Variation of Tidal Exposures and Seasons on Growth, Morphology, Anatomy and Physiology of Seagrass *Halophila ovalis* (R.Br.) Hook.f. at Seagrass Bed in Trang Province

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ชื่อวิทยานิพนธ์ ความแปรผันของระยะการโผล่พ้นน้ำ และฤดูกาล ต่อการเจริญเติบโต ลักษณะทางสัณฐาน ลักษณะทางกายวิภาค และสรีรวิทยาของหญ้าทะเล *Halophila ovalis* (R.Br.) Hook.f. บริเวณแหล่งหญ้าทะเลจังหวัดตรัง

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อ ศึกษาผลของระยะเวลาการโผล่พ้นน้ำ และฤดูกาล ต่อการเจริญเติบโต ลักษณะทางสัณฐาน ลักษณะทางกายวิภาค และสรีรวิทยาของหญ้าใบมะกรูด (*H. ovalis* (R.Br.) Hook.f.) ลักษณะดังกล่าวข้างต้นได้ถูกศึกษาใน 3 บริเวณ คือ เขตน้ำขึ้นน้ำลงตอนบน เขตน้ำขึ้นน้ำลงตอนล่าง และเขตที่จมอยู่ใต้น้ำตลอดเวลา ตั้งแต่เดือนกรกฎาคม 2555 ถึงเดือนพฤษภาคม 2556 ความหนาแน่นและมวลชีวภาพส่วนบนดินมีความแปรผันตามระยะการโผล่พ้นน้ำและฤดูกาล ขณะที่มวลชีวภาพส่วนใต้ดินไม่มีความเปลี่ยนแปลงในรอบปี หญ้าทะเลมีอัตราการเจริญเติบโตช้าที่สุดบริเวณเขตน้ำขึ้นน้ำลงตอนบนซึ่งอาจเป็นผลมาจากความเครียดจากการโผล่พ้นน้ำ ความแปรผันของลักษณะทางสัณฐานมีมากในเรื่องของขนาดใบ โดยพบใบหญ้าทะเลมีขนาดใหญ่ที่บริเวณเขตน้ำขึ้นน้ำลงตอนล่างเมื่อเทียบกับเขตน้ำขึ้นน้ำลงตอนบนและเขตที่จมอยู่ใต้น้ำซึ่งมีขนาดใบเล็กกว่า ความแปรผันของขนาดมีโซฟิลล์เซลล์ และช่องอากาศในใบพบว่ามีความสัมพันธ์กับขนาดของใบ จากการวัดประสิทธิภาพการสังเคราะห์ด้วยแสงจากการเรืองแสงของคลอโรฟิลล์พบว่า อัตราการขนส่งอิเล็กตรอนสูงสุดที่บริเวณเขตน้ำขึ้นน้ำลงมีค่ามากกว่าเขตที่จมอยู่ใต้น้ำ หญ้าทะเลบริเวณเขตน้ำขึ้นน้ำลงมีความต้องการใช้แสงสำหรับกระบวนการสังเคราะห์ด้วยแสงมากกว่าหญ้าทะเลที่จมอยู่ใต้น้ำตลอดเวลา ปริมาณคลอโรฟิลล์และแคโรทีนอยด์ในใบที่เพิ่มขึ้นบริเวณเขตที่จมอยู่ใต้น้ำเอื้อประโยชน์ในการดูดกลืนช่วงคลื่นแสงสีน้ำเงินบริเวณที่ลึก ปริมาณแอนโทไซยานินมีมากในใบหญ้าทะเลที่บริเวณเขตน้ำขึ้นน้ำลงตอนบนเมื่อเทียบกับบริเวณที่ลึกกว่าเพื่อประโยชน์ในการช่วยป้องกันความเสียหายต่อรงควัตถุชนิดอื่นจากปริมาณแสงที่มากเกินไป และมีการสะสมของแอนโทไซยานินช่วงฤดูร้อนมากกว่าฤดูฝน

Thesis Title	Variations of Tidal Exposures and Seasons on Growth, Morphology, Anatomy and Physiology of seagrass *Halophila ovalis* (R.Br.) Hook.f. at Seagrass Bed in Trang Province
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ABSTRACT

The aim of this study was to investigate the variations of tidal exposures during low tide and seasons on growth parameters, morphology, anatomy and physiology of the seagrass *H. ovalis*. All variables were examined in three different tidal zones: upper intertidal, lower intertidal and shallow subtidal areas during July 2012 to May 2013. Shoot density and above-ground biomass varied due to variations of tidal exposures and seasons whereas below-ground biomass showed no seasonal variation. Slowest growth rate of seagrass presented in the upper intertidal zone which might be affected by desiccation stress. Morphological plasticity was greatly expressed through leaf size, bigger plant leaves were found in the lower intertidal area compared to smaller leaves in the upper intertidal and subtidal areas. Variations in mesophyll and air lacunar sizes were related with plant leaf size. Photosynthetic performance of *H. ovalis* obtained by chlorophyll fluorescence data showed greater maximum electron transport rate in intertidal seagrass than those in subtidal zone. Optimum light requirement was also higher in the intertidal seagrass than those in subtidal area. Increment of chlorophyll and carotenoid contents in subtidal seagrass help plant maximize light harvesting efficiency of blue light region in deeper water. Anthocyanin content was greater in the upper intertidal seagrass than those in deeper habitats in order to protect other photosynthetic pigment from excess light, and greater anthocyanin production was found in summer months.

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LIST OF ABBREVIATIONS AND SYMBOLS

µmol photons $m^{-2} s^{-1}$	SI unit for irradiance (400-700 nm)
mol photons $m^{-2} d^{-1}$	unit of daily light exposure
Abt_F	Absorptance factor
ETR	Electron transport rate
E_{opt}	Optimum light intensity for photosynthesis
F_m	Maximum fluorescence
F_v	Variable fluorescence
LHC	Light Harvesting Complex Proteins
O.D.	Optical density
PAM	Pulse Amplitude Modulated fluorometry
PAR	Photosynthetically Active Radiation (400-700 nm)
PEA	Plant Efficiency Apparatus
PPFD	Photosynthetic Photon Flux Density (400-700 nm)
PS	Photosystem
ppt	Parts per thousand
rETR	Relative electron transport rate
RLC	Rapid light curve
Y (Φ_{PSII})	The yield of PSII photoefficiency

CHAPTER 1
INTRODUCTION

Background and rationale

Environmental changes are common phenomena regularly occur and affect all living organisms. In order to survive and maximize their reproduction success, organisms have to adapt themselves to cope with uncertain conditions of their habitat whether in large or small scale. Thus, the variability in plant or animal characteristics, especially intraspecific morphological variations is a result of adaptation processes to surrounded environments (Begon *et al.*, 2006). In marine systems, one very interesting condition is an intertidal area where a number of environmental factors rapidly changes due to tidal action such as light availability, temperature and degree of desiccation. Organisms' responses can be varied in their characteristics due to environmental changes during the day and throughout the year from the effects of seasonal changes.

Seagrasses are marine flowering plants which have adapted to live under marine environments both in tropical and temperate coastlines around the world (Short *et al.*, 2007). They provide important ecological and economical value as habitat and food source for a number of marine species (Hughes *et al.*, 2009; Waycott *et al*. 2009; Short *et al.*, 2011) as well as to stabilize sediment, which can store large amounts of inorganic carbon (Orth *et al.*, 2006). In addition, the estimate ecosystem service value of seagrass meadows in terms of nutrient cycling is $1.9 trillion per year (Waycott *et al.*, 2009).

Seagrasses distribute along the vertical gradient of the shore from the high intertidal to deep water areas where substrata are suitable and adequate light reaches the sea floor (Hemminga and Duarte, 2000). Vertical distribution limit of seagrass regarding water depth is influenced by both physical and biological factors. The result of adaptation processes underlying their zonation patterns, lead to the evolution of the variety of species combinations in seagrass meadow. Consequences of habitats heterogeneity have affected directly on growth, distribution and productivity of seagrasses (Livingston, 1984; 1998).

Light intensity as a major role control growth and distribution of plants (Livingston, 1984), the lower limit of distribution of seagrass is well known to be limited by light intensity (Dennison, 1987) which they necessarily require to have a sufficient carbon gain (Hemminga and Duarte, 2000; Ralph *et al*., 2007). Excess light intensity, however, which seagrass experience during low tide might inhibit their photosynthesis ability, which affects plant growth and survival. High light intensity can also induce plant to synthesize anthocyanin pigment, as fifteen seagrass species from intertidal and shallow subtidal waters have been documented to do so (Novak and Short, 2010). This was one of the adaptation strategies to protect other pigments from excess light and maintain their photosynthetic performance (Gould *et al*., 2000, Novak and Short 2011a). This phenomenon was also observed in some seagrass species in Thailand, especially in the small seagrass *H. ovalis*.

Temperature is one of physical factors directly correlated to light intensity and tidal cycle. Plants exposed to high irradiance during low tide are also exposed to higher temperature than those submerged in the subtidal habitat. High temperature have negatively impacts growth by altering the enzymes involved in plant photosynthesis (Bulthuis, 1987; Ralph 1998, 1999).

Desiccation is an important factor controlling the upper limit of intertidal seagrasses which each species shows different tolerances to the desiccation (Hemminga and Duarte, 2000, Tanaka and Nakaoka, 2004). Some seagrasses have increased ability to tolerate exposure events by their complex interactions of morphological features and growth strategies which help them to reduce the rate of tissue water loss, rather than only the physiological responses of individual leaves (Shafer *et al*., 2007).

The small dioecious seagrass *H. ovalis,* is a common and dominant species found along the tropical and temperate coastlines. In Thailand, this species distributed along the coasts the Gulf of Thailand and the Andaman Sea. It is the most abundant among seagrasses, which occur predominantly in tropical and subtropical areas (Short *et al*., 2007). This species has shown a broad range of distribution along the depth gradient from the uppermost intertidal to subtidal areas.

This study was to investigate the effects of environmental conditions related to tidal exposures and seasons on growth and adaptation of *H. ovalis* in different shore levels. The result of this study will be divided into two parts: 1) growth, morphological and anatomical characters responses to tidal cycles and seasons will be reported in Chapter 3 and 2) photosynthesis and pigment contents of plants will be reported in Chapter 4.

Review of literature

Characteristics and growth form of *H. ovalis*

H. ovalis is a group of paddle-blade seagrasses, belonged to the family Hydrocharitaceae (den Hartog, 1970); plants are small, delicate and extreme varied in size ranged between 1.5-4.1(-5.0) cm high. Rhizomes are smooth, white, producing two leaves and one or more roots at each node. Roots are thin, simple, covered with fine fibrous root hairs, 5-12 cm long. Leaves are thin, oval, bright to yellow green, 7-20 mm long, 4-10 mm wide, bearing 12-19 cross veins (Lewmamomont and Ogawa, 1995) with a long white petiole, 6-27 mm long.

This species exhibits mono-meristematic non-leaf replacing growth form (Fig. 1). The new leaves and rhizome segments are produce at the end of the meristem area of the rhizome. The new rhizome is always produced with the new shoot and no new leaves are produced after the turnover cycle (Short and Duarte, 2001).

Fig. 1 Rhizome segment and shoots of mono-meristematic non-leaf replacing growth form, M indicates the meristem tissue of growth.

Density, Biomass and Growth studies

Density and biomass production are often used in seagrass research as the parameter which indicates seagrass health and response to changing environmental conditions, especially along the vertical gradient of seagrass distribution and seasonal effects which are crucial factors controlling seagrass coverage. Biomass production of seagrass directly results from growth ability of the plant in response to the surrounding environment in their habitats. Change in density, biomass and growth of the small seagrass *H. ovalis* might be affected by heterogeneity of light available, desiccation and sediment characteristics present along the depth gradient. In the case of seasonal effects, *H. ovalis* might be subject to pulse turbidity events caused by heavy sediment runoff during monsoon months, the coverage of *H. ovalis* decreases with increasing sedimentation in sheltered areas where other bigger seagrass species which can better tolerate sedimentation would have an advantage (Prathep *et al*., 2010).

Rate of plant growth is a parameter which can be used to estimate the production of plant populations in ecosystems. Differences in their growth are species specific based on the biology of each plant. Seagrass bed is one of the very productive ecosystems on earth contribute the significant value of primary production of global ocean (Smith, 1981). There are many parameters can be used to measure growth rate of seagrass, for example, using the number of new leaf production rate, leaf elongation rate and the rate of biomass production. We usually notice size-dependent growth rate in seagrass, for instance, fast-growing colonizing species are often small such as *Halophila* spp. whereas slow-growing, climax species are often large such as *Enhalus acoroides* (Vichkovitten, 1998; Rattanachot and Prathep, 2011). *H. ovalis* is one of the very fast growing seagrass with rapid turnover rate compared to other bigger seagrass species as they produce new leaf shoot every 2-6 days (Duarte, 1991; Erftemeijer and Stapel, 1999; Hemminga and Duarte, 2000; Collier *et al*., 2012). As well as rapid growth rate, this species also showed the greatest horizontal rhizome elongation rate at 356 cm yr^{-1} compared the other seagrasses, 2-223 cm yr^{-1} (Marbà and Duarte, 1998). *H. ovalis* can grow in a wide range of salinity, 9-52 ppt with the upper range of salinities been observed under hypersaline conditions from a baseline of 35-55 ppt (Walker *et al*.. 1988; Hillman *et al*., 1995; Ralph, 1998) which indicated greater tolerance to salinity fluctuation compared to other seagrasses in which increase or decrease in salinity often negatively affect leaf growth and survival of leaves (Lirman and Cropper, 2003; Fernádez-Torquemada and Sánchez-Lizaso, 2005; Sánchez-Lizaso *et al*., 2008). However, *H. ovalis* still cannot tolerate prolonged exposure to low salinity at 20 ppt which cause plant mortality reached 75% within three weeks (Benjamin *et al*., 1999). Sulphide in sediment was another factor which negatively affects growth of *H. ovalis* by decreasing growth rate and average leaf weight of the seagrass by 63% and 30%, respectively (Kilminster *et al*., 2008).

Morphological study

High morphological plasticity in many seagrass species is thought to improve survival in the intertidal area where the physical environments are rapidly changes. A number of studies have described the variability of seagrass morphological characteristics. Leaf morphology is the character which is often used to indicate seagrass morphological plasticity and also rhizome characteristics. In the seagrass *H. ovalis*, high variability in their leaf size can be easily observed, the smaller leaves commonly found in the upper intertidal zone while larger leaves were found in deeper zone and also in plant at the edge of patches. McMillan (1984) suggested that seagrass populations with small leaves are more resistant to high temperature stress than large-leaved populations. Decrease in their leave size was suggested to help plant maintain the level of cell water content which is important for their physiological activities (Shafer *et al*., 2007) and also smaller leaves found in plant would be exposed to greater irradiance at the edge of patches than those in the center with higher density (Kaewsrikhaw and Prathep, 2014). Internode distance of *H. ovalis* was decreased when exposed to sulphide-rich sediment (Kilminster *et al*., 2008). *H. ovalis* decreased their leaf size in response to desiccation stress in the upper shore to maintain the level of favorable water content in their cell for supporting physiological processes and response greater in temperature hydrodynamics was the same strategy observed in other seagrasses (Procaccini *et al*., 1999; Tanaka and Nakaoka, 2004; York *et al*., 2013).

Anatomical study

Anatomical characters of seagrasses are interesting feature of those plants but are not well documented. Seagrass anatomical study in Thailand was first conducted by Meesawat *et al*. (1999), where they reported that seasonal variation affected the development of large air lacunae in the root of *E. acoroides*. Kaewsrikhaw and Prathep (2014) recently reported the variations in air lacunae of *H .ovalis*, but the variation found were not yet well understood. Some anatomical characters in seagrasses are different from those of terrestrial plants, for example, no stomata, no cortex cell layer of roots and an extremely thin layer of cuticle (Roberts and McComb., 1984; Roberts and Caperon, 1986; Enríquez, 2005; Kuo and den Hartog, 2006). Reduction of their xylem tissue with a lacunal gas transport system is one of adaptation trait to life underwater (Stevenson, 1988). The lacunar system plays an important role in gas exchange during photosynthesis (Enríquez, 2005; Kuo and den Hartog, 2006), surrounded by non-pigmented mesophyll (Enríquez, 2005). Previous studies generally assumed that arenchyma has developed to increase the internal gas space of aquatic plants. Thus, great development of this character may occur when submerged aquatic plants grow in an oxygen-poor environment (Kuo and den Hartog, 2006) to increase oxygen supply for underground parts of the seagrass. Seagrasses in the genus *Halophila* present thin leaf blades with few mesophyll cells and few lacunae (Kuo and den Hartog, 2006). Thin leaf blade allows them to absorb nutrient directly from ambient water column due to root might sometimes lack the capacity to supply total nutrient requirement for the whole plant body (Stapel *et al*., 1996). Thickened leaf and rhizome epidermal layer and reduction in lacunae with greater root hair development is found in the seagrass *Phyllospadix* spp. indicate their adaptation to live in areas expose to wave action on rocky substrates (Cooper and McRoy, 1988). Moreover, the ability of *H. ovalis* to grow in the high intertidal zone which is frequently air-exposed might be related to their ability to uptake HCO_3^- directly as an inorganic carbon source (Björk *et al*., 1997; Carr and Axelsson, 2008). Air lacunar is a character related to gas transport within plant bodies, gases can flow through above and below ground parts of the plants. Furthermore, they can maintain oxygenic conditions level in their cells when growing in surrounded anoxia sediment.

Photosynthesis and pigment studies

Photosynthesis is an important activity supporting plant growth and survival. Seagrasses evolved from land plants, so they must express their strategies to maintain their photosynthetic performance under submerged and emerged conditions during tidal change. Environmental changes in intertidal area directly affect seagrass photosynthesis, especially differences in light regimes which plants receive along the vertical gradient. The optimum light requirements for seagrass growth vary among and within species due to their unique physiological adaptation and photo-acclimation to local light environment (Lee et al., 2007). *H. ovalis* is a species which grows in various conditions along the depth gradient which means they show an ability to adjust themselves to photosynthesize well under rapidly changing environmental conditions. A number of studies on photosynthetic status of *H. ovalis* have been carried out in the past. Shallow water *H. ovalis* exhibited greater maximum electron transport rate (ETR_{max}) than deeper water plants, and the minimum light requirement of plant was also higher in shallower area (Schwarz *et al*., 2000). ETR_{max} and PSII photochemical efficiency of *H. ovalis* were greater than those of other tropical seagrasses (Beer and Björk, 2000; Ralph, 1998). Temperature was the dominating factor affecting *H. ovalis* photosynthesis, optimum temperature was 25-30 °C. Complete collapse of photosystem II electron transport system can occur when plants are exposed to extremely low or high temperatures indicated from the decline in maximum fluorescence, especially heat stress at 40 °C which negatively affected Calvin cycle enzyme activity (Ralph, 1998, 1999). Combined effects of osmotic and light stresses affected all chlorophyll *a* fluorescence parameters (Ralph, 1999).

Pigment contents of seagrasses are identical to terrestrial vascular plants. Leaf pigment contents can provide insight into the physiological performance of leaves (Sims and Gamon, 2002). Chlorophyll *a* is common to all oxygen-producing photosynthetic organisms (Casazza and Mazzella, 2002). Chlorophyll *a* and *b* function directly in photosynthesis which convert light energy and store in form of carbohydrate (Touchette and Burkholder, 2000; Tiaz and Zeiger, 2002). As seagrasses are sometimes exposed to strong light at noon during low tide, midday chloroplast clumping phenomenon in shallow water plants was suggested to play a protective role against high UVR radiation under high PAR conditions (Sharon *et al.*, 2011a). The proportional change in pigments contents would help them to photosynthesize more efficiently or prevent photodamage which mainly occurs when plants experience excessive light conditions during the tidal exposure period of a sunny day. *H. ovalis* would be able to adapt their pigment concentration in response to environmental changes: chlorophyll *a*, *b*, total chlorophyll and chl. *a*:*b* ratio, which usually investigated along with fluorescence measurement to assess the ability of a plant to adjust to different light conditions. They ranged of 10.33-11.72 µg cm^{-2} in chlorophyll *a* content, 8.07-9.07 µg cm^{-2} in chlorophyll *b* content, 18.60-20.79 µg cm^{-2} total chlorophyll content and 1.25-1.35 in chl. *a*:*b* ratio, respectively (Ralph and Burchett 1998a, b, 1999). Carotenoid is another pigment which provides two major functions, it acts as an accessory pigment and also protects other pigments from photodestructive reactions of oxygen in terms of light energy dissipation through the xanthophyll cycle when plants are exposed to excess light (Demming-Adams and Adams, 1996). Anthocyanin, a flavonoid compound, is most commonly located in the vacuole of photosynthetic and epidermal plant cells (Gould *et al.*, 2000). In most plants, anthocyanin could theoretically compete with light harvesting pigments, chlorophyll and carotenoid (Chalker-Scott, 1999). There are many factors that induce the accumulation of anthocyanin which is responsible for the red, blue and purple coloration seen in many plant leaves, flowers and fruits. Many reports on terrestrial plants have been focused on photoinduction of anthocyanin by wavelengths in the UV, visible and far-red regions, however, other factors are also reported to induce the accumulation of anthocyanin such as cold temperature, osmotic inductions, nitrogen and phosphorus deficiency, wounding and pathogen infections, the appearance of anthocyanin may be permanent or environmentally transient induced by changes in photoperiod or other signals (Chalker-Scott, 1999). We observed extensive red leaves in *H. ovalis* and Kaewsrikhaw and Prathep (2014) reported that greater anthocyanin content was found in the red leaves of *H. ovalis* plants.

Fig. 2 Leaf reddening in the seageass *H. ovalis* suggesting greater anthocyanin content.

Using chlorophyll fluorescence technique to study seagrass photosynthesis

Chlorophyll *a* fluorescence is a widely used powerful method in plant physiological studies (Maxwell and Johnson, 2000). Using Pulse Amplitude Modulated (PAM) fluorometry (Fig. 3) to measure the photosynthesis of plant is well known as a non-destructive powerful tool (Roháček, 2002) which can rapidly assess the photosynthesis status of plants under natural environments compared to the traditional methods which commonly measured as gas exchange via O_2 evolution method (Beer *et al*., 1998; Beer and Björk, 2000). The PAM machine was originally developed for physiological study in terrestrial plants in order to easily measure the status of plants more conveniently and precisely. This method, however, was adopted to use in eco-physiological study of plants in aquatic ecosystems. A number of researchers have been using this technique to assess the level of plant stresses by measuring their photosynthetic status under different environmental factors.

When plants absorb light in the range they can utilize is called photosynthetically active radiation (PAR; 400-700 nm). The energy of light excites the chlorophyll *a* molecule higher to an excited state. The excited electron then returns to the ground state throughout three processes which occur in competition: it can be used to drive the photochemical process, excess energy can be dissipated as heat called non-photochemical quenching and finally it can be re-emitted as chlorophyll fluorescence. By measuring the chlorophyll *a* fluorescence yield, any changes in photochemical efficiency and dissipate energy as heat can be estimated (Maxwell and Johnson, 2000).

Changes in the yield of chlorophyll fluorescence were first observed in 1960 by Kautsky and his co-worker. The quantum yield of photosystem II (PSII) photochemistry (Y, Φ_{PSII}) is one of the most useful quenching parameter which measures the proportion of PAR absorbed by chlorophyll associated with PSII. This parameter can be used to calculate the rate of electron transport (ETR) which is commonly an indication of overall photosynthesis using the following equation: $ETR = \Phi_{PSII} \times PPFD \times Abt_F \times 0.5$, where PPFD is the actinic photosynthetic active radiation given by an internal halogen lamp of the PAM, AF is the light absorption capacity of plant leaves with the standard value of 0.84, and 0.5 is the constant value assume the photons absorbed between PSI and PSII are in equal proportion (Belshe *et al.*, 2007, Ritchie, 2010). Abt_F of plants is species specific, many aquatic plants have Abt_F of less than 1 (Silva and Santos, 2003).

By measuring the effective quantum yield over a range of increasing light intensities given by the internal halogen lamp of the PAM, functions of ETR vs. PAR can be generated using the rapid light curve (RLC) method. This is very useful in measuring the physiological state of the plant in different contexts of light conditions and provides additional information about the efficiency (α) and capacity (ETR_{max}) for plant photosynthesis (Marshall *et al.*, 2000) and also can be used to investigate the acclimation of plant to a range of light intensities (Bautista and Necchi, 2007; Sharon *et al.*, 2011b). PAM is very useful for investigating plant stress under a range of physiological environments by measuring the maximum quantum yield (F_v/F_m) which can be used to indicate the state of the potential of PSII photochemistry.

Fig. 3 Pulse Amplitude Modulated (PAM) fluorometry;
Mini PAM, Walz, Germany.

Research questions

1. Are there any differences in growth parameters (density, biomass, P_L and rhizome elongation rate), morphology and anatomy of the seagrass *H. ovalis* subject to variations associated with tidal exposures, sites and seasonal factors?
2. Are there any differences in photosynthesis and pigment contents of the seagrass *H. ovalis* subject to variations associated with tidal exposures, sites and seasonal factors?

Hypotheses

Null hypotheses

H_{0A}: There are no differences in growth parameters, morphology and anatomy of the seagrass *H. ovalis* growing in different tidal exposures, sites and seasons.

H_{0B}: There are no differences in photosynthesis and pigment contents of the seagrass *H. ovalis* growing in different tidal exposures, sites and seasons.

Alternative hypotheses

H_{1A}: There are differences in growth parameters, morphology and anatomy of the seagrass *H. ovalis* grows in different tidal exposures, sites and seasons.

H_{1B}: There are differences in photosynthesis and pigment contents of the seagrass *H. ovalis* grows in different tidal exposures, sites and seasons.

Objectives

1. To investigate the variations in growth parameters, morphology and anatomy of the seagrass *H. ovalis* subject to variations in tidal exposures, sites and seasons.
2. To investigate the variations in photosynthesis and pigment contents of the seagrass *H. ovalis* subject to variations in tidal exposures, sites and seasons.

CHAPTER 2

RESEARCH METHODOLOGY

Study site

This study was conducted at Laem Yong Lam (YL) seagrass bed which is located in Haad Chao Mai Marine National Park, and Koh Muk (KM) which is located opposite to Laem Yong Lam. These two study sites are situated on the west coast of the Andaman Sea, Trang Province, Southern Thailand, between latitude 7º21´-7º25´ N and longitude 99º18´-99º21´ E. (Fig. 4). This area has been reported as the largest seagrass bed in Thailand which covers an area of 18 km^2 (Poovachiranon *et al.*, 2006). Nine of twelve seagrass species in Thai Waters are present along the depth gradient of this area. *H. ovalis* is one of the most dominant species present from upper intertidal to subtidal zones both in monospecific and multispecific meadows.

There is a semi-diurnal tide at the study site. The low tide event usually occurs twice a day in the morning and evening. Different tidal zones are separated using the criteria of exposure hours during low tide: 1) upper intertidal zone exposed around four hours, 2) lower intertidal zone exposed around half an hour during low tide (Apichanangkool, 2011) and 3) subtidal zone which is always submerged: the shallow subtidal zone at 4-6 meters depth was chosen to use in this study.

There are differences in distance from the shoreline of each study area as follows: 50 m, 250 m and 2200 m at the upper intertidal, lower intertidal and subtidal zones of YL; 230 m, 380 m and 760 m at the upper intertidal, lower intertidal and subtidal zones of KM, respectively. Exposure hours of the upper and lower intertidal zones of YL are 30 minutes longer than those at the upper and lower intertidal zones of KM (Fig. 5). However, the subtidal habitat of YL (ca. 6 m) is deeper than Subtidal zone of KM (ca. 4 m). Sediment characters of each treatment are characterized as follows; fine sand is found in the upper-lower intertidal zones of YL and upper intertidal zone of KM, sand is found in the lower intertidal zone of KM and silt sediment is found in subtidal zone of both study sites.

There are two predominant seasons in this area: a rainy season is influenced by the Southwest monsoon from May to October and a dry season is affected by the Northeast monsoon from November to April (Poovachiranon and Chansang, 1994).

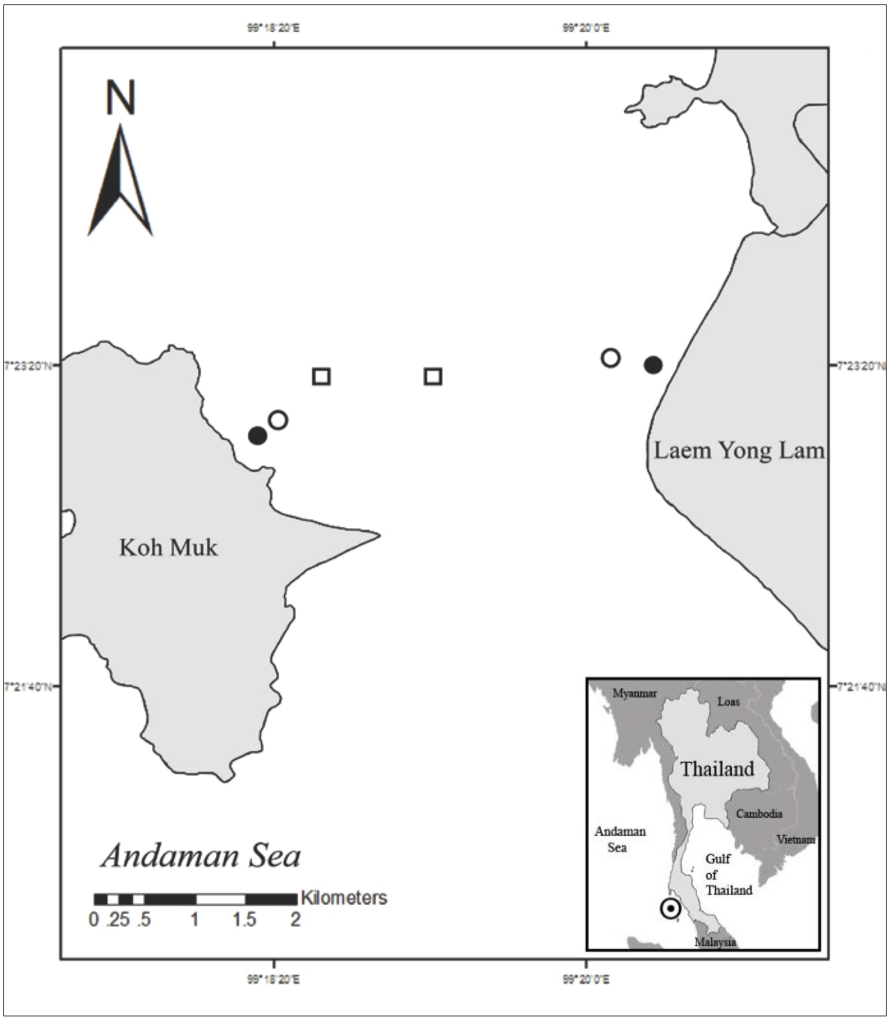


Fig. 4 Study sites at Laem Yong Lam and Koh Muk, Trang Province,Thailand: ●, ○ and ☐ represent upper intertidal zone, lower intertidal zone and subtidal zone, respectively.

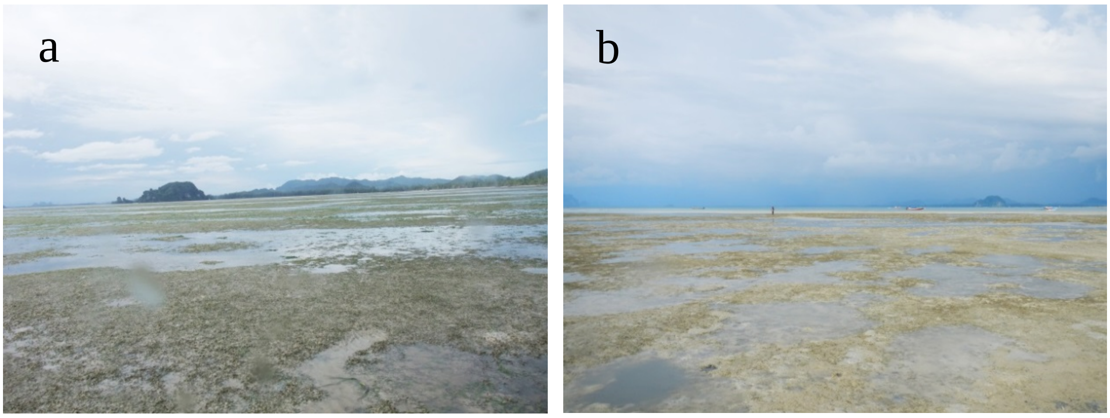


Fig. 5 The conditions of the study areas during low tide in the upper and lower intertidal zones with dense stands of seagrass: (a) Laem Young Lam and (b) Koh Muk.

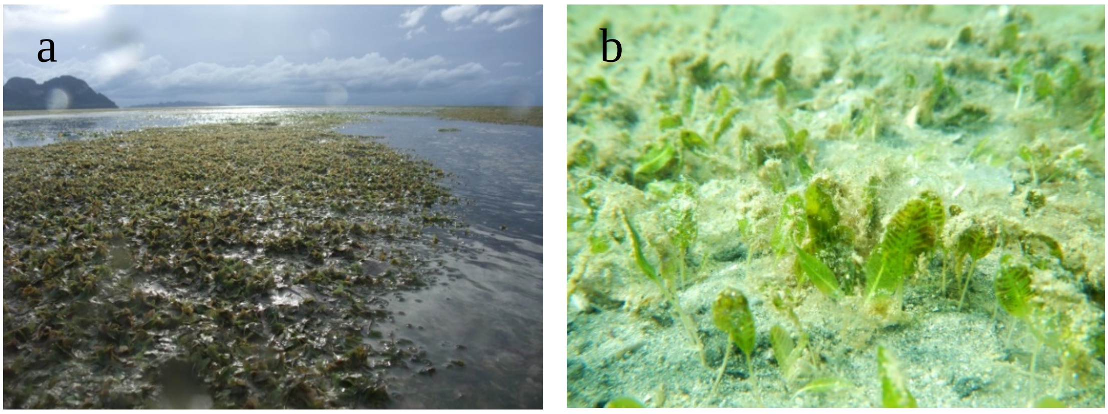


Fig. 6 Monospecific bed of seagrass *H. ovalis* (a) *H. ovalis* bed during low tide in the upper and lower intertidal zones and (b) *H. ovalis* bed in the subtidal zone.

Methods

Data samplings were carried out four times throughout the year in two different seasons from July 2012 to May 2013.

Physical parameters measurement

Environmental parameters were measured as follows:

Light intensity (Lumens ft^{-2}) and temperature (°C) were recorded using the Onset-Hobo® LI light logger (Onset Computer Corporation, USA) which was set up around 20 cm above the ground (Fig. 7a). Data were automatically collected every hour. Light data from the logger was transformed to μmol photon $m^{-2}\ s^{-1}$ following the equation from calibration with light meter 4π sensor (Li-Cor, LI-250A, LI-Cor Inc., USA), then transformed to mol photon $m^{-2}\ d^{-1}$.

$$\mu\text{mol photons } m^{-2}\ s^{-1} = 0.364\ (\text{lumens } ft^{-2}) + 379.491$$

$$\text{mol photons } m^{-2}\ d^{-1} = \mu\text{mol photons } m^{-2}\ s^{-1} \times 0.0864$$

- Salinity (ppt) was measured using a reflectometer (ATC, 0-100 ppt, XHO RSH-10ATC, ATACO, China).
- Sedimentation rate was measured using sediment traps. Eight replicates of 50 ml cylindrical plastic centrifuge tube with an aspect ratio of 4.1 (28 mm diameter) were setted up near the sea bottom at each site (Fig. 7b). Each tube of sediment sample was filtered through pre-weighted 47 mm diameter GF/C filter paper (Gacia *et al.*, 2003) which was dried at 80 °C for 5 days. Filtered Samples were dried at 80 °C for 5 days (Motter and Jones, 2013) before weighed using a 4 digits balance. The rate of sedimentation was calculated and reported as g DW $m^{-2}\ d^{-1}$.

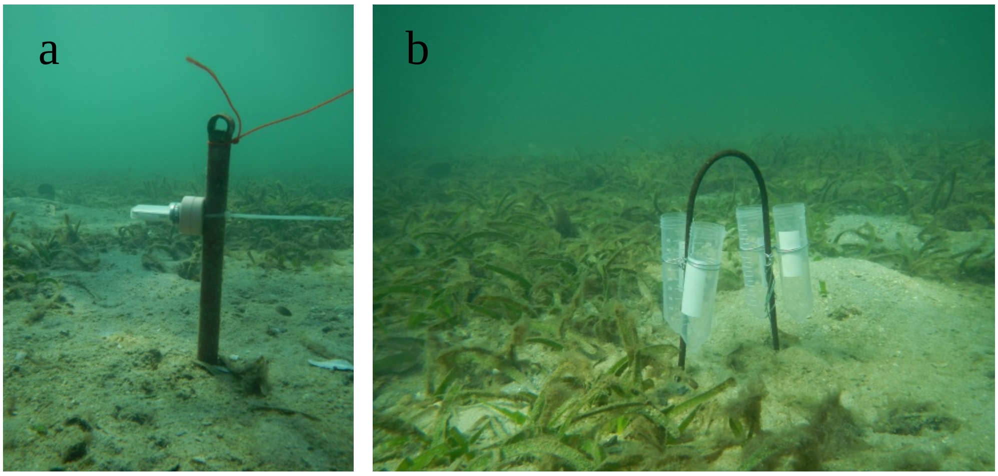
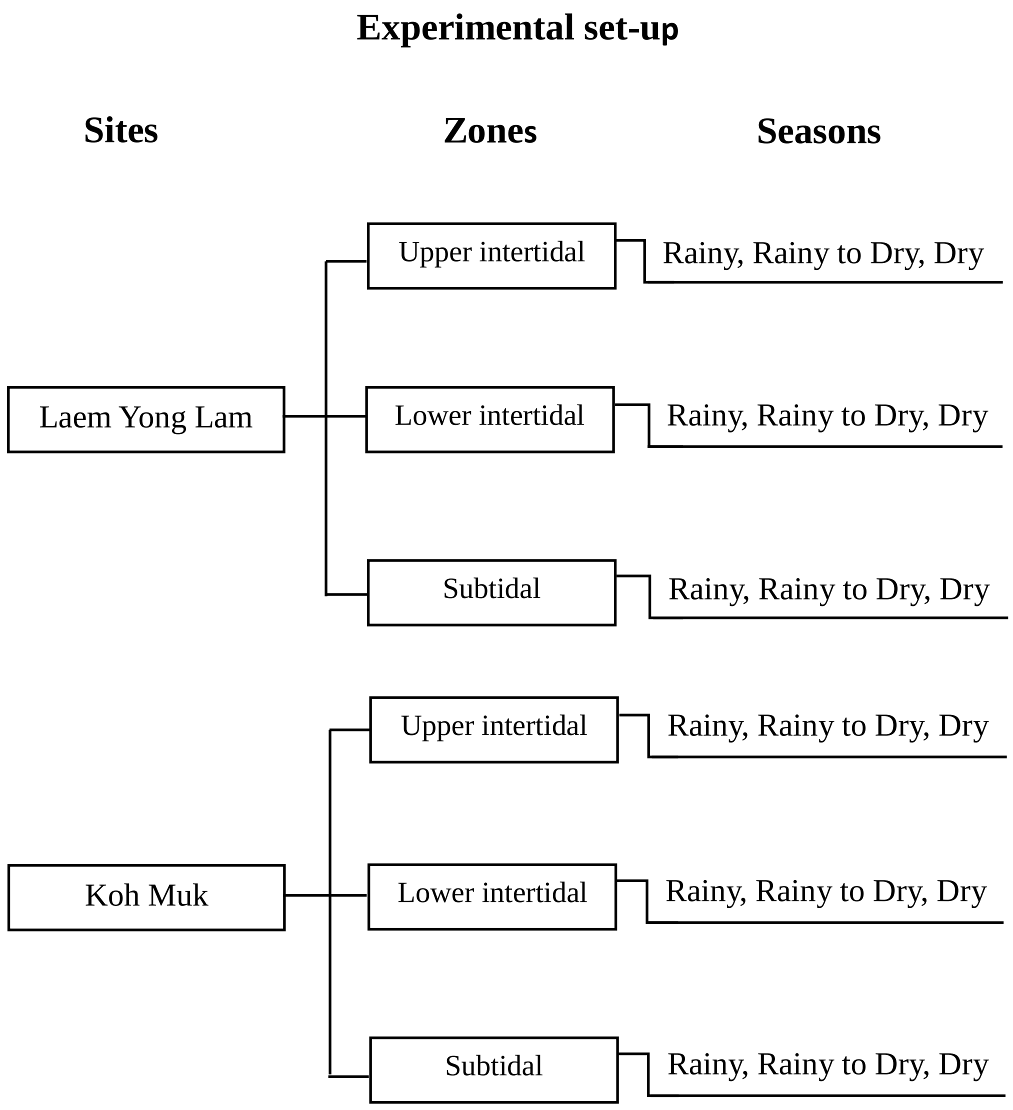


Fig. 7 Environmental parameters measurement (a) Light intensity and temperature were measured using Hobo light data logger and (b) sediment trap was near the sea bottom in the seagrass bed.

Experimental set-up

Monospecific meadows of seagrass *H. ovalis* in three different tidal zones at Laem Yong Lam and Koh Muk were used as the study areas based on the criteria of exposure time as stated above. The experimental set up was conducted to examine their growth rate, morphological characters, anatomical characters, photosynthetic status and pigment contents. The detail of methodology in each section will be shown in Chapters 3 and 4.



Measured variables

1. Growth parameters
   - density (5), biomass (20) and P_L (10)
2. Morphological characters
   - leaf width (20), leaf length (20) , leaf area (20), petiole length (20) and rhizome internode length (20)
3. Anatomical characters
   - marginal cell width (20), marginal cell length (20) , superficial cell width (20), superficial cell length (20), epidermis thickness (20), mesophyll cell width (20), mesophyll cell length (20), air lacunar width (20) and air lacunar length (20)
4. Physiological characters
   - photosynthesis: $rETR_{max}$ (9-20) and E_{ppt} (9-20)
   - Pigment contents: chlorophyll *a* content (20), chlorophyll *b* content (20), total chlorophyll content (20), chl. *a*:*b* ratio (20), carotenoid content (20) and anthocyanin content (20)

Note: The numbers in the brackets represent the number of replications in each treatment during each sampling time.

Fig. 8 Experimental set-up scheme with the number of replications of measuring variables in each treatment.

Statistical analysis

SPSS 13.0 for Windows (SPSS, Chicago, IL, USA) was used to analyze all data sets at the significance level of 0.05. A three-way ANOVA was applied to test the difference in growth rate, density, biomass, morphology, anatomy and physiology of seagrass *H. ovalis* in different tidal exposure levels, sites and seasons. Prior to performing the analysis, data were tested and transformed (Log(X), Log(X+1), X^2, $\sqrt{X}$ and $\sqrt{\sqrt{X}}$) to meet the assumptions of normal distribution. Post-hoc multiple comparisons were applied to test the differences of measuring character among treatments (Zar, 1999).

CHAPTER 3

Title: Variations of tidal exposures and seasons on growth, morphology and anatomy of seagrass *Halophila ovalis* (R.Br.) Hook.f. at seagrass bed in Trang Province

Abstract

Halophila ovalis, is a seagrass species which is reported to be distributed over a wide range of habitats along the vertical gradient of coastal areas of Thailand. Differences in tidal exposures control physical factors relating to plant growth and morphological characters. In this study, growth parameters, morphology and leaf anatomy of the seagrass *H. ovalis* was examined in plants growing in three levels of tidal exposure at Laem Yong Lam (YL) and Koh Muk (KM). The study was conducted from July 2012 to May 2013 which covering both the dry and rainy seasons. Dense patches of small plant leaves in the upper intertidal zone resulted in reduced water loss during tidal emergence times. Slowest growth rate throughout the year presented in the upper intertidal zone with P_L of 4 days and rhizome elongation rate of 3.61 mm shoot^{-1} d^{-1}. Biomass of seagrass related their leaf size and density; the biggest leaf size presented in the lower intertidal zone, 41.44-103.21 mm^2, suggested that this habitat provided better condition for plant growth than those the upper intertidal and subtidal areas. Mesophyll cell, air lacunar size also related leaf size. This study provided the overall trend of spatial and seasonal variation on plant in growth ability, morphological and anatomical characters of the seagrasss *H. ovalis* which express in response to gradient of environmental conditions in varying tidal exposures and seasons.

Keyword: *Halophila ovalis* (R.Br.) Hook.f., tidal exposure, growth rate, morphology, anatomy, response

1. Introduction

Seagrasses which are marine angiosperms, mainly inhabit the coastal habitat. They are experiencing varying conditions of physical factors which regularly change according to daily tidal cycle and also in terms of seasonal change. Seagrasses are often reported to be susceptible to coastal environmental impact (Freeman *et al.*, 2008). Intensity of light reaching the sea floor which seagrasses use for photosynthesis (Hemminga and Duarte, 2000) is documented as one of the main factors regulating plant growth and productivity. *H. ovalis* is the small seagrass species which present in different tidal levels might have an optimum depth for growth in response to stressors and limiting factors in their environment. Plant growing in shallow water may suffer excessive light which may have a negative effect on their growth (Xu *et al.*, 2011). Seasonal changes in environmental parameters are also very crucial. Plant stress due to the effects of high light intensity, temperature and desiccation in the upper shore might occur during summer months while the effect of high sediment runoff and wave exposure influence seagrass health during monsoon months. Decline in percentage cover, biomass and species composition of seagrass beds are affected by heavy sediment load which reduces subsurface light intensity (Freeman *et al.*, 2008).

Growth rate of plant is also very important parameter which can be used to calculate seagrass productivity as one of the very productive systems on earth (Hemminga and Duarte, 2000). Due to very fast growth rate, *H. ovalis* might exhibit greater productivity than those some others larger species with slower growth rate. This fast growing species would be one of the important seagrass providing high carbon stock in the coastal systems according to their rapid turnover rate.

Morphological plasticity of plants in response to different environmental regimes and many stressors are well studied in many terrestrial plants (Cornelissen *et al.*, 2003; Pérez-Haeguindeguy *et al.*, 2013).They have to adapt their external and internal structures to survive under changing environmental conditions. Morphological plasticity and adaptation of some anatomical features are present in seagrasses. From previous studies, leaf length of the seagrass *H. ovalis* transplanted from lower to upper intertidal zone were decreased, which suggested there were response to emergence stress to avoid direct exposure to air (Apichanangkool, 2011) which help the plants to maintain tissue water balance (Shafer *et al*., 2007) . The small seagrass *H. ovalis* which is distributed in a wide range of habitats showed distinct changes in their leaf size along the depth gradient or even when they presented at the same tidal level. *H. ovalis* in the higher intertidal shore which grow in different habitat types and densities defined by the position existing within the patches in the same tidal level showed significantly smaller leaf size in low density areas at the periphery of the patch which was exposed to higher stress from higher light intensity and desiccation than those at the centre of the patch. It has also been observed that the length of rhizome internode increased in the rainy season (Kaewsrikhaw and Prathep, 2014).

The review on anatomical character reported in many seagrass species whit distinctive character of having air space system which allows movement of the gases through the plant (Kuo and den Hartog, 2006). The ecological aspect of anatomical character in seagrass have been carried out in in the seagrss genus *Phyllospadix*, their maller air lacunae exhibited their anatomical adaptation to grow in exposed rocky substrate (Cooper and McRoy, 1988). Studies about the anatomical variation in *H. ovalis* are still rare (Meesawat *et al*., 1999). Another study compared the air lacunar size in *H. ovalis* leaves inhabit different habitats which found significant differences, but the reason for their anatomical changes were not clear and the authors suggested that bigger lacunar might be the consequence from the bigger leaves (Kaewsrikhaw and Prathep, 2014).

Previous study investigated the variation in morphological and anatomical characters of *H. ovalis* have been carried only in the upper intertidal areas which plant grow in tide pool and sandy habitats. It would provide more insight into their ability to response to environmental differentiation along the vertical gradient of the shore as they can grow in three tidal levels; upper intertidal, lower intertidal and subtidal zones. This study focused on the variation in growth, morphology and anatomy of the spoongrass *H. ovalis* both in the upper and lower intertidal areas which experience different levels of tidal exposure and also involved studies in the shallow subtidal area which is totally submerged. All variables were compared in different seasons throughout the year. This study will provide the basic understanding of the ecological traits of plants present under different environmental regimes essential to maintain their populations.

2. Materials and Methods

The characteristics of sampling sites, environmental measurements and experimental set-up were described in Chapter 2.

2.1 Growth parameters measurement

Growth parameters of the seagsass *H. ovalis* were examined. Density, biomass and leaf plastochrone interval (P_L) of the plants were measured as the growth parameters. Five replicates of 10 cm x 10 cm sampling plots were randomly placed on the *H. ovalis* patch. The numbers of leaf pair were counted and converted into square meter unit. Above-ground and below-ground biomass were examined after drying at 60 °C for 3 days and converted to the unit of g DW m^{-2}. Plant Growth rate was investigated using the plastochrone method (Short and Duarte, 2001) in order to know how long it takes for the plant to produce a new leaf pair. *In situ* experimental growth measurements were set up at three different tidal zones. Rhizome between the second and the third youngest leaf pairs of a plant was marked using colored-thread and all samples were collected after leaving them in the natural condition for 5-7 days. Samples were brought back to determine the numbers of new leaf pairs and the rhizome elongation rate (Fig. 9). Productivity of *H. ovalis* was estimated from the data of plant biomass and P_L in each treatment; productivity = total biomass m^{-2} x $(365/P_L)$.

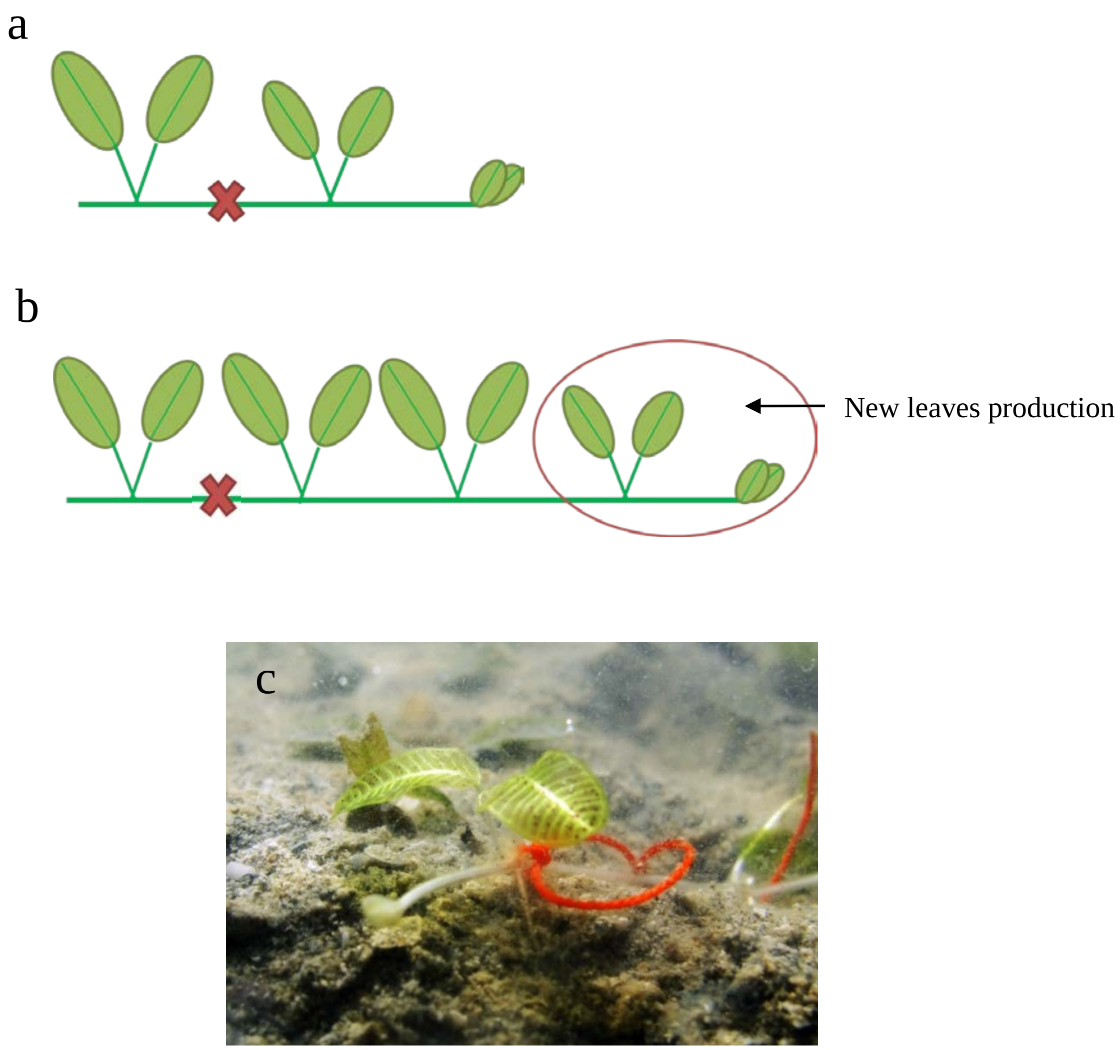


Fig. 9 *H. ovalis* growth measurements: (a) Rhizome between the second and the third youngest leaf pairs of *H. ovalis* was marked to determine the growth rate (b) the new leaf production of plant after left in the natural condition and (c) rhizome of *H. ovalis* was marked by red thread in the field to measure their growth rate.

2.2 Morphological measurements

A single leaf of the third youngest leaf pair of *H. ovalis* was used to examine the standard morphological characters. Leaf width, leaf length and leaf area of the plants were measured by analyzing the picture of plant leaves taken under a stereo microscope (Olympus, DP71) using the freeware image analysis software, ImageJ. Petiole length and rhizome internode length between the second and the third youngest leaf pairs were measured using a digital caliper (Fig. 10).

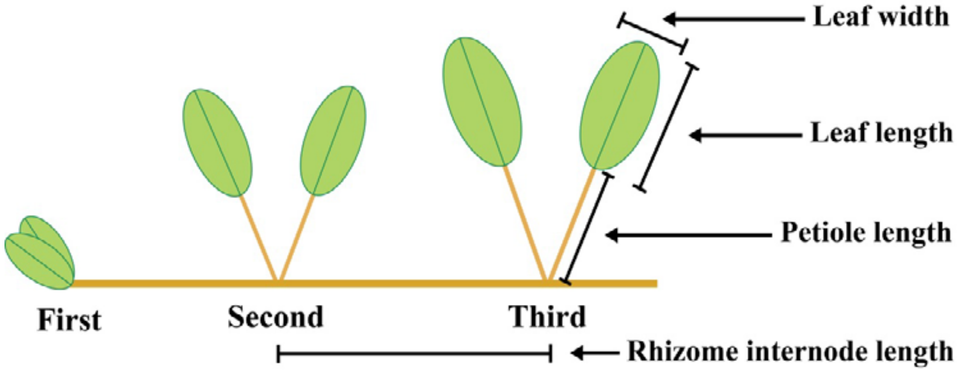


Fig. 10 Morphological characters were measured: leaf width, leaf length, petiole length and rhizome internode length (Kaewsrikhaw and Prathep, 2014).

2.3 Anatomical measurements

Hand sectioning was applied on another single leaf which was the third youngest leaf pair of the plant. Photographs of leaf surface and cross section views were taken under a compound microscope (Olympus, DP72) and later analyzed using the freeware image analysis software, ImageJ. Marginal cell width, marginal cell length, superficial cell width, superficial cell length, epidermis thickness, mesophyll cell width, mesophyll cell length, air lacunar width and air lacunar length were the measured characters (Fig. 11).

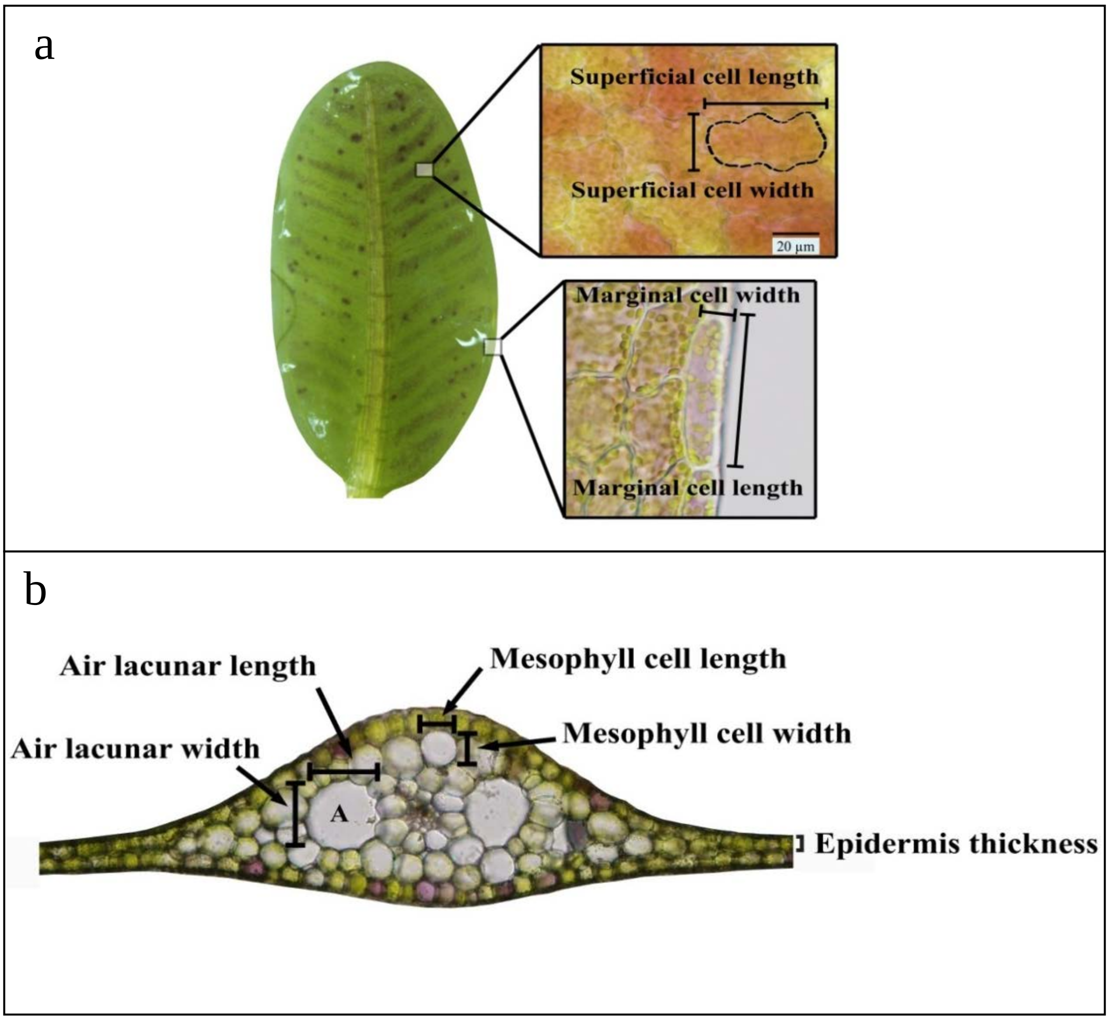


Fig. 11 Anatomical characters were measured (a) superficial cell width, superficial cell length, marginal cell width and marginal cell length and (b) epidermis thickness, mesophyll cell width, mesophyll cell length, air lacunar width and air lacunar length (Kaewsrikhaw and Prathep, 2014).

2.4 Statistical analyses

Three-way ANOVA was employed to test for differences in growth parameters, morphology and anatomy of the seagrass *H. ovalis* among different tidal exposure levels, sites and seasons at a significance level of 0.05 using SPSS statistical analysis program version 13.0 for Windows (SPSS, Chicago, IL, USA). Normality test and when necessary appropriate data transformation ((Log(X), Log(X+1), X^2 and $\sqrt{\sqrt{X}}$)) were applied to all data sets prior to the test with ANOVA model. Non-parametric Kruskal Wallis test was applied to test for differences among data sets with non-normal distribution. Post hoc multiple comparison were applied to test the differences of measuring character among treatments (Zar, 1999).

3. Results

3.1. Environmental parameters

3.1.1. Daily light exposure (photosynthetically active radiation; PAR)

There were significant differences in PAR among zones, sites and months (Kruskal Wallis test, $p<0.001$). The average PAR throughout the year was 90.28±2.80 mol photons m^{-2} d^{-1}(mean±S.E.). The highest PAR was in the upper intertidal zone of KM in July 2012 (rainy season), 128.62±19.02 mol photons m^{-2} d^{-1} whereas the lowest PAR was in the subtidal zone of YL in May 2013 (rainy season), 42.51±1.11 mol photons m^{-2} d^{-1}. There were no significant differences of PAR between the upper and lower intertidal zones within the same site in each month. PAR in the subtidal zone was always significantly lower than those in the upper and lower intertidal zones. Overall PAR in May 2013 was significantly lower than the rest of the year (Fig. 12; Table 1).

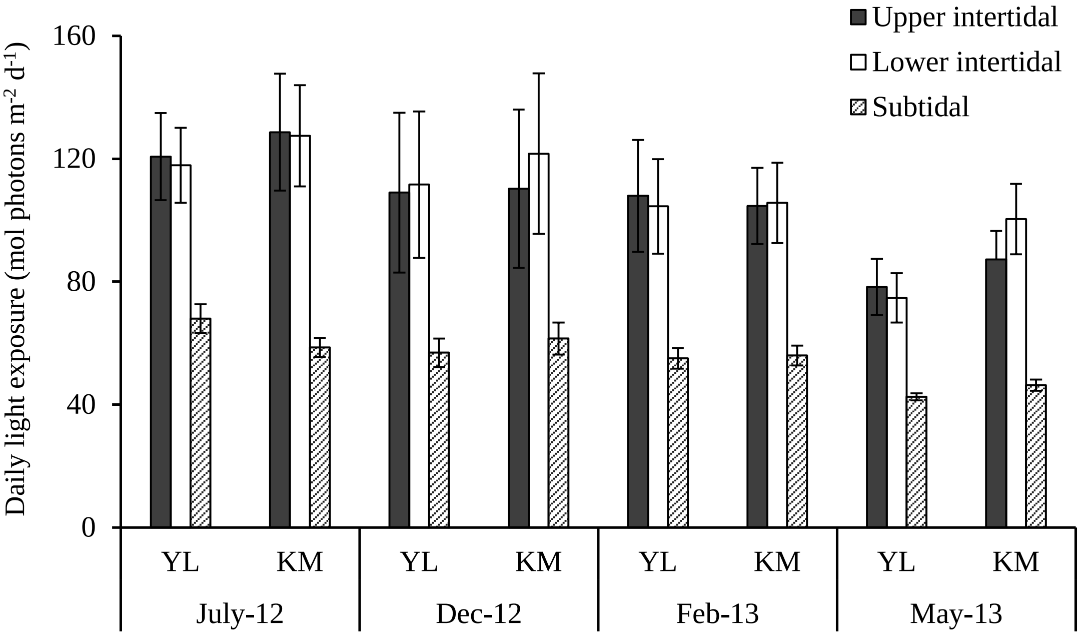


Fig. 12 Daily light exposure (PAR) measured from 7.00 a.m. to 6.00 p.m. in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=18-48).

3.1.2. Temperature

There were significant differences in mean temperature among zones, sites and months (Kruskal Wallis test, *p*<0.001). The average mean temperature throughout the year was 29.95±0.05 °C. The highest temperature was in the upper intertidal zone of YL in February 2013 (dry season), 30.61±0.25 °C whereas the lowest temperature was in the subtidal zone of YL in May 2013 (rainy season), 29.50±0.16 °C. Overall temperature was significantly greater in February 2013 and significantly lower in July 2012 and May 2013. There was no significant difference in temperature in temperature among three zones in each site and month. The overall range of temperatures found in this study however were very low, only 29 to 31°C (Fig. 13; Table 1).

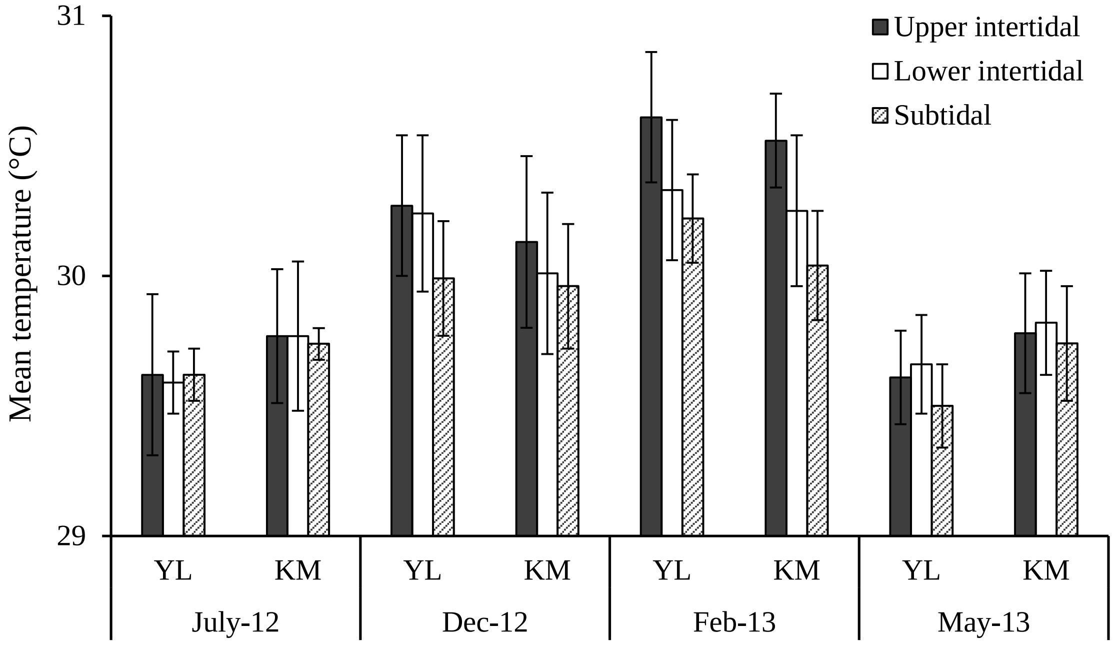


Fig. 13 Mean temperature measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=48).

3.1.3. Salinity

There were significant differences in salinity among zones, sites and months (Kruskal Wallis test, $p<0.001$). The average salinity throughout the year was 30.6±0.1 ppt. The greatest salinities were in the upper and lower intertidal zones of YL in May 2013, 31.7±0.3 ppt. The lowest salinities were in the upper-lower intertidal zones of YL in July 2012, upper intertidal zone of KM in July 2012, upper-lower intertidal zones of YL in December 2012, lower intertidal zone of YL in February 2013 and lower-subtidal zones of KM in February 2013, 30.0±0.0 ppt. There was no pattern of salinity among zones, between sites and among months (Fig 14; Table 1).

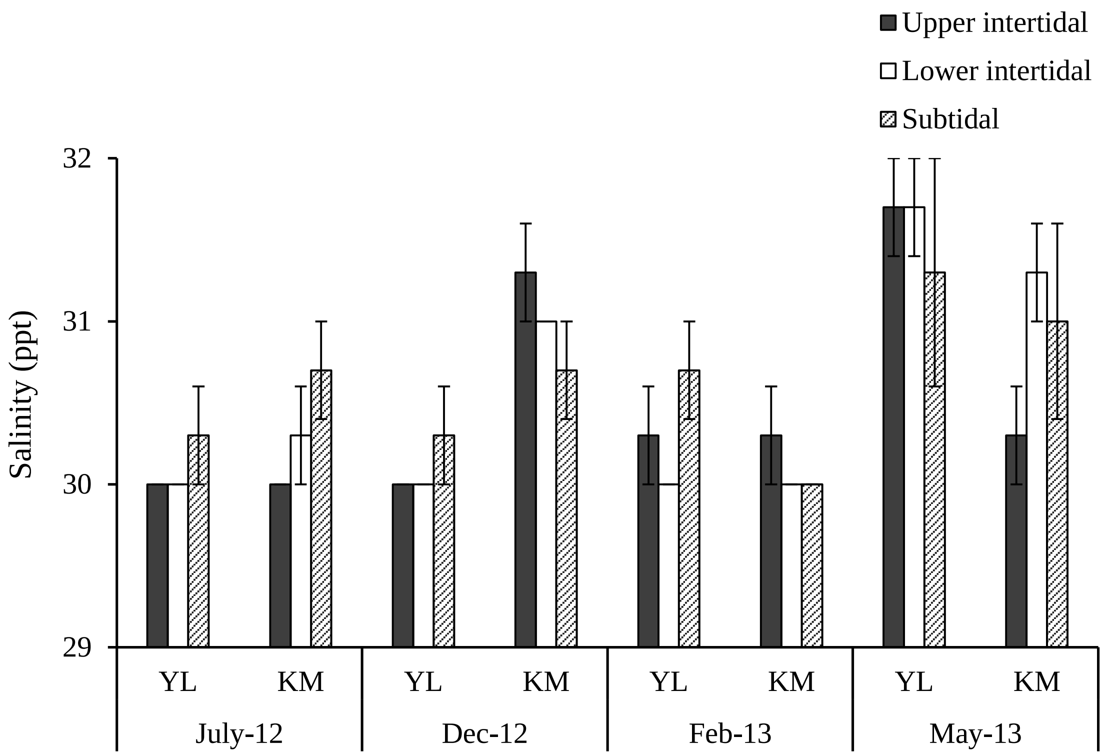


Fig. 14 Salinity measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=3).

3.1.4. Sedimentation rate

There were significant differences in the sedimentation rate among zones, sites and months (Kruskal Wallis test, $p<0.001$). The average sedimentation rate throughout the year was 1342.75±134.38 g DW m^{-1} d^{-1}. The highest sedimentation rate was in the lower intertidal zone of KM in May 2013, 7291.87±642.92 g DW m^{-1} d^{-1}, whereas the lowest sedimentation rate was in the subtidal zone of KM in February 2013, 31.59±4.10 g DW m^{-1} d^{-1}. Sedimentation rate of both study sites in May 2013 was significantly greater than during the rest of the year. There were differences in sediment characters in each treatment: the type of sediment in the lower intertidal zone of KM was characterized to be the sandy sediment which differs from the others which were characterized to be the fine sand and silt sediment types (Fig.15; Table 1).

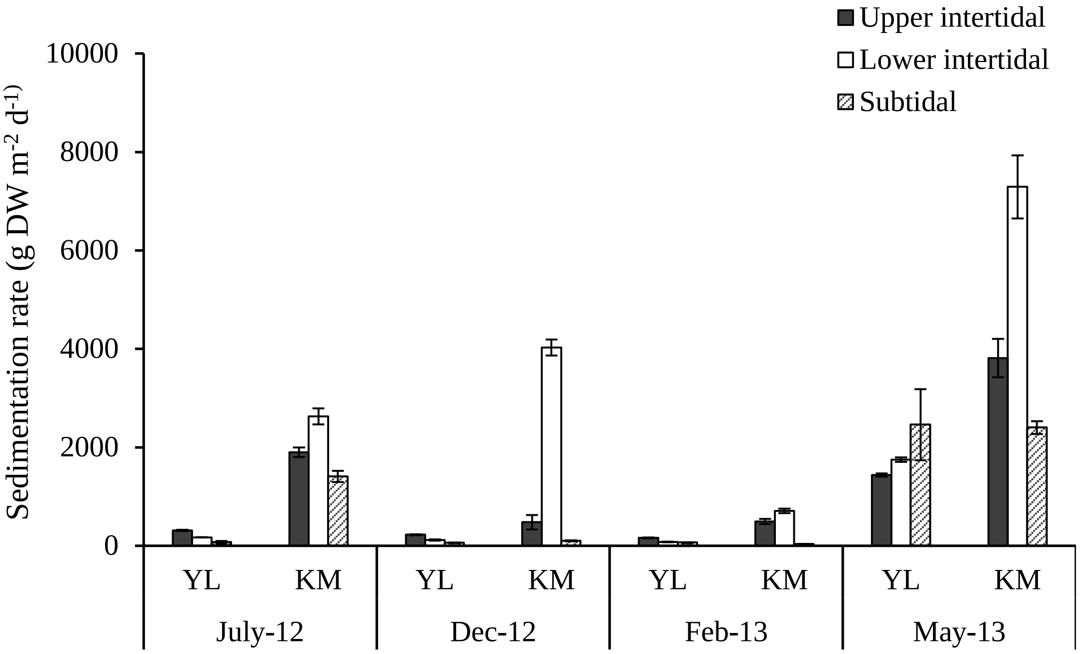


Fig.15 Sedimentation rate measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=8).

Table 1. Summary of statistical analyses of daily light exposure, temperature, salinity and sedimentation rate showing the results of non-parametric Kruskal Wallis test: *Z* = zone, *S* = site and *M* = month.

Variables	mean±S.E.	Min-Max	Z	S	M	Z x S	Z x M	S x M	Z x S x M
			Chi-square (Kruskal Wallis test)						
Daily light exposure (mol photons m^{-2} d^{-1})	90.28±2.80	32.91-644.67	148.615***	1.603	29.419***	151.552***	184.278***	35.736***	193.163***
Temperature (°C)	29.95±0.05	25.15-37.59	3.431	0.509	24.251***	4.684	37.605***	30.404***	45.629**
Salinity (ppt)	30.6±0.1	30-33	0.561	0.498	20.845***	2.101	26.126**	33.652***	44.202**
Sedimentation rate (g DW m^{-2} d^{-1})	1342.75-134.28	13.09-9978.37	25.606***	38.537***	83.384***	72.827***	119.465	129.117***	181.727***

* $p<0.05$, ** $p<0.01$ and *** $p<0.001$

2. Growth study

3.2.1. Shoot density

There were significant differences in shoot density of *H. ovalis* affected by zones and months (p=0.009). The highest density of plant shoots was in the upper intertidal zone of YL in May 2013 (rainy season), 9540±1606 leaf pairs m^{-2}. The lowest density of plant was in the subtidal zone of YL in February 2013 (Dry season), 1260±178 leaf pairs m^{-2}. There was no significant difference in shoot density between upper and lower intertidal zones during the same month except at KM in October 2012 (rainy season), YL in February 2013 and YL in May 2013 when shoot density in the lower intertidal zone was significantly lower than those in the upper intertidal zone. The lowest density in each month was always in the subtidal zone (Fig. 16; Table 2).

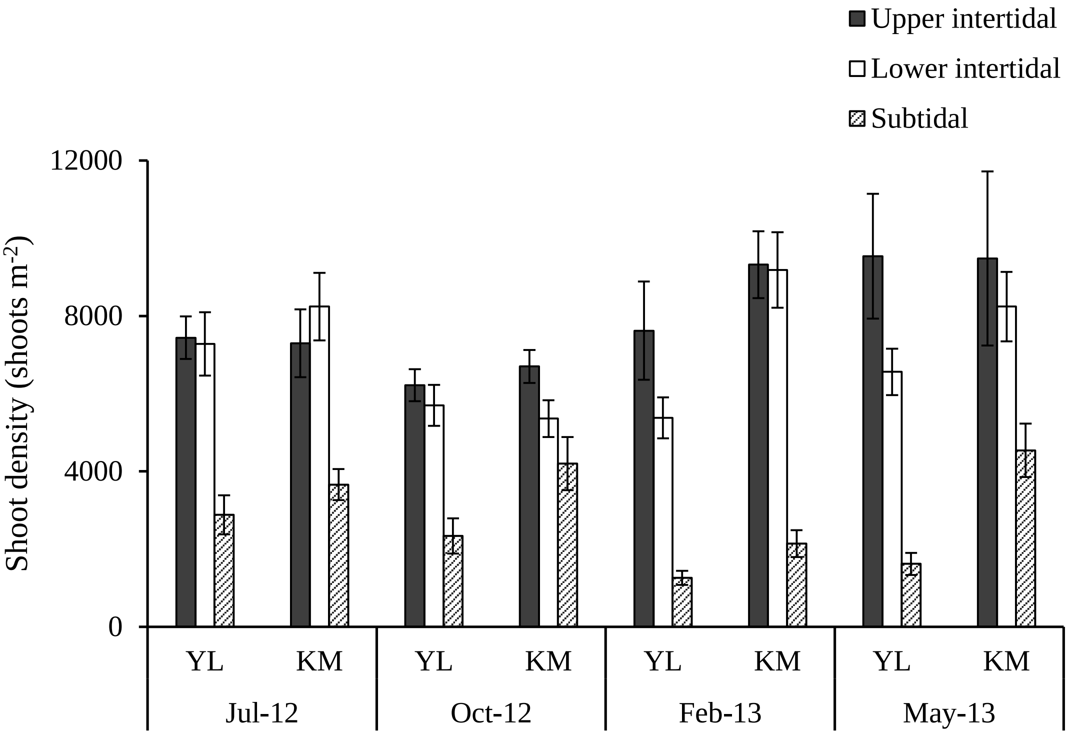


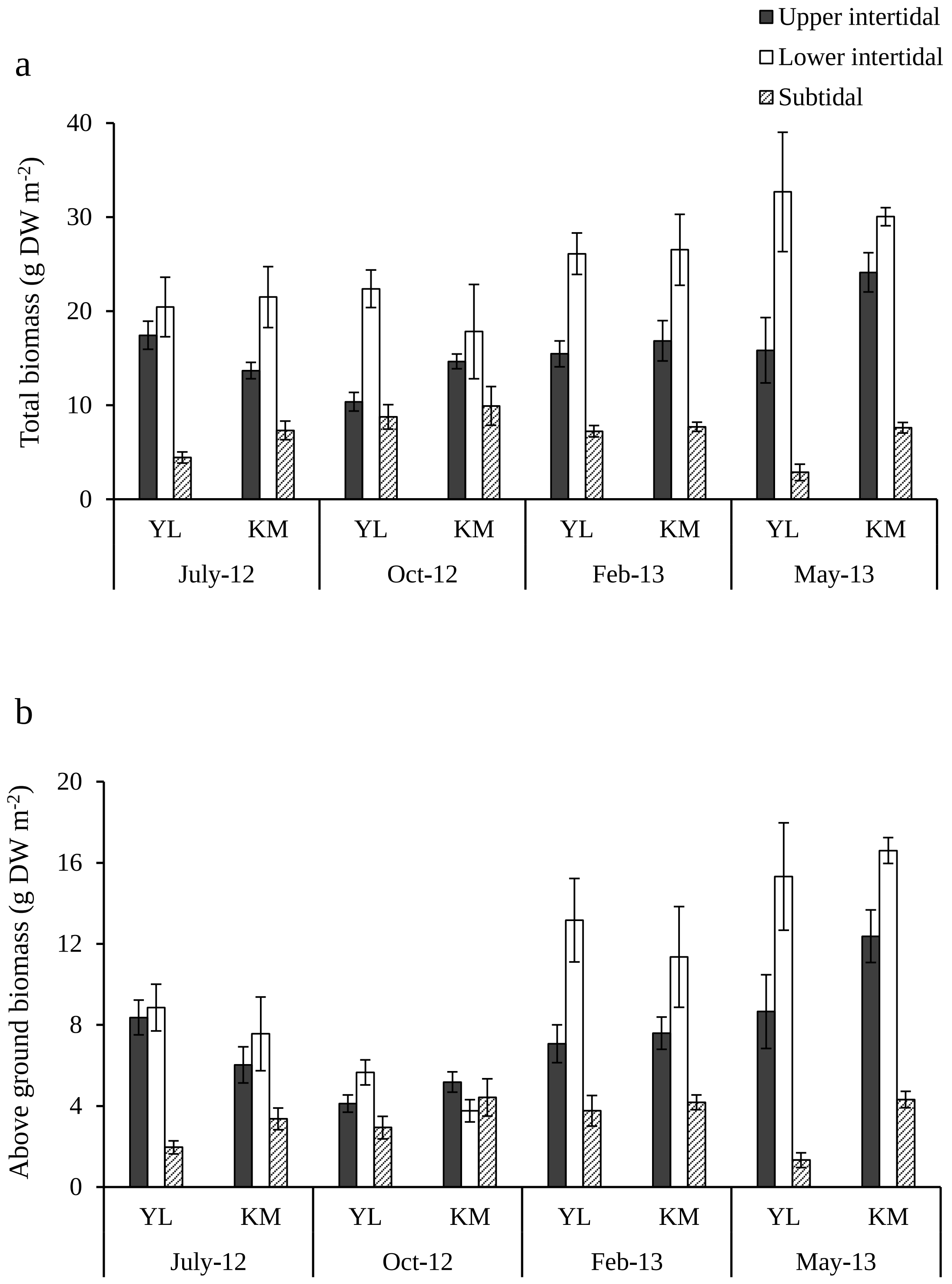
Fig. 16 Shoot density of *H.ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=5).

3.2.2. Biomass

There were significant differences in total biomass of *H. ovalis* among zones, sites and months (*p*=0.037) and there were significantly differences in above-ground (leaf + petiole) affected by zones, sites and months (*p*<0.001, *p*=0.017 and *p*<0.001). The greatest total biomass was in the lower intertidal zone of YL in May 2013, 32.676±6.331 g DW m^{-2}. The lowest total biomass was in the subtidal zone of YL in May 2013, and in the lower intertidal zone of KM in May 2013, 16.604±0.637 g DW m^{-2}. The lowest total and above-ground biomasses were in the subtidal zone of YL in May 2013, 2.872±0.877 g DW m^{-2} and 1.330±0.363 g DW m^{-2}, respectively. Generally, significantly greater total and above-ground biomasses of plants were found in the lower intertidal zone, moderate biomass found in the upper intertidal zone and significantly lower biomass presented in the subtidal zone. However, there were no clear patterns of plant biomass between YL and KM. Seasonal variation in total and above-ground biomasses were also observed: significantly greater plant biomass occurred in May 2013 whereas there was significantly lower plant biomass in October 2012 (Fig. 17a and 17b; Table 2).

There were significant differences in below-ground biomass (rhizome + root) affected by zones (*p*<0.001). The greatest below-ground biomass was in the lower intertidal zone of YL in May 2013, 17.352±3.824 g DW m^{-2}, whereas the lowest below-ground biomass was in the subtidal zone of YL in May 2013, 1.542±0.575 g DW m^{-2}. Significantly greater below-ground biomass of plants was always in the lower intertidal zone and significantly lower biomass was in the subtidal zone. No significant seasonal variation in below-ground biomass was observed in this study (Fig. 17c; Table 2).

The ratios between above-ground and below-ground biomasses were significantly affected by zones and months (*p*=0.012 and *p*<0.001). The greatest ratio was in the subtidal zone of KM in February 2013, 1.38±0.41 whereas the lowest ratio was in the lower intertidal zone of YL in October 2012, 0.36±0.06. Above-ground and below-ground biomasses ratios in May 2013 were higher than those in October 2012 (Fig. 17d; Table 2).


■ Upper intertidal
□ Lower intertidal
▨ Subtidal
a
Total biomass (g DW m-2)
40
30
20
10
0
YL
KM
YL
KM
YL
KM
YL
KM
July-12
Oct-12
Feb-13
May-13
b
Above ground biomass (g DW m-2)
20
16
12
8
4
0
YL
KM
YL
KM
YL
KM
YL
KM
July-12
Oct-12
Feb-13
May-13

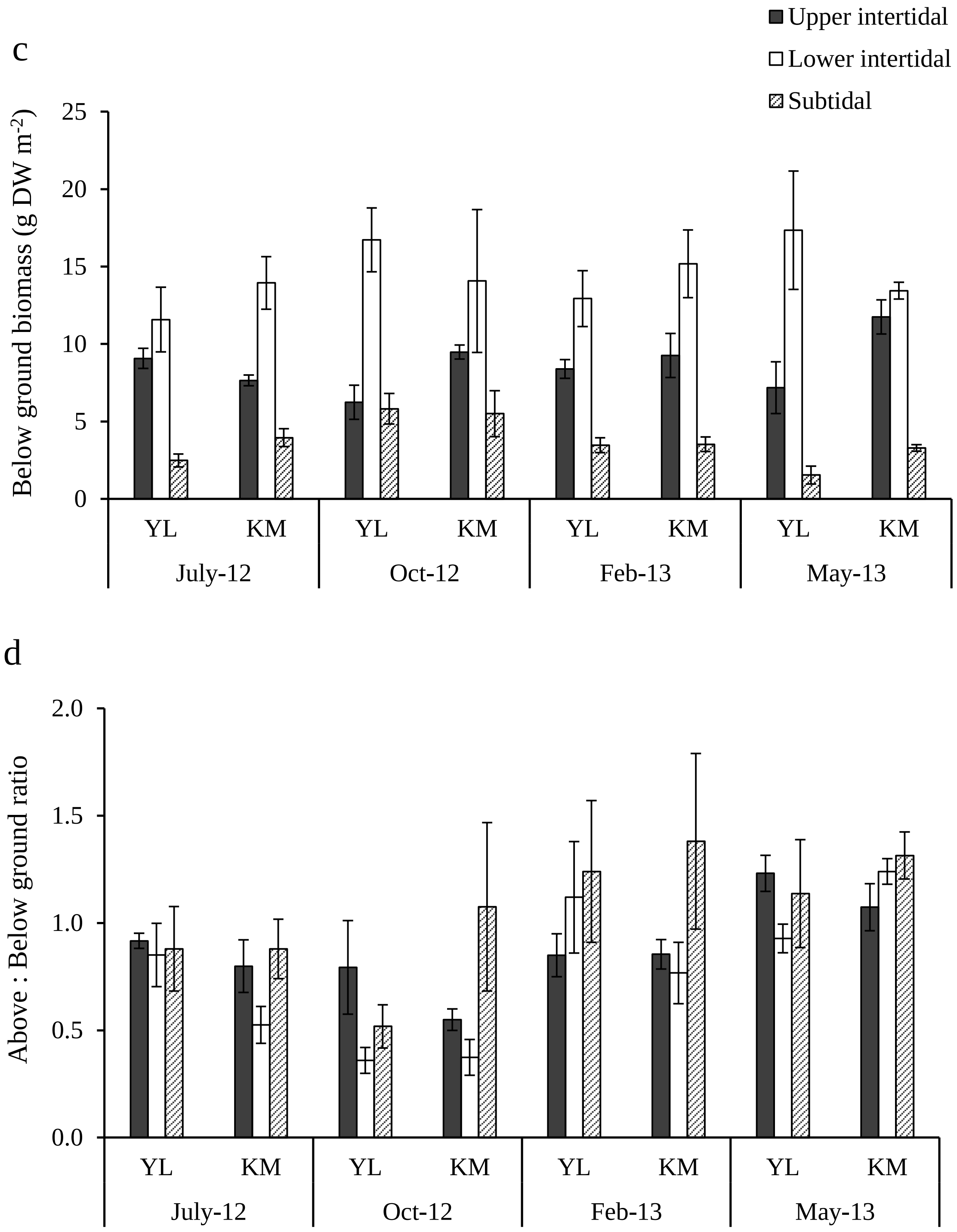


Fig. 17 Biomass of *H. ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=5); (a) total biomass, (b) above-ground biomass, (c) below-ground biomass, (d) above-ground:below-ground biomass and (e) productivity.

3.3 Growth rate: Plastochrone interval (P_L) and rhizome elongation rate

There were significant differences in P_L among zones, sites and months (Kruskal Wallis test, $p<0.001$,). The longest P_L was in the upper intertidal zone of KM in July 2012, 5.07±0.48 days leaf pair^{-1}, whereas the shortest P_L was in the subtidal zone of YL in July 2012, 2.10±0.10 days leaf pair^{-1}. P_L of *H. ovalis* in the upper intertidal zone of both study sites in July 2012 and KM in October 2012 were significantly longer than those plants growing in the lower intertidal and subtidal zones. The slowest P_L throughout the year was in the upper intertidal zone, 4.05±0.39 days leaf pair^{-1} (Fig. 18a; Table 2).

There were significant differences in rhizome elongation rate among zones, sites and months (Kruskal Wallis test, $p=0.005$,). The greatest rhizome elongation rate was in the subtidal zone of KM in October 2012, 9.06±1.02 mm shoot^{-1} day^{-1} whereas the lowest rhizome elongation rate was in the upper intertidal zone of YL in May 2013, 2.38±0.17 mm shoot^{-1} day^{-1}. Significantly greater rhizome elongation rate was found in the subtidal zone of KM in February and May 2013 and also in the lower intertidal zone of KM in February 2013 while the rhizome elongation rate was significantly lower in the upper intertidal zone of YL in July 2012 and May 2013, respectively (Fig. 18b; Table 2).

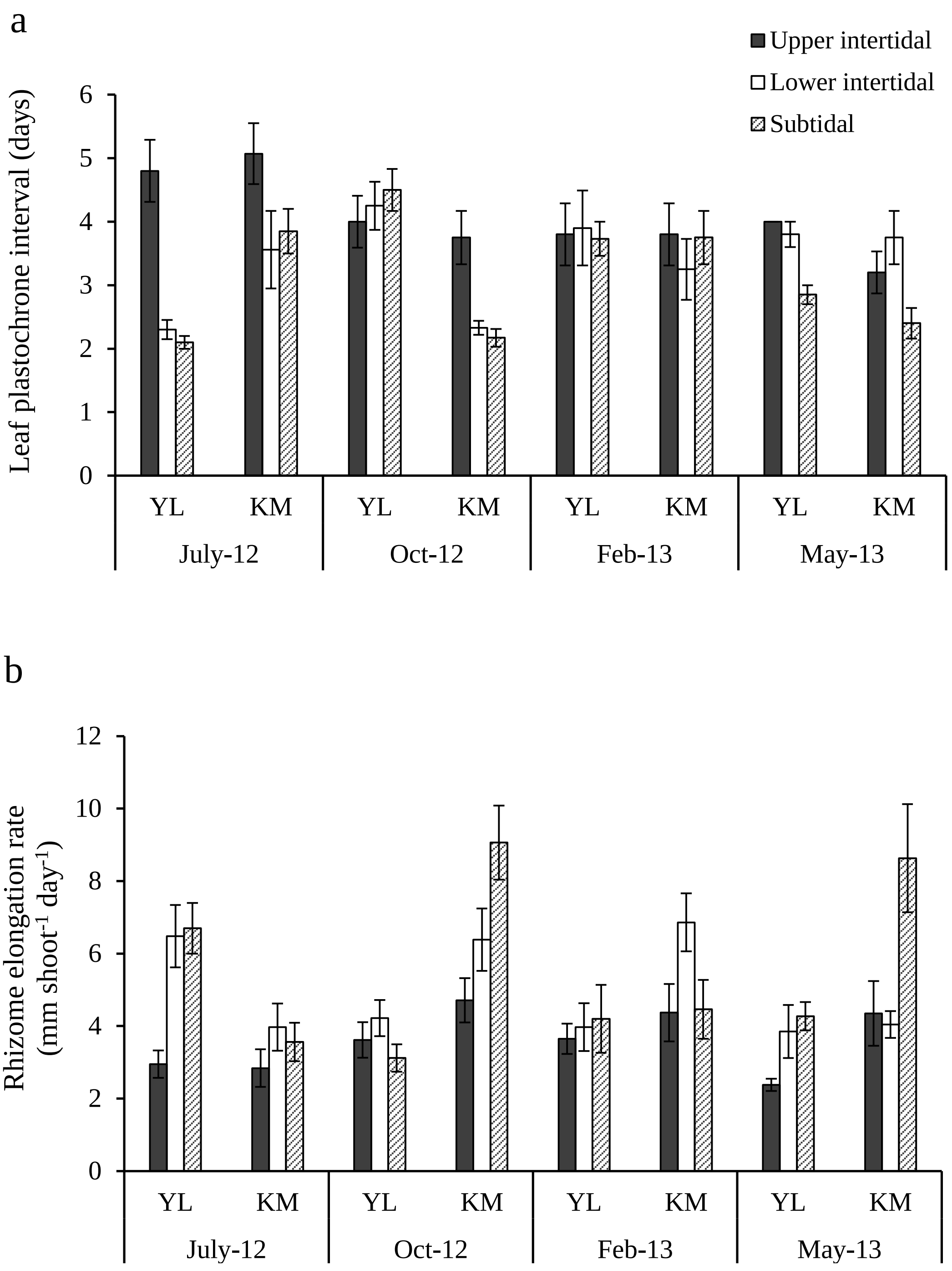


Fig. 18 Growth rate of *H. ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=10); (a) leaf plastochrone interval and (b) rhizome elongation rate.

3.4 Productivity

There were significant differences in productivity of *H. ovalis* affected by zones and sites (p<0.001). The greatest productivity was in the lower intertidal zone of YL in July 2012, 3243.193±501.976 g DW m^{-2} y^{-1}, whereas the lowest productivity was in the Subtidal zone of YL in May 2013, 367.817±112.351 g DW m^{-2} y^{-1}. Significantly greater productivity was in the lower intertidal zone while the significantly lower productivity was in the subtidal zone (Fig.19; Table 2).

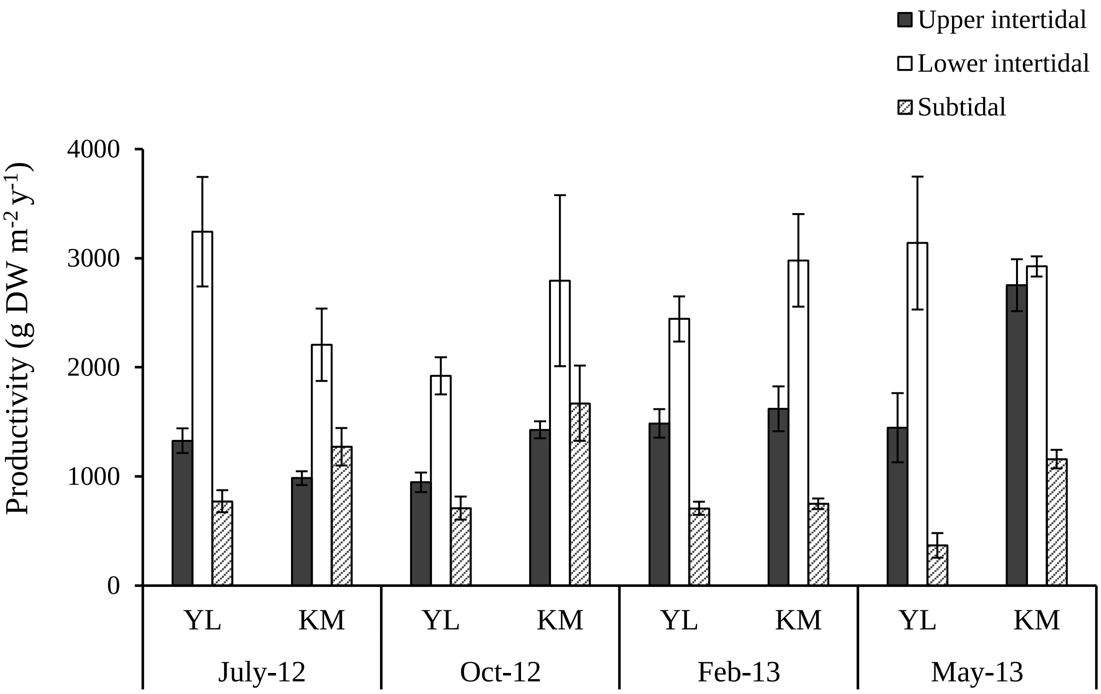


Fig. 19 Productivity of *H. ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=5).

Table 2. Summary of statistical analyses of shoot density, biomass and growth rate on *H. ovalis* showing the results of three-way analysis of variance (Z x S x M) and for any possible combination of factors: Z = zone, S = site and M = month and Kruskal Wallis test .

Variables	Transformation method	Z	S	M	Z x S	Z x M	S x M	Z x S x M
				F-statistic and Chi-square (Kruskal Wallis test)				
Density (leaf pairs m^{-2})	X	81.156***	10.493**	2.960*	0.905	3.047**	1.090	1.163
Total biomass (g DW m^{-2})	Log(X)	148.005***	8.183**	1.677	6.305**	7.028***	4.073**	2.350*
Above-ground biomass (g DW m^{-2})	Log(X)	92.987***	5.849*	12.584***	10.020***	8.192***	4.652**	1.204
Below-ground biomass (g DW m^{-2})	$\sqrt{X}$	110.972***	2.620	0.999	1.324	1.973	0.528	1.970
Above-ground : Below-ground ratio	Log(X)	4.672*	0.008	14.156***	2.365	0.902	0.916	0.858
P_L (days)	n	17.385***	4.321*	2.147	21.809**	39.234***	36.295***	89.363***
Rhizome elongation rate (mm shoot $^{-1}$ day^{-1})	Log(X)	16.711**	10.510**	2.154	0.351	2.753*	15.202***	3.213**
Productivity (g DW m^{-2} y^{-1})	Log(X)	108.577***	23.024***	0.315	9.975***	5.865***	7.435***	2.100

n = non-parametric Kruskal Wallis test; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

3.5 Morphological characters

There were significant differences in leaf width, leaf length and leaf area among zones, sites and months ($p<0.001$). There were significantly differences in petiole length affected by zones, sites and months ($p<0.001$, $p=0.018$ and $p<0.001$) and there were significantly differences in rhizome internode length among zone, sites and months ($p=0.002$). The highest leaf width, leaf length and leaf area were in the lower intertidal zone of KM in May 2013, 7.99±0.12 mm, 15.66±0.38 mm and 103.21±2.75 mm, respectively. The lowest leaf width, leaf length and leaf area were in the upper intertidal zone of YL in October 2012, 4.51±0.14 mm, 8.70±0.29 and 32.10±2.16 mm, respectively. The longest petiole length was in the upper intertidal zone of YL in December 2012, 17.83±0.86 mm and the shortest petiole length was in the subtidal zone of KM in February 2013, 11.19±0.56 mm. The longest internode length was in the lower intertidal zone of KM in February 2013, 25.21±1.13 mm and the shortest internode length was in the upper intertidal zone of KM in December 2012, 12.21±0.78 mm (Fig. 20; Table 3). Two types of plant leaf were observed in the subtidal zone in this study; very thin small leaves with no anthocyanin production contrasting with thicker and bigger leaves with a red stripe. It might have some genetic difference between these two characters in plants of the same species which colonized the same environment.

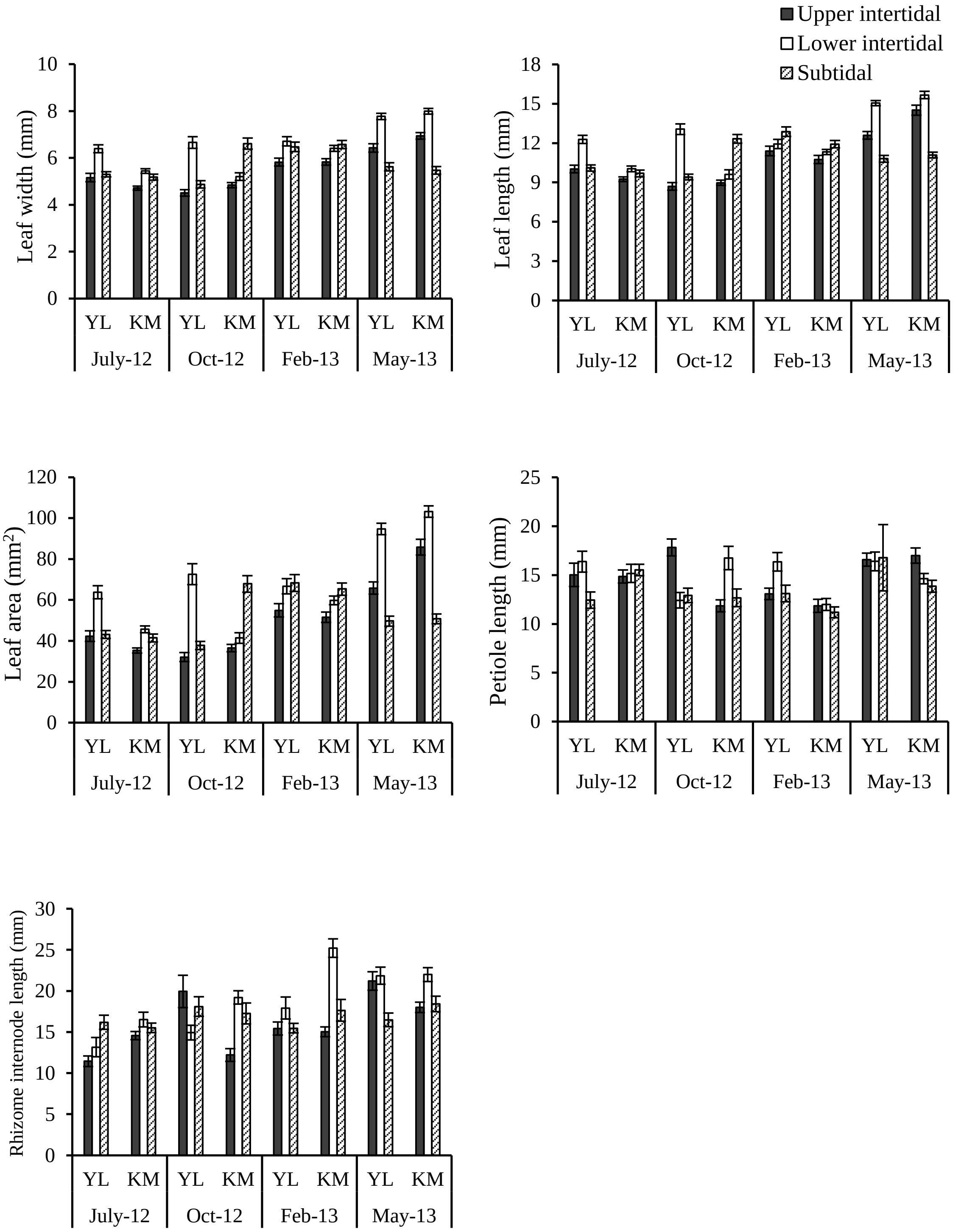


Fig. 20 Morphological characters of *H. ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=20).

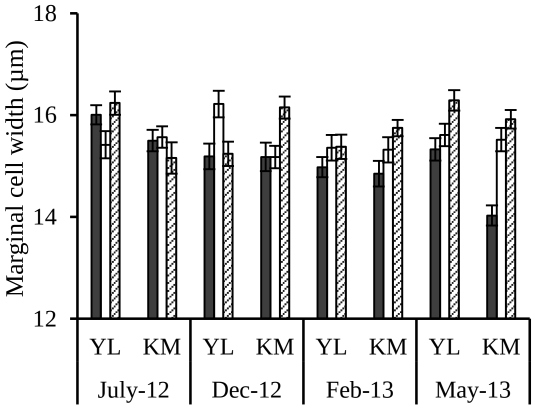
Table 3. Summary of statistical analyses of morphological characters showing the results of three-way analysis of variance (*Z* x *S* x *M*) and for any possible combination of factors: *Z* = zone, *S* = site and *M* = month.

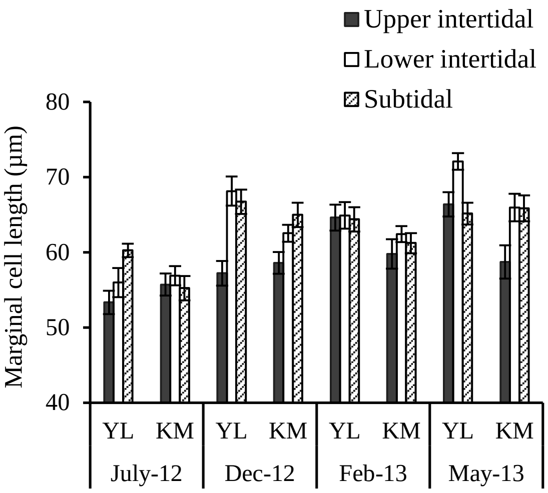
Variables	Transformation method	Z	S	M	Z x S	Z x M	S x M	Z x S x M
F-statistic								
Leaf width (mm)	Log(X)	87.544***	0.290	98.902***	20.799***	24.330***	7.104***	11.821***
Leaf length (mm)	Log(X)	65.190***	5.672*	137.552***	25.714***	33.309***	13.049***	15.391***
Leaf area (mm^2)	Log(X)	83.598***	0.376	128.308***	25.970***	30.450***	10.019***	14.415***
Petiole length (mm)	Log(X)	17.700***	5.650*	10.866***	2.381	4.600***	3.778*	0.352
Internode length (mm)	Log(X)	30.941***	3.644	31.819***	6.902**	6.176***	5.699**	3.627**

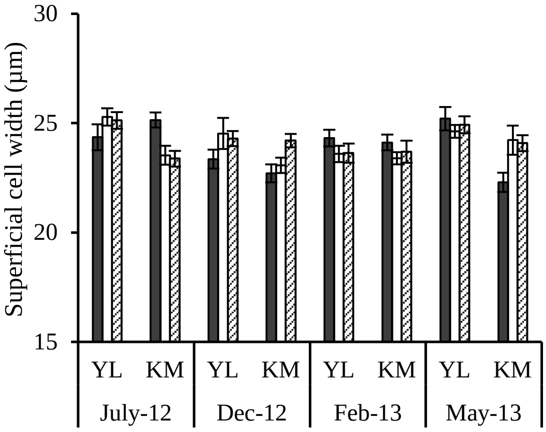
* $p<0.05$, ** $p<0.01$ and *** $p<0.001$

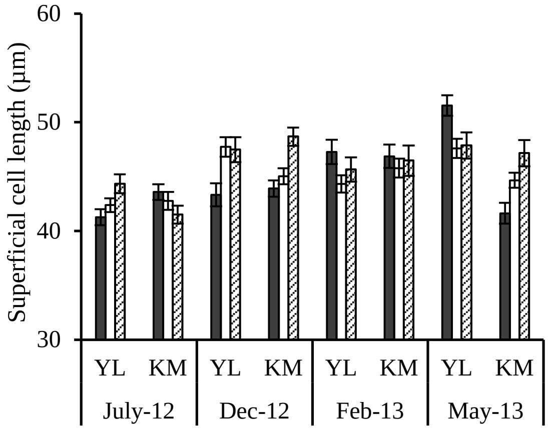
3.6 Anatomical characters

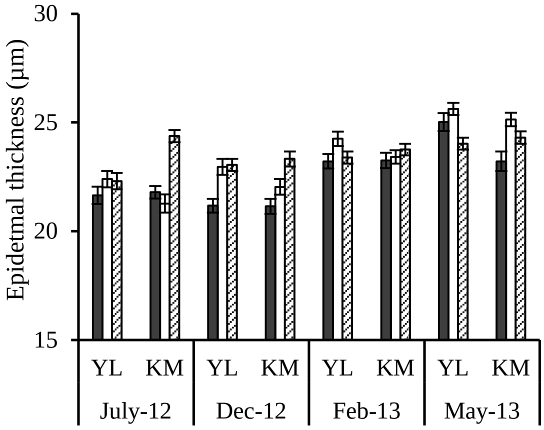
There were significant differences in marginal cell width, marginal cell length, superficial cell width, superficial cell length, epidermis thickness, mesophyll cell width, mesophyll cell length, air lacunar width and air lacunar length among zones, sites and months ($p<0.05$). The greatest marginal cell width and length were in the subtidal and lower intertidal zones of YL in May 2013, 116.29±0.20 µm and 72.09±1.12 µm, whereas the lowest values were in the subtidal zone and lower intertidal zone of YL in May 2013, 14.03±0.20 µm and 53.37±1.55 µm, respectively. The greatest superficial cell width and length were in the lower intertidal zone of YL in July 2012 and upper intertidal zone of YL in May 2013, 25.28±0.39 µm and 51.54±0.95 µm, whereas the lowest values were in the upper intertidal zone of KM in May 2013 and subtidal zone of YL in July 2012, 22.99±0.44 µm and 41.51±0.82, respectively. The greatest epidermal thickness was in the lower intertidal zone of YL in May 2013, 25.62±0.28µm, whereas the lowest thickness was in the upper intertidal zone of KM in December 2012, 21.14±0.35 µm. The greatest mesophyll cell width and length were in the lower intertidal zone of YL in May 2013, 57.05±1.65 µm and 66.71±1.77 µm whereas the lowest values were in the upper intertidal zone of KM in July 2012 and upper intertidal zone of YL in December 2012, 30.72±0.99 µm and 47.29±1.84 µm, respectively. The greatest air lacunar width and length were in the lower intertidal zone of YL in May 2013, 138.48±4.60 µm and 157.61±4.08 µm, whereas the lowest values were in the upper intertidal zone of KM in July 2012 and upper intertidal zone of YL in December 2012, 47.42±2.17 µm and 80.91±3.47 µm, respectively. There were no clear patterns in most anatomical characters among zones, sites and months. However, the most greatly varied anatomical characters were mesophyll cell width-length and air lacunar width-length which were significantly greater in the lower intertidal zone of both study sites in May 2013, whereas these characters were significantly smaller in the upper intertidal and subtidal zones in December 2012 (Fig. 21; Table 4).

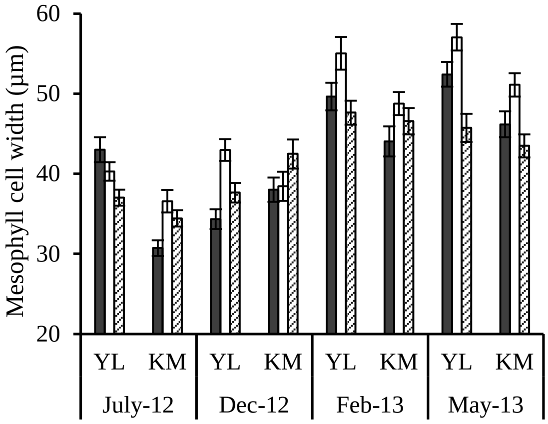

Marginal cell width (µm)
18
16
14
12
YL KM YL KM YL KM YL KM
July-12
Dec-12
Feb-13
May-13


■ Upper intertidal
□ Lower intertidal
▨ Subtidal
Marginal cell length (µm)
80
70
60
50
40
YL KM YL KM YL KM YL KM
July-12
Dec-12
Feb-13
May-13


Superficial cell width (µm)
30
25
20
15
YL KM YL KM YL KM YL KM
July-12
Dec-12
Feb-13
May-13


Superficial cell length (µm)
60
50
40
30
YL KM YL KM YL KM YL KM
July-12
Dec-12
Feb-13
May-13


Epidetmal thickness (µm)
30
25
20
15
YL KM YL KM YL KM YL KM
July-12
Dec-12
Feb-13
May-13


Mesophyll cell width (µm)
60
50
40
30
20
YL KM YL KM YL KM YL KM
July-12
Dec-12
Feb-13
May-13

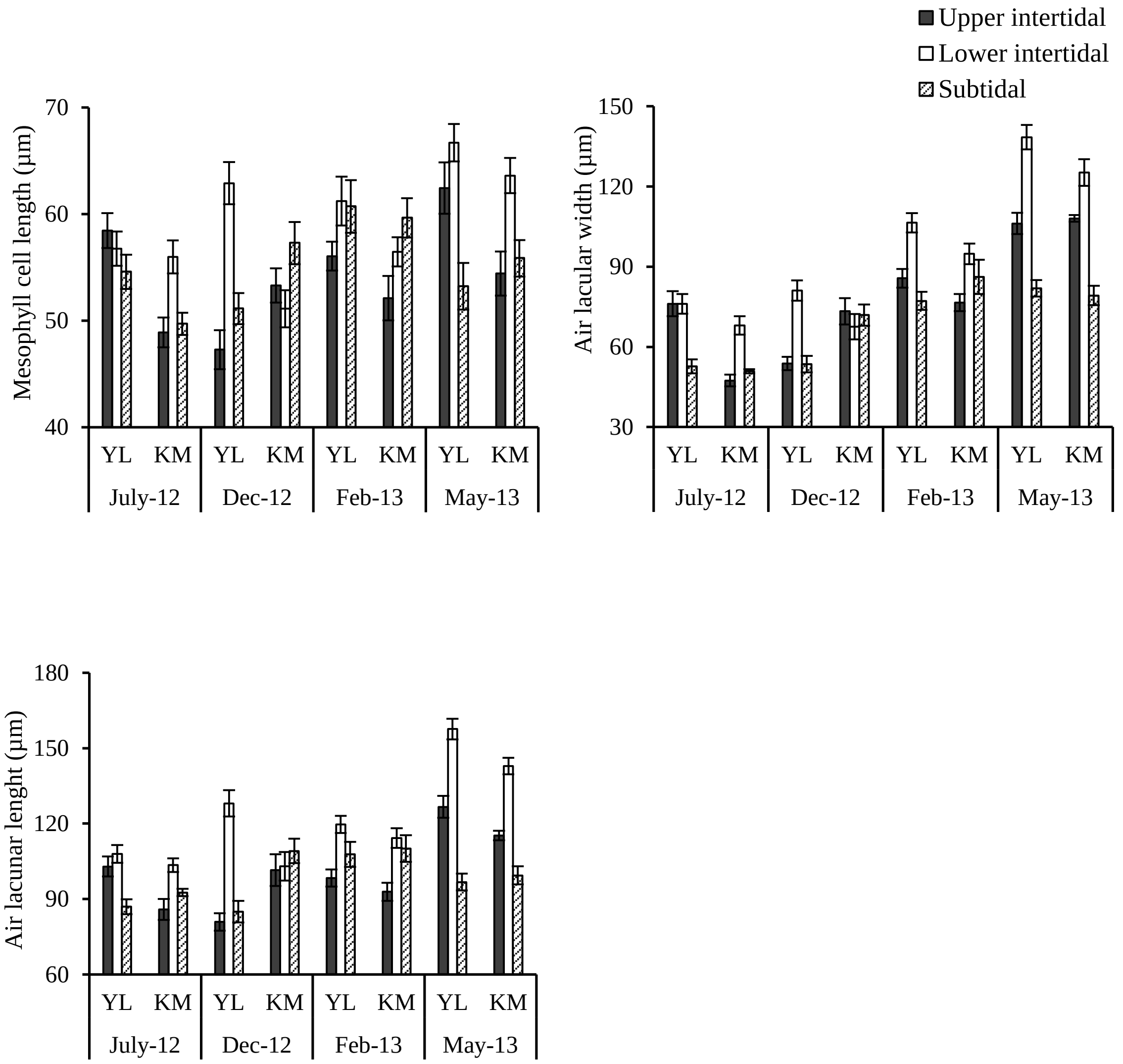


Fig. 21 Anatomical characters of *H. ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=20).

Table 4. Summary of statistical analyses of anatomical characters showing the results of three-way analysis of variance (Z x S x M) and for any possible combination of factors: Z = zone, S = site and M = month.

Variables	Transformation method	Z	S	M	Z x S	Z x M	S x M	Z x S x M
				F-statistic				
Marginal cell width (µm)	X^2	15.181***	7.527**	2.914*	1.685	3.850**	2.806*	4.987***
Marginal cell length (µm)	X	16.933***	15.874***	37.966***	0.308	3.049**	1.621	3.066**
Superficial cell width (µm)	X	0.557	19.401***	4.239**	0.242	2.486*	2.187	3.824**
Superficial cell length (µm)	Log(X)	3.590*	7.675**	21.158***	1.050	3.349**	8.172***	6.112***
Epidermis thickness (µm)	X^2	18.774***	1.561	63.077***	11.879***	7.599***	2.513	2.478*
Mesophyll cell width (µm)	X	19.181***	32.458***	110.183***	6.676**	3.717**	8.617***	3.587**
Mesophyll cell length (µm)	X	18.207***	13.849***	13.584***	5.634**	5.042***	2.104	6.064***
Air lacunar width (µm)	$\sqrt{\sqrt{X}}$	73.804***	2.157	144.688***	6.560**	6.601***	8.308***	5.784***
Air lacunar length (µm)	$\sqrt{\sqrt{X}}$	53.379***	1.753	30.804***	10.126***	7.711***	1.513	3.428***

* $p<0.05$, ** $p<0.01$ and *** $p<0.001$

4. Discussion

Growth parameters, morphological and anatomical characters of the seagrass *H. ovalis* were spatially and temporally different. Generally, shoot density of the plants in the upper and lower intertidal zones were not different. Higher density of plant in these two habitats might be a beneficial trait of the plants in which they form dense patches to protect their leaves from being directly exposed to the air and intense sunlight during low tide. This could help maintain the normal water balance which is essential for many physiological processes such as cell enlargement and gas exchange in plant leaves (Taiz and Zeiger, 2002). Dramatically decreased shoot density in the subtidal zone might help plants reduce respiratory demand in shorter canopy species (Ralph *et al*., 2007). The average density of seagrass in subtidal habitat in this study was 1260-4540 leaf pairs m^{-2} whereas the previously reported density from Indonesia was 1099 leaf pairs m^{-2} (Erftemeijer and Stapel, 1999).

Above-ground biomass in this study was 1.96-16.60 g DW m^{-2}. Greater above-ground biomass of the plant in the lower intertidal zone is related the bigger plant leaf size while there was a significantly lower plant biomass presented in the subtidal zone related to plant density. Seasonal variation was likely to impact the above-ground biomass of seagrass growing in the upper intertidal zone as more stressors are present there. In February 2013, above-ground biomass per unit area increased with increasing plant density in order to protect the meadow from desiccation stress in summer month. Biomass continued to increase in May 2013 when the extreme exposure time was also reduced compared to previous months. The studies in the

Philippine reported the above-ground biomass of this species at 54.80 g DW m^{-2} (Duarte and Chiscano, 1999) which was significantly higher than the recent study.

Below-ground part in this study was 1.542-17.352 g DW m^{-2} with the greatest below-ground biomass in the lower intertidal zone which is related plant size. Rhizome of seagrass in this area was bigger than those in the upper intertidal and subtidal zones. This below-ground biomass was in the range of previous work at 21.10 g DW m^{-2} (Duarte and Chiscano, 1999) reported from the Philippines and 30-72 g DW m^{-2} reported in the South West coast of Thailand (Supanwanid, 1996; Nakaoka and Aioi, 1999). Below-ground biomass of the plants show the same spatial pattern as above-ground biomass, but no seasonal variation was observed as *H. ovalis* reproduces throughout the year by the rhizome branching. The variation in total biomass was mainly affected by change in above-ground biomass and this parameter showed the same monthly variation as above-ground biomass. This species might not exhibit the seasonal energy storage because of their thin small rhizome and the ability to reproduce faster than other seagrasses. Rapid growth rate helps them to maintain their population throughout the year which plays an important role in maintaining the abundance of this seagrass species in the wide range of environmental conditions for their habitat.

The fastest leaf P_L in this study was 2 days whereas the longest time plant use to produce new leaf pair was up to 5 days compared to average P_L from previous at 2.2 days (Duarte *et al*., 1998). The average of plant growth rate in term of P_L and rhizome elongation rate throughout the year was significantly lower in plant grow in the upper intertidal zone, desiccation stress might be the factor that affect plant growth at the higher intertidal level whereas seagrass grow in the lower intertidal zone and subtidal zone showed the greater growth rate. Rhizome elongation rate in this study ranged from 2.38-9.06 mm shoot^{-1} d^{-1} while the previous report was at 10 mm shoot^{-1} d^{-1} (Marbà and Duarte, 1998). Horizontal rhizome elongation rate was suggested to be density dependent, thus looser density of plant at the subtidal zone provided more space available for seagrass to grow faster and extend longer horizontal rhizome than those extending within the dense meadow (Marbà and Duarte, 1998).

The greater annual productivity of *H. ovalis* found in the lower intertidal zone which is related their shoot density and leaf size as high density and biggest leaves found in the lower intertidal zone whereas significantly lower density found in the subtidal zone. Productivity of *H. ovalis* in this study was range from 367.187-3243.19 g DW m^{-2} y^{-1} compared with the average productivity in previous study in the range 410-5694 g DW m^{-2} y^{-1} (Hillman *et al*., 1995; Nakaoka and Aioi, 1999; Huong *et al*, 2003), and up to 3243 g DW m^{-2} y^{-1} in uniform stand for shallow water *H. ovalis* (Hillman *et al*., 1995). Productivity of this species also greater than some other tropical seagrasses reported from mixed seagrass meadow in the Philippines waters such as 1595.8 g DW m^{-2} y^{-1} in *Thalassia hemprichii*, 204.4 g DW m^{-2} y^{-1} in *Cymodocea rotundata* and 215.5 g DW m^{-2} y^{-1} in *Enhalus acoroides*, (Duarte *et al*., 1988).

Significantly bigger in leaf size was found in the lower intertidal zone indicating better conditions in this area where the level of variability of the physical factors was not as extreme as in the upper zone where the smallest leaves were observed. Thus the seagrasss growing in this area exhibited more healthy condition than those in the upper and lower end of the distribution. The advantage of downsizing their leaf size in the upper intertidal zone was to minimize the rate of water loss (Shafer *et al*., 2007). Significantly bigger leaf size in the upper and lower intertidal zones were found in the early rainy season, May 2013 when the physical conditions were better than those the other period as desiccation stress was reduced due to cloud cover and humidity. There were 4.51-7.99 in width and 8.70-15.66 in length of *H. ovalis* leaves in the present study, this was in the normal range of leaf size from previous reports in the Tropical Atlantic Ocean range 5-20 mm wide (Short *et al*., 2010), but smaller than those reported from China where *H. ovalis* leaves range in range from 11.0-14.2 mm in width and 19.3-26.5 in length (Xu *et al*., 2011). The larger leaves of *H. ovalis* in China might due to the response of the plant to expand their leaf area in order to increase the ability of capture light in the lower irradiance area as China is situated greater distance from the equator than our study site.

We cannot clearly conclude on the source of variation in mesophyll cell size and air lacunar size because of lack of sufficient available experimental evidence. The trends in the sizes of these two characters showed the same trend as leaf size both spatially and seasonally. Thus their variation might be the consequence of leaf size because this anatomical character is one of the allometric factors reported also in terrestrial plants (Cornelissen *et al*., 2003).

CHAPTER 4

Title: Variations of tidal exposures and seasons on photosynthesis and pigment contents of seagrass *Halophila ovalis* (R.Br.) Hook.f. at seagrass bed in Trang Province

Abstract

The physiological parameters of the seagrasss *Halophila ovalis* were investigated in plants growing at different tidal levels and in different seasons. Maximum relative electron transport rate ($rETR_{max}$) and optimum light requirement for plant photosynthesis (E_{opt}) were measured using the chlorophyll *a* fluorescence technique (PAM). Pigment contents of plants were also measured spectrophotometrically. Lowering of the $rETR_{max}$ in the subtidal zone might occur as a consequence of sulphide toxicity which increases the seagrass respiration rate. Faster rate of CO_2 diffusion in the gas phase during emergence condition combined with high irradiance supported higher photosynthetic rates of the seagrass in the upper intertidal zone. E_{opt} of seagrass in the upper and lower intertidal habitats (402.20-817.61 µmol photons m^{-2} s^{-1}) were higher than those in the subtidal zone (295.61-518.41 µmol photons m^{-2} s^{-1}) which was in the range well below the measured light available at the study sites (full sunlight directly overhead is about 2200 µmol photons m^{-2} s^{-1}). This is not consistent with the straightforward hypothesis that light limited seagrass photosynthesis in subtidal area. Enhanced chlorophyll and carotenoid content of seagrass in the subtidal zone are necessary to increase light harvesting ability in blue wavelengths which penetrate much deeper in water than green and red light. Much higher anthocyanin content was found in the upper intertidal zone, the average was 0.43±0.04 µg cm^{-2}, while the lower and subtidal zones were only 0.15±0.02 µg cm^{-2} and 0.16±0.04 µg cm^{-2}, respectively. Greater anthocyanin content was found in the upper intertidal seagrass suggesting its role as a photoprotective pigment in the plants.

Keyword: *Halophila ovalis* (R.Br.) Hook.f., tidal exposure, photosynthesis, pigment

1. Introduction

Photosynthetic organisms are thought to be mainly affected by differences in light regimes, temperature (Bulthuis, 1987; Dennison, 1987; Ralph and Burchett, 1995) and the chemistry of the carbon sources available for photosynthesis (Beer and Waisel, 1979; Leuschner *et al*., 1998) in order to store energy as carbohydrate molecules (Taiz and Zeiger, 2012). Leaf pigment contents also provides valuable information about physiological status of plants (Gitelson and Marzlyak, 2004; Liu *et al*., 2011). Seagrasses are angiosperms which have adapted from growing on land to life under marine conditions. Therefore, they still contain all the same basic pigments for photosynthetic activity as terrestrial plants. Chloroplasts in submerged aquatic plants are often well developed in the epidermal cells (Kirk, 2011). Chlorophyll *a* is the main photosynthetic pigment which the plants use to absorb light in the range of photosynthetically active radiation (PAR; 400-700 nm) while the accessory pigments supporting the light harvesting system are chlorophyll *b* and carotenoid in light harvesting complex proteins (LHC). Beside, acting as accessory pigments carotenoid is sometimes known to provide photoprotective role in plants exposed to excess light intensity by a mechanism known as the xanthophyll cycle (Demming-Adams and Adams, 1996).

H. ovalis is a small seagrass which is distributed in high fluctuating environment areas where the ability to adjust their photosynthetic ability to best fit the changing environmental conditions across the tidal cycle is an advantage in the face of fluctuating light intensity, temperature, salinity, turbidity and sediment characteristics. Moreover, the impacts of seasonal change also influence those factors. In most studies PAM (Pulse Amplitide Modulated fluorometry) machines or PEA (Plant Efficiency Apparatus) are used as convenient means of estimating photosynthetic activity from fluorescence data and are usually used in conjunction with chlorophyll pigment content measurements (Ralph, 1999; Ralph, 2000; Major and Dunton, 2002; Enríquez, 2005). From observations in the field, plants also exhibit red leaf coloration which may relate to enhanced anthocyanin production after exposure to one or more stressors (Ragavan *et al.* 2013). Some studies suggested that anthocyanin serves an important function in the protection of plant leaves from high irradiance and for maintaining high levels of plant photosynthesis (Novak and Short, 2011a, b).

The aim of this study focused on the variations of photosynthetic capability of the seagrass *H. ovalis* measured using chlorophyll *a* fluorescence technique and the pigment contents of the seagrass in different tidal levels and seasons.

2. Materials and Methods

The characteristics of sampling sites, environmental measurements and experimental set-up were described in Chapter 2. The intensity of irradiance in the range of photosynthetically active radiation (PAR) in different water depth was predicted using the model developed by Ritchie (2014, personal communication).

2.1 Photosynthetic measurement

Photosynthetic efficiency of photosystem II (PSII) was measured using PAM fluorometry (Mini-PAM; Walz, Effeltrich, Germany) which assess the electron flow through PSII based on the chlorophyll *a* fluorescence emitted from chlorophyll *a* molecules in the PSII complex. The photosynthetic light response curve was examined from plant leaves at each study site. Samples were stored in the dark for at least 10 minutes prior to applying the series of actinic light intensities given by the internal halogen lamp of PAM fluorometer at 9 levels of PPFD (0, 170, 281, 421, 577, 853, 1156, 1738 and 2522 µmol photons m^{-2} s^{-1}) using the standard leaf absorption factors of plant leaves of 0.84 ($Abt_F = 0.84$). This is the default value used by Walz Software. This kind of measurements is called the rapid light curve method (RLC). Because the actinic light source was a quartz halogen lamp with a color temperature (6000 K) similar to sunlight, the spectrum of the light source was very similar to sunlight. Calculations of rETR assume that the absorptance (Abt_F) of leaves is 0.84 which is the default setting used in Walz software.

rETR is calculated as

$\text{rETR} = Y \times E \times 0.5 \times 0.84$

where, Y is the Yield (ΦPSII), E is the irradiance (mol photons m^{-2} s^{-1}, 0.5 is the allocation factor for PSII vs. PSI and 0.84 is the standard default absorptance factor ($\text{Abt}_F = 0.84$). The least square fit of the simple exponential decay function of *Y*, and nonlinear least square fit of the rETR as a photosynthesis-irradiance curve (*P-I*) model to the RLCs (Waiting-in-line function) $\text{ETR} = (\text{ETR}_{max} \text{ x } E/E_{opt}).e^{1-E/Eopt}$ (Ritchie and Bunthawin, 2010), then the maximum relative electron transport rate (rETR_{max}) and optimum light requirement (E_{opt}) of this seagrass were examined.

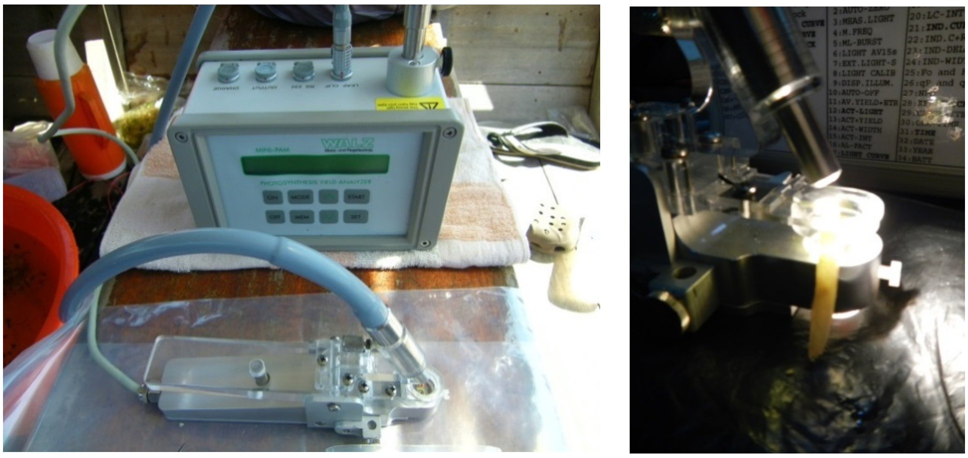


Fig. 22 Photosynthetic measurement by RLC method was performed using PAM fluorometry in dark-adapted leaves of the seagrass *H. ovalis*.

2.2 Pigment analysis

The third youngest leaf pair was used for the analysis of pigment contents. Chlorophyll *a*, chlorophyll *b*, carotenoid and anthocyanin of *H. ovalis* leaves were extracted using the specific solvents. The whole leaf of the plants were measured for the leaf area using image processing software (ImageJ) prior to putting into 1 ml of the solvent. Chlorophyll and carotenoid were extracted using DMF, and anthocyanin was exacted using acidified methanol. Samples were stored in darkness at 4 °C for 10 days in case of chlorophyll and carotenoids extractions, and overnight in the same condition in case of anthocyanin extraction. Samples were spectrophotometrically measured at the specific wavelength for each pigment analysis, and the values of measurement were used to calculated pigment content following the equations of Porra (2002), Mancinelli (1984), and Wellburn (1994) and the results were reported as µg cm^{-2} of projected leaf surface area as follows;

$$\text{Chlorophyll } a\ (\mu\text{g cm}^{-2}) = 11.65A_{664} - 2.69A_{647}$$

$$\text{Chlorophyll } b\ (\mu\text{g cm}^{-2}) = 20.81A_{647} - 4.53A_{664}$$

$$\text{Carotenoids } (\mu\text{g cm}^{-2}) = (1000A_{480} - 0.89\mathrm{C_a} - 52.02\mathrm{C_b})/245$$

$$\text{Anthocyanin } (\mu\text{g cm}^{-2}) = \mathrm{A}_{530} - 0.25A_{657}$$

Where, *A* is the absorbance represented the optical density (O.D.) at the particular wavelength, $\mathrm{C_a}$ is the chlorophyll *a* determination (µg cm^{-2}) and $\mathrm{C_b}$ is the chlorophyll *b* determination (µg cm^{-2}).

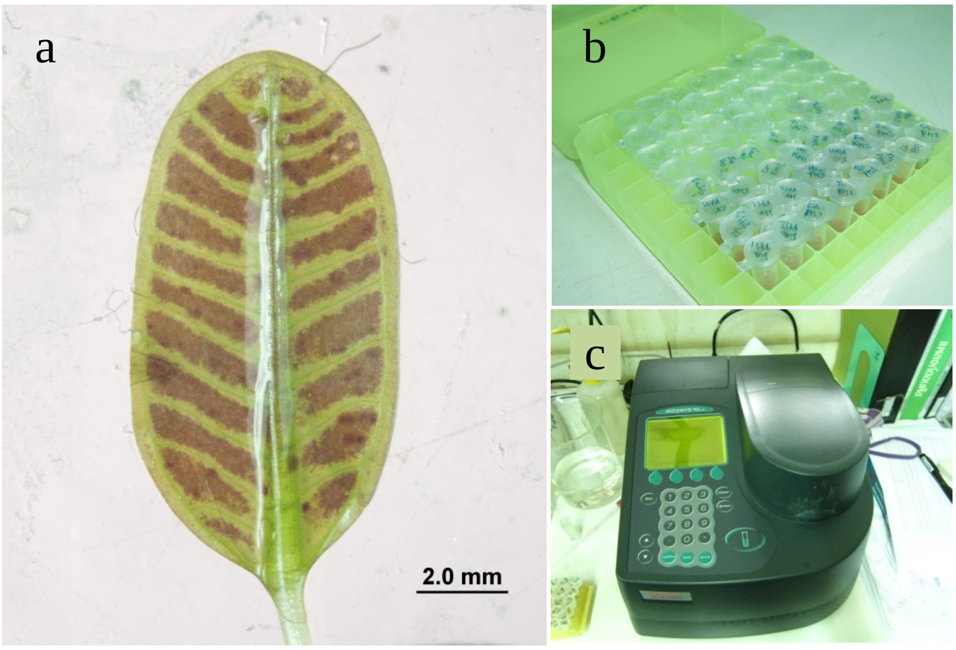


Fig. 23 Pigment analysis; (a) seagrass leaves were collected for pigment extraction (b) the whole leaf of seagrass was directly immerged in 1 ml of extractant and (c) optical densities of the samples were measured using a spectrophotometer.

2.3 Statistical analyses

Three-way ANOVA was employed to test the differences in maximum relative electron transport rate, saturating irradiance and pigment content of the seagrass *H. ovalis* among different tidal exposures, sites and seasons at the significance level of 0.05 using SPSS statistical software version 13.0 for Windows (SPSS, Chicago, IL, USA). Normality test and data transformation ((Log(X), Log(X+1) and $\sqrt{X}$) were applied to all data sets prior to the test with ANOVA model. The non-parametric Kruskal Wallis test was applied to test for differences among data sets with non-normal distribution. Post-hoc multiple comparison were employed to test the differences of measuring variables among treatments (Zar, 1999).

3. Results

3.1 Irradiance with depth in clear seawater

There were differences in the wavelength regions of PAR irradiance (400-700 nm) in different water depths. High intensity of irradiance at blue light region (455-492 nm) penetrated through the sea bottom in every water depth. The red light (622-780 nm) region was significantly lower with increasing water depths, nearly 100% of red light was depleted at 4 and 6 meter depth which are the depth of subtidal zones where *H. ovalis* was found growing (Fig. 24).

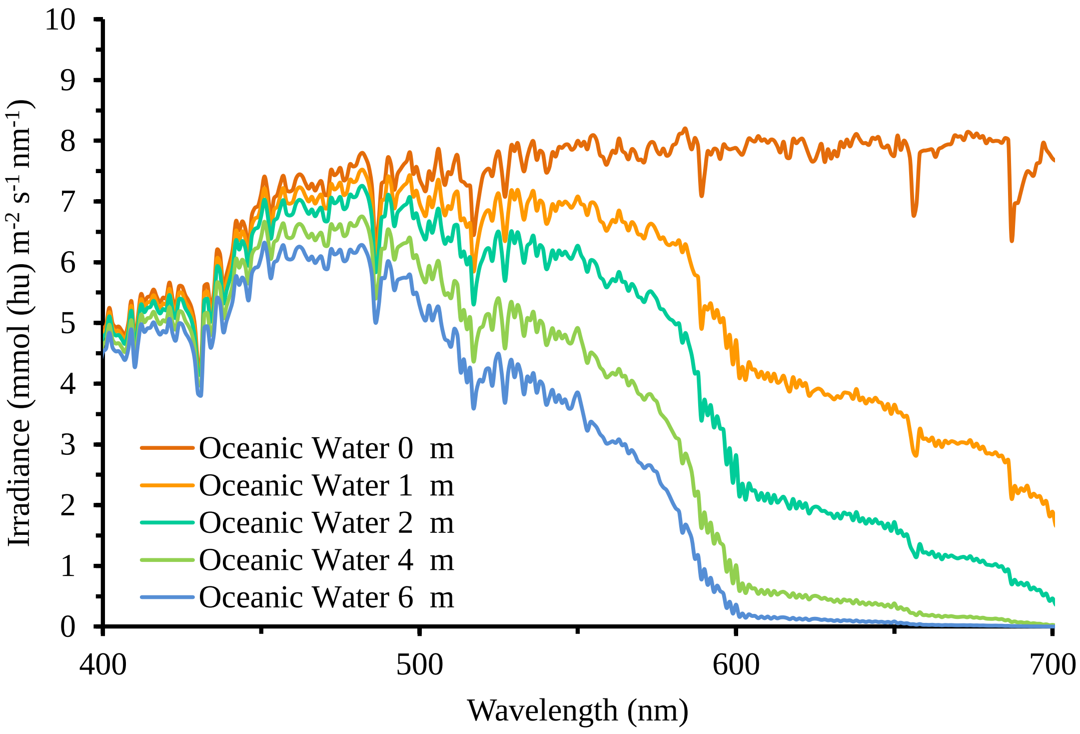


Fig. 24 Predicted intensity of irradiance in range of photosynthetically active radiation (PAR) in different water depths.

3.2 Photosynthetic responses of the seagrasss H.ovalis

3.2.1 Maximum relative electron transport rate (rETR$_{max}$)

There were significant differences in rETR$_{max}$ among zones, sites and months (p<0.001). The highest rETR$_{max}$ was in the upper intertidal zone of YL in July 2012, 11.75±1.14 µmol e^{-1} m^{-2} s^{-1} whereas the lowest rETR$_{max}$ was in the subtidal zone of YL in December 2012, 1.85±0.17 µmol e^{-1} m^{-2} s^{-1}. Significantly greater rETR$_{max}$ were found in the upper intertidal zone of YL in December 2012 and upper intertidal zone of KM in February 2013. Significantly lower rETR$_{max}$ were found in the subtidal zone of KM in July 2012, subtidal zone of YL in December 2012, subtidal zone of YL and KM in February 2013 and subtidal zone of YL and KM in May 2013 (Fig. 25a; Table 5).

3.2.2 Optimum light intensity for photosynthesis (E$_{opt}$)

There were significant differences in E$_{opt}$ among zones, sites and months (p<0.001). The highest E$_{opt}$ was in the upper intertidal zone of YL in December 2012, 817.61±60.72 µmol photons m^{-2} s^{-1}, whereas the lowest E$_{opt}$ was in the subtidal zone of KM in December 2012, 295.61±23.65 µmol photons m^{-2} s^{-1}. Significantly greater and lower in E$_{opt}$ were presented at the upper intertidal and subtidal areas, respectively (Fig. 25b; Table 5).

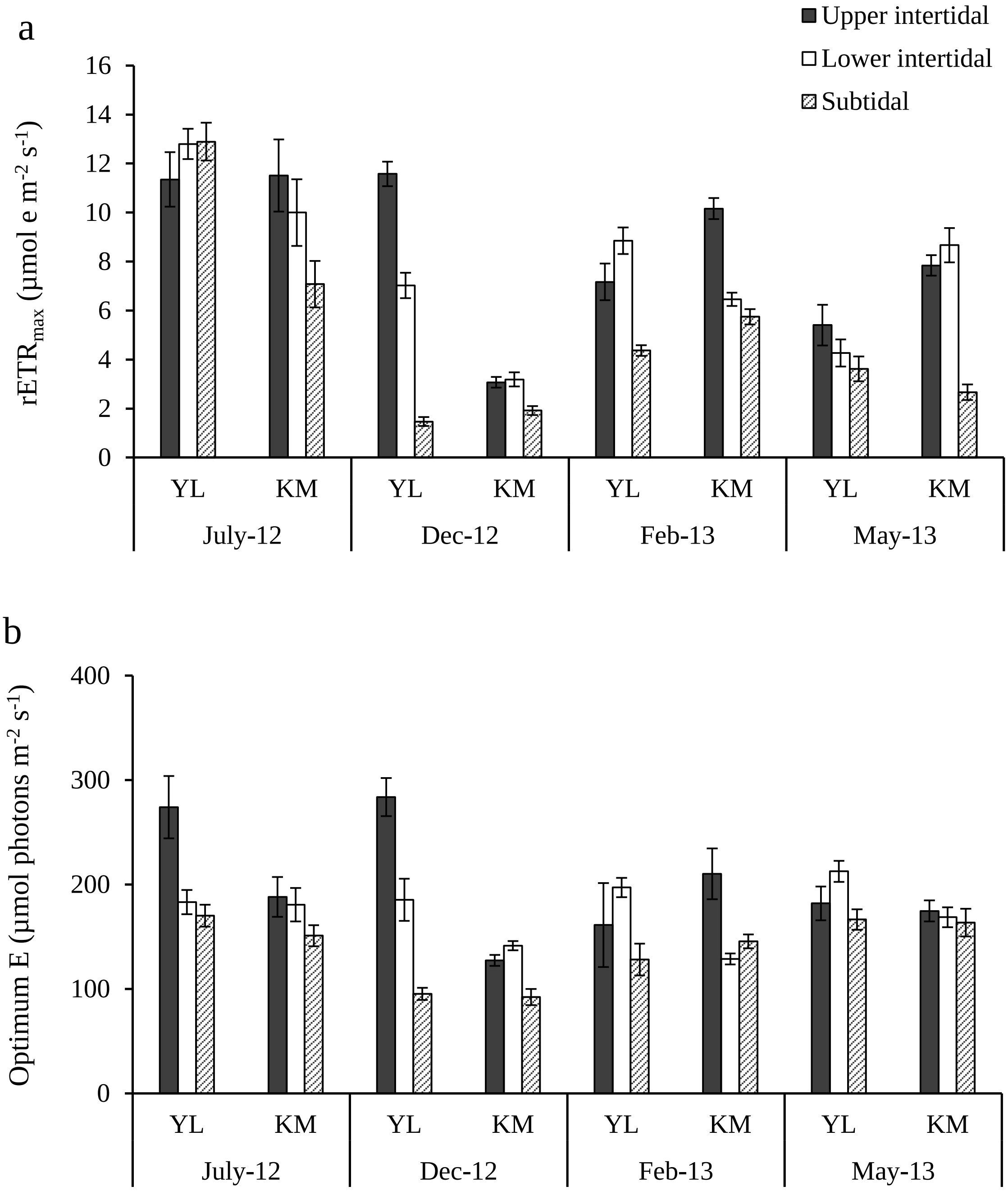


Fig. 25 Photosynthetic parameters of *H. ovalis* measured in three tidal zones at two sites during July 2012 to May 2013 (mean±S.E., n=9-20); (a) maximum relative electron transport rate and (b) optimum irradiance for plant photosynthesis.

3.3 Leaf pigment contents of the seagrass H. ovalis

3.3.1 Chlorophyll content

There were significant differences in chlorophyll *a* content among zones and months ($p<0.001$ and $p=0.001$). The greatest chlorophyll *a* content was in the subtidal zone of KM in December 2012, 11.28±0.31 µg cm^{-2} and the lowest content was in the upper intertidal zone of KM in July 2012, 7.49±0.50 µg cm^{-2}. Chlorophyll *a* content in the subtidal zone was significantly greater than the others in December 2012 at both study sites and in February 2013 at YL while the significantly lower chlorophyll *a* content was in the upper intertidal zone of KM in July 2012 and in the upper intertidal zone in December 2012 (Fig 26a; Table 5).

There were significant differences in chlorophyll *b* and total chlorophyll contents among zones and months ($p<0.001$). The greatest chlorophyll *b* and total chlorophyll contents were in the subtidal zone of KM in December 2012, 5.60±0.20 µg cm^{-2} and 16.88±0.48 µg cm^{-2}, respectively. The lowest chlorophyll *b* content was in the upper intertidal zone of YL in December 2012, 3.34±0.16 µg cm^{-2} and the lowest total chlorophyll content was in the upper intertidal zone of KM in December 2012, 11.22±0.73 µg cm^{-2}. Chlorophyll *b* and total chlorophyll contents in the subtidal zone were significantly greater than the others in December 2012 at both study sites and in February 2013 at YL while the significantly lower chlorophyll *b* and total chlorophyll contents was in the upper intertidal zone (Fig 26b and 26c; Table 5).

There were significant differences in Chl. *a*:*b* ratio among zones and months ($p<0.001$). The highest Chl. *a*:*b* ratio was in the upper intertidal zone of YL in December 2012, 2.42±0.08 whereas the lowest Chl. *a*:*b* ratio was in the subtidal zone of KM in February 2013, 1.93±0.01 (Fig 26d; Table 5).

3.3.2 Carotenoid content

There were significant differences in carotenoid content among zones and months (*p*=0.007 and *p*<0.001). The greatest carotenoid content was in the subtidal zone of KM in December 2012, 5.13±0.16 µg cm^{-2}, whereas the lowest carotenoid content was in the lower intertidal zone of YL in July 2012, 2.68±0.19 µg cm^{-2}. Carotenoid content in the subtidal zone was significantly greater than the others at KM in December 2012 and YL in February 2013 (Fig26e; Table 5).

3.3.3 Anthocyanin content

There were significant differences in anthocyanin content among zones, sites and months (*p*<0.001). The greatest anthocyanin content was in the upper intertidal zone of YL in February 2013, 0.81±0.07 µg cm^{-2}, whereas the lowest anthocyanin content was in the subtidal zone of YL in May 2013, 0.02±0.00 µg cm^{-2} (Fig 26f; Table 5).

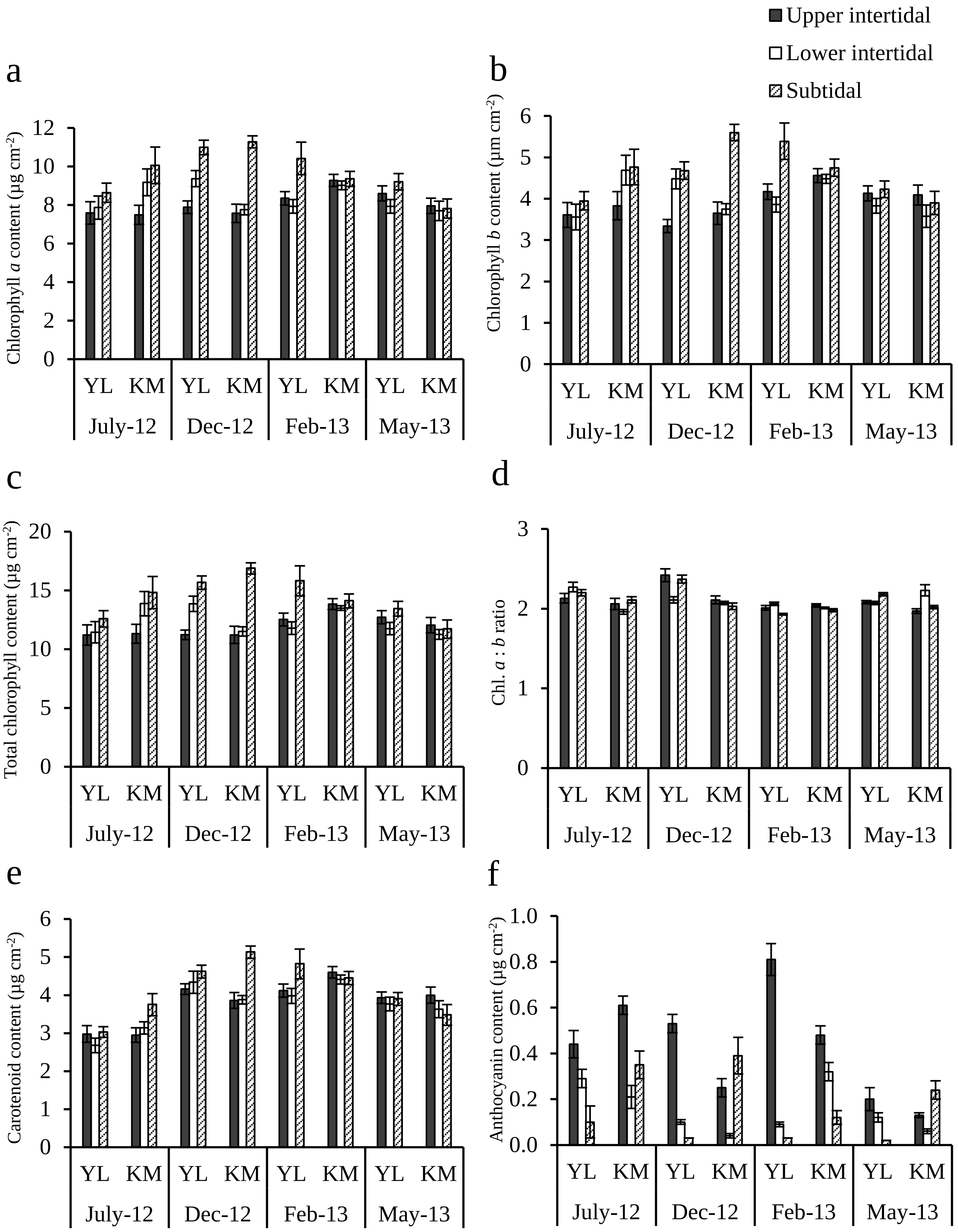


Fig. 26 Pigment content of *H. ovalis* measured in three tidal zones in two sites during July 2012 to May 2013 (mean±S.E., n=20); (a) chlorophyll *a* content, (b) chlorophyll *b* content (c) total chlorophyll content (d) Chl. *a*:*b* ratio, (e) carotenoid content and (f) anthocyanin content.

Table 5. Summary of statistical analyses of photosynthetic characters and pigment content showing the results of three-way analysis of variance (Z x S x M) and for any possible combination of factors: Z = zone, S = site and M = month and Kruskal Wallis test.

Variables	Transformation method	Z	S	M	Z x S	Z x M	S x M	Z x S x M
F-statistic and Chi-square (Kruskal Wallis test)								
rETR$_{max}$ (µmol e m^{-2} s^{-1})	n	64.017***	1.019	97.646***	67.311***	173.712***	147.860***	251.173***
E$_{opt}$ (µmol photons m^{-2} s^{-1})	n	68.190***	18.282***	27.362***	94.720***	104.276***	60.746***	162.552***
Chlorophyll *a* content (µg cm^{-2})	$\sqrt{X}$	23.956***	0.048	5.967**	0.271	4.506***	3.854*	2.028
Chlorophyll *b* content (µg cm^{-2})	$\sqrt{X}$	18.905***	3.356	6.793***	0..006	4.728***	3.534*	3.304**
Total chlorophyll content (µg cm^{-2})	$\sqrt{X}$	22.679***	0.221	5.991**	0.140	4.545***	3.648*	2.416*
Chlorophyll *a*:*b* ratio	n	0.037	36.487***	53.031***	37.926***	80.352***	108.802***	168.371***
Carotenoid content (µg cm^{-2})	n	9.850**	0.810	122.395***	11.100*	145.030***	130.395***	163.964***
Anthocyanin content (µg cm^{-2})	n	137.201***	12.787***	27.889***	218.963***	204.478***	44.213***	213.301***

n = non-parametric Kruskal Wallis test; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

4. Discussion

Photosynthetic activities at the individual leaf level of *H. ovalis* varied with tidal levels and seasons. Generally, higher maximum relative electron transport rates ($rETR_{max}$) were found in the intertidal seagrass and significantly lower rates were found in the subtidal seagrass. The results showed the same pattern as the study of Campbell *et al.* (2007) who found higher ETR_{max} was generally found for intertidal compared with subtidal plants. Greater $rETR_{max}$ in the intertidal area might be supported by higher irradiance together with readily available carbon source due to rapid diffusion of CO_2 in air which compared to the diffusion limited availability in water (Silva *et al.*, 2005). Gaseous CO_2 available during low tide has been shown in a previous study to support photosynthetic rates which were three times higher using CO_2 gas than in seawater (Beer and Waisel, 1979). Although, maximum photosynthesis is water dependent, the thin flexible petiole of *H. ovalis* allowed plant leaves to lie on moist sand and keep a favorable level of water content for supporting photosynthesis during emergence (Björk *et al.*, 1999; Silva *et al.*, 2005), unlike in bigger species such as *Z.ostera noltii* where more net photosynthesis was reduced more than 50% by a 30% decrease in leaf water content after 5 hours exposure to air (Leuschner *et al.*, 1998).

The lower ability to photosynthesize might relate to physiological process of seagrasses which are affected by sulphide-rich conditions which usually occur in subtidal sediments. Sulphide toxicity is known to reduce photosynthesis and increase plant respiration rate (Goodman *et al.*, 1995; Holmer and Bondgaard, 2001). In this study, $rETR_{max}$ of the seagrass *H. ovalis* was in the range between 1.85-11.75 µmol e m^{-2} s^{-1} which was much lower than those reported in the same seagrass species in other tropical regions: 44.7-200.0 µmol e m^{-2} s^{-1} in intertidal seagrass (Ralph *et al.*, 1998; Beer and Björk, 2000; Schwarz *et al.*, 2000; Beer *et al.*, 2006 Campbell *et al.*, 2007) and 20.8-143.2 µmol e m^{-2} s^{-1} in subtidal seagrass (Schwarz *et al.*, 2000; Campbell *et al.*, 2007). Diurnal change of ETR_{max} of *H. ovalis* investigated in an upper intertidal pool occurred during submergence in pre-noon hours and reached a maximum value of approximately 100 µmol e m^{-2} s^{-1} which is two times greater than those in emerged leaves because of a low degree of photoinhibition (Beer *et al.,* 2006) as a large proportion of incident light is absorbed by the protective layer of water (Ralph and Burchett, 1995).

Most studies have indicated that seagrass saturate at very low light intensity (Hillman *et al*. 1995). E_{opt} of *H. ovalis* in this study was 295.61-817.61 µmol photons m^{-2} s^{-1} which is in the range that has been reported and is typical of a shade plant. E_{opt} of *H. ovalis* in this study was generally higher in the intertidal zone (401.20-817.61 µmol photons m^{-2} s^{-1}) than in subtidal zone (295.61-518.41 µmol photons m^{-2} s^{-1}), our result showed the same trend with previous studies which reported that E_{opt} of intertidal *H. ovalis* ranged from 193.1-947.0 µmol photons m^{-2} s^{-1}, whereas in plants growing in subtidal areas showed the E_{opt} at 83.96-630.80 µmol photons m^{-2} s^{-1} which varied greatly among study sites even when they were measured in plant growing in the same tidal level (Campbell *et al*., 2007). Such results might be altered by differences in the local environmental conditions in each area. Intertidal *H. ovalis* showed an E_{opt} of 242 µmol photons m^{-2} s^{-1} while 10 m depth subtidal plants were saturated at 114 µmol photons m^{-2} s^{-1} (Schwarz *et al*., 2000). However, E_{opt} values found in this study were still lower than light availability in their subtidal habitat, 491.98-785.97 µmol photons m^{-2} s^{-1} suggesting that light was not the limiting factor for seagrass photosynthesis in our study.

Greater chlorophyll *a, b,* total chlorophyll and carotenoid contents in the subtidal zone allowed plants to maximize light harvesting efficiency in blue wavelengths region which penetrated through the subtidal habitat while light in red wavelengths were likely to disappear at a depth of 6 meters (Fig. 1). Long exposure time of seagrasses in the upper intertidal zone might induce photodamage action in photosynthetic pigment, they might better invest their energy to accumulate the photoprotection pigment and have lower chlorophyll content than in deeper areas. Chorophyll *a, b* and total chlorophyll contents in plants found in our study were similar to those found by Ralph (1999, 2000). The Chl *a*:*b* ratio in this study (1.93-2.42) were higher than those of Ralph’s studies (1.20-1.60) where plants were growing in lower natural light conditions under 120-150 m^{-2} s^{-1} µmol photons which might indicate the acclimation mechanism to absorb light by increasing chlorophyll *b* content.

Greater anthocyanin content was generally found in the upper intertidal zone, reflecting the well known importance of anthocyanin synthesis in protection against UV irradiance (Trocine *et al*., 1981; Holton and Cornish, 1995; Close and Beadle, 2003). The photoprotection purpose of anthocyanin accumulation is one of the responses to natural high light stress typically found in higher plants. In this study, intertidal seagrasses were exposed to high light levels of 865.14-1488.70 µmol photons m^{-2} s^{-1} which is higher than irradiances reported to induce photoprotective processes in *H. ovalis* for at least the first 60 minutes exposure to 500 µmole photons m^{-2} s^{-1} and photodamage appeared after 120 minutes exposure to 1,000 µmole photons m^{-2} s^{-1} (Ralph and Burchett, 1995). Long time exposure to high irradiance for 4 hours during summer low tide would have been long enough to inhibit the physiological activities of seagrass, thus synthesis of a flavonoid pigments such as anthocyanin would be advantageous to the plant. This correlates with the extensive red leaves of *H. ovalis* plants with more than 80% coverage of anthocyanin on leaf surfaces mainly presented in the upper intertidal zone, whereas the plants in the subtidal zone showed only less than 20% coverage of small red spots and some leaves were completely green with no observable anthocyanin. Clearly higher anthocyanin content was present in the dry season, (February 2013) only in seagrass growing in the upper intertidal zone of YL. This may be because YL has longer exposure period than the upper intertidal plants at KM. The lowest anthocyanin content of both study sites occurred in the early rainy season (May 2013). Reduced anthocyanin content may be caused by the interaction effects of lower light intensity, temperature and higher sediment runoff which might reduce light condition in this month by high suspended sediment in the water column.

CHAPTER 5

CONCLUSION

In conclusion, environmental change as the consequences of tidal exposures and seasonal variations influenced growth parameters, morphology, anatomy and physiology of the seagrass *H. ovalis*.

Variations in shoot density and leaf size suggesting the benefit of maintaining the favorable leaf water content as smaller leaves can reduce the rate of water loss in emergence condition. High density and biggest leaf size of lower intertidal seagrass resulted in greatest above-ground biomass and also annual productivity of plant in this area. Seasonal change in above-ground biomass affected the seasonal change of total biomass production while seasonal variation in below-ground biomass was not observed. Slowest growth rate presented in *H. ovalis* grow in the upper intertidal zone which might be affected by desiccation stress.

The biggest leaf size found in the lower intertidal zone indicate better condition for plant growth as less stress than the upper intertidal and better light condition than deeper area, thus the variations of internal structures, mesophyll cell size and air lacunar size are likely to present as the allometric factor relate to plant size of seagrass leaves.

Intertidal seagrass showed the better photosynthesis than those seagrass which always submerged in subtidal area suggesting the benefit of high light level combined with readily inorganic carbon source in air which quickly diffused into plant cells. Data derived from light response curves suggested that light was not limiting factor for plant growth due to light available still greater than the optimum light requirement of *H. ovalis* in three tidal zones. If light intensity sufficient for plant photosynthesis, variation in photosynthetic pigment would not reveals photoacclimation of seagrass. Increment in chlorophyll and carotenoid contents in the subtidal seagrass leaves provide benefit to maximize light harvesting efficiency in blue wavelength region at deeper water where red spectrum of light likely to disappear. High anthocyanin content in the upper intertidal seagrass leaves was suggested to serve the protection function against long time exposure to high irradiance during low tide in order to maintain the high level of photosynthesis by protecting the other photosynthetic apparatuses. Greater anthocyanin content in the dry season due to the long exposure period than the others and lower anthocyanin in early rainy season involved light deprivation caused by high sedimentation rate.

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List of publication and proceeding

- Kaewsrikhaw, R. and Prathep, A. 2014. The effect of habitats, densities and seasons on morphology, anatomy and pigment content of the seagrass *H. ovalis* (R.Br.) Hook.f. at Haad Chao Mai National Park, Southern Thailand. Aquatic Botany. 116: 69-75.
- Kaewsrikhaw, R. and Prathep, A. Variations of tidal exposures and seasons on growth, morphology and anatomy of seagrass *Halophila ovalis* (R.Br.) Hook.f. at seagrass bed in Trang Province (submitted manuscript).
- Kaewsrikhaw, R. and Prathep, A. Variations of tidal exposures and seasons on photosynthesis and pigment content of seagrass *Halophila ovalis* (R.Br.) Hook.f. at seagrass bed in Trang Province (submitted manuscript).