations (Short et al., 2007). It is one of the most important seagrass species in Thailand (Juntaban et al., 2015). This seagrass species had the highest coverage in Indo-Pacific bioregion including Thailand providing diverse and economic fauna (Nienhuis et al., 1989; Prathep et al., 2010; Unsworth et al., 2010; Unsworth et al., 2012). Due to large

leaf blades, water flow inside *E. acoroides* beds is significantly small which results in high sedimentation rate (Komatsu et al., 2004). These facts prevent erosion and create favorable environment for other seagrass species, benthos in the sediments, epiphytes and juvenile marine animals (Nienhuis et al., 1989; Komatsu et al., 2004; Unsworth et al., 2010; Unsworth et al., 2012). Because of *E. acoroides* numerous functions and factors, it had the highest important value index based on relative covering species, relative frequency of species and relative diversity of species (Dewi and Sukandar, 2017). However, distribution throughout intertidal areas, *E. acoroides* meadows were highly affected from harsh environments, such as salinity fluctuation, resulted in decline of the meadows (Unsworth et al., 2010; Unsworth et al., 2012). At Bolinao, Philippines, salinity value was usually constant (salinity of 28 to 34) but can be decreased to salinity of 20 after fresh water influx (Rollon, 1998). In Thailand, *E. acoroides* is commonly found in the vicinity of mangrove forests and river mouths (Chansang and Poovachiranon, 1994). These habitats are prone to salinity fluctuations due to the freshwater from inland that decreases salinity (Vichkovitten, 1998). A previous study reported that salinity can drastically change within the range from salinity of 29.3 to 35.7 in the *Enhalus acoroides* habitat at Laem Yong Lam, in Haad Chao Mai National Park, Trang Province, Thailand (Rattanachot and Prathep, 2011). The aim of the present study was to provide the information on the physiological responses of *E. acoroides* to hyposaline and hypersaline conditions. Experiments were conducted to investigate the effects of different levels of salinity and exposure times on photosynthetic activity, pigment contents, water content and ion concentrations, under laboratory-controlled conditions.

1.2 Materials and Methods

1.2.1 Plant material

Fully ripe seeds of *Enhalus acoroides* were collected from Ban Pak Khlong (7°36'01.8"N and 99°16'22.3"E, Trang Province, Thailand) during the lowest tidal range in March 2016. The samples were transported to the Bo Hin Farmstay seagrass seedling bank (seagrass seedling nursery, under conservation and restoration of seagrass resources project, Marine and Coastal Conservation Center No. 6, Trang, Thailand). The seeds were cultured in plastic containers with natural seawater (salinity range: salinity of 30-35) under ambient light. The seagrass seedlings were grown for 2 months before transporting to the laboratory at the Department of Biology, Prince of Songkla University.

1.2.2 Experimental design

Seagrass seedlings were transferred into 15 glass tanks (30 cm x 30 cm x 30 cm), each filled with 200 seedlings and 20 liters of artificial seawater (Marinium ® reef sea salt, Mariscience, Thailand) at a salinity of 30 containing 0.01 mg l^{-1} $NaNO_3$ (Riedel-de Haen) and 0.001 mg l^{-1} KH_2PO_4 (Fluka-Garantie). They were allowed to acclimate for 7 days before experimental manipulation of salinity. The water in the tanks was oxygenated with air pumps. Photosynthetically Active Radiation (PAR) at 45 µmol photon m^{-2} s^{-1} was provided from the LED lights on a 12 h light : 12 h dark cycle and the temperature was maintained at 26°C in temperature-controlled room.

After 7 days, the seagrass seedlings were sudden transferred to salinity of 10, 20 (hyposaline conditions), 30 (control), 40 and 50 (hypersaline conditions) water, with 3 replicate tanks for each salinity level. Nutritional supplements (0.01 mg l^{-1} $NaNO_3$ and 0.001 mg l^{-1} KH_2PO_4) were added to all tanks. In this step, the culture conditions were as during the acclimation period. The plantlets were randomly rotated around the tank every day in order to minimize differently photon acquired in each

area. The water was half removed and added every 3 day in order to maintain sufficient nutritions and water quality.

1.2.3 Chlorophyll fluorescence measurement

Chlorophyll fluorescence (maximum quantum yield of photosystem II) (F_v/F_m) was measured from three replicates 0, 1, 2, 7 (rapid response), 10 (intermediate response) and 20 (late response) days after treatment (DAT), counting the days after the plants were exposed to different salinities. Chlorophyll fluorescence was assessed using pulse amplitude modulated (PAM) fluorometer (Mini-PAM, WALZ, Germany). Before measuring maximum quantum yield of photosystem II, the leaves (2^{nd} leaf of seedling) were dark-adapted for 15 min using dark leaf clips (accessories for Mini-PAM, WALZ, Germany). The maximum quantum yield of photosystem II was calculated by the following formula (Murchie and Lawson, 2013):

$$F_v/F_m = (F_m\text{-}F_o)/F_m$$

F_o: minimum value for chlorophyll fluorescence in the dark state
F_m: maximum value for chlorophyll fluorescence in the dark state
F_v: maximum variable chlorophyll fluorescence

1.2.4 Measurement of leaf absorbance

Three replicates were collected at 0, 1, 2, 7, 10 and 20 DAT. The light absorption ability of the leaf (2^{nd} leaf of seedling) was analyzed by measuring the incident light in the air (LI-250A, LI-COR®Bioscience, USA). The leaf was then placed on the light sensor and the amount of light transmitted through the leaf was measured. The leaf absorption factor was calculated as following (Serrano et al., 2000; Ducruet et al., 2012):

$$\text{Absorbance} = \log \frac{I_0}{I}$$

I_0: the intensity of the incident light

I: the intensity of the transmitted light.

1.2.5 Pigment content measurement

Three replicates were collected at 0, 1, 2, 5 (rapid response), 10 (intermediate response) and 20 (late response) DAT. The pigments (total chlorophyll and carotenoids) were extracted by grinding each fresh leaf (2^{nd} leaf of seedling) in 80% acetone solution under dim light. After centrifuging at 3000 rpm for 2 min, the supernatant solution was collected and absorbances at 470, 646 and 663 nm were determined using a spectrophotometer (DS-11 Spectrophotometer, DeNovix, USA). Pigment contents were calculated based on fresh mass of leaf by the following formulae (Lichtenthaler and Wellburn, 1983):

Chlorophyll *a* (Chl *a*) ($\mu g\ ml^{-1}$) $= 12.21\ (A_{663}) - 2.81\ (A_{646})$

Chlorophyll *b* (Chl *b*) ($\mu g\ ml^{-1}$) $= 20.13\ (A_{646}) - 5.03\ (A_{663})$

Total chlorophyll = Chl *a* + Chl *b*

$$\text{Carotenoid } (\mu g\ ml^{-1}) = \frac{1000\ (A_{470}) - 3.27\ (\text{Chl a}) - 104\ (\text{Chl b})}{229}$$

1.2.6 Analysis of Na^+ and K^+ accumulation in plant tissue

Three replicates were collected at 0 (rapid response), 10 (intermediate response) and 20 (late response) DAT. Sodium ion (Na^+) and potassium ion (K^+) concentrations in leaf and root were determined. The plant materials (all leaves and all roots of seedlings) were cleaned with tap water and dried at 60°C for 72 hours. The samples were digested in 1 ml of HNO_3 at 95°C for 2 hours. After that, the sample solution was filtered with Whatman® filter paper (no.1) and diluted to 10 ml with deionized water. The content of sodium ions was determined by inductively coupled

plasma optical emission spectrometry (ICP-OES, Optical Emission Spectrometer Optima 4300 DV, PerkinElmer Inc., USA). The ion concentration was calculated based on dried mass of each sample (modified from Marin-Guirao et al., 2013).

1.2.7 Estimation of relative water content

Three replicates were collected at 0, 1, 2, 5, 10, and 20 DAT for the determination of relative water content in leaves and roots (all leaves and all roots of seedlings). The samples were weighed before and after drying at 60° C for 72 hours. The turgid weight (TW) was obtained by dissecting small *E. acoroides* leaf and root pieces (0.6 cm^2) and placed in closed 1.5 ml tubes filled with 1 ml de-ionized water. These were maintained in darkness for 4 h at 4°C and the pieces were removed excess water and weighed (Sandoval-Gil et al., 2014). The relative water content of the sample was calculated as follows (Back et al., 1992; Sandoval-Gil et al., 2014)

$$\text{Relative water content} = \frac{\text{FW-DW}}{\text{TW-DW}} \text{ x } 100$$

FW: sample fresh weight

DW: sample dry weight

TW: sample turgid weight

1.2.8 Statistical analysis

All statistical tests were performed at 95% confidence level using SPSS software, version 16.0 (SPSS Inc., USA). The studied parameters were tested for assumptions of normality and homogeneity of variance with the Kolmogorov-Smirnov and Levene's tests, respectively. The maximum quantum efficiency of photosystem II, Na^+ and K^+ ion concentrations, water content, pigment content, and leaf absorbance were analyzed with factorial two-way analysis of variance (ANOVA), testing the effects of two fixed factors (i.e., manipulated salinity and exposure time) on the physiological responses of *E. acoroides*. If the salinity, time, or their interaction were significant according to ANOVA, then the least significant difference

(LSD) was calculated to assess for statistical significance (post-hoc test). All the data from measurements are shown as mean ± standard error.

1.3 Results

1.3.1 Effects of salinity on photosynthesis (maximum quantum yield of PSII)

At the beginning of the experiment, there were no differences in the maximum quantum yield of PS II of *E. acoroides* leaves between the salinity treatments (Figure 1). Salinity, time and the interaction between salinity and time had significant effects on maximum quantum yield values of *E. acoroides* leaves (Table 1). The maximum quantum yield with salinity of 20 treatment remained unchanged over time and did not differ from the control (salinity of 30) (Figure 1). However, with the lowest salinity (salinity of 10) and the highest salinity (salinity of 50) treatments, the maximum quantum yield values started to decrease at 2 DAT and 10 DAT, respectively, and gradually decreased until the end of the experiment. At the end of the experiment, the maximum quantum yield of *E. acoroides* leaves from salinity of 10 and 50 were comparable (Figure 1).

1.3.2 Effects of salinity on leaf absorbance

The absorbance of *E. acoroides* leaves did not differ between treatments (salinity of 10, 20, 30, 40 and 50) at 0 DAT (Figure 2A). The statistical analyses revealed that salinity and time had significant effects on leaf absorbance of *E. acoroides*, but no interaction between salinity and time was detected (Table 1). At salinity of 30, 40 and 50 treatments, the leaf absorbance remained unchanged over time when compared to 0 DAT (Figure 2A). The obvious change was observed with the hyposalinity (salinity of 10 and 20): the leaf absorbance significantly decreased to a minimum at 7 and 10 DAT, respectively (Figure 2A).

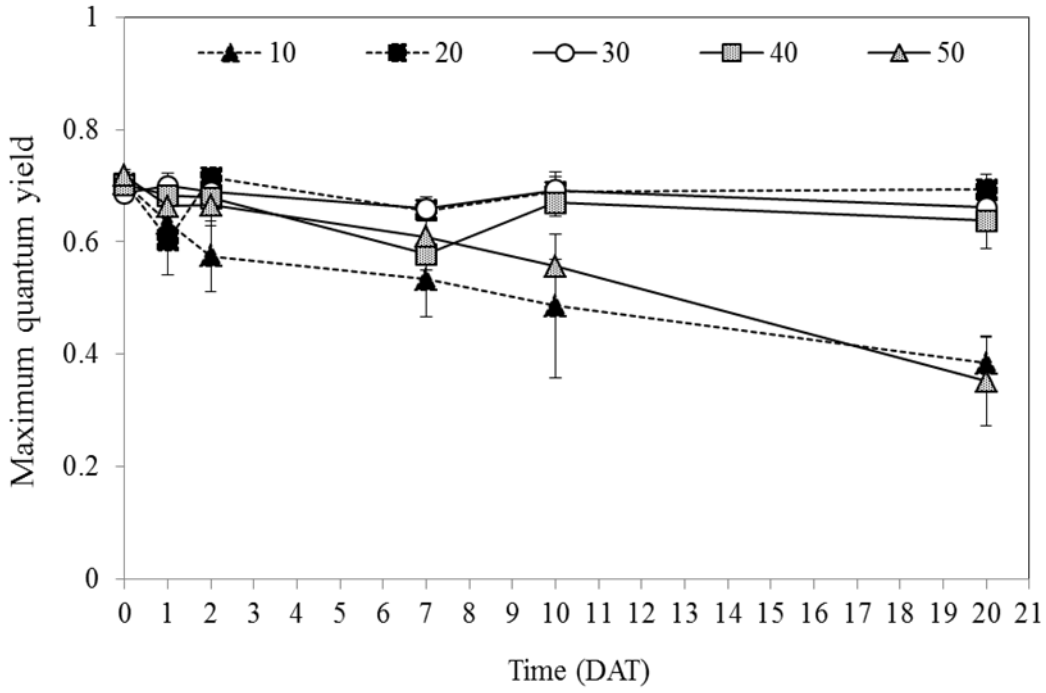

Figure 1: Maximum quantum yield of PSII (F_v/F_m) for *Enhalus acoroides* leaves with each salinity treatment (10, 20, 30 (control), 40 and 50) at different days after treatment (DAT). Values are means ± S.E.; n=3.

1.3.3 Effects of salinity on pigment contents: total chlorophyll (chlorophyll *a* +*b*) and carotenoid

At the beginning of the experiment, plants in all of the salinity treatments (salinity of 10, 20, 30, 40 and 50) had similar total chlorophyll and carotenoid contents (0.103-0.163 and 0.031-0.058 mg g^{-1} by fresh weight, respectively) (Figure 2B and 2C). Salinity and time significantly affected total chlorophyll but no interaction between salinity and time was detected (Table 1). Carotenoid content of *E. acoroides* was not affected by salinity, time and their interaction (Table 1). Variations in total chlorophyll contents were observed with salinity of 20, 40 and 50 treatments, although there was no clear trend (Figure 2B). The drastic reducing change was only observed with the salinity of 10 treatment. Chlorophyll content significantly decreased at 10 DAT and reached its lowest values at 10-20 DAT within the range 0.038-0.054 mg g^{-1} by fresh weight (Figure 2B). The

chlorophyll content with salinity of 30 treatment (control) did not significantly differ from salinity of 20, 40 and 50 treatment ($p = 0.746$, $p = 0.187$ and $p = 0.065$, respectively) but did give significant differences ($p = 0.009$) from the salinity of 10 treatment at 20 DAT (Figure 2B).

The variations in carotenoid content followed the trends of total chlorophyll content (Figure 2C). Plants in the control group (salinity of 30) had significantly increasing trend of total carotenoids. The carotenoid content at 20 DAT did not significantly differ between the salinity of 30 (control) treatment and the salinity of 20, 40 and 50 treatments ($p = 0.406$, $p = 0.180$ and $p = 0.156$, respectively) but significantly differed from the salinity of 10 treatment ($p = 0.032$) (Figure 2C).

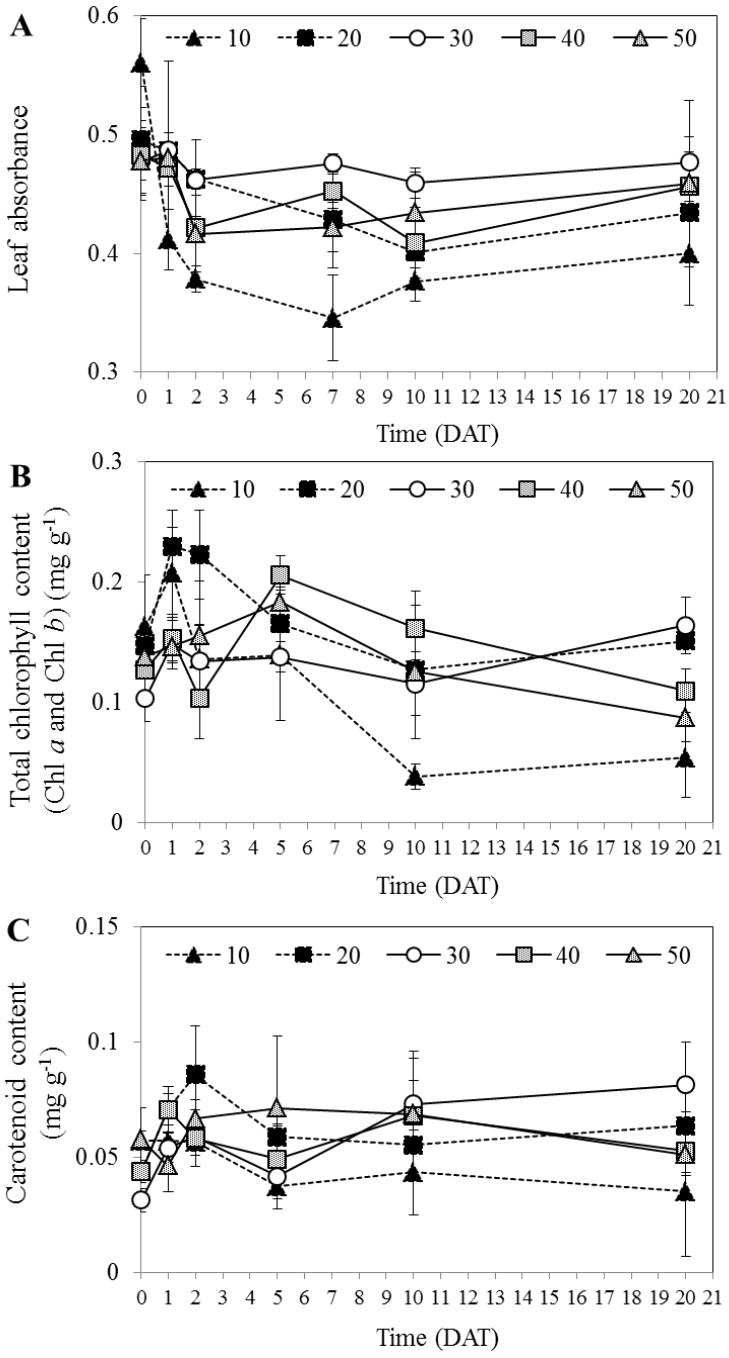

Figure 2A-C: Leaf absorbance, total chlorophyll content and carotenoid content of *Enhalus acoroides*. Leaf absorbance of *E. acoroides* (2A), total chlorophyll content (mg g^{-1} fresh weight) (2B) and carotenoid content (mg g^{-1} fresh weight) (2C) of *E. acoroides* leaves with each salinity treatment at different days after treatment (DAT). Values are means ± S.E.; n=3.

1.3.4 Effects of salinity on the ion concentrations (Na^+, K^+) and the K^+/Na^+ ratio in leaf and root tissues

Salinity and time had significant effects on Na^+ and K^+ concentrations of *E. acoroides* leaves, but no interaction of salinity and time was detected (Table 1). Na^+ concentration in the leaves did not change during exposure to salinity of 10, 20 or 30 (Figure 3A). In contrast, the Na^+ concentration in leaves significantly increased by 20 DAT when exposed to the hypersalinity (salinity of 40 or 50) (57.84 ± 11.65 and 56.49 ± 6.41 mg g^{-1} by dry weight, respectively) from the initial 0 DAT values (25.74 ± 6.33 and 29.56 ± 6.93 mg g^{-1} by dry weight, respectively) (Figure 3A). The Na^+ concentrations in roots did not significantly change during exposure to the salinity of 10, 20, 30 or 40 (Figure 3B). However, with the extreme salinity (salinity of 50), the Na^+ concentration dramatically increased by 20 DAT (85.89 ± 23.32 mg g^{-1} by dry weight) from the initial at 0 DAT (43.14 ± 16.34 mg g^{-1} by dry weight) (Figure 3B).

K^+ concentration in leaves with salinity of 10, 20, 30 and 40 treatments did not significantly change during the study period. However, the K^+ concentration in leaves with salinity of 50 treatment significantly increased at 10 DAT (13.97 ± 1.52 mg g^{-1} by dry weight) from the initial on 0 DAT (7.31 ± 1.44 mg g^{-1} by dry weight, $p = 0.009$) but decreased to the initial value by 20 DAT (Figure 3C). There was an effect of salinity levels on K^+ concentrations in the roots but no effect from exposure time was detected (Table 1). Nevertheless, on comparing at the same exposure time, there was no detectable effect from hyposaline (salinity of 10 and 20) or hypersaline (salinity of 40 and 50) treatments on K^+ concentration in the roots relative to the control (salinity of 30) (Figure 3D).

Salinity, time and the interaction of salinity and time had significant effects on the K^+/Na^+ ratio in leaves (Table 1). However, salinity did not influence the K^+/Na^+ ratio in roots, but there were effects of exposure time and an interaction of salinity and exposure time (Table 1). The K^+/Na^+ ratio in leaves, with any of the salinity treatments, did not change on 0-10 DAT, but the K^+/Na^+ ratios in leaves with salinity of 30, 40 and 50 decreased by 20 DAT (Figure 3E). Similarly, the K^+/Na^+ ratio in the roots, with any of the salinity treatments did not change on 0-10 DAT, but the ratio in the roots with salinity of 10 and 20 decreased by 20 DAT when compared

to 10 DAT, whereas the ratios in the roots with salinity of 30, 40 and 50 decreased by 20 DAT when compared to 0 and 10 DAT (Figure 3F).

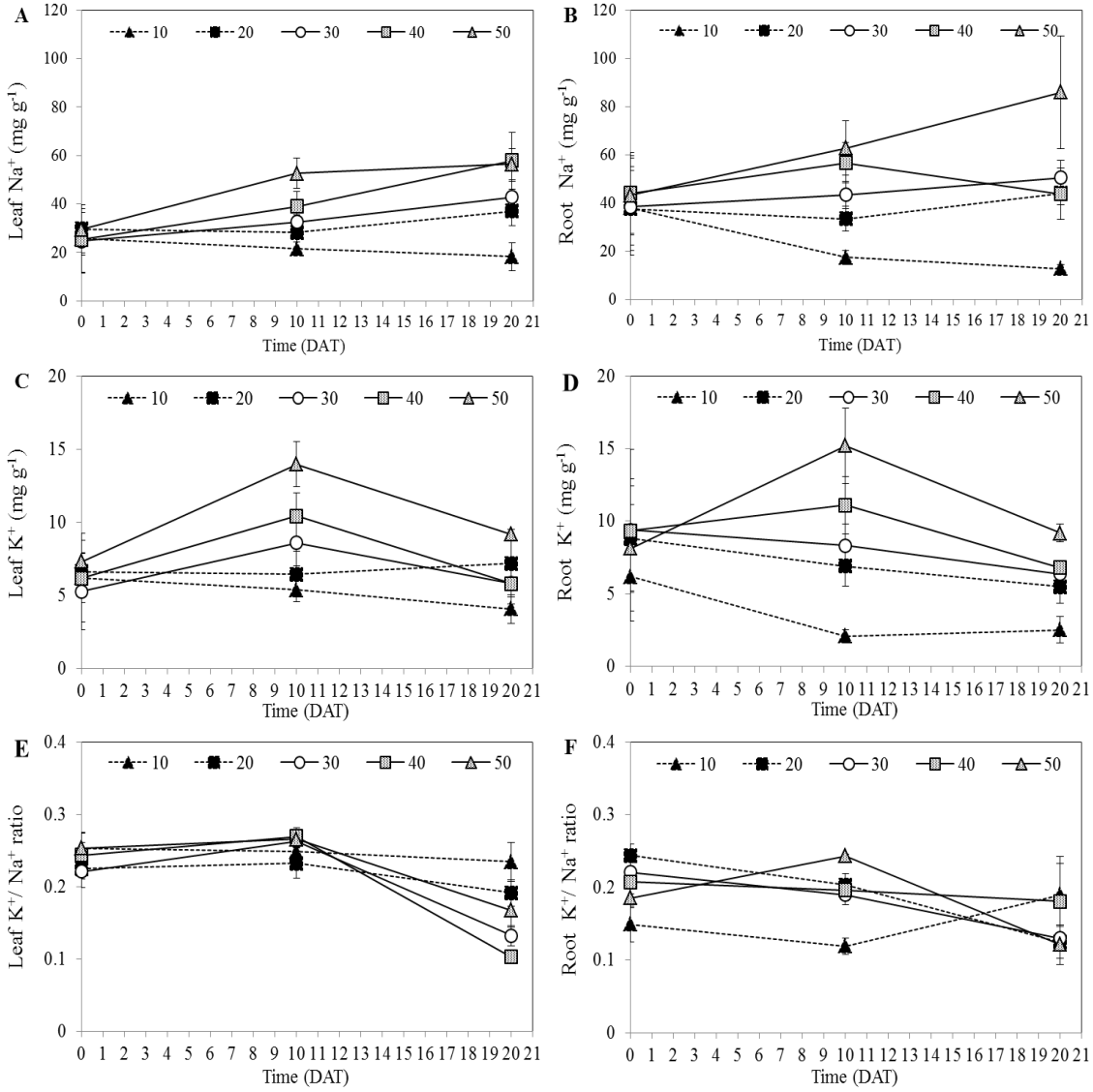

Figure 3A-F: Ion contents and their ratio in different *Enhalus acoroides* tissues. Na^+ (mg g^{-1} dry weight) of *E. acoroides* leaves (3A) and roots (3B), K^+ (mg g^{-1} dry weight) of *E. acoroides* leaves (3C) and roots (3D), K^+/Na^+ ratio of *E. acoroides* leaves (3E) and roots (3F) with each salinity treatment at different days after treatment (DAT). Values are means ± S.E.; n=3.

1.3.5 Effects of salinity on relative water content in leaf and root tissues

The relative water contents in the leaves and roots with salinity of 10, 20, 30, 40 and 50 treatments were not statistically different at 0 DAT (ranges 62.53 - 70.89% and 61.19-65.19%, respectively) (Figure 4A and B). Salinity had significant effects on the relative water content in leaves and roots of *E. acoroides* but no time and the interaction between salinity and time was detected (Table 1). There were fluctuations in relative water content in the leaves during the early stage of the experiments (1-5 DAT) (Figure 4A). All of the salinity treatment leaves showed the same relative water content at 0 DAT and did not change until 20 DAT compared to the initial state (Figure 4A, Table 1).

The salinity of 10 and 20 treatments increased the relative water content of the roots at 20 DAT from the initial at 0 DAT (Figure 4B). While the relative water content of the salinity of 30, 40 and 50 roots remained similar throughout the experiments. The final relative water content (20 DAT) in the control roots (salinity of 30) was significantly different from those with the salinity of 10 ($p = 0.012$) but not different from those with the salinity of 20, 40 and 50 ($p = 0.087$, $p = 0.778$ and $p = 0.641$) (Figure 4B).

Our result showed that *E. acoroides* responded to both hypo- and hypersalinity by negative changing in the maximum quantum yield and total chlorophyll content more than other parameters (leaf absorbance, carotenoid content, ion concentration and relative water content). The maximum quantum yield and total chlorophyll content were decreased at salinity of 10 (45.43 and 66.87%, respectively) and 50 (50.88 and 36.72%, respectively).

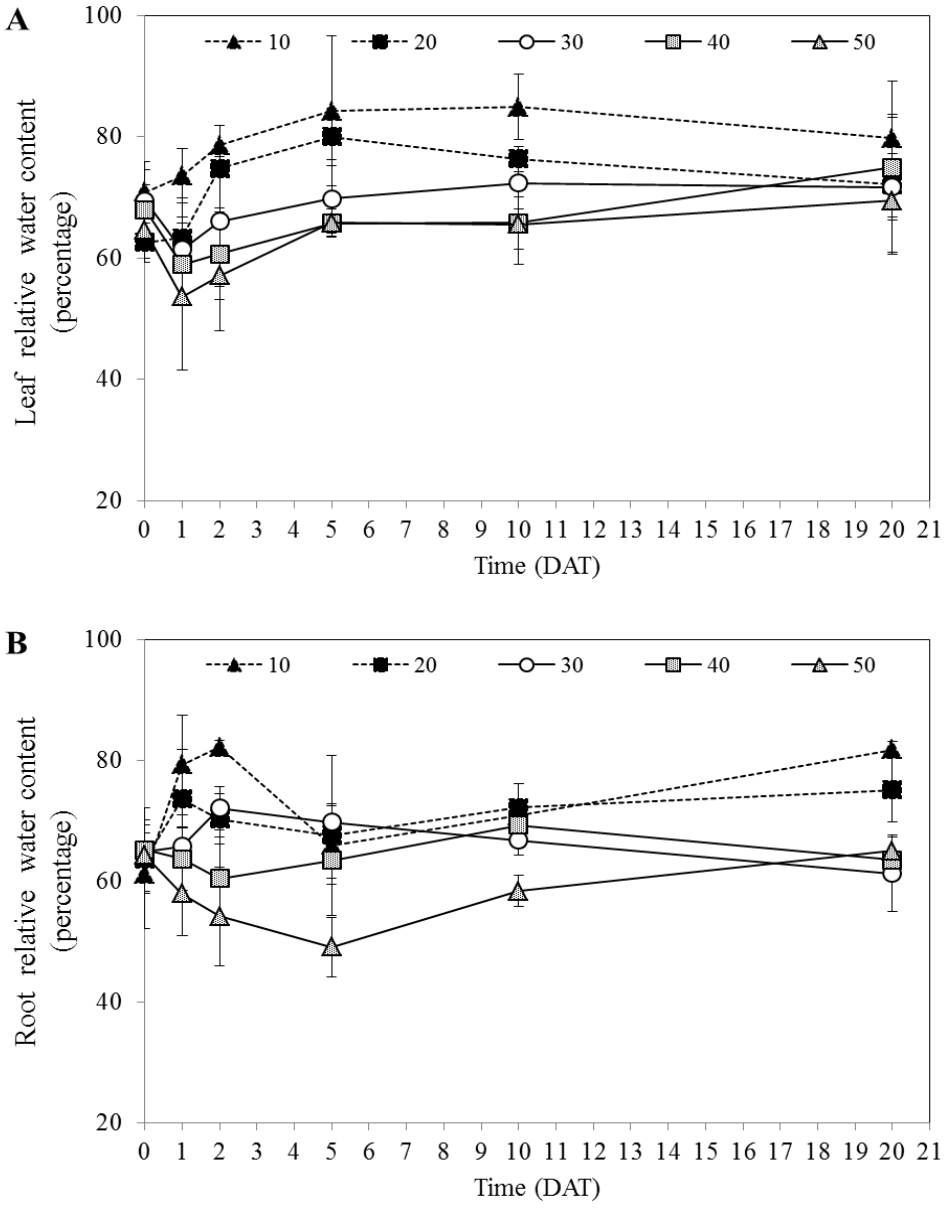

Figure 4A-B: Percentage relative water content in leaves and roots of *Enhalus acoroides*. Leaves (4A) and roots (4B) with each salinity treatment at different days after treatment (DAT). Values are means ± S.E.; n=3.

Table 1: Summary of the two-way ANOVA testing the effect of salinity treatment (10, 20, 30, 40 and 50) and time (0, 1, 2, 5 or 7, 10 and 20 days after treatment) on physiological responses of *Enhalus acoroides*

Parameter	**Source of variation**	***df***	**MS**	***F***	**p-Value**
Maximum quantum yield	Salinity	4	0.06	11.05	**<0.001**
of PSII	Time	5	0.05	8.59	**<0.001**
	Salinity x Time	20	0.01	2.68	**0.002**
	Error	60	0.01		
Leaf absorbance	Salinity	4	0.01	2.8	**0.034**
	Time	5	0.02	4.75	**0.001**
	Salinity x Time	20	0	0.95	0.533
	Error	60	0		
Total Chlorophyll	Salinity	4	0.01	2.6	**0.045**
(Chl *a* + Chl *b*)	Time	5	0.01	4.26	**0.002**
	Salinity x Time	20	0	1.63	0.076
	Error	60	0		
Carotenoid	Salinity	4	0	0.89	0.476
	Time	5	0	1.04	0.401
	Salinity x Time	20	0	0.73	0.782
	Error	60	0		
Leaf Na	Salinity	4	776.33	3.81	**0.013**
	Time	2	899.91	4.42	**0.021**
	Salinity x Time	8	223.14	1.1	0.394
	Error	30	203.74		
Root Na	Salinity	4	2022.63	4.06	**0.010**
	Time	2	198.66	0.4	0.675
	Salinity x Time	8	516.47	1.04	0.432
	Error	30	498.74		

Bold text; p-Value significant ($p<0.05$)

Table 1: Summary of the two-way ANOVA testing the effect of salinity treatment (10, 20, 30, 40 and 50) and time (0, 1, 2, 5 or 7, 10 and 20 days after treatment) on physiological responses of *Enhalus acoroides* (continued)

Parameter	**Source of variation**	***df***	**MS**	***F***	**p-Value**
Leaf K	Salinity	4	29.94	3.53	**0.018**
	Time	2	33.66	3.97	**0.030**
	Salinity x Time	8	8.73	1.03	0.436
	Error	30	8.48		
Root K	Salinity	4	65.37	3.51	**0.018**
	Time	2	31.11	1.67	0.205
	Salinity x Time	8	14.27	0.77	0.635
	Error	30	18.64		
Leaf K/Na ratio	Salinity	4	0	2.92	**0.037**
	Time	2	0.03	38.78	**<0.001**
	Salinity x Time	8	0	3.76	**0.004**
	Error	30	0		
Root K/Na ratio	Salinity	4	0	1.17	0.346
	Time	2	0.01	5.39	**0.010**
	Salinity x Time	8	0.01	2.59	**0.028**
	Error	30	0		
Leaf relative water content	Salinity	4	677.61	4.79	**0.002**
	Time	5	318.04	2.25	0.061
	Salinity x Time	20	46.39	0.33	0.996
	Error	60	141.48		
Root relative water content	Salinity	4	628.12	6.66	**<0.001**
	Time	5	93.71	0.99	0.429
	Salinity x Time	20	93.27	0.99	0.488
	Error	60	94.31		

Bold text; p-Value significant ($p<0.05$)

1.4 Discussion

Our results indicate that both hypo- and hypersaline conditions affected several physiological processes of the seedlings of *Enhalus acoroides*. Seagrasses respond to various stresses by adjusting or changing photosynthetic apparatus, including salinity stress (Touchette, 2007). We observed reductions in the maximum quantum yield with salinity of 10 and 50, corresponding to results from previous studies in other seagrass species (Murphy et al., 2003; Pages et al., 2010; Griffin and Durako, 2012; Zarranz-Elso et al., 2012; Howarth and Durako, 2013a; Salo et al., 2014; Sandoval-Gil et al., 2014; Cambridge et al., 2017). Hyposaline conditions lead to down-regulation of photosynthesis or damage to photosynthetic machinery, as indicated by the maximum quantum yield reduction in several seagrass species such as *Ruppia maritima* (Murphy et al., 2003), *Halophila johnsonii* Eiseman (Griffin and Durako, 2012), *Cymodocea nodosa* (Zarranz-Elso et al., 2012) and *Zostera marina* (Salo et al., 2014). Similarly, reduced maximum quantum yield in hypersaline conditions has been recorded in *R. maritima* (Murphy et al., 2003), *C. nodosa* (Pages et al., 2010), *Thalassia testudinum* (Howarth and Durako, 2013a), *Posidonia oceanica* (Sandoval-Gil et al., 2014) and *Posidonia australis* (Cambridge et al., 2017). However, the degrees of stress response differ by species, the salinity treatments (intensity and duration), or even ecotypes (Salo et al., 2014). For example, salinity of 55 had no significant effect on the maximum quantum yield in *T. testudinum, Halodule wrightii* and *R. maritima* (Koch et al., 2007), while salinity of 43 caused a decline in the maximum quantum yield of *P. oceanica* (Sandoval-Gil et al., 2014). The same species has varied responses to salinity dependent on the habitat (Salo et al., 2014). *Z. marina* originally habituated at high salinity had decreasing the maximum quantum yield with exposure to hyposaline condition (< salinity of 9) while individuals originally habituated at low salinity had decreasing the maximum quantum yield only at more extreme hyposalinity (< salinity of 2) (Salo et al., 2014). The decline of the maximum quantum yield in *E. acoroides* suggests that extreme hypo- and hypersaline conditions impose stress on photosynthesis (Garrote-Moreno et al., 2015). Our results indicate that *E. acoroides* in hypo-salinity condition (salinity of 10) have shown greater negative photosynthetic activity than that in hyper-salinity

condition (salinity of 50) and *E. acoroides* can tolerate salinity ranging from salinity of 20 to 40 at least 20 days according to no significant the maximum quantum yield changes have been observed. However, decreasing in the maximum quantum yield might be another long-term strategy of plants to dissipate excess photons due to stress acquired. Plants have safety valves to avoid damages occurring to photosynthetic apparatus (Niyogi, 2000).

The maximum quantum yield on *E. acoroides* had similar trend as leaf absorbance and total chlorophyll. Hyposalinity (salinity of 10) decreased leaf absorbance and total chlorophyll content in *E. acoroides* more than other salinity treatments. The decline of total chlorophyll content in *E. acoroides* with the hyposaline conditions is consistent with a study of *H. johnsonii* (Kahn and Durako, 2008). Similarly, *T. testudinum* at low salinities (salinity of 16, incubated at 24 hours) increased leaf reflectance and decreased chlorophyll content resulting in reduced light absorption (Thorhaug et al., 2006). The decrease of total chlorophyll content with hypersaline conditions has also been observed in other studies in *T. testudinum* (Howarth and Durako, 2013b) and *C. nodosa* (Sandoval-Gil et al., 2014). In addition, a study of *T. testudinum* with high salinity (salinity of 50) has shown increased leaf reflectance which indicates decreased light absorption (Durako and Howarth, 2017). Decline of photosynthetic pigments is considered a general response to stress in plants. However, the decreases in photosynthetic activity, photosynthetic pigments and absorbance observed in our study might be one of the photoprotective mechanisms to alleviate oxidative stress under salinity shift. Salinity stress induces generation of reactive oxygen species (ROS) in plant cells, which may lead to oxidative stress (Luo and Liu, 2011). Down-regulation of light utilization decreases ROS production from the chloroplast and might consequently reduce photo-oxidative damage (Luo and Liu, 2011).

Seagrass takes up both nutrients and ions from bulk water via leaves and from porewater via roots (Stapel et al., 1996). Effects of salinity on the ion concentration accumulations in seagrasses depend on salinity variations, different types of organelles in the plant tissues, and exposure time (Garrote-Moreno et al., 2016). The analysis of ion concentrations in leaves and roots of *E. acoroides* at high salinity showed Na^+ accumulation in leaves in the short-term, similar to those in

P. oceanica, C. nodosa (Garrote-Moreno et al., 2015), *T. testudinum* and *H. wrightii* (Garrote-Moreno et al., 2014). Nevertheless, the increases in Na^+ ion concentration at the end of experiment in leaf tissues at hyper salinities were more pronounced than those in roots. This contradicts the results from previous studies in *T. testudinum* and *H. Wrightii* which Na^+ ion concentrations in leaves were lower than those in rhizomes (Garrote-Moreno et al., 2014). Higher percentage Na^+ concentration change in leaf tissues than that in root tissues at 10 DAT suggests deceased photosynthetic activity. K^+/Na^+ ratio is also considered as a salinity stress descriptor. The higher the K^+/Na^+ ratio, the more tolerance the salinity stress (Garrote-Moreno et al., 2015). Hypersaline conditions had no significant effect on K^+/Na^+ ratio in the leaves of *E. acoroides* in the short- and medium-term (up to 10 days after treatment). This suggests that *E. acoroides* are able to regulate ion balance in short and medium periods. We did not observe competition of Na^+ and K^+ transports in hypersalinity since the decrease in K^+/Na^+ was driven by increasing Na^+ alone, not by K^+ reduction.

Both hypo- and hypersaline conditions affected the water content in tissues by ion accumulation and osmotic adjustments. Under hypersaline conditions, seagrasses can reduce the water potential of their tissues by the accumulation of osmotically-active solutes within the cell, by turgor regulation (i.e. cell-wall hardening processes) or even by cell water efflux (Sandoval-Gii et al., 2012; Cambridge et al., 2017). In *E. acoroides*, hypersaline conditions reduced water content both in the leaves and in the roots which corresponds to a study in *P. australis* (Cambridge et al., 2017). Nevertheless, these were only short-term responses as the osmotic adjustment successfully took place to maintain the cell water content, by increasing the osmotic forces for water uptake (Passioura and Munns, 2000; Touchette, 2007; Garrote-Moreno et al., 2014). Hypo-osmotic shock leads to increased cell volume, turgor pressure and rate of water influx (Takahashi et al., 1997), and accordingly the water content both in leaves and in roots of *E. acoroides* increased when exposed to hyposalinity. However, the minor changes in relative water content observed in our study, although statistically significant, had only slight effects on the stress in seagrass.

Our results indicate that the photosynthetic machinery of *E. acoroides* seedlings was more sensitive to hyposaline (salinity of 10) than to hypersaline

(salinity of 50) conditions, because hyposalinity caused rapid reduction in photosynthesis which persisted until the end of the experiment. Extreme salinities (salinity of 10 and 50) seemed not to be able to recover after decreasing while intermediate salinity levels (salinity of 20 and 40) were able to recover to the initial values. The mechanisms of photosynthetic stress differ between hyposaline and hypersaline conditions. It has been suggested that photodamage in hyposaline conditions may be attributed to decreased cellular ion contents, including the ions necessary as photosynthetic cofactors (Touchette, 2007). However, this might not be the cause of a decline in photosynthesis observed in our experiment since Na^+ and K^+ in hyposaline treatments did not change. This might suggest that hyposalinity conditions inhibit electron transport and increase ROS leading to oxidative damage in chloroplasts, electron flow in photosystem II blocking, and photodamage to the reaction center (Jahnke and White, 2003; Luo and Liu, 2011). On the contrary, it has been suggested that hypersalinity affects photosynthetic efficiency by changing the abundance and ultrastructure of chloroplasts, inhibiting the activity of enzymes associated with carbon assimilation (Cambridge et al., 2017), and disturbing the permeability of ions (principally Na^+ and Cl^-) across the thylakoid membrane (Touchette, 2007). However, the photosynthetic systems appeared more resistant to increased Na^+ with hypersaline conditions.

In conclusion, this research found adverse effects of hypo- and hypersaline conditions and the duration of exposure to them, and the photosynthetic effects could be used as markers to detect *E. acoroides* stress in response to salinity changes. Both natural and anthropogenic disturbances to salinity should be closely monitored in order to effectively protect the fragile *E. acoroides* communities. Our results showed that *E. acoroides* seedlings have higher sensitivity to hyposaline condition. Therefore, this seagrass may be more affected by a sudden decrease in salinity brought about by heavy rainfall and freshwater inputs during monsoon season and extreme weather events which are predicted to become more frequent in global change scenarios.

CHAPTER 2

GENE EXPRESSION OF *ENHALUS ACOROIDES* TO SALT STRESS

2.1 Introduction

Seagrass meadow is one of the important components of the coastal ecosystems (Garrote-Moreno et al., 2014). The ecosystems are highly dynamic as a result of fluctuation of abiotic factors such as light intensity, salinity, temperature and hypoxic conditions which influence seagrass viability (Vergeer et al., 1995; Blakesley et al., 2002; Trevathan et al., 2011). Salinity stress, an abiotic stress, brings about induction of photoinhibition and reactive oxygen species (ROS) which consequently reduce the photosystem efficiency (Vasilikiotis and Melis, 1994; Saibo et al., 2009).

Photosynthesis is often damaged in seagrasses exposed to hypo- and hypersaline conditions with different degrees of damage depending on the exposure time to the stress and salinity level (Touchette, 2007). *Enhalus acoroides*, one of the most important seagrass species in Thailand, indicated that hypo- and hypersalinity conditions affected the decline of photosynthesis (the maximum quantum yield of photosystem II (F_v/F_m) and total chlorophyll content measurement). The physiological photosynthetic indicators are highly sensitive to salinity shifts (Kongrueang et al., 2018).

Photosynthesis is negatively affected by several pathways such as the inhibition of the electron flow between photosystem I (PSI) and II (PSII), the change of the pigment concentration and composition and the change of photosynthetic enzymes (Touchette, 2007; Salo et al., 2014). Salinity stress disturbs photosynthesis both in short and long terms as well. It can lead to carbon assimilation decrease in the short term which consequently balk the plant growth after exposure to salinity in a few hours. In the long term, salinity stress shifts salt accumulation in the leaves and decreases the chlorophyll and carotenoid contents leading to negative photosynthetic effects (Acosta-Motos et al., 2017).

The increase of salinity stress can decrease photosynthetic efficiency which mainly affects the chlorophyll content reduction (Baek et al., 2005; Karimi et al., 2005; Touchette, 2007). Furthermore, inhibition of electron flow, decreasing photosystem function, reducing rubisco amount and activity, and changing in chloroplast ultrastructure are also mechanisms to inhibit photosynthesis (Kirst, 1989; Ziska et al., 1990; Stoynova-Bakalova and Toncheva-Panova, 2004; Touchette 2007). In macroalgae species, salinity stress inhibits both PSI and II due to ion (Na^+ and Cl^-) induction to the toxic level across the thylakoid membrane (Gilmour et al., 1982; Gilmour et al., 1985; Xia et al., 2004; Touchette, 2007).

Analysis in photosynthetic process genes, salinity stress alters light reaction-related genes, including *light-harvesting chlorophyll a/b-binding* (*LHC*) *proteins* (a type of photosynthetic pigment-related genes), *ribulose-1,5-bisphosphate carboxylase/oxygenase* (*RuBisCO*), *RuBisCO activase* (*RCA*), *PSII reaction center protein D1* (*psbA*) and *D2* (*psbD*) (Zhang et al., 2012; Zhang et al. 2016).

Light-harvesting chlorophyll a/b-binding (LHC) proteins are important proteins in photosynthetic process which are abundant in thylakoids of chloroplasts. Their prominent function is collecting the light and transferring the solar energy to photosystem reaction centers via excitons (Baker, 2008; Gururani et al., 2015; Kong et al., 2016). In higher plants, the *LHC* gene family is composed of two major subfamilies: *LHCA* (or *LHCI*) which encodes light harvesting complex of PSI and *LHCB* (or *LHCII*) which encodes light harvesting complex of PSII (Green and Durnford, 1996; Jansson, 1999; Dekker and Boekema, 2005; Daum et al., 2010; Kong et al., 2016). The compositions of PSI and PSII antenna complexes are quite different in terms of bound-chlorophyll type. LHCA is bound with chlorophyll a while LHCB is mostly bound with chlorophyll b (Xu et al., 2012; Rochaix, 2014; Gururani et al., 2015). *Zostera marina* showed that salinity stress can affect the expression of *ZmLhca* (in PSI) and *ZmLhcb* (in PSII) genes under different salinity stress conditions. It is suggested that extreme high salinity condition greatly reduced most of the *ZmLhc* transcripts while low salinity appeared less impact to the abundances of the transcripts (Kong et al., 2016).

In addition, PSII–LHCII supercomplex contains major redox components which is composed of D1–D2 (or PsbA–PsbD) heterodimer (Rochaix, 2014; Gururani et al., 2015).

Osmotic stress due to salinity stress causes a reduction in CO_2 assimilation rate. This can be observed through a decreased abundance of Rubisco and RCA (Parihar et al., 2015). Rubisco is an essential enzyme catalyzing carbon assimilation in higher plants and algae. It is under regulation of RCA during photosynthesis. At proper condition, RCA is abundant in chloroplasts. On the other hand, under the stress, RCA is reduced (Bayramov and Guliyev, 2014).

The study of abiotic stress on seagrass meadows was suggested to be measured by molecular indicators as they provide the evidence earlier than morphological and physiological measurements (Hoffmann and Daborn, 2007; Macreadie et al., 2014). Nowadays, molecular indicators have not been widely performed in seagrass. Molecular indicators should be a new era in management of seagrass ecosystems (Hasegawa et al., 2000; Macreadie et al., 2014).

The aim of the present study was to provide the information on the photosynthesis gene expression of *E. acoroides* to hyposaline and hypersaline conditions. Experiments were conducted to investigate the effects of different levels of salinity and exposure times on (*LHCB*, *psbA*, *psbD* and *RCA*) under laboratory-controlled conditions.

2.2 Materials and Methods

2.2.1 Plant materials

In March 2016, *Enhalus acoroides* ripe seeds were taken from Ban Pak Khlong (7°36'01.8"N, 99°16'22.3"E), Trang Province, Thailand. The seeds were cultivated with natural seawater (salinity approximately between 30 and 35) under natural light at the seagrass seedling bank, Bo Hin Farmstay (seedlings seagrass nursery under conservation and restoration of seagrass resources project, Marine and Coastal Conservation Center No. 6, Trang, Thailand). The two months old seagrass seedlings were transferred to the laboratory at the Department of Biology, Faculty of Science, Prince of Songkla University.

2.2.2 Experimental design

The seagrass seedlings were acclimatized in 20 liters of artificial seawater (Marinium ® reef sea salt, Mariscience, Thailand) having salinity 30 with 0.01 mg/L $NaNO_3$ (Riedel-de Haen) and 0.001 mg/L KH_2PO_4 (Fluka-Garantie) in glass tanks (30 cm x 30 cm x 30 cm) for 7 days. The seedling culture was illuminated with LED white light at a photon flux density (PPFD) of 45 µmol photon m^{-2} s^{-1} with 12 h dark: 12 h light cycle in 26°C controlled room and the water in the tanks was oxygenated.

After the acclimation period, the seagrass seedlings were cultured at salinity [10, 20] (hyposaline condition), 30 (control), [40 or 50] (hypersaline condition) with three replicate tanks per each salinity treatments while other conditions were strictly maintained as the acclimation period.

Leaves and roots of ten random seedlings of each tank were excised at 0, 1, 5, 10 and 20 days after treatment (DAT). The tissues were temporarily kept in liquid nitrogen and subsequently preserved at -80°C for RNA extraction.

2.2.3 RNA extraction and cDNA synthesis

Total one hundred milligrams fresh weight of leaf or root tissues were equally pooled from ten different seagrass seedlings. The tissue was ground into powder with liquid nitrogen and RNA was extracted using the RNAprep Pure Kit (For Polysaccharides & Polyphenolics-rich plant) (TIANGEN) according to the manufacturer's instruction. The RNA yield and quality were determined by spectrophotometer (DS-11 Spectrophotometer, DeNovix, USA) and agarose gel electrophoresis. The RNA samples were stored at -80°C.

One microgram of the total RNA was converted into 50 ul of the first-strand cDNA using the *AccuPower*® RT Pre Mix (BIONEER) with random-hexamer primer and further amplified using the *AllInOneCycler*™ Thermal Block (BIONEER) according to the manufacturer's protocol. The cDNA samples were stored at -20°C

2.2.4 Primer design

18s primer pairs (reference gene) were designed from *Enhalus acoroides* (NCBI accession number JQ041644.1) while *RCA* primer pairs were designed from conserved regions of *Oryza punctata* and *Oryza sativa* (NCBI accession number KX455915.1 and U74321.1, respectively) *and LHCB* primer pairs were designed from conserved regions of *Zea mays* and *Oryza sativa* Japonica (NCBI accession number NM_001148812.1 and D00641.1, respectively). The conserved regions alignments were performed by using the software MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumer et al., 2016) and primers were generated by using the software Primer3 (Rozen and Skaletsky, 2000). In addition, *psbA* and *psbD* primers were directly selected from Dattolo et al. (2014).

2.2.5 Quantitative polymerase chain reaction (qPCR)

The qPCR was performed in a 96-well plate (white plate, BIO-RAD) with CFX Connect™ Real-Time PCR Detection System and BIO-RAD CFX manager (BIO-RAD, USA) using *AccuPower*® 2X *GreenStar*™ qPCR MasterMix (BIONEER). All reactions were performed in 3 technical replicates and sterile water was added as the negative control in 96-well reaction plate. For *18s*, *RCA*, *LHCB*, *psbA* and *psbD* genes, the thermal cycling consisted of 5 min at 95 °C and 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. The melting curve was measured by heating from 65 to 95 °C.

2.2.6 Gene Expression analysis

For gene expression analysis, *18s* was used as the reference gene for the internal control. The gene expression was analyzed from the relative expression ratio which can be calculated by $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

$\triangle C_T$	=	C_T (target gene) - C_T (reference gene)
$\triangle\triangle C_T$	=	$\triangle C_T$ (sample) - $\triangle C_T$ (control)
Expression ratio	=	$2^{-\Delta\Delta C}{}_T$

C_T: the threshold cycle for reference or target amplification

$\triangle C_T$: the difference in threshold cycles for target and reference

Table 2: Sequence primer of reference and target gene

Gene	**Sequence primer (5'-3')**		**PCR product size (bp)**	**Efficiency (%)**
18s	Forward	AACAATACCGGGCTCTACGA	172	92.02
	Reverse	CCCAACCCAAAGTCCAACTA		
RCA	Forward	TCAAGAAGGGGAAGATGTGC	275	95.05
	Reverse	GGTGGGAGCCCAGTAGAACT		
LHCB	Forward	GAGGCCGTGTGGTTCAAG	308	94.08
	Reverse	AAGAAGCCGAACATGGAGAA		
psbA	Forward	GACTGCAATTTTAGAGAGACGC	137	90.22
	Reverse	CAGAAGTTGCAGTCAATAAGGTAG		
psbD	Forward	CCGCTTTTGGTCACAAATCT	162	101.34
	Reverse	CGGATTTCCTGCGAAACGAA		

2.3 Results

Effects of salinity on gene expression (*LHCB*, *RCA*, *psb*A and *psb*D gene)

To identify molecular indicators of photosynthetic apparatus in the leaf, we identified the quantitative gene expression profiles of *LHCB*, *RCA*, *psbA* and *psbD*.

The statistical analyses revealed that salinity and time had significant effects on the expression ratio of *LHCB* gene in *Enhalus acoroides* leaves but no interaction between salinity and time effect was detected (Table 3). *LHCB* transcript showed decreasing trend at extreme hypo- and hypersalinity treatments, however, they were not significant different when compared to the control. The transcript at salinity of 40 at 1 DAT (3.708 ± 0.664) was the only one that showed significant induction when compared to the control. However, it was appeared that salinity of 10 at 10 DAT showed statistically significant difference from salinity of 20 and 40 at the same time (Figure 5).

The statistical analyses revealed that salinity, time and interaction between salinity and time had significant effects on the expression ratio of *RCA* gene in *E. acoroides* leaves (Table 3). The expression of *RCA* transcript did not respond at all salinity treatments (10, 20, 30, 40 and 50) at 0-10 DAT. However, at 20 DAT of hypo- and hypersalinity treatments showed increment of the transcript. At salinity of 10, 40 and 50 treatment, the expression of *RCA* transcript significantly increased and showed the highest value at 20 DAT (25.403 ± 1.529, 30.030 ± 21.701 and 164.158 ± 34.721, respectively) when compared to the control treatment (0 DAT) (Figure 6).

The statistical analyses revealed that salinity and time had significant effects on the expression ratio of *psbA* gene in *E. acoroides* leaves but no interaction between salinity and time was detected (Table 3). Generally, the expression of *psbA* transcript was not changed at all salinity treatments and times. Nevertheless, the expression of *psbA* transcript of salinity of 50 at 20 DAT significantly increased and showed the highest value (3.230 ± 1.176) (Figure 7). The *psbA* transcript at salinity of 40 at 10 DAT showed significant highest value (1.964 ± 1.096) when compared to different salinity treatments at the same time (Figure 7).

The statistical analyses revealed that time and interaction between salinity and time had significant effects on the expression of *psbD* transcript in *E. acoroides* leaves but no salinity effect was detected (Table 3). The expression of *psbD* transcript did not respond to salinity of 20 treatments.). The expression of *psbD* transcript of salinity of 10 and 50 treatments significantly increased to highest value at 20 DAT (28.374 ± 11.991 and 12.178 ± 3.558, respectively) when compared with control treatment (salinity of 30 at 20 DAT) (Figure 8).

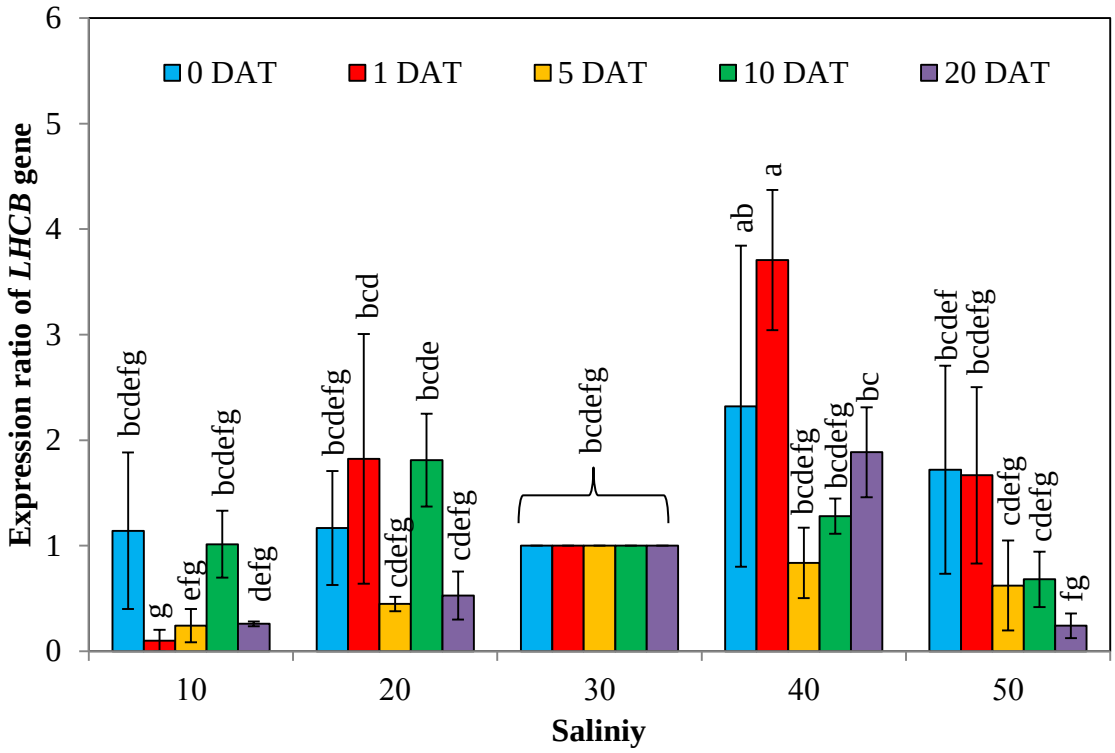

Figure 5: Expression ratio of *LHCB* gene for *Enhalus acoroides* leaves with each salinity treatment (10, 20, 30 (control), 40 and 50) at different days after treatment (DAT). Values are means ± S.E.; n=3.

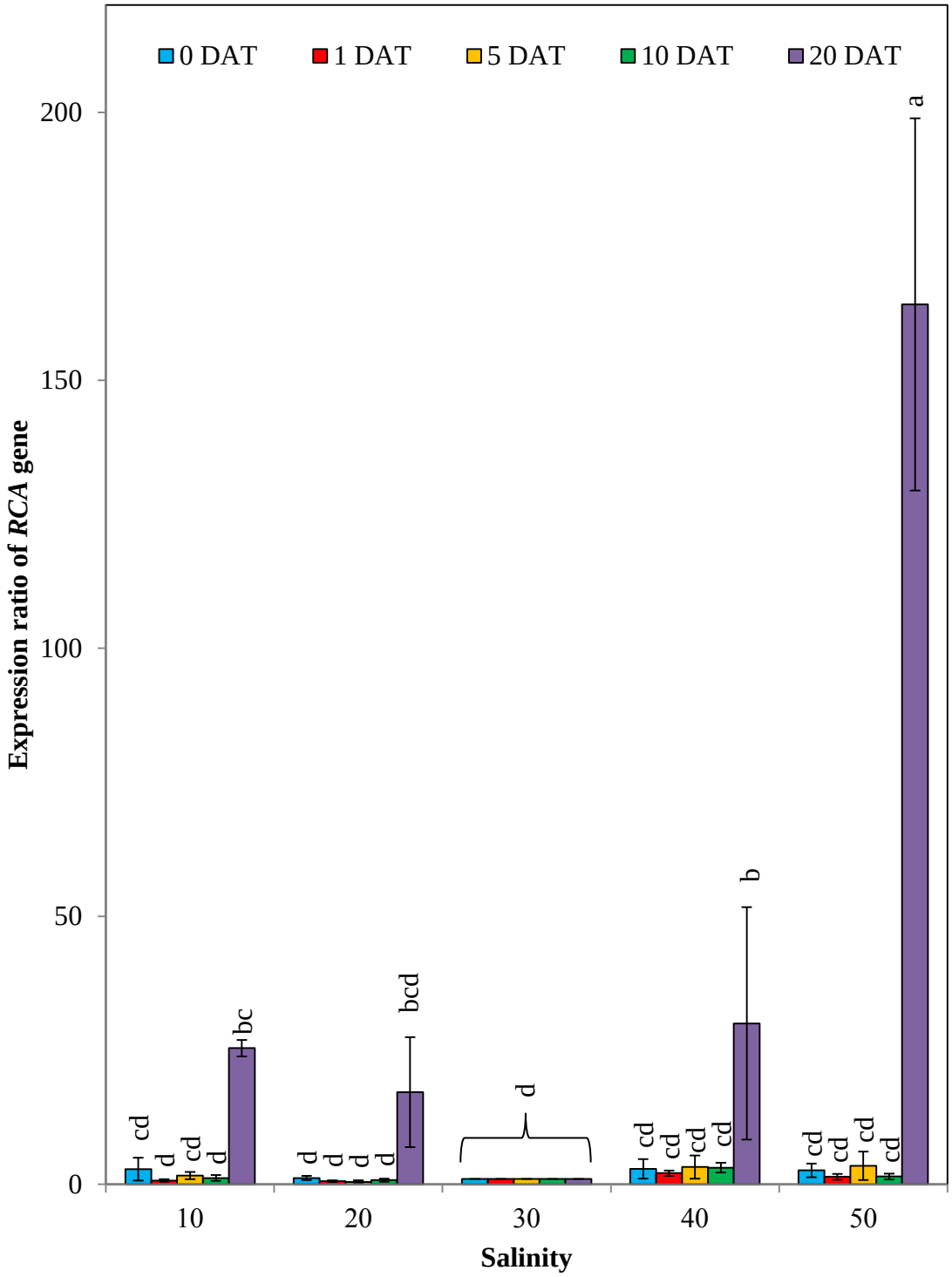

Figure 6: Expression ratio of *RCA* gene for *Enhalus acoroides* leaves with each salinity treatment (10, 20, 30 (control), 40 and 50) at different days after treatment (DAT). Values are means ± S.E.; n=3.

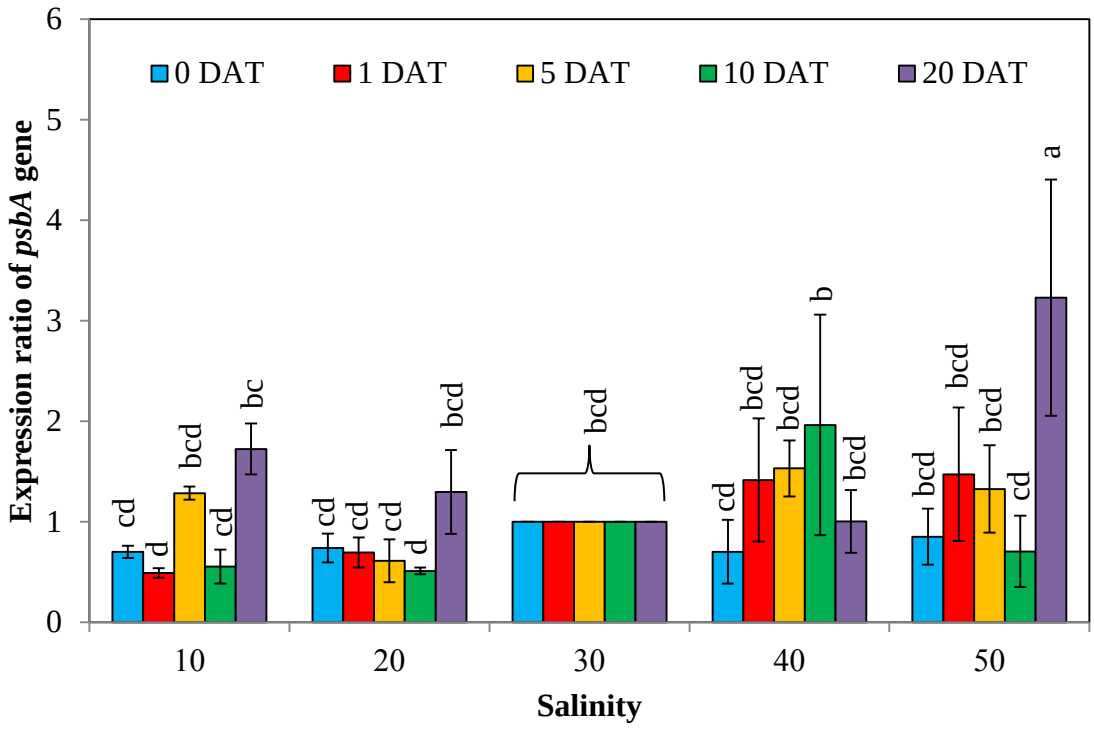

Figure 7: Expression ratio of *psbA* gene for *Enhalus acoroides* leaves with each salinity treatment (10, 20, 30 (control), 40 and 50) at different days after treatment (DAT). Values are means ± S.E.; n=3.

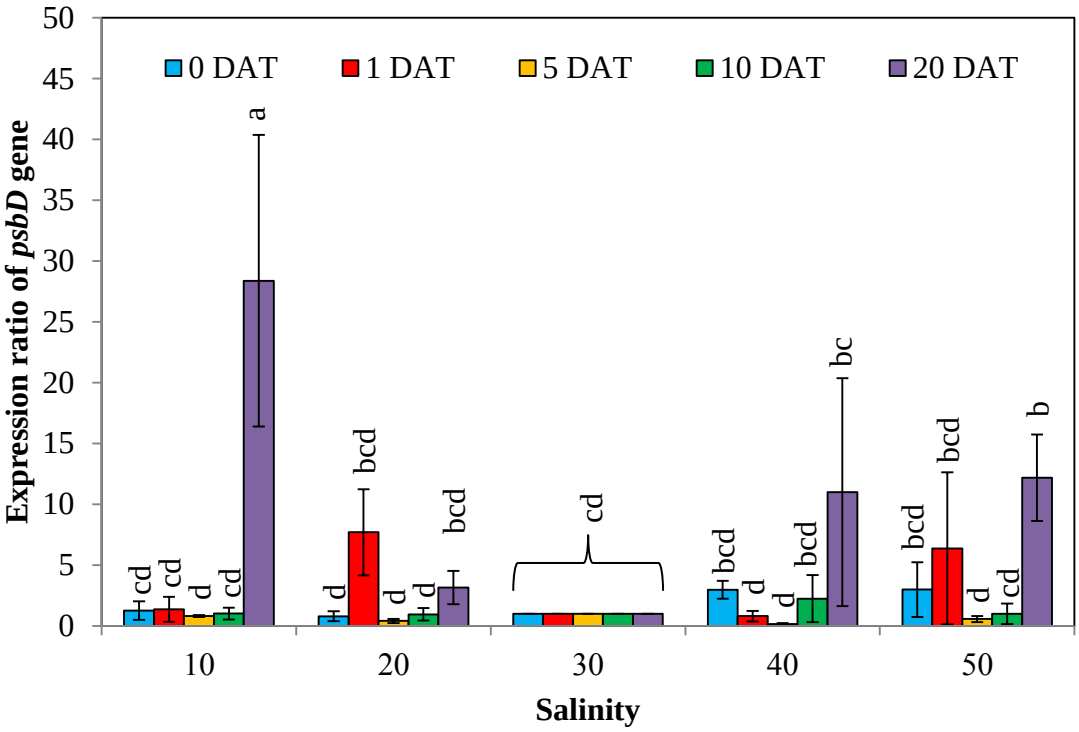

Figure 8: Expression ratio of *psbD* gene for *Enhalus acoroides* leaves with each salinity treatment (10, 20, 30 (control), 40 and 50) at different days after treatment (DAT). Values are means ± S.E.; n=3.

Table 3: Summary of the two-way ANOVA testing the effect of salinity treatment (10, 20, 30, 40 and 50) and time (0, 1, 5, 10 and 20 days after treatment) on gene expression of *Enhalus acoroides*

Parameter	Source of variation	*df*	MS	*F*	p-Value
LHCB gene	Salinity	4	4.277	4.668	**0.003**
	Time	4	2.875	3.137	**0.022**
	Salinity x Time	16	1.044	1.139	0.348
	Error	50	0.916		
RCA gene	Salinity	4	2756.725	12.707	**<0.001**
	Time	4	6315.758	29.112	**<0.001**
	Salinity x Time	16	2591.69	11.946	**<0.001**
	Error	50	216.946		
psbA gene	Salinity	4	1.365	2.564	**0.050**
	Time	4	1.598	3.003	**0.027**
	Salinity x Time	16	0.826	1.551	0.119
	Error	50	0.532		
psbD gene	Salinity	4	66.021	1.773	0.149
	Time	4	280.412	7.532	**<0.001**
	Salinity x Time	16	80.044	2.15	**0.020**
	Error	50	37.231		

Bold text; p-Value significant ($p<0.05$)

2.4 Discussion

Our result indicated the photosynthetic-related genes, *LHCB* (light-harvesting chlorophyll *a*/*b* binding protein of PSII) showed down-regulation trend in *Enhalus acoroides* leaves at extreme hypo- and hypersaline conditions while the expression of *RCA* gene was increase in *E. acoroides* leaves at both hypo- and hypersaline conditions. Additionally, *psbA* (PSII reaction center D1) genes were up-regulated in *E. acoroides* leaves to extreme hypersalinity conditions. Finally, *psbD* (PSII reaction center D2) genes were up-regulated in *E. acoroides* leaves to extreme hypo- and hypersalinity conditions.

The previous studies suggested that LHCB is an important component in photosynthesis and adaptation which is sensitive to environmental stresses (Ganeteg et al., 2004; Kovacs et al., 2006; Loukehaich et al., 2012). The *LHCB* transcription was mainly repressed by salinity stress (Liu and Shen, 2004; Liu et al., 2006; Wen et al., 2011; Kong et al., 2016). The *LHCB* transcription of *E. acoroides* did not change markedly under salinity stress when compared to the control, probably because *E. acoroides* can tolerate to a wide salinity range at a particular period. The expression ratio of *LHCB* gene in *E. acoroides* leaves decreasing trend at hypo- and hypersaline conditions corresponded to the previous studies in green alga and other seagrass species (Wen et al., 2011; Kong et al., 2016). It was found that *Dunaliella salina* negatively exhibited *Lhcb3* at low salt stress (Wen et al., 2011). Furthermore, hypoosmotic shock decreased the LHCII phosphorylation proteins in *D. salina* (Liu and Shen, 2004; Liu et al., 2006). On the other hand, high salinity conditions reduced *LHC* gene expression in *Arabidopsis* species (Seki et al., 2002). The present result partially agrees to that in *Zostera marina* which most of the *LHCB* transcripts were repressed at hypersaline condition. However, *ZmLhcb1.2* showed similar pattern to our result as it significantly reduced both in hypo- and hypersalinity (Kong et al., 2016). In *E. acoroides*, the distinct *LHCB* transcript depletion at extreme hyposalinity treatment (salinity of 10) when compared to salinity of 20 and 40 could explain the drastic reduction of maximum quantum yield of PSII (Kongrueang et al., 2018). This relationship between *LHCB* transcript and maximum quantum yield of PSII could also be found at extreme hypersalinity (Kongrueang et al., 2018). *Arabisopsis* also

confirmed that lacking *Lhcb* transcripts reduced maximum quantum yield (Andersson et al., 2003). This explains the cooperation of PSII core and Lhcbs (Minor antenna proteins CP24 and CP26 affect the interactions between photosystem II) (Bianchi et al., 2008). *LHCB* transcript reduction trend at hypo- and hypersaline conditions also explains the previous physiological result from our group. It has been shown that total chlorophyll content and leaf absorbance were dramatically reduced during the time course (Kongrueang et al., 2018). This similar chlorophyll content reduction appeared in antisense *Arabidopsis* plants (Andersson et al., 2003). However, this could simply explain the reduction of the light that was captured (Andersson et al., 2003).

Hypo- and hypersaline conditions (salinity of 10, 40 and 50) increased the expression ratio of *RCA* gene in *E. acoroides* leaves at the end of the experiment. The previous studies have been shown hypersaline conditions up-regulated *RCA* gene in *Oryza sativa* (Parker et al., 2006), *Brachypodium distachyon* (Bayramov et al., 2014), *Vitis vinifera* (Cramer et al., 2007), *Thellungiella halophile* (Pang et al., 2010). The early response of *RCA* increment has been shown in water deficit treatment but hypersaline treatment appeared a little bit later (Cramer, 2007). In terrestrial plants, increment of *RCA* may be a mechanism to tolerate salt stress at long-term period in plants by reducing stomatal conductance and subsequent lowering CO_2 levels (Parker et al., 2006). However, there might be different RCA-related salt stress tolerance regulations in seagrass. In contrast, hypersaline showed *RCA* decline in glycophyte *Arabidopsis thaliana* (Pang et al., 2010). The decrease of RCA is related to photosynthetic activity by reducing Calvin cycle activity (decreasing the photosynthetic CO_2 assimilation) (Pang et al., 2010). The increase of RCA may be necessary to maintain the rubisco activity at high level even in low CO_2 concentration (Ghaffari et al., 2014; Yousuf et al., 2015). Therefore, the increase of *RCA* transcripts in our result might be related to the increase of CO_2 assimilation due to the stress conditions (Li et al., 2011; Deeba et al., 2012; Abreu et al., 2014).

E. acoroides leaves at the extreme hypersaline condition increased the expression ratio of *psbA* gene. In contrast, salt stress reduced *ZmpsbA* transcript in *Zea mays* (Huo et al., 2016). The PSII D1 is one of the core proteins of the PSII reaction center which is the target of the photodamage from the excitation of excess energy after exposure to salt stress (Allakhverdiev et al., 2002; Takahashi and Badger,

2011; Suo et al., 2017). Previous study in *Posidonia oceanica*, the inhibition of the translations of the PSII core proteins D1 (*psbA*) is related to the inhibition of the photosystem II repair cycle which is necessary to recover photodamage of PSII (Aro et al., 1993; Marin-Guirao et al., 2016). Salt stress inhibited both the transcription of *psbA* gene and the translation to pre-D1 protein, which may inhibit the photosystem II repair cycle (Aro et al., 1993; Allakhverdiev et al., 2002; Al-Taweel et al., 2007; Murata et al., 2007; Yang et al., 2007; Marin-Guirao et al., 2016). Therefore, the increase of D1 protein synthesis are necessary to increase the PSII repair efficiency in salt-stressed plants (Allakhverdiev et al., 2002; Takahashi and Badger, 2011; Suo et al., 2017), corresponding to the present result which showed the increase of *psbA* gene in *E. acoroides* leaves at the extreme hypersalinity condition.

The abiotic stress affects photoinhibition by damaging D1 PS II reaction center protein while light induces the damage of another PS II reaction center protein, D2 (encoded by *psbD*) and internal antenna protein CP43 (encoded by *psbC*) (Christopher and Mullet, 1994; Giardi et al., 1997; Nagashima et al., 2004). In addition, *Chlamydomonas* has shown D2 may play an important role in the regulation of the D1 protein (Erickson et al., 1986). The decrease of *psbD* leads to the damage of PSII (Surzycki et al., 2007). Hypo- and hypersaline conditions (salinity of 10, 40 and 50) increased the expression ratio of *psbD* transcript in *E. acoroides* leaves at the end of the experiment. The previous studies have also been shown salinity stress up-regulated *psbD* in *Arabidopsis* (Nagashima et al., 2004) and *Solanum lycopersicum* (Li et al., 2015). Many other abiotic stresses (such as: cold, high light and hyperosmotic stresses) activate *psbD* transcription in *Arabidopsis* (Nagashima et al., 2004). In contrast, the previous study in *Robinia pseudoacacia* indicated the expression of *RppsbD* was down-regulated in salinity treatment (Chen et al., 2017) and *Chlamydomonas reinhardtii* showed the reduction of D2 protein content (6-30%) after exposed to high NaCl concentration treatment (Neelam and Subramanyam, 2013). The increase of the *psbD* transcription is one of the plant protection mechanisms from the abiotic stress by producing D2 protein of PSII and enhancing recovery PSII reaction center from damage (Nagashima et al., 2004; Kiss et al., 2012).

In this present study, the photosynthesis-related gene expression of *E. acoroides* leaves, including *LHCB*, *RCA*, *psbA* and *psbD* genes, was affected by salinity stress differently.

CHAPTER 3

CONCLUSIONS

From this study, it can be conclude that:

Salinity stress has several effects to *Enhalus acoroides* in physiological response and gene expression. We observed that hyposalinity has more effect to the seagrass species than hypersalinity.

With respect to the physiological response, it appeared that both hypo- and hypersalinities affected the photophysiological responses of *E. acoroides* as reducing the maximum quantum yield of photosystem II and total chlorophyll content.

With respect to the photosynthesis-related gene expression, it appeared that *light-harvesting antenna* (*LHCB*) gene expression appeared reducing trend in both hypo- and hypersalinities. Moreover, *Rubisco activase* (*RCA*), *PSII reaction center D1* (*psbA*), and *PSII reaction center D2* (*psbD*) showed induction at the late stage of treatments. However, the transcript induction was more pronounced in *RCA*.

There is also strong correlation between physiological response and gene expression as we observed the relationship among *LHCB*, maximum quantum yield and chlorophyll content.

We suggest the future works that would help to understand effect of salinity to photosynthesis as following:

1.1 Investigation of physiological response and gene expression during recovery period.

1.2 Investigation other light-harvesting antenna genes.

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Appendix

Paper publication

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Pimpanit Kongrueang, Pimchanok Buapet and Peerapat Roongsattham*

Physiological responses of *Enhalus acoroides* to osmotic stress

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Abstract: This study aims to examine photophysiological and osmotic responses in seedlings of the seagrass *Enhalus acoroides* after exposure to different salinity levels. Seagrass seedlings were grown for 20 days in control (salinity 30), hyposaline (salinity 10 and 20) and hypersaline (salinity 40 and 50) conditions. The present study showed that both hypo- and hypersaline conditions affected the photophysiology of *E. acoroides* seedlings, reducing the maximum quantum yield of photosystem II (F_v/F_m) and total chlorophyll content. The photosynthetic system appeared to be more sensitive to hyposaline than to hypersaline conditions as shown by immediate declines in F_v/F_m and total chlorophyll content. Hyposaline conditions increased the water content in roots. The increase in tissue Na^+ content induced by hypersalinity did not affect photosynthetic integrity and was more pronounced in leaves than in roots. It is concluded that the ionic homeostasis of *E. acoroides* seedlings is less affected by short-term hypersalinity than by hyposalinity. The K^+/Na^+ ratios in leaves with hypersalinity decreased by 20 days after treatment. Additionally, the photosynthetic efficiency (F_v/F_m and total chlorophyll content) is highly sensitive to salinity shifts and can be used as a marker for short-term acclimation to salinity stress in this seagrass species.

Keywords: *Enhalus acoroides*; osmotic stress; photosynthesis; salinity stress; seagrass.

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Introduction

The coastal areas are dynamic environments with frequent shifts in irradiance, salinity and temperature which disturb seagrass growth (Vergeer et al. 1995, Blakesley et al. 2002, Trevathan et al. 2011). Natural phenomena and anthropogenic disturbances, such as heavy rainfall, fresh water inflows, storms, changes in watersheds or wastewater disposal, and decline of freshwater input due to consumption by agriculture, can lead to dramatic salinity changes in some coastal areas and estuaries, especially in areas adjacent to the shore (Adams and Bate 1994, Tomasko and Hall 1999, Fernandez-Torquemada and Sanchez-Lizaso 2005, Thorhaug et al. 2006, Chollett et al. 2007, Touchette 2007). For example, wastewater from desalination plants increased the salinity of some Mediterranean coastal areas from 37 to up to 44, or even 90 (Fernandez-Torquemada and Sanchez-Lizaso 2005).

Each seagrass species has a different optimal salinity ranging from salinity of 20–42 (Les and Cleland 1997, Collier et al. 2014). Nevertheless, rapid changes in salinity result in stress in this group of plants (Tyerman 1982, Tyerman et al. 1984). Salinity stress alters seagrass biochemical and physiological processes, which may subsequently affect their growth, reproduction and survival (Touchette 2007). Hyposaline and hypersaline conditions have been shown to negatively affect the photosynthetic activity of *Halophila johnsonii* during medium-term exposures (15 days; Fernandez-Torquemada et al. 2005). *Cymodocea nodosa* under high salinity conditions for long periods (47 days) showed small reductions in photosynthetic rate that indicated *C. nodosa* can tolerate hypersaline conditions more readily than *Posidonia oceanica*, which grows better in stable salinity (Sandoval-Gil et al. 2012a). Additionally, a prolonged exposure to salinity stress may dramatically increase the mortality rate (Kahn and Durako 2008, Griffin and Durako 2012).

High salinity affects plant homeostasis by two means: 1. osmotic stress by removing water from plant tissues and 2. ionic toxicity by altering ion concentrations and metabolic processes, especially growth and photosynthesis (Munns and Tester 2008, Cambridge et al. 2017). In contrast, hyposaline conditions lead to hypo-osmotic stress in plants resulting from ion efflux from vacuoles

and compatible solute (osmoprotectant) degradation (Bisson and Kirst 1995, Griffin and Durako 2012). Sudden hypo-osmotic conditions also increase turgor pressure, and consequently trigger hypo-osmotic shock (Takahashi et al. 1997, Walley et al. 2007, Beauzamy et al. 2014) by a steady decrease in plant cell osmolarity (Felix et al. 2000). The osmotic responses to unfavorable salinity are energy-demanding processes and may increase the total energy requirements of the plants, thus decreasing growth and fitness (Fernandez-Torquemada and Sanchez-Lizaso 2005, Touchette 2007, Griffin and Durako 2012). Short-term high salinity pulses have been shown to increase Cl^- and Na^+ ion concentrations and to deplete K^+ and Ca^{2+} ions from the leaves and rhizomes of seagrass species. Many K^+ transporters have high affinities to Na^+, thus they serve as Na^+/K^+ symporters. Therefore, relatively high Na^+ levels in the environment can affect K^+ influx efficiencies in marine plants (Touchette 2007, Garrote-Moreno et al. 2014). K^+ is necessary for managing the osmotic balance, as an auxiliary participating in biological reactions, and as a co-factor of enzymatic reactions (Touchette 2007). Thus, a decline of K^+ uptake negatively affects plant growth (Touchette 2007). The K^+/Na^+ ratio in plants has been proposed as a proxy for salinity tolerance (Lopez and Satti 1997). Seagrass species that are tolerant to hypersaline conditions have been shown to be able to maintain their K^+/Na^+ ratio (Garrote-Moreno et al. 2014).

Enhalus acoroides is distributed along the coastal areas and in estuaries in the tropical Indo-Pacific regions that have salinity fluctuations (Short et al. 2007). It is one of the most important seagrass species in Thailand (Juntaban et al. 2015). This seagrass species has the highest coverage in the Indo-Pacific bioregion, including Thailand, providing a habitat for a diverse and economically important fauna (Nienhuis et al. 1989, Prathep et al. 2010, Unsworth et al. 2010, 2012). Due to their large leaf blades, water flow inside *E. acoroides* beds is significantly reduced, which results in high sedimentation rate (Komatsu et al. 2004). These facts prevent erosion and create a favorable environment for other seagrass species, benthos in the sediments, epiphytes and juvenile marine animals (Nienhuis et al. 1989, Komatsu et al. 2004, Unsworth et al. 2010, 2012). *Enhalus acoroides* had the highest importance value index based on the relative cover of the species, and the relative frequency and diversity of other species, implying that it affects numerous ecological functions of the seagrass bed (Dewi and Sukandar 2017). However, the distribution of *E. acoroides* throughout intertidal areas was greatly affected by harsh environmental conditions, such as salinity fluctuation, resulting in a decline of the meadows (Unsworth et al. 2010, 2012). At Bolinao, Philippines, salinity was usually constant (28–34) but could be decreased to 20 after fresh water influx (Rollon 1998). In Thailand, *E. acoroides* is commonly found in the vicinity of mangrove forests and river mouths (Chansang and Poovachiranon 1994). These habitats are prone to salinity fluctuations due to the freshwater from inland (Vichkovitten 1998). Rattanachot and Prathep (2011) reported that salinity can drastically change within the range 29.3–35.7 in the *E. acoroides* habitat at Laem Yong Lam, in Haad Chao Mai National Park, Trang Province, Thailand. The aim of the present study was to provide information on the physiological responses of *E. acoroides* to hyposaline and hypersaline conditions. Experiments were conducted to investigate the effects of different salinities and exposure times on photosynthetic activity, pigment content, water content and ion concentrations, under laboratory-controlled conditions.

Materials and methods

Plant material

Fully ripe seeds of *Enhalus acoroides* (L.f.) Royle were collected from Ban Pak Khlong (7°36′01.8″N and 99°16′22.3″E, Trang Province, Thailand) during the lowest tidal range in March 2016. The samples were transported to the Bo Hin Farmstay seagrass seedling bank (seagrass seedling nursery, under conservation and restoration of seagrass resources project, Marine and Coastal Conservation Center No. 6, Trang, Thailand). The seeds were germinated in plastic containers with natural seawater (salinity range: 30–35) under ambient light. The seagrass seedlings were grown for 2 months before being transported to the laboratory at the Department of Biology, Prince of Songkla University.

Experimental design

Seagrass seedlings were transferred into 15 glass tanks (30 × 30 × 30 cm), each containing 200 seedlings and 20 l of artificial seawater (Marinium® reef sea salt, Mariscience, Thailand) at a salinity of 30 with 0.01 mg l^{-1} $NaNO_3$ (Riedel-de Haen) and 0.001 mg l^{-1} KH_2PO_4 (Fluka-Garantie). They were allowed to acclimate for 7 days before experimental manipulation of salinity. The water in the tanks was oxygenated with air pumps. Photosynthetically Active Radiation (PAR) at 45 µmol photon m^{-2} s^{-1} was provided from

LED lights on a 12 h light:12 h dark cycle and the temperature was maintained at 26°C in a temperature-controlled room.

After 7 days, the seagrass seedlings were transferred directly into salinities of 10, 20 (hyposaline conditions), 30 (control), 40 or 50 (hypersaline conditions), with three replicate tanks for each salinity. Nutritional supplements (0.01 mg l^{-1} $NaNO_3$ and 0.001 mg l^{-1} KH_2PO_4) were added to all tanks. During this step, the culture conditions were as during the acclimation period. The plantlets were randomly rotated around the tank every day in order to minimize variations in irradiance with position in the tank. Half the water was removed and replaced every 3 days in order to maintain sufficient nutrition and water quality.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence (maximum quantum yield of photosystem II; F_v/F_m) was measured using a pulse amplitude modulated (PAM) fluorometer (Mini-PAM, WALZ, Germany) in three replicate seedlings on 0, 1, 2, 7, 10 and 20 days after treatment (DAT), counting the days after the plants were exposed to different salinities. Before measuring F_v/F_m, the leaves (2nd leaf of seedling) were dark-adapted for 15 min using dark leaf clips. F_v/F_m was calculated by the following formula (Murchie and Lawson 2013):

$$F_v/F_m = \frac{F_m - F_o}{F_m}$$

F_o: minimum value for chlorophyll fluorescence in the dark state

F_m: maximum value for chlorophyll fluorescence in the dark state

F_v: maximum variable chlorophyll fluorescence.

Measurement of leaf absorbance

Three replicate seedlings were collected at 0, 1, 2, 7, 10 and 20 DAT. The light absorption ability of the 2nd leaf of each seedling was analyzed by measuring the incident light in the air (LI-250A, LI-COR®Bioscience, USA). The leaf was then placed on the light sensor and the amount of light transmitted through the leaf was measured. The leaf absorbance was calculated as (Serrano et al. 2000, Ducruet et al. 2012):

$$\text{Absorbance} = \log \frac{I_0}{I}$$

I_0: irradiance of the incident light

I: irradiance of the transmitted light.

Pigment content measurement

Three replicate seedlings were collected at 0, 1, 2, 5, 10 and 20 DAT. The total chlorophyll and carotenoids were extracted by grinding the 2nd leaf of each seedling in 80% acetone under dim light. After centrifuging at 604 *g* for 2 min, the supernatant was collected and absorbances at 470, 646 and 663 nm were determined using a spectrophotometer (DS-11 Spectrophotometer, DeNovix, USA). Pigment contents were calculated based on fresh mass of leaf by the following formulae (Lichtenthaler and Wellburn 1983):

$$\text{Chlorophyll } a \text{ (Chl } a\text{) } (\mu g\ ml^{-1}) = \left[12.21\ (A_{663}) - 2.81\ (A_{646})\right]$$

$$\text{Chlorophyll } b \text{ (Chl } b\text{) } (\mu g\ ml^{-1}) = \left[20.13\ (A_{646}) - 5.03\ (A_{663})\right]$$

$$\text{Total chlorophyll} = \text{Chl } a + \text{Chl } b$$

$$\text{Carotenoid } (\mu g\ ml^{-1}) = \frac{[1000\ (A_{470}) - 3.27\ (\text{Chl } a) - 104\ (\text{Chl } b)]}{229}$$

Analysis of Na^+ and K^+ accumulation in plant tissue

Three replicate seedlings were collected at 0, 10 and 20 DAT. Sodium and potassium ion concentrations in leaf and root were determined. The plant materials (all leaves and all roots of seedlings) were cleaned with tap water and dried at 60°C for 72 h. The samples were digested in 1 ml of HNO_3 at 95°C for 2 h. After that, the solution was filtered with Whatman® filter paper (no.1) and diluted to 10 ml with deionized water. The content of sodium and potassium ions was determined by inductively coupled plasma optical emission spectrometry (ICP-OES Optical Emission Spectrometer Optima 4300 DV, PerkinElmer Inc., USA). The ion concentration was calculated based on dried mass of each sample (modified from Marin-Guirao et al. 2013).

Estimation of relative water content

Three replicate seedlings were collected at 0, 1, 2, 5, 10 and 20 DAT for the determination of relative water content

in all leaves and roots of seedlings. The samples were weighed before and after drying at 60°C for 72 h. The turgid weight (TW) was obtained by placing small leaf and root pieces (0.6 cm^2) in closed 1.5-ml tubes filled with 1 ml de-ionized water. These were maintained in darkness for 4 h at 4°C, after which the pieces were removed, excess water drained from them, and weighed (Sandoval-Gil et al. 2014b). The relative water content of the sample was calculated as (Back et al. 1992, Sandoval-Gil et al. 2014b):

$$\text{Relative water content} = \frac{FW - DW}{TW - DW} \times 100$$

FW: sample fresh weight
DW: sample dry weight
TW: sample turgid weight

Statistical analysis

All statistical tests were performed using SPSS software, version 16.0 (SPSS Inc., USA). The studied parameters were tested for assumptions of normality and homogeneity of variance with the Kolmogorov-Smirnov and Levene's tests, respectively. F_v/F_m, Na^+ and K^+ ion concentrations, water content, pigment content, and leaf absorbance were analyzed with factorial two-way analysis of variance (ANOVA), testing the effects of two fixed factors (i.e. manipulated salinity and exposure time) on the physiological responses of *Enhalus acoroides*. If the salinity, time, or their interaction were significant according to ANOVA, then the least significant difference (LSD) was calculated to assess statistical significance (post-hoc test). All the data from measurements are shown as mean ± standard error.

Results

Effects of salinity on photosynthesis (maximum quantum yield of PS II)

At the beginning of the experiment, there were no differences in the maximum quantum yield of photosystem II (F_v/F_m) of *Enhalus acoroides* leaves among the salinity treatments (Figure 1). Salinity and time had significant effects on F_v/F_m values of *E. acoroides* leaves, and the interaction between salinity and time was also significant (Table 1). The F_v/F_m at salinity 20 remained unchanged over time and did not differ from the control (Figure 1).

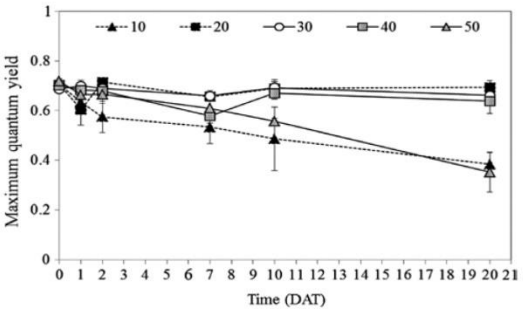

Figure 1: Maximum quantum yield of PSII (F_v/F_m) for *Enhalus acoroides* leaves with each salinity treatment (10, 20, 30 (control), 40 and 50) at different days after treatment (DAT). Values are means ± S.E.; n = 3.

However, at the lowest salinity (10) and the highest salinity (50), the F_v/F_m started to decrease at 2 DAT and 10 DAT, respectively, and gradually decreased until the end of the experiment. At the end of the experiment, the F_v/F_m of *E. acoroides* leaves at salinities of 10 and 50 were comparable (Figure 1).

Effects of salinity on leaf absorbance

The absorbance of *E. acoroides* leaves did not differ among treatments at 0 DAT (Figure 2A). The statistical analyses revealed that salinity and time had significant effects on leaf absorbance, but no interaction between salinity and time was detected (Table 1). At salinities of 30, 40 and 50, leaf absorbance remained unchanged over time when compared to 0 DAT (Figure 2A). The obvious change was observed with the hyposalinity treatments (salinities 10 and 20): leaf absorbance significantly decreased to a minimum at 7 and 10 DAT, respectively (Figure 2A).

Effects of salinity on pigment content: total chlorophyll (chlorophyll *a* + *b*) and carotenoid

At the beginning of the experiment, plants in all salinity treatments had similar total chlorophyll and carotenoid contents (0.103–0.163 and 0.031–0.058 mg g^{-1} fresh weight, respectively; Figure 2B,C). Salinity and time significantly affected total chlorophyll and no interaction between salinity and time was detected, but the carotenoid content of *Enhalus acoroides* was not affected by salinity, time or their interaction (Table 1). Variations in total chlorophyll

Table 1: Summary of the two-way ANOVA testing the effect of salinity treatment (10, 20, 30, 40 and 50) and time (0, 1, 2, 5 or 7, 10 and 20 days after treatment) on physiological responses of *Enhalus acoroides*.

Parameter	Source of variation	*df*	MS	*F*	p-Value
Maximum quantum yield of PSII	Salinity	4	0.06	11.05	**<0.001**
	Time	5	0.05	8.59	**<0.001**
	Salinity × time	20	0.01	2.68	**0.002**
	Error	60	0.01		
Leaf absorbance	Salinity	4	0.01	2.80	**0.034**
	Time	5	0.02	4.75	**0.001**
	Salinity × time	20	0.00	0.95	0.533
	Error	60	0.00		
Total chlorophyll (Chl *a* + Chl *b*)	Salinity	4	0.01	2.60	**0.045**
	Time	5	0.01	4.26	**0.002**
	Salinity × time	20	0.00	1.63	0.076
	Error	60	0.00		
Carotenoid	Salinity	4	0.00	0.89	0.476
	Time	5	0.00	1.04	0.401
	Salinity × time	20	0.00	0.73	0.782
	Error	60	0.00		
Leaf Na	Salinity	4	776.33	3.81	**0.013**
	Time	2	899.91	4.42	**0.021**
	Salinity × time	8	223.14	1.10	0.394
	Error	30	203.74		
Root Na	Salinity	4	2022.63	4.06	**0.010**
	Time	2	198.66	0.40	0.675
	Salinity × time	8	516.47	1.04	0.432
	Error	30	498.74		
Leaf K	Salinity	4	29.94	3.53	**0.018**
	Time	2	33.66	3.97	**0.030**
	Salinity × time	8	8.73	1.03	0.436
	Error	30	8.48		
Root K	Salinity	4	65.37	3.51	**0.018**
	Time	2	31.11	1.67	0.205
	Salinity × time	8	14.27	0.77	0.635
	Error	30	18.64		
Leaf K/Na ratio	Salinity	4	0.00	2.92	**0.037**
	Time	2	0.03	38.78	**<0.001**
	Salinity × time	8	0.00	3.76	**0.004**
	Error	30	0.00		
Root K/Na ratio	Salinity	4	0.00	1.17	0.346
	Time	2	0.01	5.39	**0.010**
	Salinity × time	8	0.01	2.59	**0.028**
	Error	30	0.00		
Leaf relative water content	Salinity	4	677.61	4.79	**0.002**
	Time	5	318.04	2.25	0.061
	Salinity × time	20	46.39	0.33	0.996
	Error	60	141.48		
Root relative water content	Salinity	4	628.12	6.66	**<0.001**
	Time	5	93.71	0.99	0.429
	Salinity × time	20	93.27	0.99	0.488
	Error	60	94.31		

Values in bold face: p-value significant ($p < 0.05$).

contents were observed with salinities of 20, 40 and 50, although there was no clear trend (Figure 2B). Major reductions were only observed at a salinity of 10; chlorophyll content significantly decreased at 10 DAT and remained low to 20 DAT (Figure 2B). The chlorophyll content at salinity 30 (control) did not differ significantly from salinities

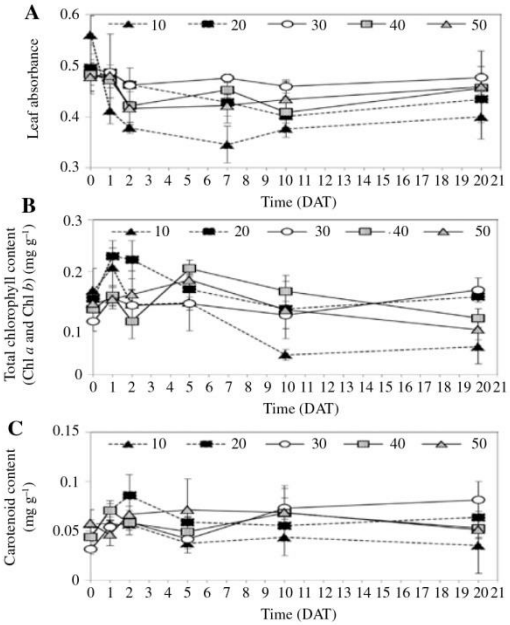

Figure 2: (A–C) Leaf absorbance, total chlorophyll content and carotenoid content of *Enhalus acoroides*.
Leaf absorbance of *E. acoroides* (A), total chlorophyll content (mg g^{-1} fresh weight) (B) and carotenoid content (mg g^{-1} fresh weight) (C) of *E. acoroides* leaves with each salinity treatment at different days after treatment (DAT). Values are means ± S.E.; n = 3.

of 20, 40 and 50 but was significantly different from the salinity of 10 at 20 DAT (Figure 2B).

Effects of salinity on ion concentrations (Na^+, K^+) and K^+/Na^+ ratio in leaf and root tissues

Salinity and time had significant effects on Na^+ and K^+ concentrations of *Enhalus acoroides* leaves, but no interaction of salinity and time was detected (Table 1). Na^+ concentration in the leaves did not change during exposure to salinities of 10, 20 or 30 (Figure 3A). In contrast, the Na^+ concentration in leaves had increased by 20 DAT when exposed to salinities of 40 and 50 (Figure 3A). The Na^+ concentrations in roots did not change during exposure to salinities of 10, 20, 30 or 40 (Figure 3B). However, at salinity 50, the Na^+ concentration had increased by 20 DAT (Figure 3B).

K^+ concentration in leaves at salinities of 10, 20, 30 and 40 did not change during the study period. However, the K^+ concentration in leaves at a salinity of 50 had increased at 10 DAT but decreased to the initial value by 20 DAT (Figure 3C). There was an effect of salinity level on K^+ concentrations in the roots but no effect of exposure time was detected (Table 1, Figure 3D).

Salinity, time and their interaction had significant effects on the K^+/Na^+ ratio in leaves (Table 1). However, salinity did not influence the K^+/Na^+ ratio in roots, but there were effects of exposure time and the interaction of salinity and exposure time (Table 1). In all of the salinity treatments, the K^+/Na^+ ratio in leaves did not change between 0 and 10 DAT, but the ratio had decreased by 20 DAT at salinities of 30, 40 and 50 (Figure 3E). Similarly, the K^+/Na^+ ratio in the roots did not change between 0 and 10 DAT with any of the salinity treatments, but this ratio had decreased by 20 DAT with all salinities of 10 and 20 (Figure 3F).

Effects of salinity on relative water content in leaf and root tissues

Salinity had significant effects on the relative water content in both leaves and roots of *Enhalu acoroides* but there was no effect of time or the interaction between salinity and time (Table 1). There were fluctuations in relative water content in the leaves during the early stage of the experiments (1–5 DAT; Figure 4A). In all of the salinity treatments, the relative water content of leaves did not change compared to the initial state until 20 DAT (Figure 4A, Table 1). At salinities of 10 and 20, the relative water content of the roots had increased by 20 DAT, but remained similar throughout the experiments at salinities of 30, 40 and 50 (Figure 4B). The final relative water content (20 DAT) in the control roots (salinity 30) was significantly different from those at a salinity of 10 ($p = 0.012$) but not different from those at salinities of 20, 40 and 50 (Figure 4B).

Discussion

Our results indicate that both hyposaline and hypersaline conditions affected several physiological processes of the seedlings of *Enhalus acoroides*. Seagrasses respond to various stresses, including salinity stress, by adjusting or changing the photosynthetic apparatus (Touchette 2007). We observed reductions in the maximum quantum yield of photosystem II (F_v/F_m) with salinities of 10 and 50, in agreement with results from previous studies in other

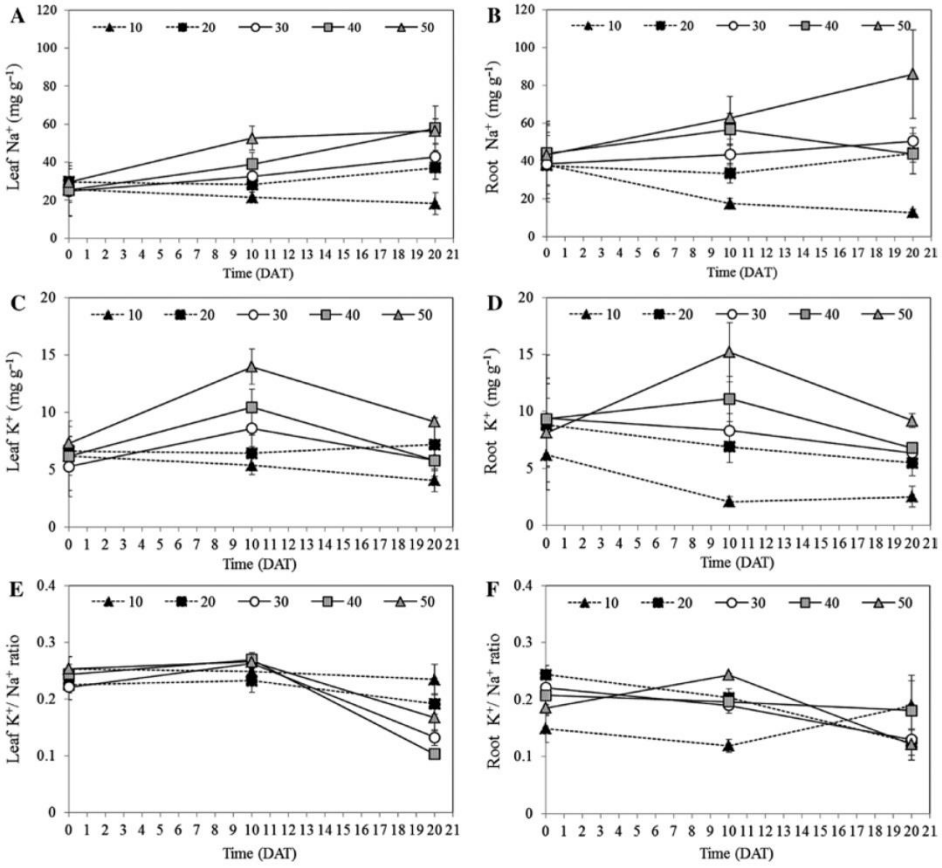

Figure 3: (A–F) Ion contents and their ratio in different *Enhalus acoroides* tissues.
Na^+ (mg g^{-1} dry weight) of *E. acoroides* leaves (A) and roots (B), K^+ (mg g^{-1} dry weight) of *E. acoroides* leaves (C) and roots (D), K^+/Na^+ ratio of *E. acoroides* leaves (E) and roots (F) with each salinity treatment at different days after treatment (DAT). Values are means ± S.E.; n = 3.

seagrass species. Hyposaline conditions lead to down-regulation of photosynthesis or damage to the photosynthetic machinery, as indicated by the F_v/F_m reduction in several seagrass species such as *Ruppia maritima* (Murphy et al. 2003), *Halophila johnsonii* (Griffin and Durako 2012), *Cymodocea nodosa* (Zarranz-Elso et al. 2012) and *Zostera marina* (Salo et al. 2014). Similarly, reduced F_v/F_m in hypersaline conditions has been recorded in *R. maritima* (Murphy et al. 2003), *C. nodosa* (Pages et al. 2010), *Thalassia testudinum* (Howarth and Durako 2013a), *Posidonia oceanica* (Sandoval-Gil et al. 2014a) and *Posidonia australis* (Cambridge et al. 2017). However, the degrees of stress response differ with species, the salinity treatments (intensity and duration), or even ecotypes (Salo et al. 2014). For example, a salinity of 55 had no significant effect on F_v/F_m in *T. testudinum*, *Halodule wrightii* and *R. maritima* (Koch et al. 2007), while a salinity of 43 caused a decline in the F_v/F_m of *P. oceanica* (Sandoval-Gil et al. 2014a). The same species has varied responses to salinity dependent on the habitat (Salo et al. 2014). *Zostera marina* originally acclimatized to high salinity had decreasing F_v/F_m with exposure to hyposaline conditions (salinity <9), while individuals acclimatized to low salinity showed decreased F_v/F_m only at more extreme hyposalinity (salinity <2; Salo et al. 2014). The decline of F_v/F_m in *E. acoroides* suggests that extreme hypo- and hypersaline conditions impose stress on photosynthesis (Garrote-Moreno et al. 2015). Our results indicate that *E. acoroides* in hyposaline conditions (salinity 10) show greater negative photosynthetic activity than in hypersaline conditions (salinity 50), and that *E. acoroides* can tolerate salinity ranging from 20 to 40 for at least 20 days, since no significant changes in F_v/F_m

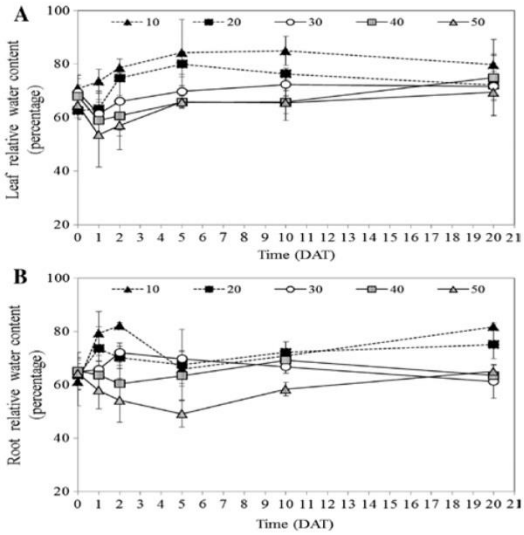

Figure 4: (A–B) Percentage relative water content in leaves and roots of *Enhalus acoroides*.
Leaves (A) and roots (B) with each salinity treatment at different days after treatment (DAT). Values are means ± S.E.; n = 3.

were observed. However, decreasing the F_v/F_m might be another long-term strategy of plants to dissipate excess photons acquired due to stress. Plants have safety valves to avoid damage occurring to the photosynthetic apparatus (Niyogi 2000).

The F_v/F_m of *Enhalus acoroides* had similar trends as for leaf absorbance and total chlorophyll. Hyposalinity (salinity 10) decreased leaf absorbance and total chlorophyll content in *E. acoroides* more than the other salinity treatments. The decline of total chlorophyll content in *E. acoroides* with hyposaline conditions is consistent with a study of *Halophila johnsonii* (Kahn and Durako 2008). Similarly, *Thalassia testudinum* at low salinities (salinity 16, incubated for 24 h) had increased leaf reflectance and decreased chlorophyll content, resulting in reduced light absorption (Thorhaug et al. 2006). A decrease in total chlorophyll content with hypersaline conditions has also been observed in other studies with *T. testudinum* (Howarth and Durako 2013b) and *Cymodocea nodosa* (Sandoval-Gil et al. 2014a). In addition, a study of *T. testudinum* at high salinity (50) also showed increased leaf reflectance (Durako and Howarth 2017). Decline of photosynthetic pigments is considered to be a general response to stress in plants. However, the decreases in photosynthetic activity, photosynthetic pigments and absorbance observed in our study might be one of the photoprotective mechanisms to alleviate oxidative stress after salinity shift. Salinity stress induces the generation of reactive oxygen species (ROS) in plant cells, which may lead to oxidative stress (Luo and Liu 2011). Down-regulation of light utilization decreases ROS production from the chloroplast and might consequently reduce photo-oxidative damage (Luo and Liu 2011).

Seagrasses take up both nutrients and ions from the bulk water via leaves and from porewater via roots (Stapel et al. 1996). The effects of salinity on the ion concentration in seagrasses depend on salinity variations, different types of organelles in the plant tissues, and exposure time (Garrote-Moreno et al. 2016). The analysis of ion concentrations in leaves and roots of *Enhalus acoroides* at high salinity showed the accumulation of Na^+ in leaves in the short-term, similar to those in *Posidonia oceanica* and *Cymodocea nodosa* (Garrote-Moreno et al. 2015), *T. testudinum* and *Halodule wrightii* (Garrote-Moreno et al. 2014). Nevertheless, the increases in Na^+ ion concentration at the end of experiment at hypersalinities were more pronounced in leaf tissues than in roots. This contradicts the results from previous studies in *Thalassia testudinum* and *H. wrightii*, in which Na^+ ion concentrations in leaves were lower than those in rhizomes (Garrote-Moreno et al. 2014). The higher percentage Na^+ concentration change in leaf tissues than in root tissues at 10 DAT suggests deceased photosynthetic activity. K^+/Na^+ ratio is also considered to be a salinity stress descriptor. The higher the K^+/Na^+ ratio, the more tolerant to salinity stress (Garrote-Moreno et al. 2015). Hypersaline conditions had no significant effect on K^+/Na^+ ratio in the leaves of *E. acoroides* in the short- and medium-term (up to 10 days after treatment). This suggests that *E. acoroides* are able to regulate ion balance over short and medium periods. We did not observe competition of Na^+ and K^+ transport in hypersaline conditions since the decrease in K^+/Na^+ was driven by increasing Na^+ alone, not by K^+ reduction.

Both hypo- and hypersaline conditions affected the water content in tissues through ion accumulation and osmotic adjustments. Under hypersaline conditions, seagrasses can reduce the water potential of their tissues by accumulating osmotically-active solutes within the cell, by turgor regulation (i.e. cell-wall hardening processes) or even by cell water efflux (Sandoval-Gil et al. 2012a,b, Cambridge et al. 2017). In *Enhalus acoroides*, hypersaline conditions reduced water content in both the leaves and the roots, and this agrees with a study in *Posidonia australis* (Cambridge et al. 2017). Nevertheless, these were only short-term responses, since the osmotic adjustment took place successfully to maintain the cell water content by increasing the osmotic forces for water uptake (Passioura and Munns 2000, Touchette 2007, Garrote-Moreno

et al. 2014). Hypo-osmotic shock leads to increased cell volume, turgor pressure and rate of water influx (Takahashi et al. 1997) and, accordingly, the water content in both leaves and roots of *E. acoroides* increased when exposed to hyposalinity. However, the minor changes in relative water content observed in our study, although statistically significant, had only slight effects on the stress in this seagrass.

Our results indicate that the photosynthetic machinery of *Enhalus acoroides* seedlings was more sensitive to hyposaline (salinity 10) than to hypersaline (salinity 50) conditions, because hyposalinity caused a rapid reduction in photosynthesis which persisted until the end of the experiment. The seagrasses exposed to extreme salinities (salinities of 10 and 50) seemed not to be able to recover after an initial decrease while those exposed to intermediate salinity levels (salinities of 20 and 40) were able to recover to the initial values. The mechanisms of photosynthetic stress differ between hyposaline and hypersaline conditions. It has been suggested that photodamage in hyposaline conditions may be attributed to decreased cellular ion contents, including of the ions necessary as photosynthetic cofactors (Touchette 2007). However, this might not be the cause of the decline in photosynthesis observed in our experiment since Na^+ and K^+ in hyposaline treatments did not change. This might suggest that hyposaline conditions inhibit electron transport and increase ROS leading to oxidative damage in chloroplasts, blocking of electron flow in photosystem II, and photodamage to the reaction center (Jahnke and White 2003, Luo and Liu 2011). As an alternative, it has been suggested that hypersalinity affects photosynthetic efficiency by changing the abundance and ultrastructure of chloroplasts, inhibiting the activity of enzymes associated with carbon assimilation (Cambridge et al. 2017), and disturbing the permeability of ions (principally Na^+ and Cl^-) across the thylakoid membrane (Touchette 2007). However, the photosynthetic systems appeared more resistant to increased Na^+ with hypersaline conditions.

In conclusion, this research found adverse effects of both hypo- and hypersaline conditions and the duration of exposure to them, and the photosynthetic effects could be used as markers to detect *Enhalus acoroides* stress in response to salinity changes. Both natural and anthropogenic disturbances to salinity should be closely monitored in order to effectively protect the fragile *E. acoroides* communities. Our results showed that *E. acoroides* seedlings have higher sensitivity to hyposaline conditions. Therefore, this seagrass may be more affected by a sudden decrease in salinity brought about by heavy rainfall and freshwater inputs during monsoon seasons and extreme weather events, which are predicted to become more frequent in global change scenarios.

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