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Seagrass responses to interacting abiotic stresses

Yayu A. La Nafie



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**Yayu Anugrah La Nafie
2016**



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Seagrass responses to interacting abiotic stresses

DOCTORAL THESIS

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CHAPTER 1

General Introduction



1.1 INTRODUCTION

1.1.1 What are seagrass meadows and where do they occur?

Seagrasses are clonal marine flowering plants (angiosperms) that consist of a bundle of leaves, rhizomes with roots (Fig.1.1), flowers and seeds, and obtain their energy via photosynthesis. However, they are unlike terrestrial plants that possess strong, supportive stems and trunks to overcome the force of gravity on land. Instead, seagrass leaves possess air spaces (lacunae) that can create buoyancy to make them to stand upright while being flexible enough when exposed to waves currents (Hemminga and Duarte 2000). The most commonly found seagrass are those with ribbon-like leaves, whilst the others can have, e.g. oval-shaped, oblong or cylindrical leaves. Leaves are often present in bundles on seagrass rhizomes, a bundle of leaves can contain 1 (e.g. in *Syringodium*) to over 10 leaves (such as in *Amphibolis*). In contrast, most *Halophila* species comprise of a pair of petiolate-leaves (Hemminga and Duarte 2000). Leaves can be as long as 8 meters (such as in *Zostera caulescens*, Lee and Lee 2003) but can also be as short as 1 cm (such as in *Halophila ovalis*, Hemminga and Duarte 2000).

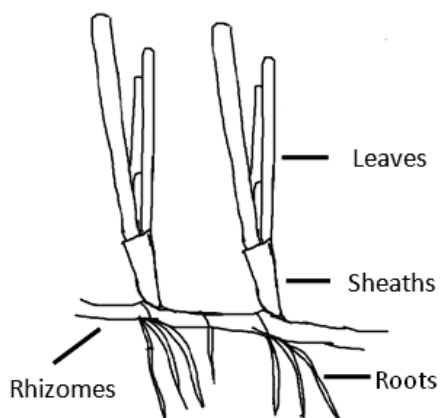


Figure 1.1 A typical seagrass shoot, consisting of a bundle of leaves, sheaths, roots and rhizomes

Seagrasses can be found in shallow coastal areas in the tropics and in temperate regions and may form monospecies or mixed species meadows. Seagrass depth distribution depends on light availability in the water and can range from 1m (in turbid systems) to 30-61 metres in very clear waters (Beer and Waisel 1982; Erftemeijer and Stapel 1999; Coles et al. 2011). In addition,

the upper depth distribution is generally determined by desiccation during exposure or hydrodynamic stress (waves/currents) (Erftemeijer 1993; van Katwijk et al. 2000; van der Heide et al. 2010). The global distribution of seagrasses can be categorized into 6 geographic bioregions for seagrass in the world, namely 1) temperate North Atlantic, 2) tropical Atlantic, 3) Mediterranean, 4) temperate North Pacific, 5) tropical Indo-Pacific, and 6) temperate southern oceans (Fig. 1.2). The diversity of seagrass of the Indo-Pacific region is considered to be very high (24 species) compared to other tropical regions like the Caribbean Sea, Bermuda, the Bahamas, Gulf of Mexico and both the tropical coasts of the Atlantic (10 species). Only 4 species of all global seagrass are native European. On a global scale, seagrasses are however declining (Orth et al. 2006; Short et al. 2007; Waycott et al. 2009).

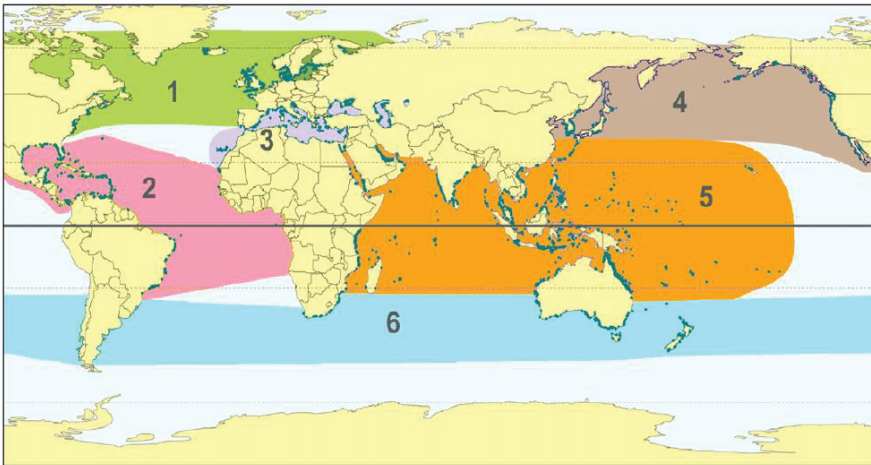


Figure 1.2 Seagrass distribution (blue dots) and their geographic bio-regions: 1) temperate North Atlantic (green-shaded), 2) tropical Atlantic (pink-shaded), 3) Mediterranean (purple-shaded), 4) Temperate North Pacific (brown-shaded), 5) tropical Indo-Pacific (orange-shaded), 6) temperate southern oceans (blue-shaded) (source: Short et al., 2007).

1.2 WHY CARE ABOUT SEAGRASS?

1.2.1 Seagrass Ecosystem services and functions

Seagrass ecosystems belong to the most productive ecosystems in both the marine and terrestrial environment and may be regarded as equivalent to the

tropical rainforest in terms of their productivity, complexity and the diversity of the organisms associated with them. They typically have a high species richness (Hemminga and Duarte 2000), as they can act as feeding, breeding and nursery grounds for numerous marine organisms including economically valuable ones, such as shrimps, scallops and finfish (Watson et al. 1993; Unsworth et al. 2010; Barbier et al. 2011). For example, the prawn industry in an area of North Queensland, Australia, was estimated to yield 178 ton annually, equivalent to landed values of US\$ 1.1 million annually (Watson et al. 1993), whereas in Indonesia, seagrass related fisheries can be worth approximately a minimum of US\$230 million (Unsworth et al. 2010). Apart from its economic importance for fisheries, seagrass ecosystems can also act as water purifier as they can increase water clarity via nutrient uptake and suspended particle deposition. They can remove nutrients both from the sediment and water column (Stapel et al. 1996; Duarte 2002; Orth et al. 2006) and with the algae (the epiphytes) colonizing their leaves, nutrients are further removed from the water column (Cornelisen and Thomas 2006). When the plants decompose, nutrients that were incorporated into the seagrass and algae tissue can slowly be released back into the water column, or be buried in the sediment and hence removed from the nutrient cycle (Romero et al. 2006). Seagrass also can act as carbon sink and can store as much as 83.000 metric tonnes of carbon per square kilometer (Fourqurean et al. 2012). Physically, seagrass also can act as coastal protection by their ability to attenuate wave energy hence stabilizing the sediment (Fonseca and Fisher 1986, Bouma et al. 2005). This ability can also be beneficial to their neighboring ecosystems. For example, in the tropics, seagrass interact with mangrove and coral reef ecosystems (Gillis et al. 2014). As seagrass slows water movement, they cause suspended sediment to settle, thereby decreasing water turbidity, sustaining light intensity for plants' photosynthetic activity. This of course will also benefit downstream coral ecosystems. Overall, this creates positive feedbacks that stabilize the environment (Gillis et al. 2014).

1.2.2 Seagrasses are declining

Despite all seagrass functional values, we are losing the seagrasses in an alarming rate (Orth et al. 2006; Short et al. 2007; Waycott et al; 2009). It has been reported from most parts of the world, that losses are sometimes due to natural causes, e.g., high-energy storms and "wasting disease". More commonly, loss resulted from eutrophication (Burkholder et al. 2007) as a result of increasing nutrient runoffs due to fertilizer usages from agriculture and aquaculture. Changes in land use, that increase turbidity of the water such as land reclamation, logging, sand-mining, also impose threats to tropical

seagrasses (Freeman et al. 2008; van Katwijk et al. 2011; Christianen et al. 2012). In addition to that, the world's population is increasing, and more than 50% of them live within 60 km from the coasts (Waycott et al. 2009). This may lead to increasing human impacts on seagrass meadows that are continuing to destroy or degrade these coastal ecosystems and decrease their yield of natural resources (Waycott et al. 2009). All of these activities have serious consequences for marine biodiversity and for the human population that (directly) depends on the seagrass ecosystem services and functions. We still lack information of how seagrass can cope with these environmental threats, regardless of their cause being anthropogenic or natural. Obtaining more insight in such mechanisms would be highly useful for managing our seagrass ecosystem, and for preventing any further seagrass loss.

1.3 STRESSES TO SEAGRASS THAT MAY CAUSE DECLINE

1.3.1 Nutrients

Seagrasses need nutrients -like all other plants do- to be able to maintain their high productivity. However, nutrient levels can be stressful to seagrass either by being too low or too high.

In the case of oligotrophic conditions, nutrients are a stress because they limit seagrass growth and photosynthetic performance (Agawin et al. 1996). Oligotrophic conditions are often occurring in tropical waters where nutrients in the water column sometimes cannot be detected (Erftemeijer et al. 1994, Stapel et al. 1996, Vonk et al. 2008b). However, seagrass in oligotrophic waters are flourishing and some may wonder how these plants can survive in such low nutrient concentrations. The availability of high light intensity in oligotrophic tropical environments can sustain seagrass photosynthetic activity (Ralph et al. 2007). Seagrass' ability in taking up nutrients by both their leaves and roots (Short and McRoy 1984, Stapel et al. 1996, Lee and Dunton 1999) benefits seagrass in oligotrophic environments, as these uptakes are sometimes considered to fulfill the nutrient demand for seagrass (Stapel et al. 1996). In addition to that, recent studies show that seagrass is also able to take up dissolved organic nitrogen in addition to dissolved inorganic nitrogen (Vonk et al. 2008a, Van Engeland et al. 2010). In other cases in the tropics, low nutrient condition is a stress to seagrass as occurred in seagrass of Cape Bolinao, the Philippines (Agawin et al. 1996). After an *in situ* nutrient enhancement experiment it was found that seagrass growth, leaf length, shoot length, and biomass were increased. A nutrient limitation was also shown in Green Island,

Great Barrier Reef, Australia, where seagrass distribution and biomass were increased due to an increasing nutrient availability from the tourism and the installation of a sewage outfall (Baxter 1990). We lack information of how a temperate seagrass can survive when nutrients are available in low concentrations. In this thesis, we studied how temperate seagrass (*Zostera noltii*) can get enough nutrients in oligotrophic conditions (**chapter 2**) which further can be used in the efforts in nutrient reduction of temperate waters.

In the case of eutrophic conditions, further nutrient enrichment generally results in negative effects on seagrass growth and survival, either directly due to the toxic effect of nitrate (Burkholder et al. 1992) or ammonium (van Katwijk et al. 1997, Brun et al. 2002), or indirectly due to algal overgrowth that can cause light reduction and enhanced organic matter fluxes to the sediment (Short et al. 1995, Brun et al. 2003). Many studies have reported that seagrasses are disappearing around the globe due to eutrophication (Short and Wyllie-Echeverria 1996, Green and Short 2003, Short et al. 2006, Waycott et al. 2009, Short et al. 2006). This eutrophication is mainly due to fertilizer usage and sewage release (Short and Wyllie-Echeverria 1996, Cabaço et al. 2008). The sediment nutrient pool in seagrass ecosystems can result in positive effects in seagrass growth. It can increase seagrass density (Agawin et al. 1996) leaf length and biomass (Short et al. 1984, Lee and Dunton 2000), therefore creating a positive feedback on seagrass sediment trapping capacity and sediment stabilization capacity, hence reducing sediment erosion due to high hydrodynamic stress. However, we lack information of how sediment nutrients affect the mechanical properties of seagrass, while these traits are important in knowing how seagrasses or an organism can cope with highly dynamic environment. In this thesis we studied how increasing sediment nutrient pools affect mechanical properties (in addition to their morphological properties), on two slow-growing tropical seagrass species (**chapter 3**).

1.3.2 Light

As primary producers, seagrasses need sufficient light to conduct photosynthesis (Ralph et al. 2007). Light controls seagrass distribution horizontally and vertically (Ralph et al. 2007). Depending on the light availability, seagrasses can be found in 30-61 m water depth (Beer and Waisel 1982, Stapel and Erftemeijer 1999, Coles et al. 2011). In the tropics, high light intensity is occurring all year-long, so that – as long as the water is clear – seagrasses have more than enough light to conduct photosynthesis. However, due to the increasing anthropogenic activities in the coastal areas that lead to water turbidity, (e.g. intensive logging, agriculture such as e.g. palm-oil plantations, aquaculture, sewage discharge, beach-sand and coral mining), light

has become a stress causing seagrass decline (Sloan and Sughandy 1994, Freeman et al. 2008, van Katwijk et al. 2011). Another cause of light attenuation is by natural events (e.g. storm events) that increase turbidity in the coastal areas. Many studies have looked at seagrass morphological and physiological response to sediment enrichment. However, despite the ecological importance, we have no information how seagrass respond mechanically to sediment enrichment. Hence, in this thesis we studied how two slow-growing tropical seagrass species respond morphologically and mechanically to light stress (**Chapter 3**).

1.3.3 Hydrodynamics

Hydrodynamics are important for seagrass dispersal and seedling distributions (Koch 1994). They can reduce the diffusive boundary layer hence maintaining CO₂ supply for sustaining seagrass productivity (Koch 1994) and increase nutrient uptake (Thomas and Cornelisen 2003, Morris et al. 2008). Hydrodynamics are also important for nutrient transport from outside seagrass ecosystems into seagrass meadows. Despite these positive effects, the physical forces due to increasing hydrodynamics are also a stress to seagrass survival and growth (van Katwijk and Hermus 2000, Schanz and Asmus 2003). Flow velocity can directly reduce plant growth morphometry (Schanz and Asmus 2003, Peralta et al. 2007, de los Santos et al. 2010) and can result in mechanical failure (including loss of tissues or even uprooting) (Schanz and Asmus 2003, van Katwijk and Hermus 2000). They can also increase turbidity, hence decreasing light intensity, and affect seagrass productivity. Hence, hydrodynamics are an important factor that determines the existence of seagrass ecosystems. Especially since the environment is continually changing (e.g. due to the climate change) it is necessary to include hydrodynamic effects in seagrass research. In this thesis, we present how hydrodynamics can influence biomechanical and morphological properties of tropical and temperate seagrass species (**Chapter 4, 5**).

1.3.4 Interacting stresses (Nutrients and waves)

In their natural condition, seagrasses are not merely experiencing single stress. They may encounter multiple stresses such as water dynamics and nutrient resource availability. There are many studies conducted on the effect of single stress on seagrass (e.g. van Katwijk and Hermus 2000, Schanz and Asmus 2003, Brun et al. 2006, 2010, Peralta et al. 2007) but relative little is known on the effect of multiple stresses that are likely to co-occur such as waves and nutrients. As they occur in coastal ecosystems, seagrasses are prone

to hydrodynamic stress interacting with nutrient stress, and this interaction is expected to rise due to both the increasing storm frequency as a result of climate change and anthropogenic activities near the coast. Seagrass response to high nutrients as a single stress had been studied by many scholars. They showed that nutrient enrichment may cause positive response (Perez et al. 1991, Brun et al. 2002), negative response (Burkholder et al. 1992, van Katwijk et al. 1997, Brun et al. 2002) or may have no response (Harlin and Thornemiller 1981, Lee and Dunton 2000). However, we lack information on how interacting stress of hydrodynamics and nutrient stress would impact on seagrass. Hence, in this thesis, we show how waves interacting with nutrients affect seagrass mechanical and morphological properties in tropical species in the field (sheltered versus exposed) (**chapter 4**), and in a laboratory experiment on temperate species **chapter 5**).

1.4 WHAT ARE RELEVANT RESPONSE PARAMETERS WHEN STUDYING STRESS TO SEAGRASS ?

Seagrass response to stresses can be observed through changes in their physiological traits (e.g. through their tissues nutrient content), survival, physical traits (e.g. in biomass, growth and morphologies) and in their mechanical traits (e.g. how seagrass can cope with physical forces before they break). Hence, this thesis focuses on how environmental individual and multiple stresses can influence seagrass physiological response (**chapter 2**), morphological, and biomechanical response (**chapter 3, 4 and 5**) and survival (**chapter 5**).

1.4.1 Physiological response (Uptake capacity and tissues' nutrient content)

Seagrasses show different responses to stresses they encounter. One of these responses is their capability to take up nutrients from the water column. Early studies show that seagrasses take up inorganic nutrients from the sediment as well as from the water column (Stapel et al. 1996, Short et al. 1994). Only recently it was shown that seagrass can also take up organic nitrogen (Vonk et al. 2008a). In addition to that, Van Engeland et al. (2010) showed that seagrass take up organic nitrogen based on the complexities of the nitrogen compound. We are aware that seagrasses are confronted with different nitrogen compound pools in their systems. However, we lack knowledge on how seagrass uptake responds physiologically to these nitrogen compound pools. Hence, we measured this in **chapter 2**.

1.4.2 Seagrass morphology

Seagrass can respond to their environments through developmental plasticity (Peralta et al. 2007). Seagrass in high nutrient environment may grow better (e.g. by having longer and wider leaves, Short et al. 1984) compared with those in limited nutrient environment. Seagrass can show varieties of morphometric responses to (abiotic) stresses that sometime are difficult to predict. However, seagrass (aboveground) morphology is a prominent feature that would be easy to use to indicate seagrass response to stress. In addition, through morphological features, we may be informed that there is a high level of interconnection among ecological, physiological and developmental features of an organism (Koehl and Wainwright 1985). In this thesis, we measured seagrass morphology in response to environmental stress in **chapter 3, 4, and 5**.

1.4.3 Seagrass mechanical properties

Biomechanical properties are basically the response of an organism to survive physical stresses. Studying biomechanical properties requires an engineering approach. This approach has long been used in many marine algae (Koehl and Wainwright 1977, Koehl 1984, 2000, Armstrong 1987, Dudgeon and Johnson 1992, Dames et al, 2013), corals (Koehl. 1984, 1988), followed by terrestrial plants (Onoda et al. 2011, Anten et al. 2005) and freshwater plants (Puijalon et al 2008). The instrument used to measure mechanical properties is called the tensometer. It measures the strength, stiffness and toughness of a piece of material or tissue, for instance in a force-extension test (Koehl and Wainwright 1985). After a tissue or a material is placed between the upper and lower grip of the tensometer, it will be pulled slowly apart by applying specific velocity of the tensometer with a specific force (e.g. 5N). When the material breaks, the machine directly records the breaking force (F_{MAX}), i.e. the maximum force (N) needed to break a material, and also the extensibility of the material (L_T), or the breaking strain (dimensionless; $mm\ mm^{-1}$). When material length and width were input to the computer prior to the mechanical measurement, the machine can also directly record the material specific force-to-tear (F_{TS}). This trait is also known as breaking stress (Nmm^{-2}) or as the result of breaking force (F_{MAX}) divided by the cross-sectional area (ca) (i.e. thickness x width) of the tissue. When the value of F_{TS} is high, it means that the material/tissue is strong, and when the value of L_T is high, it means that the material/tissue is extensible. Sometimes a material only posses one or two prominent mechanical response(s), which may (or may not) depend on its morphology. Recently, a few studies on seagrass mechanical properties have started to emerge: in mesocosm and field studies to see the effect of nitrate and

light) (Kopp 1999) and in correlative field studies (Patterson et al 2001; de los Santos 2012; 2013)). However, we are still lacking information on how seagrass responds mechanically to environmental stress. Hence, we measured seagrass mechanical response to nutrient and light stress (independently) (**chapter 3**) and interactively to wave-nutrient stress (**chapter 4 and 5**).

1.5 OBJECTIVES OF THIS THESIS

The objective of this thesis is to answer the question “*How do seagrasses respond physiologically, morphologically and mechanically to single and multiple abiotic stresses, and what are the ecological consequences of these responses*”? It is hypothesized that environmental conditions affect seagrass physiological performance (through nutrient uptake capacity and their tissues nutrient concentration), the survival, morphological as well as mechanical responses. These responses can be resource, (intra and inter) species, and spatially specific. We tested the response by exposing seagrass to low nutrient stresses in a laboratory experiment (**Chapter 2**), conducting an in situ experiment with nutrient and light stress (**Chapter 3**), and conducting a field observation with a matrix of anthropogenic impact (nutrient source) and wave exposure (**Chapter 4**). In addition, we also studied how seagrass responds to interacting stresses of nutrient and hydrodynamics (**Chapter 5**).

The hypotheses are explained further in the **chapters 2 to 5** and are summarized in Table 1.1. In the last chapter (**Chapter 6**) discussion is provided to answer the main questions of how seagrass would respond to (various) environmental stresses, and the potential ecological consequences.

Table 1.1 Summary of questions and hypotheses in this thesis

Stress	Response parameter	Questions	Hypothesis	Chapter
Low (in)organic nutrients	Nutrient uptake; nutrient tissue concentration	How does temperate seagrass <i>Zostera noltii</i> response to (low) nitrogen compound pools	It is expected that the presence of other nitrogen components would limit the uptake of ammonium.	2
Nutrient Light	Morphology, Mechanical	How does light and (sediment) nutrients <i>independently</i> affect morphological and mechanical properties of slow-growing tropical seagrass?	Seagrass would respond negatively to low lights, and positively to high nutrients. No significant differences in responses between two slow-growing seagrass species.	3
Anthropogenic/ Pristine vs Exposure/ sheltered	Morphology, Mechanical	How plastic are 2 tropical climax species? Have inter-and intra-specific plasticity a similar magnitude? How do site differences influence plasticity of both climax species?	The slowest biggest tropical seagrass species <i>E. acoroides</i> is expected to be more plastic and have a higher breaking stress than <i>T. hemprichii</i> . Sheaths are expected to have higher breaking stress (stronger) than the leaves, due to the structural function of the sheaths in protecting young leaves by wave absorbance. Site differences cause difference responses for different species of seagrass.	4
Hydrodynamic X nutrient	Morphology, Mechanical, Biomass, Growth, Survival	How do high nutrient stress <i>interacting</i> with waves affect seagrass?	Interacting stress of high nutrients and hydrodynamics may create greater impact to seagrass than the sum of both individual stresses.	5

CHAPTER 2

Uptake of nitrogen from compound pools by the seagrass *Zostera noltii*

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ABSTRACT

*In nature, seagrasses are confronted with a compound pool of low concentrations inorganic and organic nitrogen-containing substances of varying bioavailability. Nevertheless, the majority of research on nitrogen acquisition by seagrasses has been largely limited to studies addressing a single nitrogen substrate at a time. Using a combination of one of ^{15}N -labelled substrates and one ^{14}N -labelled background substrate, we investigated how the rate of nitrogen uptake by the seagrass, *Zostera noltii* varies with nitrogen background. Leaf and root mediated uptake were studied separately for different combinations of inorganic (ammonium, nitrate) and organic substrates (urea, glycine). Ammonium uptake rates were higher than for the other substrates. However, substrate uptake was not dependent on the background nutrient. Similar patterns and uptake rates were found for above- and belowground plant parts. The dependence of uptake rate on substrate type, combined with an independence of nutrient background is explained as difference in uptake capacity, rather than substrate preference. For the dual labeled (^{15}N and ^{13}C) urea and glycine, strong relationships existed between nitrogen and carbon uptake, but with deviations from expectations under complete uptake of the molecules. Overall, this study indicates that at realistically low ambient concentrations, seagrasses can simultaneously use inorganic and organic sources for their nitrogen needs, and do not distinguish between substrates. In other words, they take up whatever is available.*

Keywords: Nitrogen uptake; seagrass; *Zostera noltii*; isotope label; inorganic nitrogen; organic nitrogen

2.1 INTRODUCTION

Like all plants, seagrasses need nitrogen (N) to maintain their high productivity. However, unlike many terrestrial plants, the resorption of N from the senescent leaves is very limited and a lot of N is lost due to the high leaf detachment (Stapel and Hemminga 1997; Romero et al. 2006). This makes seagrasses strongly dependent on external nutrient sources (Short and McRoy, 1984) from the sediment and water column (Short and McRoy 1984; Stapel et al. 1996; Touchette and Burkholder 2000).

Nitrogen is available to seagrasses as a mixture of compounds, of which some are expected to be more immediately useful to them than others. Usually, affinities for ammonium are higher than for nitrate in kinetic uptake experiments (e.g. Hasegawa et al. 2005; Alexandre et al. 2010), which is generally attributed to additional costs associated with nitrate reduction (Turpin 1991). If this increased affinity for ammonium is inherent to the organism, and exists without external stimulus (and literature shows at least that this property is very common in seagrasses in general (Touchette and Burkholder 2000), and in *Zostera noltii* in particular (Alexandre et al. 2010)), it could be called a 'constitutive preference'. In addition, nutrient-nutrient interactions have been reported, where nitrate uptake is down-regulated under increasing ammonium availability (Alexandre et al. 2010). In their uptake experiments, Alexandre and co-workers (2010) could also demonstrate an up-regulation of ammonium uptake by *Zostera noltii* under increased nitrate concentrations, which they attributed to a signaling function of nitrate in the ammonium metabolism. The latter mechanism could be addressed as an 'induced preference', where ammonium uptake is stimulated by an external factor.

Whereas for a long time nitrogen research has solely focused on dissolved inorganic nitrogen (DIN) uptake by seagrasses (e.g. Stapel et al. 2001; Cornelisen and Thomas 2004), recent studies suggest that seagrasses are also able to use dissolved organic matter as a nitrogen source. This enables them to shortcut N cycling (Barron et al. 2006; Evrard et al. 2006; Vonk et al. 2008a). Similar to terrestrial plants (Harrison et al. 2007), seagrasses exhibit distinct uptake rates for different organic nitrogen substrate, that seem to be related to the substrate's bioavailability, molecular complexity and/or chemical stability of the molecules (Vonk et al. 2008a; Van Engeland 2011, 2013). For instance, urea is a very simple molecule that provides two amine groups per molecule. Amino acids with chemically very stable phenyl-groups may be less prone to breakdown and uptake. It is currently not clear if any nutrient-nutrient interactions exist in the uptake dynamics of organic nitrogen.

In oligotrophic coastal systems (usually in tropical regions), effective use of nitrogen sources are vital to maintain a high productivity, whereas in eutrophic areas (usually in temperate regions) nitrogen overloading may occur (e.g., Touchette and Burkholder 2007). Recently, it was discovered that dissolved organic nitrogen pools in coastal waters are relatively high and not refractory, even in oligotrophic systems (Bronk et al. 2007). In oligotrophic systems, the availability of additional nitrogen sources may help to explain the high productivity of seagrass systems. In eutrophic systems, the availability of additional nitrogen sources may form an additional threat. Therefore, we aim to (1) quantify uptake rates of each of the dominant nitrogen sources, i.e., inorganic and organic nitrogen, and (2) detect whether the availability on one of these sources affects uptake rates of the other sources. We studied this in a temperate seagrass species, *Zostera noltii*, as this species usually occurs in meso- or eutrophic situations (e.g. Cadiz Bay, Brun et al. 2003; and Wadden Sea, Dolch et al. 2013), but can also be found in oligotrophic lagoons (Honkoop et al. 2008). We tested this in the lower range of nutrient concentrations as observed in Cadiz Bay in summer (Van Engeland et al. 2013).

Using stable isotope labelling, we investigated uptake by the temperate seagrass, *Zostera noltii* Horneman, of ^{15}N nitrogen from different inorganic (ammonium, nitrate) and organic substrates (urea and glycine) as a function of the presence of one of the other substrates as background (^{14}N). By adding fairly low concentrations, we focused on nutrient interactions in uptake at nutritional conditions that are realistic for the source population of the studied plants (Cadiz Bay, Spain). Dual labeling (^{13}C and ^{15}N) was used to track potential dissolved organic carbon uptake.

2.2 MATERIALS AND METHODS

2.2.1 Biological material and experimental setup

Shoots of *Zostera noltii* Horneman were collected from an intertidal meadow of Cadiz Bay (Southern Spain, 36°29'19.79"N; 6°15'53.05"E), brought to the lab in a cool box, wrapped in moist paper, and then boxed in an ice-chest to be transported to the Netherlands. The plants arrived after two days and were immediately put in a tank with 2 μm filtered water from Oosterschelde (south-west Netherlands) under controlled temperature (19°C) and light (278 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) conditions. Inorganic nitrogen concentration in the tank were as in Oosterschelde ($\text{NH}_4^+ = 4.7\mu\text{mol N}$, $\text{NO}_x^- = 1.17\mu\text{mol N}$, $\text{DON} = 20.1\mu\text{mol N}$). After an acclimation period of two full days, plants were cut into single complete shoots (with leaves, rhizomes and roots) and gently cleaned from epiphytes with a razor blade to minimize microbial degradation by e.g. free

living bacteria, exo-enzymes etcetera, van Engeland et al. 2011). This enabled us to focus on the ability of the seagrass itself to process or use nitrogen forms, rather than facilitation by better equipped micro-organisms. Six days after harvest in Cadiz bay, the plants were incubated in a climate-controlled room (temperature 19°C and lights 254 mol photons m⁻² s⁻¹) in 250 ml plastic cups. Plants (2 - 3 shoots) were left intact with their belowground and aboveground parts submersed in separate cups (Van Engeland et al. 2011) (Fig. 2.1). As the plants would protrude out of the water, the cups were filled almost until the edge to prevent desiccation, while exchange of water between cups was prevented. We used artificial seawater (constituents from Merck and Sigma-Aldrich) that we manually prepared to exclude unintended nutrient addition (modified F2 medium containing only the major constituents, without the nitrogen salts; see for instance De Brouwer et al. 2005), and to minimize interference of microorganisms (e.g. competition for nutrients, remineralisation). In this setup, either the aboveground or the belowground tissue received a nutrient treatment, and were incubated for approximately 3 hours under continuous bubbling to prevent local depletion and the built-up of concentration gradients. For logistic reasons the labelling of the aboveground and belowground tissues were performed on consecutive days.

At the start of the experiment, the plants received a combination of one heavy isotope labelled nitrogen substrate (¹⁵N 99% pure ¹⁵N, Cambridge Isotope Laboratories) at a concentration of 1 µM, and one background substrate in the light isotope form (¹⁴N) at 1 µM (both added with a pipet). The substrates were ammonium, nitrate, urea, and glycine (Cambridge Isotope Laboratories). Urea and glycine (amino acid) also contained isotope labelled carbon (¹³C, universally labelled 99%, Cambridge Isotope Laboratories) to track potential carbon uptake. The different substrate combinations are given in Table 2.1. These nutrient concentrations are similar to those found in the water column of Cadiz bay (Van Engeland et al. 2013) and for ammonium and nitrate in the range commonly found in the water column of seagrass ecosystems (0 – 8 µM and 0 – 3.2 µM, respectively; Touchette and Burkholder 2000). Control treatments were performed where only the substrate was added without background. Each nutrient treatment was replicated 5 times. Since the cups were relatively small, there may have been substrate depletion during the experiment. However, because the objective of this study is to determine the uptake capacity of nitrogen from a pool of nitrogen, rather than quantifying their uptake kinetics, this was not a problem.

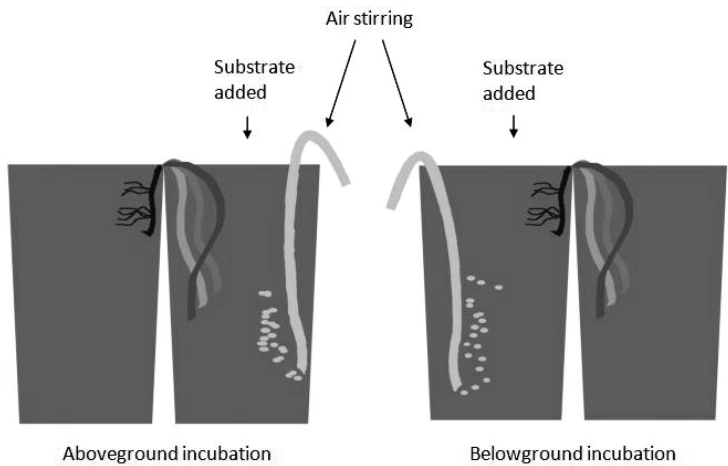


Figure 2.1 Experimental setup with two cups containing the aboveground and belowground parts of intact *Zostera noltii* plants. Bubbling was used to stir the water in order to prevent concentration gradients from developing during the incubation.

After the incubation, plants were rinsed and cleaned with artificial seawater containing only the nutrient background, and dabbed with paper tissues. Aboveground and belowground parts were separated and immediately stored in glass vials at -20 °C. Later they were freeze-dried for 48 hours. Dried samples were weighed and ground to a homogenous powder for further analysis.

Table 2.1 Experimental design showing concentrations of non-labelled-background and labelled-substrate applied to both above and belowground tissues. Values should be interpreted as “background substrate concentration – labelled substrate concentration” in micromolar.

Background concentration (non-labelled) (μM)	Substrate concentration (isotope labelled) (μM)			
	NH ₄ ⁺	NO ₃ ⁻	Urea	Glycine
None (control)	0 - 1	0 - 1	0 - 1	0 - 1
NH ₄ ⁺	1 - 1	1 - 1	1 - 1	1 - 1
NO ₃ ⁻	1 - 1	1 - 1	1 - 1	1 - 1
Urea	1 - 1	1 - 1	1 - 1	1 - 1
Glycine	1 - 1	1 - 1	1 - 1	1 - 1

2.2.2 Sample and data treatment

Dried samples were analyzed for their nitrogen and carbon content, and nitrogen and carbon isotope composition using Thermo EA 1112 elemental analyzer coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer with a ConFlo II interface (EA-IRMS). Specific ^{15}N uptake rates ($V_{15\text{N}}$; expressed $\mu\text{mol N g DW}^{-1} \text{ h}^{-1}$; DW = dry weight) were calculated as :

$$V_{15\text{N}} = \left((AF_{sa} - AF_{bg}) * F_N \right) / (M_N * time),$$

where AF_{sa} and AF_{bg} are the ^{15}N fraction in the sample and the natural isotope fraction in the plant tissue, respectively. F_N is the nitrogen fraction in the sample's dryweight (gN gDW^{-1}), M_N is the molar mass of nitrogen (14 g mol^{-1}) and time is the length of the incubation period (hours). These specific ^{15}N uptake rates were converted to specific N uptake rates using the ^{15}N fraction in the substrate ($F_{\text{substr}}^{15\text{N}}$) :

$$V_N = V_{15\text{N}} / F_{\text{substr}}^{15\text{N}}$$

This fraction was 1 for all substrate-background combinations, except those where the labeled substrate (^{15}N) and the non-labeled background (^{14}N) were the same ($F_{\text{substr}}^{15\text{N}} = 1/2$). Total N uptake (ρ_N ; $\mu\text{mol N}$) after incubation was calculated for individual treatment as:

$$\rho_N = V_N * time * DW_{sa}$$

where DW_{sa} is the sample's dryweight (g).

Depletion was calculated as the percentage of available substrate (N_{added}) that was taken up:

$$Depletion = (\rho_N / N_{\text{added}}) \times 100$$

Note that in the treatment where the substrate (^{15}N) was also added as background (^{14}N), this background was also taken into account. Similar formulas were used for the carbon uptake rates from the organic molecules.

2.2.3 Statistical analysis

Treatment and background effects were tested through variance analysis (ANOVA). When needed, asymmetry in distribution per group was compensated by log-transformations. Regression analysis (ordinary least square) was performed to compare carbon and nitrogen uptake from the organic substrates.

2.3 RESULTS

2.3.1 Seagrass DIN and DON uptake independency on nutrient background

Our results showed different uptake rates for different substrates (Fig. 2.2) with similar patterns for above- and belowground tissues. Variance analyses per substrate, indicated systematically higher uptake rates in aboveground than in belowground tissues (always $p < 0.01$), except for the labelled glycine addition. In the leaf-mediated substrate uptake, only substrate type exhibited a significant effect (ANOVA; $F_{3, 80} = 75$, $p < 0.001$), but the background type did not (ANOVA; $F_{4, 80} = 75$, $p > 0.05$). Tukey HSD tests indicated differences between all labelled substrates ($p < 0.01$), except between nitrate and urea. Root-mediated uptake rates were significantly affected by both the substrate type and the background type, but the latter effect was very weak (ANOVA; $F_{3, 80} = 28$, $p < 0.001$; ANOVA; $F_{4, 80} = 2$, $p < 0.05$; respectively). Ammonium uptake rates were higher than for the other substrates (Tukey, always $p < 0.5$), and a significant difference existed between nitrate and urea uptake rates (Tukey, $p < 0.05$). From these analyses it is clear that substrate uptake showed no clear dependence on the presence and type of a background substrate (Fig.2.2).

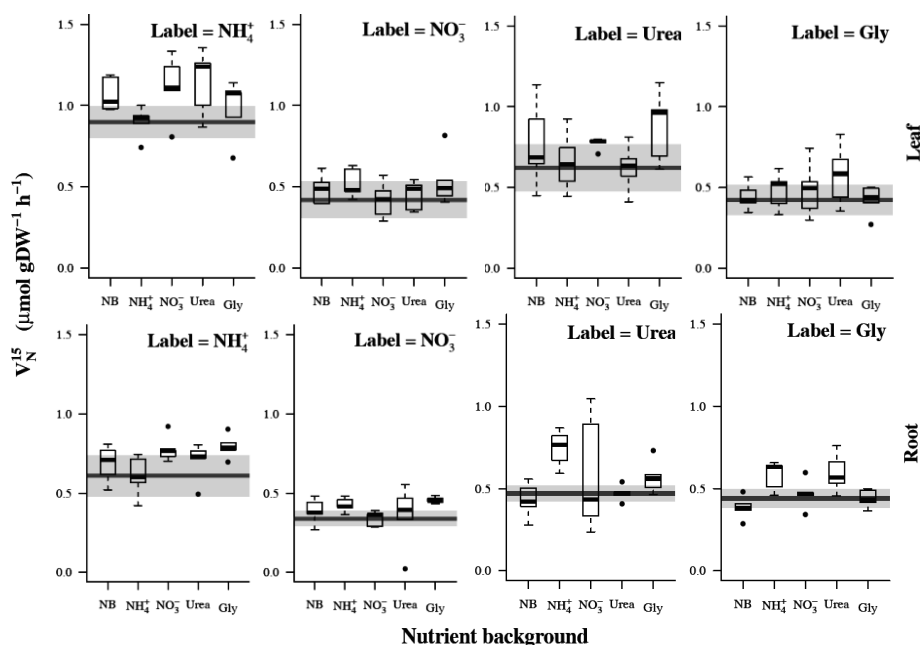


Figure 2.2 Boxplots of the ^{15}N specific uptake rates for the different labelled substrates (grouped in separate graphs) in different backgrounds of nitrogen containing substances (horizontal axis) in leaves (upper panels) and roots (lower panels). Both the labelled (^{15}N) and background (^{14}N) were added in final concentration of $1\mu\text{M}$. The small lines, boxes, whiskers and dots indicate median, interquartile range (IQR), $1.5 \times \text{IQR}$ and outliers (deviation from median larger than $1.5 \times \text{IQR}$). The thick horizontal lines and grey zones indicate the mean and ($\pm$) standard deviation of the uptake rate for the ^{15}N substrate in a background of the same substances in ^{14}N form. NB = No Background (indicating no ^{14}N -nutrient added).

Considering the small volumes and low (but realistic) concentrations used, it is imperative that we investigated the potential for depletion. Substrate depletion was significantly affected by the tissue type and labelled substrate, but not by the background substrate (Table 2.2). The amounts of ammonium taken up represent a considerable fraction of the added amounts (Fig. 2.3), indicating a strong potential for depletion-related underestimation of the corresponding uptake rates. This is supported by the similar degrees of depletion in ammonium, with and without ammonium background (i.e. doubling of the ammonium concentration “visible” to the plant). The fraction taken up for the other substrates were far less (Fig. 2.3). Hence, depletion-related underestimation of the uptake rates are not likely for these substrates.

Table 2.2 Analysis of variance (ANOVA) table for the substrate depletion, indicating the degrees of freedom of the F statistic (df), the value of the F statistic, and the corresponding probability value.

Factor	Df	F	P
Intercept	1, 160	1633	< 0.001
Label (L)	3, 160	70	< 0.001
Tissue (T)	1, 160	62	< 0.001
Background (B)	4, 160	0.5	0.7
L x T	3, 160	5.8	< 0.001
L x B	12, 160	1.4	0.2
T x B	4, 160	1	0.4
L x T x B	12, 160	0.5	0.9

2.3.2 Carbon versus nitrogen uptake from organic N-sources

For the dual-labelled glycine and urea, a strong linear relationship existed between carbon and nitrogen uptake (Linear regression per substrate; only the slope coefficients were significant $p < 0.05$; Fig. 4.4). If the organic molecules would be taken up intact, one could expect that the total uptake of carbon and nitrogen by the plants occurred in proportions dictated by the C:N ratio or the substrates. This hypothetical uptake is in figure 3 indicated by the lines. The observed C:N ratios of this uptake were clearly lower than expected from the C:N ratios in the substrates (lines in Fig. 4.4), indicating preferential nitrogen uptake over carbon uptake. However, C:N ratio of uptake was stronger for the more carbon-rich glycine than for urea (R^2 values of 99% and 88%, from the respective regression analyses).

2.4 DISCUSSION

In nature, nitrogen is available to marine macrophytes as a mixture of inorganic and organic molecules. In coastal and estuarine areas the dissolved organic nitrogen constitutes 13-18% of the nitrogen pool (except dissolved N₂; Berman and Bronk 2003) of which substantial parts can be non-refractory (Bronk et al. 2007). It is currently established that dissolved organic matter also serves as an effective source of nitrogen to marine macrophytes (Vonk et al. 2008a; Van Engeland et al. 2011). Our study supports these findings and demonstrates organic nitrogen uptake by *Zostera noltii* under conditions of a strongly reduced microbial community (epiphyte removal and artificial seawater). Our ammonium uptake rates are slightly higher than those reported by Morris et al. (2008) for *Zostera noltii* shoots from the same source population under low current conditions (data not shown). Our uptake rates for the aboveground tissue are also in the same range as those reported by Van Engeland et al. (2011) for the same substrates, but somewhat higher for the belowground tissue. Variability between substrates also resembles those reported by Van Engeland et al. (2013) for the same seagrass species and by Vonk et al. (2008a) for tropical species. These similarities with literature show that our data are of good quality. In addition, our study takes research in organic nitrogen uptake by marine macrophytes one step further by considering the role of organic substrates in nutrient-nutrient interactions in nitrogen uptake by a temperate seagrass species.

2.4.1 Seagrass DIN and DON uptake independency on nutrient background

With regard to the inorganic nitrogen substrates, our results agree with earlier studies that show higher uptake rates for ammonium than for nitrate (Touchette and Burkholder 2000; Alexandre et al. 2010; Van Engeland et al. 2011) and organic N-sources (Vonk et al. 2008a; Van Engeland et al. 2011, 2013). This effectively results in a 'constitutive preference' for ammonium over the other substrates. If all substrates are supplied in the same concentrations (like in this study), ammonium is taken up in higher amounts than the others. As the presence of a background substrate did not affect the uptake rates of the labelled substrate in any of the treatments (Fig. 2.2; comparison within panels), no down- or up-regulation was observed that favored one nitrogen source over the others (i.e. an induced preference). This contrasts with the findings of (Alexandre et al. 2010), who showed an inhibition effect of ammonium on nitrate uptake, and stimulated ammonium uptake under higher nitrate concentrations.

Considering the low (but close to ambient) nitrogen concentrations applied in our study, we may have not reached certain threshold concentrations to induce inhibition or stimulation of substrate uptake. It is likely that, at these low concentrations *Zostera noltii* is “programmed” to take up whatever nutrients it can find. Clearly, our experiment was conducted in nutritional conditions characteristic of the quasi-linear part of the Michaelis-Menten curve for uptake of nitrogen sources.

Under nutritionally poor conditions, other seagrass species also seem to take up nutrients from whatever source is available. *Posidonia oceanica* in Revellata Bay (Corsica) seems to take up inorganic nitrogen according to the available water column concentrations (Lepoint et al. 2002). However, although the same applies to *Phyllospadix iwatensis*, this species still exhibits a preference for ammonium as revealed by its uptake affinities (Hasegawa et al. 2005). Inorganic nitrogen concentrations in the latter study varied so much that they simply drowned out the difference in affinities. This shows the value of kinetic studies in unravelling nutrient preference mechanisms. To summarize, *Zostera noltii* exhibits a constitutive and induced preference for ammonium under higher nutritional conditions (Alexandre et al. 2010), but only a constitutive preference at lower nutritional conditions (this study). Apart from that, the eventual contribution of different sources in the overall nitrogen acquisition may further depend on the relative concentrations of the different sources.

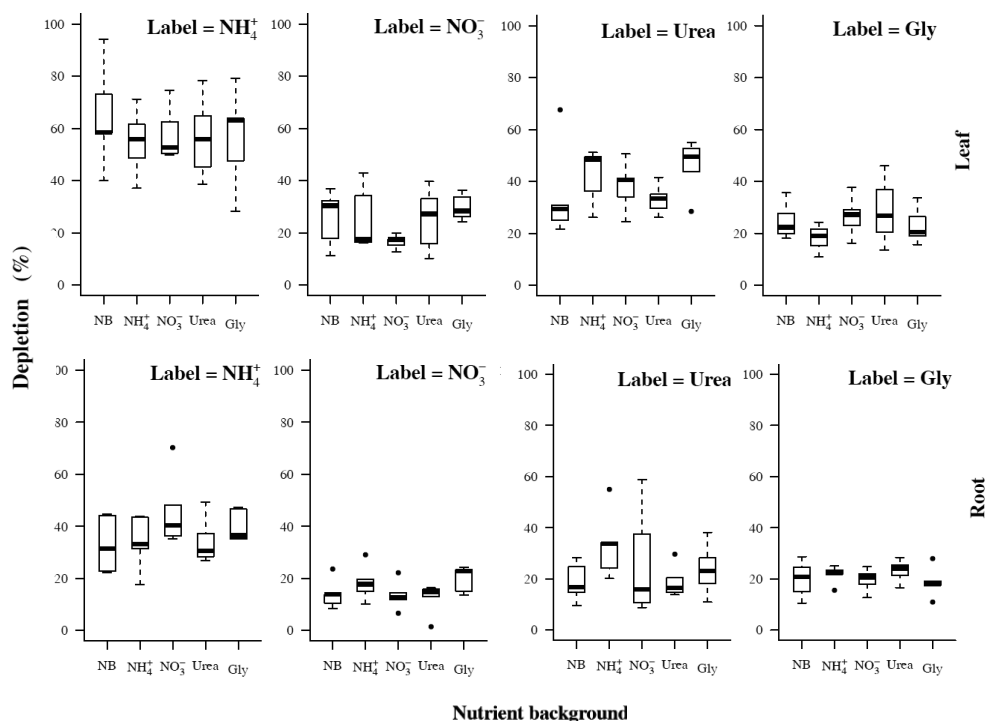


Figure 2.3 Total N uptake as a percentage of the added substrate Nin leaves (upper panels) and roots (lower panels). The small lines, boxes, whiskers and dots indicate median, interquartile range (IQR), 1.5×IQR and outliers (deviation from median larger than 1.5×IQR). Background N is not taken into account unless the background was the same n species as the substrate.

Due to the strong depletion in the labelled ammonium additions, the true ammonium uptake rates may have been underestimated, although they were roughly similar to those reported by Morris et al. (2008) for *Zostera noltii* from the same source population under low current conditions. Underestimating uptake rates due to depletion would imply that a potential down- or up-regulation of ammonium uptake could remain undetected. However, since the up-regulation, demonstrated by Alexandre et al. (2010) was more pronounced at substrate concentrations of 25 μM than at 5 μM , we consider such an effect at concentrations of 1 μM would not likely to occur.

The organic nitrogen substances in our study did not have any effect on the uptake of any nitrogen source, nor were their uptake rates influenced by the presence of another substrate. Considering that the pattern in uptake rates for the aboveground tissue was similar to that found by Van Engeland et al. (2011), it probably reflects a ‘constitutive preferential’ order from ammonium as most

preferred, to urea, nitrate and glycine as least preferred (note however that the differences with glycine were not statistically significant in our study). Whether an inducible preference mechanism exists in *Zostera noltii* involving organic nitrogen substances remains an open question. Note however, that amino acids concentrations of 1 μM are really at (or beyond) the upper limit of the observed range for seagrass ecosystems (e.g. Hansen et al. 2000). This implies that the chance of not detecting and existing role for amino acids in the down-regulation of the uptake of some nitrogen source is much smaller than the change that such a role actually exists.

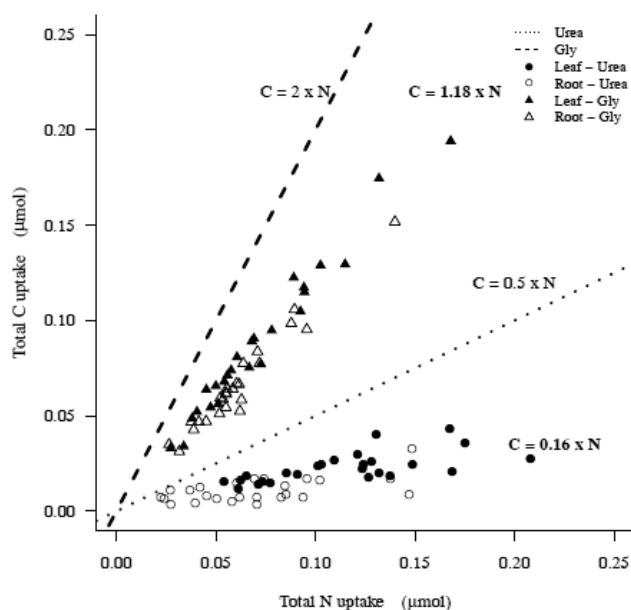


Figure 2.4 Total uptake of substrate C versus substrate N for the two organic substrates. The theoretically expected relationship between C and N uptake, derived from the substrate C:N ratios are for urea and glycine shown by the dotted and dashed line, respectively. These calculations assumed absence of fractionation. Root and leaf-mediated uptake are for both substrates indicated with different symbols (*cf.* legend in figure). Equations carbon uptake (C) as function of nitrogen uptake (N) are given for the theoretical lines (normal font) and the empirical data (bold font). In the latter case, only the slope coefficients were significant in the linear regression (*cf.* text).

2.4.2 Carbon versus nitrogen uptake from organic N-sources

A strong relationship existed between nitrogen and carbon uptake from organic molecules in *Zostera noltii*. However, the uptake C:N ratios were lower than expected from the respective molecule C:N ratios, indicating occurrence of carbon loss. Several reasons can be put forward for this partial decoupling: 1) remineralisation outside the plant with subsequent uptake of the products (NH_4^+ and dissolved inorganic carbon), 2) uptake of the entire molecule with subsequent loss of a part of the carbon, or 3) remineralisation outside plant by epiphytic bacteria (in the boundary layer) with transport of the products influenced by boundary layer effects (i.e. coupling through limited physical transport after external remineralisation).

The fact that the uptake C:N ratio is stronger for the more carbon-rich glycine (C:N=2) than for urea (C:N=0.5) seems to support remineralisation (see Harrison et al. 2007; von Felten et al. 2008), considering that coupled uptake implies a specific uptake mechanism which is most likely not directly dependent on the molecule's C:N ratio. However, explanation 1 is problematic in the sense that the produced DIC would still enter a large background pool (micromolar versus millimolar concentrations). In contrast, explanation 3 does not suffer from this problem as remineralisation within the boundary layer would cause less dilution losses of labelled DIC in the unlabelled DIC background pool. Present study does however not provide an affirmative answer to the mechanisms causing constant uptake C:N ratios that deviate from theoretical expectations.

2.4.3 Summarising conclusion

Overall, this study suggests that at low ambient concentrations, *Zostera noltii* exhibits a 'constitutive preference' for ammonium over other (in)organic nitrogen sources, in-line with findings from kinetic studies. However, contrary to the demonstrated ammonium-nitrate interaction in nitrogen uptake by *Zostera noltii* at higher nitrogen concentrations, no similar regulation seems to exist in lower ambient concentrations, indicating that in low-nutrient environments *Zostera noltii* takes whatever (in)organic nutrients are available.

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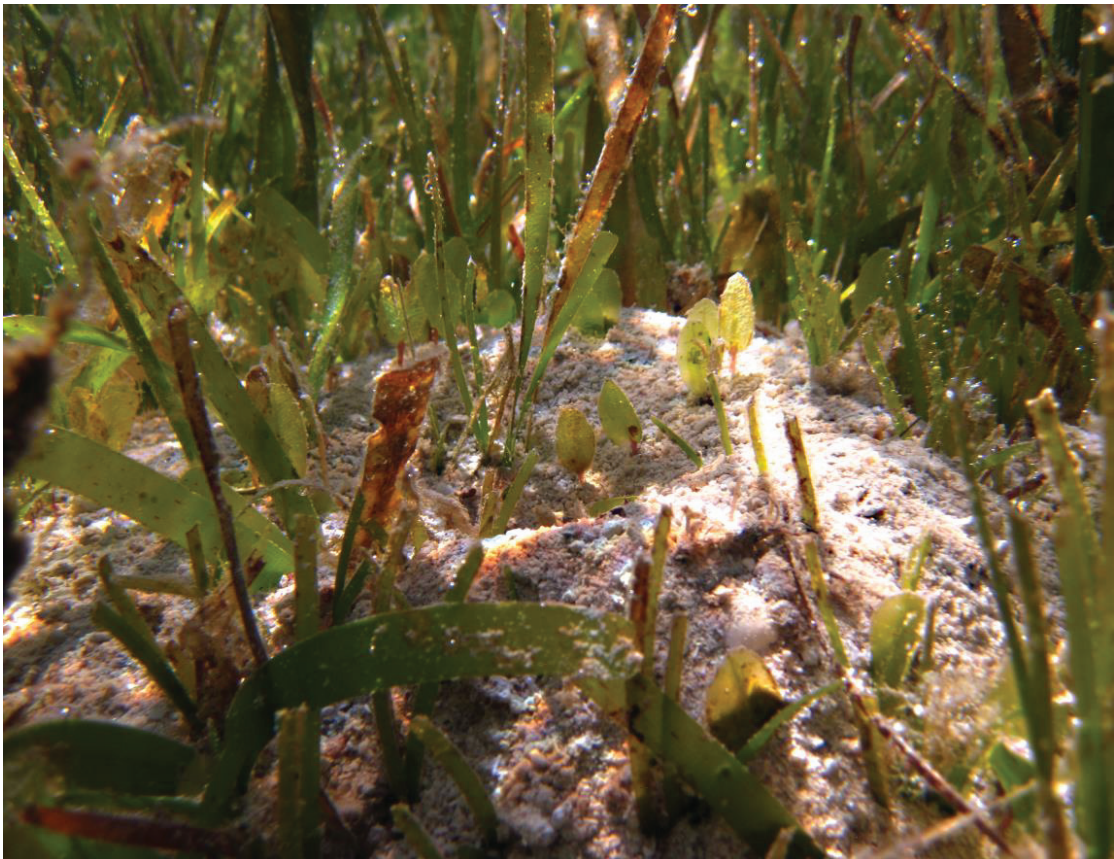
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CHAPTER 3

Biomechanical response of two fast-growing tropical seagrass species subjected to *in situ* shading and sediment fertilization

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ABSTRACT

Although seagrasses experience strong hydrodynamic forces, little is known about their biomechanical response in spite of the potential importance for their ecological success. We investigated how light reduction and sediment-nutrient enrichment affect biomechanical and morphological properties of two short-lived tropical seagrass species: *Halophila ovalis* and *Halodule uninervis*. A 50-day manipulative field experiment of shading and sediment-nutrient enrichment versus a natural population (control) showed that both shading and nutrient enrichment made the leaves of *Halophila ovalis* weaker (lower F_{TS}) and more elastic (lower E_T). As the absolute breakability of leaves (F_{MAX}) was not affected by either of the treatments, this implies that these changes in strength and stiffness resulted from the increase in leaf dimensions under nutrient enrichment (i.e., longer, wider and thicker leaves) and shading conditions (i.e., thicker leaves). In contrast, the biomechanical properties of *H. uninervis* leaves were less responsive and only became more extensible under shading whilst their biomechanics did not change under sediment nutrient enrichment. This limited response of *H. uninervis* might be due to the lack of morphological response in this species since leaves only became longer under nutrient enrichment. When comparing both species across treatments under shading (after normalizing them with their controls), *H. ovalis* became significantly weaker compared to *H. uninervis*, and the latter became more extensible. Under nutrient enrichment, *H. ovalis* became significantly more elastic compared *H. uninervis*. Overall we found that (i) biomechanical properties can be affected by environmental conditions, (ii) the responses were species specific, and (iii) seagrass morphology (leaf thickness and width) affected by environmental conditions will influence seagrass biomechanical properties. Further experimental studies on seagrass biomechanics are needed as present understandings of the acclimation of these properties and the consequences for species functioning are only starting to emerge.

Keywords : Biomechanics; Morphology; Tropical seagrass; Shading; Sediment-fertilization; Indonesia.

3.1 INTRODUCTION

Seagrasses are clonal plants that live in estuarine and shallow coastal areas in the tropics and sub-arctic regions (den Hartog 1970; Short et al. 2007), where they are highly acknowledged for their ecological, physical and economical values (Costanza et al. 1997; Barbier et al. 2011). However, they are facing increasing threats causing their global decline (Waycott et al. 2009). One important threat originates from increased sediment run-off due to, for instance, deforestation or erosion of agricultural grounds (Freeman et al. 2008). In tropical regions, such run-off may have 2 important impacts on nearby seagrass meadows: *i*) reduced light availability during the monsoon season when sediment is in suspension and *ii*) sediment nutrient enrichment due to sediment settling on the sea floor after the rainy periods, when the water is calmer. Due to the global change process, storm frequencies may increase (Young et al. 2011) enhancing turbidity events and low light conditions. In addition, sediment nutrient enrichment may intensify if ongoing anthropogenic land-use changes continue to enhance the run-off volumes and, with the increased use of fertilizers increasing the soil nutrient levels (Freeman et al. 2008). Being rooted plants, seagrasses cannot escape from these environmental stressors, which may lead to seagrass decline (Short and Neckles 1999; Waycott et al. 2009) unless seagrasses can acclimate to them.

Given the great importance of light for photosynthetic organisms, many studies have focused on the effect of light on seagrasses. In general, light reduction decreases seagrass growth (Lee and Dunton, 1997; Peralta et al., 2002; Collier et al., 2007), survival (Collier et al. 2011) and shoot density (Lee and Dunton, 1997). Morphological changes related to light alteration can be a bit more diverse. Light reduction often enhances leaf length (e.g. in *Halodule pinifolia*, Longstaff and Dennison 1999) and width (e.g. in *Thalassia testudinum*, Lee and Dunton 1997; in *Posidonia oceanica*, Dalla Via et al. 1998), enabling plants to collect more light. However, sometimes the opposite effect has been observed, which might be explained by the need of decreasing the respiratory demand of the shoot (Gordon et al. 1994); in other cases no changes in leaf width (Gordon et al. 1994), or morphology in general, were recorded (Ochieng et al. 2010).

Although less studied than light, many studies have also been focussed on the effect of increased nutrient loading on seagrass growth, morphology and survival. In general, nutrient enrichment of the water column has a clear negative effect on seagrass growth and survival either directly due to the toxic effect of nitrate (Burkholder et al. 1992) or ammonium (van Katwijk et al. 1997; Brun et al. 2002; Christianen et al. 2011) or indirectly due to algal overgrowth, causing light deprivation and enhanced organic matter fluxes to the sediment

(Short et al. 1995; Apostolaki et al. 2009; Thomsen et al. 2012). However, in oligotrophic areas, sediment nutrient enrichment may stimulate seagrass growth and affect their morphology (Lee and Dunton 1997; Short 1983). Most studies have however focussed on nutrient enrichment in the water column, whereas relative few studies have looked at the effect of an increase in sediment nutrients (but see Erftemeijer and Middelburg 1993; Short, 1983; Peralta et al. 2003). These studies on sediment enrichment showed a much lower sensitivity to nutrient enrichment than from water column enrichment studies (Hemminga 1998; Short 1983).

Despite that there are many studies focussing on the effect of light reduction and nutrient enrichment on seagrass growth, morphology, physiology and/or population demography, there is virtually no information on how such conditions affect the biomechanical properties of seagrass shoots (but see Kopp 1999; La Nafie et al. 2012). Biomechanical properties can be used to characterize the strength of organisms, i.e. the resistance against mechanical damage, according to mechanical principles (Niklas 1992) and can hence serve as a tool to provide insights in how organisms can cope with physical forces imposed on their tissues (Niklas 1992; Patterson et al. 2001). Given the high density of water when compared to air (almost 800 times denser), marine organisms receive larger drag forces than terrestrial organisms, making the biomechanical response especially important for organisms living in dynamic aquatic environments (Niklas 1992). Like the morphology, the biomechanical properties are also variable under differential environments indicating their plasticity, e.g. wave-exposed plants may be short but having extensible and tough leaves (de los Santos et al. 2013).

Biomechanical studies have been conducted in many terrestrial plants (Anten et al. 2005; Onoda et al. 2011), and algae (Koehl, 2000; Denny and Gaylord 2002). In seagrasses only few studies measured biomechanical properties (Kopp 1999; Patterson, 2001; de los Santos et al. 2012; 2013; La Nafie et al. 2012), in studies of nutrient effects (but were only tested in mesocosm studies; Kopp 1999; La Nafie et al. 2012) and in correlative field studies (Patterson et al. 2001; de los Santos et al. 2012; 2013). Under mesocosm conditions, leaves of the temperate seagrasses *Zostera marina* and *Z. noltii* became weaker following nutrient enrichment in the water column, causing them to break easily (Kopp 1999; La Nafie et al. 2012) and leading to a reduced plant survival (La Nafie et al. 2012). Intra- and interspecific differences in biomechanical properties as well as spatial and seasonal effects have been identified, showing ecological implications for dispersal distances and susceptibility for herbivory (Patterson et al. 2001; de los Santos et al. 2012; 2013).

However, to our knowledge, nothing is known about the effect of light reduction and sediment-nutrient enrichment on the biomechanical properties of seagrasses. Also interspecific variability in environmental responses to such factors was never tested. Hence we conducted a manipulative field experiment to investigate both the biomechanical and the morphological responses of two fast-growing tropical seagrasses subjected to *i)* light reduction and *ii)* sediment-nutrient enrichment, as these factors are expected to become increasingly important in the coming era. Moreover, we aimed to compare two co-existing species with contrasting morphologies since interspecific differences in biomechanical traits can have important ecological implications (e.g. de los Santos et al. 2013). Given the very few studies available for biomechanical properties in seagrasses, we do not wish to pose testable hypotheses, albeit it might be speculated that both manipulative treatments will weaken the leaves of both species.

Our results will provide ecologically relevant information for situations of increased light limitation and for situations of nutrient enrichment. In addition, this study will provide a base for hypotheses on interactive effects, which will likely occur in nature as well. This can then be tested in follow-up studies using multiple levels of treatments and varying combinations in order to generate ecologically meaningful results on interactive effects.

3.2 MATERIALS AND METHODS

3.2.1 Experimental field location

An *in situ* manipulative experiment was conducted at Bone Batang island, which is one of the (more than) 200 islands occurring at Spermonde archipelago in South-Sulawesi, Indonesia (Fig. 3.1). The island is *ca.* 15 km from the south-western part of the mainland Makassar, and *ca.* 30 km from the shelf-edge and extend from north to south within the geographical range of 5°00'47.66" S - 119°19'35.12" E. The island is an un-inhabited sand bar covering an area of *ca.* 5,000 m², although during high tide only a few square meters remain emerged. The area is surrounded by a large coral reef flats inhabited by an extensive seagrass meadows with a mixed species composition (Fig. 3.1). Like many other islands in the Spermonde archipelago, Bone Batang island experiences a semi-diurnal tidal cycle. Apart from small-scale sand-mining by local islanders from nearby islands, the island does not experience anthropogenic impacts causing nutrient enrichment or turbidity-induced shading, making it an ideal place for our manipulative experiment.

Tissue N-content was measured, as this provides a better indication of nutrient limitation than sediment nutrient concentrations (e.g. van Katwijk et al.

2011). The seagrasses at Bone Batang island are nitrogen-limited based on the low seagrass tissue N contents (less than 2%; Duarte 1990; Vonk and Stapel 2008) indicating a typical oligotrophic tropical island. At the south-east part of the island, experimental plots were constructed in its subtidal and extensive seagrass meadows. Meadows were composed of 5 seagrass species (shoot density between brackets, in shoots m^{-2} ; mean $\pm$ SD, $n = 5$): *Cymodocea rotundata* (52 ± 17), *Enhalus acoroides* (56 ± 24), *Halophila ovalis* (48 ± 22), *Halodule uninervis* (248 ± 23) and *Thalassia hemprichii* (112 ± 26), rendering a total density of 516 ± 37 shoots m^{-2} . In this study we specifically focused on the two pioneer species, i.e., *Halophila ovalis* and *Halodule uninervis*, that have a short leaf-life span (see section 3.2.2) to be able to detect changes in leaf properties in our 50-day experimental period

3.2.2 Study species

H. ovalis and *H. uninervis* were selected because of *i*) their short leaf-life spans, *ii*) their capacity to respond quickly to experimental treatments and *iii*) they largely differ in their morphologies, with *H. ovalis* leaves being oval and *H. uninervis* leaves being ribbon - like shaped. Leaf age (leaf longevity) for *H. ovalis* is *ca.* 12 days (Duarte 1991) and in the range of 25 to 50 days for *Halodule* species (Duarte 1991; Hemminga 1999). The shoot age (shoot longevity) for *H. ovalis* and *H. uninervis* are 73 days and 69 days, respectively (Duarte 1991). Overall, this implies that an experimental period of around 50 days should be sufficiently long to detect changes in leaf morphology and biomechanical properties. *H. ovalis* commonly occurs on sandy (not muddy) sediments, whereas *H. uninervis* can occur in all sediment types (Green and Short 2003).

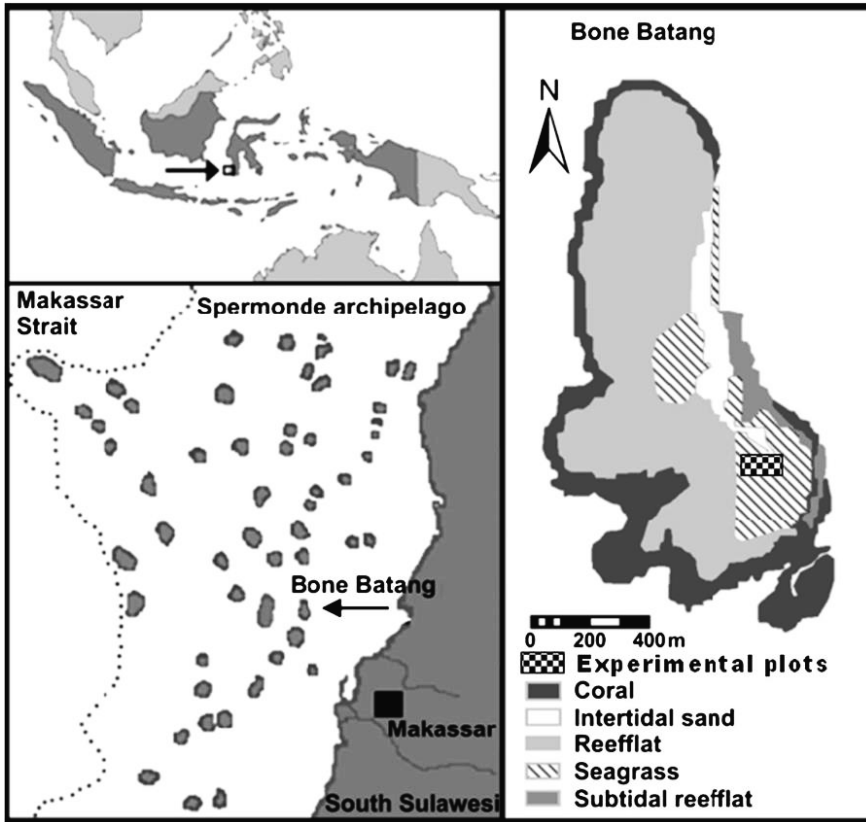


Figure 3.1 Map indicating the location of the experiment at Bone Batang island, in the Spermonde archipelago, South Sulawesi, Indonesia (modified from Vonk et al. 2008b).

3.2.3 Experimental design

The *in situ* experiment consisted of 2 treatments: i) sediment-nutrient enrichment and ii) shading plus a control (i.e., no sediment-nutrient enrichment and no shading) that was used to compare to both treatments. Experimental and control plots were selected in areas with similar species composition and shoot density (see section 3.2.1) Plots were marked with poles (1.5 m high) constructing a square of 2 x 2 m. A minimum distance of at least 10 m between plots was kept to ensure no interference among the treatments and the control. All plots were in the same depth, thus receiving the same tidal inundation. Five plots per treatment plus five plots per control (15 plots in total) were set up. A full factorial design including a test for interactive effect of shading and nutrients was outside the scope of our objectives, and not feasible without putting treatments too close together, therefore this treatment was not used in

this study. Shading was obtained by allocating dark-green nets (mesh size of *ca.* 1 – 2 mm) on the poles covering 2 x 2 m of the area, with a 50 cm distance from the bottom hence currents can run freely under the shading nets. Wave reduction by the nets is negligible because of its open structure, and because the nets could move along with the waves. At the beginning, during (5 times along the experimental time) and at the end of the experiment, light was measured by using a Li-Cor radiometer (model Li-250A) indicating that shading treatment reduced by more than 80% ($185 \pm 46 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) the light availability ($952 \pm 238 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, mean $\pm$ 1SE). Light measurements were conducted right underneath the shades and in the water *ca.* 50 cm from the bottom (similar to the height of the shades) at the controls. Enriched plots were left unshaded and nutrients were applied into the sediment. Fertilization was done by using a slow-release commercial fertilizer (N:P:K ratio of 18:9:3; osmocote®). In each fertilized plot (un-shaded), two kilograms of osmocote® was applied (0.5 kg m^{-2}) by spreading 5 pockets (made from small mesh-sized material) filled with *ca.* 400 grams each, in a regular pattern over the plot. Fertilizer bags were buried in the sediment *ca.* 5 cm deep in order to mimic sediment enrichment, where species without roots (algae/epiphytes) cannot access to the added nutrients. The five control plots had no shading and no nutrient addition, but were also marked by putting up 4 poles for each control to differentiate them with the treated plots. Plots were monitored weekly to verify that nutrient bags and shading equipments were correctly placed. Experiment lasted for 50 days. This duration of the experiment was based on the short leaf-life span of *Halophila ovalis* and *Halodule uninervis* (Duarte 1991; Hemminga et al. 1999; Green and Short 2003; Bujang et al. 2008) (see section 3.2.2).

3.2.4. Sampling

Once experimental period ended, plants were harvested from the centre of the plot by using a spade. The entire specimen (leaves with rhizomes and roots) sampled was then cleaned from sediment, pooled per treatment, kept in a cool box and transported to the laboratory at Hasanuddin University, in Makassar. Upon arrival, plants were placed in 3 big containers filled with seawater (salinity of 30) and air bubbled in a controlled temperature room (29°C). Selected shoots were wrapped in moist paper (tissues damped with sea-water), laid out horizontally, placed in sealed plastic bags to avoid desiccation and transported to the University of Cádiz (Spain) in a cool box. Upon arrival in less than 72 hours from the sampling event, plants were directly placed into 3 aquariums with air bubbled salt-water (salinity of 30) and controlled temperature (29°C). The next day, the morphological and biomechanical properties of the leaves were measured (please note that a leaf is composed by

the leaf blade and the leaf sheath, in this regard, when we refer to leaf, it means the leaf blade). Given the fast transport, effects on tissues are expected to be small and not causing treatment effects.

3.2.5 Measuring morphological & biomechanical properties

For measurements, 5 healthy looking seagrass leaves (free of herbivores bites and holes) were selected per treatment plus 5 for the control and cleaned carefully by gently scraping off the epiphytes. Leaf length, width and thickness were measured with a ruler (cm), a digital caliper (mm) and a dial thickness gauge (mm; Mitutoyo®), respectively. Leaves were cut off at the junction between the sheath and the blade. To measure their biomechanical properties, leaves were individually clamped into 5-Newton (N) grips (model 2712) of the tensometer (Instron® model 3342) with the mountings 10 mm apart. The leaves were stretched at a velocity of 5 mm min⁻¹, while the extension (δ , mm) and the force (F, N) (Fig. 3.2) were recorded every 0.1 second until the leaf blades broke, recording both the maximum force that the leaf can bear before breaking (F_{MAX} , N), and the maximum extensibility that the leaf can experience before breaking (δ_{MAX} , mm) (Fig. 3.2). From the force-extension curve (Fig. 3.2) and the morphology of the specimens, we obtained 4 mechanical properties (Table 3.1; Fig. 3.2): The *specific force-to-tear* or strength (F_{TS} , N mm⁻²) is the maximum force (F_{MAX} , N) per unit of cross-sectional area (CA, mm²) needed for breaking the tissue. This is the equivalent to the property known as ‘tensile or breaking stress’. The *elongation-to-tear* or extensibility (L_T , mm mm⁻¹ or %) corresponds to the increase in length (δ_T) from the original specimen length (L_0) that occurs before it breaks as the result of the tensile force applied to it. This property is also called ‘ultimate elongation’ or ‘breaking strain’. *Young's modulus of elasticity* for tension or stiffness (E_T , N mm⁻²) represents the resistance to deformation. It is calculated by taking the initial slope from the force against extension graph (F per δ) as well as considering the initial length (L_0) and the cross-sectional area of the specimen (CA). The *Specific work-to-tear* or toughness (W_{TS} , kJ m⁻³) is the work per unit of volume needed to break the specimen. It is calculated as the area under the force-extension curve (W, kJ) standardized by the specimen volume (V, m³; calculated by approximating a leaf to a rectangular body, i.e., $V = CA * L_0$).

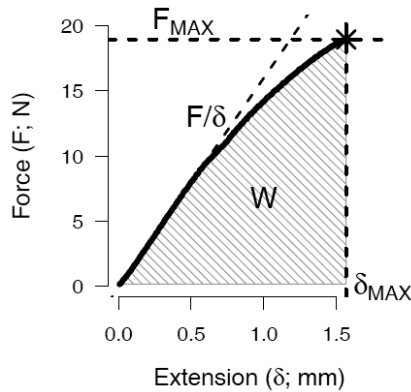


Fig. 3.2 A typical force (F ; N) – extension (δ ; mm) curve, showing the forces applied to the tissue with the extension experienced by the tissue. Graph also shows the maximum force that the tissue can bear before breaking (breaking force, F_{MAX}), the maximum extensibility of the tissue before breaking (breaking extension, δ_{MAX}) and the slope of the curve (F/δ), used to calculate the modulus of elasticity in tension (E_T).

3.2.6 Statistical analysis

As we did not have a full factorial design, we tested pair-wise the effect of sediment-nutrient enrichment and shading relative to control conditions by applying independent sample t -test to the studied seagrass properties. When Levene's test showed an equal variances ($p > 0.05$), then we used the result of the t -test for equality of means. However, when Levene's test was significant ($p < 0.05$) we used the result of the t -test for equality of means but by not assuming a homocedasticity (Field 2009). To compare the biomechanical and morphological properties between species (*H. ovalis* and *H. uninervis*), we also conducted independent sample t -test. In addition to that, normalization of the biomechanical properties of each treated samples was conducted by dividing each variable data by the control. Data for all variables were presented as means $\pm$ 1 SE and differences were considered to be significant when $p < 0.05$.

3.3 RESULTS

3.3.1. Morphological properties

Shading significantly increased the leaf thickness and volume of *H. ovalis* (Fig. 3.3), whereas morphological properties of *H. uninervis* did not show any response under shading treatment (Fig. 3.3). Nutrient enrichment caused morphological responses in both seagrass species where *H. ovalis* leaves

became significantly longer ($p = 0.043$), wider ($p = 0.025$), and thicker ($p = 0.016$) and thus leading to a bigger leaf dimension ($p = 0.008$) when compared to the control (Fig 3.3). In case of *H. uninervis*, only the leaf length increased ($p = 0.006$) in response to nutrient enrichment (Fig. 3.3). Differences in morphological properties between species were obvious because they have different leaf morphologies: *H. ovalis* is oval-shaped leaf (hence commonly known as spoon- or paddle - seagrass) and *H. uninervis* is a ribbon-like seagrass. *H. ovalis* leaves were significantly wider, thicker and shorter than *H. uninervis* leaves (all $p < 0.05$).

3.3.2 Biomechanical properties

Both shading and nutrient enrichment made the leaves of *Halophila ovalis* weaker (F_{TS} ; $p = 0.001$ and 0.024 , respectively) and more elastic (E_T ; $p = 0.035$ and 0.047 , respectively) (Fig. 3. 4). *Halodule uninervis* leaves, however, became significantly more extensible (L_T ; $p = 0.005$) under shading (Fig. 3.4), whereas sediment-nutrient treatments had no effect on its biomechanical properties (Fig. 3.4). For both species, the absolute breaking stress (F_{MAX}) was however not affected by shading (*H. ovalis* = 6.33 ± 0.50 N and *H. uninervis* = 3.42 ± 0.27 N) or nutrient enrichment (*H. ovalis* = 6.92 ± 0.55 N and *H. uninervis* = 2.79 ± 0.42 N) (data not shown). The comparison of the two species across treatments (after normalizing them with their controls) revealed that, under shading, *H. ovalis* became significantly weaker (lower F_{TS}) compared to *H. uninervis*. The latter became more extensible (higher L_T) than *H. ovalis* under shading (both $p < 0.05$). Under nutrient treatment, *H. ovalis* became significantly more elastic (lower E_T) compared to *H. uninervis* (Table 3.2, Fig. 3.4; note different axes for both species).

Table 3.1 Biomechanical properties of the seagrasses *Halophila ovalis* and *Halodule uninervis* were measured per individual leaf (F_{MAX} ; L_T ; W) and subsequently expressed per unit tissue cross-section area (F_{TS} ; E_T) or per tissue volume (W_{TS}). Abbreviations used stand for: F_{MAX} (N) is the maximum force that the leaf can bear before breaking; CA (mm²) is the cross-sectional area of the leaf (i.e., width*thickness; mm²); L_0 (mm) is the initial length of the leaf; δ_{MAX} (mm) is the maximum extensibility of the leaf experienced before breaking; F (N) is the force applied to the leaf; F/δ (Nmm⁻¹) is the slope of the forceextensibility curve; W (kJ) is the amount of energy needed to break the leaf; V (m³) is the volume of the leaf ($CA * L_0$).

Variables	Units	Equation	Description
Specific force – to – tear (F_{TS}) ($\Leftrightarrow$ tensile or breaking stress) (strong vs weak)	N mm ⁻²	$F_{TS} = \frac{F_{MAX}}{CA}$	F_{TS} is the force needed per unit of a cross section area to break a material. A material is stronger/ weaker compare to other material when it has higher/lower value of F_{TS}
Elongation – to – tear (L_T) ($\Leftrightarrow$ extensibility or breaking strain) (extensible vs less extensible)	%	$L_T = \frac{L_0 - \delta_{MAX}}{L_0} * 100$	L_T is the capability of linear deformation. A material is stretchy (extensible)/ less stretchy when it has higher/lower value of L_T
Young's modulus of elasticity for tension (E_T) ($\Leftrightarrow$ stiffness) (stiff vs elastic)	N mm ⁻²	$E_T = \frac{L_0}{CA} \cdot \frac{F}{\delta}$	E_T is the capability of a material to resist deformation. The ratio of normal stress to normal strain measured within the elastic range. A material is stiffer/ flexible when it has higher/lower value of E_T .
Specific work- to – tear (W_{TS}) ($\Leftrightarrow$ toughness) (tough vs brittle)	kJ m ⁻³	$W_{TS} = \frac{W}{V}$	W_{TS} is the capability of a material to absorb energy before breaking per unit of volume. A material is tougher/ brittle when it has higher/ lower value of W_{TS} .

3.4 DISCUSSION

3.4.1. Treatment effects

Morphological and growth responses of seagrass species to environmental factors like shading and eutrophication have been studied extensively (for references see Introduction). The effect on biomechanical properties have however been neglected even though they can strongly respond to abiotic conditions and may affect plant survival and performance (La Nafie et al. 2012). To our knowledge, this is the first study showing for seagrasses how environmental stresses i.e., shading and increased nutrient status, can affect both the biomechanical and morphological properties of tropical seagrasses under field conditions. Present results showed that leaf biomechanics of *H. uninervis* remained largely unaffected by shading or sediment nutrient enrichment, but *H. ovalis* leaves became weaker under the influence of both stresses (separately). However, leaves did not break more easily (high F_{TS}) because the cross-sectional area increased simultaneously.

H. ovalis experiencing nutrient enrichment had weaker (lower F_{TS}) and more elastic (lower E_T) leaves. This agrees with the recent study by La Nafie et al. (2012; for seagrass *Z. noltii*) and Lamberti-Raverot and Puijalon (2012; for freshwater plant) that under high water column nutrient condition, plants produce weaker leaves. Present results showed that this effect also occurs under sediment enrichment and in tropical species. However, contrasting to La Nafie et al. (2012), the absolute force that *H. ovalis* leaves can bear before breaking (F_{MAX}) showed no significant differences with the control. This may imply that leaf mechanical resistance may result from acclimation to any environmental changes by morphological changes. The leaf dimensions (leaf length, width, and thickness) of *H. ovalis* were indeed larger compared to the control treatment. In other words, although the leaf tissues of this species became weaker (low F_{TS}) under nutrient enrichment treatment, this was compensated by the increased dimensions of the leaves, and thus the absolute breakability of the leaves (F_{MAX}) was not affected. Increased leaf dimensions may indicate that nutrients are used for growth (cf Lee and Dunton 1997; Short 1983), confirming that our study area is still relatively oligotrophic as indicated by the relatively low leaf tissue nutrients (Duarte 1990; Vonk and Stapel 2008). *H. uninervis* leaves were also longer under nutrient enrichment, but we found no effects on width and thickness. Neither the absolute breakability (F_{MAX}) nor the strength per cross-sectional area (F_{TS}) of the leaves from this seagrass species were influenced by nutrient enrichment.

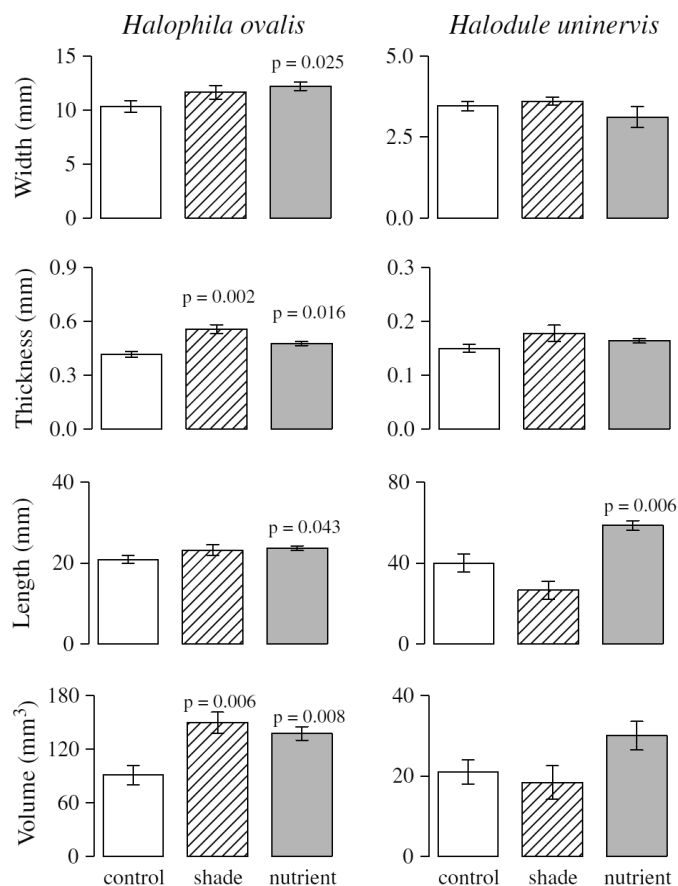


Figure 3.3 Morphological properties of *Halophila ovalis* and *Halodule uninervis* leaves under shading and nutrient-enrichment compared to control. Bars represent mean values $\pm$ 1 SE. Significant differences are indicated by p values as obtained from an independent sample t -test, and always indicate the differences between a treatment and the control; there is no comparison between treatments (Please note that axis scales are adapted for each species).

Under light deprivation *H. ovalis* leaves were also weaker (less strong; lower F_{TS}) and more elastic (lower E_T) when compared to the control. Leaves were thicker under shading, but length and width were similar, hence resulting in higher CA values. Light deprivation effect on leaf thickness has to our knowledge been poorly studied in detail (Ralph et al. 2007). However, as hypothesized by Enriquez (2005), there may be a positive effect of leaf thickness in light absorption efficiency. Like in the nutrient enrichment treatments, shading had no effect on the absolute force needed to break the

leaves (F_{MAX}), because leaves became weaker per cross-sectional area (i.e. lower F_{TS}), but also thicker. In *H. uninervis* leaves, the morphological properties were unaffected by shading nor did the leaves break easier or became weaker (F_{MAX} and F_{TS} remained the same). The *H. uninervis* leaves did however become more extensible (L_T) than control plants under shading. Morphological and biomechanical properties can vary through seasons, particularly in temperate zones (e.g. Kopp 1999; Patterson et al. 2001; de los Santos et al. 2013). In tropical zones, seasonality is relatively less and particularly affects intertidal seagrass (Brouns 1987; Erftemeijer and Herman 1994). Our experiment was carried out subtidally during the transition of the dry to the wet season with mild weather conditions. Hence, we do not expect seasonality to have affected the morphological and biomechanical properties we studied during the 50 days experimental treatment, nor to have interfered with the treatments we imposed.

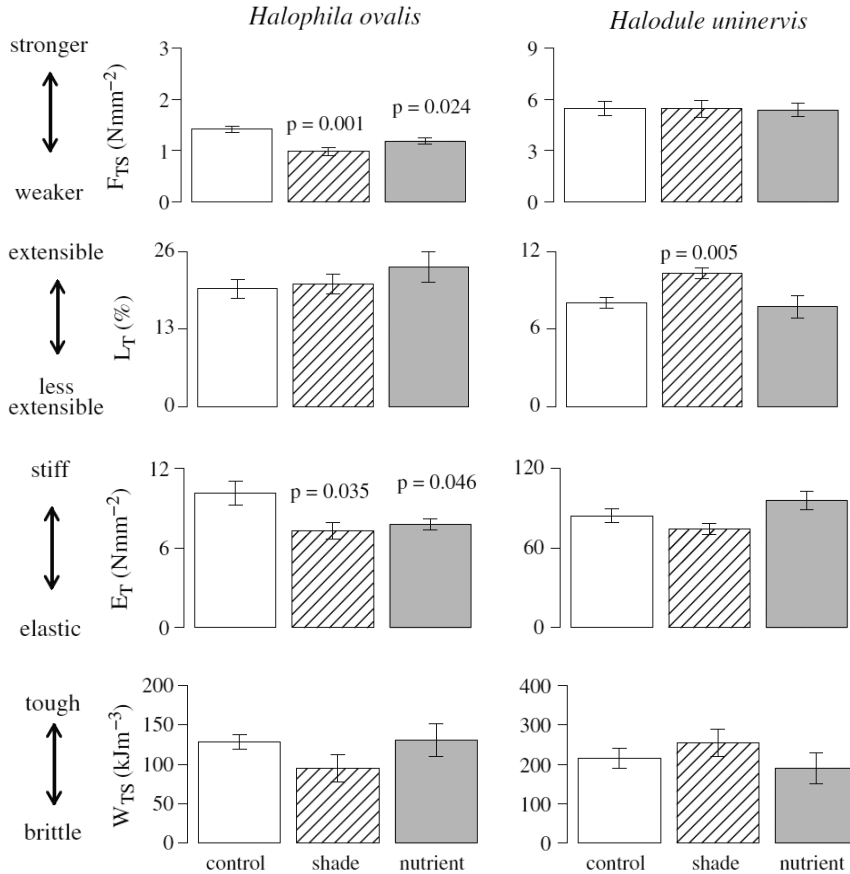


Figure 3.4 Biomechanical properties of *Halophila ovalis* and *Halodule uninervis* leaves under shading and sediment-nutrient enrichment treatments, compared to control. Bars represent mean values $\pm$ 1SE. Significant differences are indicated by p values as obtained from an independent sample t test, and always indicate the difference between a treatment and the control; there is no comparison between treatments. Abbreviations of variables stand for : F_{TS} = specific force-to-tear (N mm⁻²); L_T = elongation-to-tear (%); E_T = Young's modulus of elasticity for tension (N mm⁻²); W_{TS} = specific work-to-tear (kJ m⁻³).

3.4.2 Species comparison and ecological implications

The morphology of the two species is greatly different, with *H. ovalis* having an oval-shaped leaves and *H. uninervis* having a more typical ribbon-like leaves (Fig. 3.3; note different axis for both species). As a result, overall the *H. ovalis* leaves are wider, thicker and shorter than those of *H. uninervis*. Both

species also showed significant differences in their biomechanical responses. Under shading, *H. ovalis* leaves became relatively weaker (lower F_{TS}) compared to *H. uninervis*. The latter became more extensible (higher L_T) than *H. ovalis* under shading. Under nutrient enrichment, *H. ovalis* became significantly more elastic (lower E_T) compared to *H. uninervis*.

The ecological implications of these inter-species differences in biomechanical and morphological responses to shading and nutrient enrichment are difficult to predict, as the absolute breakability (F_{MAX}) of both plants was not affected by the treatments. Only the elasticity in tension of *H. ovalis* leaves increased under the influence of both shading and nutrient enrichment, but the ecological implications on drag may be expected to be minor in such small and highly seagrass species (*cf.* Bouma et al. 2005). In this regard, biomechanical properties of seagrass leaves are ecologically relevant as storms can cause healthy leaves to break and thus damage the seagrass beds (Fonseca et al. 2007). In *H. ovalis*, the increased leaf weakness of the tissues was counterbalanced by the increased leaf dimensions (width and thickness) when subjected to both treatments. In a previous study with temperate seagrass *Zostera noltii*, water nutrient enrichment caused a decrease in both F_{MAX} and F_{TS} , whereas the cross-sectional area remained invariable (La Nafie et al. 2012; and unpublished results). The differential morphological response recorded in both studies (La Nafie et al. 2012 and the present) can be due to several factors, for example system nutrient loading, however this remains speculative. In general, plant biomechanics are affected by environmental conditions (this study, Kopp 1999; La Nafie et al. 2012) where nutrient enrichment and light reduction may covary in nature. In temperate regions, environmental condition effects will be more noticeable due to seasonality (de los Santos et al. 2013). In addition, variability of biomechanical properties between reproductive shoots may also occur (Patterson et al. 2001). These variabilities were all demonstrated by standard deviations per treatment in the various studies mentioned. From our results we could hypothesize that *H. ovalis* leaves would become even wider and thicker under both stressors, and as a consequence, specific tissue strength (F_{TS}) may reduce further. However, other interaction may occur, also depending on the degree of light reduction or nutrient enrichment. Follow-up studies using multiple levels of treatment may elucidate interactive effects of both stressors.

Differential species responses may lead to shifts in species composition under environmental change. For instance, competitive interactions may be affected by a broad range of parameters such as light capturing efficiency (Gordon et al. 1994; Lee and Dunton 1997; Longstaff and Dennison 1999), capacity for nutrient uptake (Morris et al. 2008; Vonk and Stapel 2008), inherent growth rate (Marba and Duarte 1998) tissue longevity and construction costs (Dalla Via et al. 1998; Longstaff and Dennison 1999; Puijalon et al. 2011). The

present study indicates that in addition to these factors, biomechanical properties is also an important factor that needs further attention, because *i)* species can change their biomechanical properties in response to environmental conditions and *ii)* species differences are huge and *iii)* changes in biomechanical properties may affect seagrass survival and affect the avoidance and tolerance strategies, as shown in marine and fresh water macrophytes (Puijalon et al. 2011; La Nafie et al. 2012). Thus, biomechanical properties may have an important value as indicator of the seagrass health and their competitiveness capacity. This is especially relevant given the broad range of hydrodynamic, nutritional and light conditions at which seagrass meadows can occur.

Table 3.2 Results of the student *t* – test of biomechanical properties comparing the two species *Halophila ovalis* (Ho) and *Halodule uninervis* (Hu) from each treatment. Data for each variable and for each species is normalized by dividing each variable within a treatment by the control. For a more detailed explanation of the abbreviations, see table 1. Asterisks (*) indicate significant differences of means between species per treatment when $p < 0.05$. EXP = Experiment; CTR = Control; Nutrient = sediment-nutrient enrichment.

	<i>p</i> value (<i>H. ovalis</i> - <i>H. uninervis</i>)				
	$(F_{TS})_{EXP}/(F_{TS})_{CTR}$	$(L_T)_{EXP}/(L_T)_{CTR}$	$(E_T)_{EXP}/(E_T)_{CTR}$	$(W_{TS})_{EXP}/(W_{TS})_{CTR}$	$(F_{MAX})_{EXP}/(F_{MAX})_{CTR}$
Shading	0.021*	0.036*	0.086	0.073	0.242
Nutrient	0.121	0.230	0.007*	0.589	0.369

3. 5 CONCLUSIONS

We showed that biomechanical properties of two tropical seagrass species (*Halophila ovalis* and *Halodule uninervis*) differentially responded to *in situ* shading and sediment nutrient enrichment over a 50-day experiment. Whereas *H. uninervis* remained largely unaffected by shading or sediment nutrient enrichment, *H. ovalis* became weaker under the influence of both stresses (separately), but the leaves did not break easier because the cross-sectional area simultaneously increased. This indicates that (i) biomechanical properties can be affected by environmental conditions, which may make these traits a valuable potential bio-indicator of seagrass health status, (ii) responses are species specific, which may give some ecological advantage of some species against others, and leading to a shift in species composition under changing environments, which require further studies and (iii) seagrass morphology (leaf thickness and width) affected by environmental conditions, will in turn influence the seagrass biomechanical properties.

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CHAPTER 4

Biomechanical properties of two slow-growing tropical seagrass species: plasticity and relation to morphometry

(Submitted)

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ABSTRACT

*Seagrasses are acknowledged to have large morphological plasticity in relation to environmental conditions but little is known about their mechanical plasticity. We investigated how mechanical properties of two slow-growing seagrass species i) vary between species, plant parts and amongst locations, and ii) how this variation is related to plant morphometry. We sampled two climax seagrass species *Enhalus acoroides* and *Thalassia hemprichii* from four Indonesian islands covering a gradient of anthropogenic activities (relatively pristine versus anthropogenic impacted areas) and wave exposure (sheltered versus exposed). *Enhalus acoroides* had a higher F_{MAX} value (absolute force needed to break a material) than *T. hemprichii*, indicating that *E. acoroides* leaves were less easy to break than *T. hemprichii* leaves. It resulted not only from having a larger dimension (higher cross sectional area = thickness $\times$ width), but also from bearing stronger tissues (higher F_{TS} , specific force to tear; i.e., the maximum force needed to break a material per its cross sectional area). Sheaths were more extensible than leaves, which was probably due to the softer meristematic tissues that form the sheaths that still bear elastic cell walls. Amongst locations, *E. acoroides* acclimated to local conditions by modifying both morphological and biomechanical properties, while *T. hemprichii* were less responsive to location. However, in *E. acoroides* we did not found a clear correlation between variation in biomechanical properties and morphometry with the anthropogenic influence or wave exposure. Overall, both species showed morphological and biomechanical acclimation capacities within the studied environmental range, although *E. acoroides* showed a higher plasticity than *T. hemprichii*.*

Keywords: *Biomechanical properties; seagrass morphometry; tropical seagrass species; *Enhalus acoroides*; *Thalassia hemprichii**

4.1 INTRODUCTION

Seagrasses, marine flowering plants with an anchoring system and a bundle of leaves, are found in a wide range of environments. A single species of seagrass may occur in contrasting conditions, ranging from shallow intertidal to deep subtidal habitats (Hemminga and Duarte 2000), from clear to relative turbid waters (Vermaat et al. 1997), and from calm to wave-exposed hydrodynamic conditions (van Katwijk and Hermus 2000; Schanz and Asmus 2003). The physico-chemical heterogeneity that seagrasses encounter in their habitat may have an anthropogenic origin (Mann 2000). Thriving under such variety of conditions requires seagrasses to present high intra-specific plasticity to accommodate this environmental heterogeneity.

The morphological plasticity in leaf traits of seagrasses, such as cross-section area or length, has been intensively studied in response to the availability of resources such as light and nutrients (Gordon et al. 1994; Longstaff and Dennison 1999) and the mechanical loads they encounter due to water motion (Schanz and Asmus 2003; Bouma et al. 2009; de los Santos et al. 2010). Such morphological plasticity enables seagrasses to cope with adverse conditions and highly dynamic environments. More recently, biomechanical approach has been incorporated into the study of seagrass leaf traits. Indeed, seagrasses in temperate regions show plasticity in biomechanical and morphological properties depending on the environmental conditions, such as hydrodynamics and nutrients. For instance, mechanical properties of seagrass leaves have shown to vary with nutrient loads, where high nutrient concentrations weakened the leaves of *Zostera marina* (Kopp 1999) and *Z. noltii* (La Nafie et al. 2012). In addition, the seagrass *Cymodocea nodosa* showed a considerable spatial and seasonal plasticity in morphological and mechanical traits of leaves to accommodate environmental heterogeneity, so that wave-exposed individuals presented short but extensible and tough leaves (de los Santos et al. 2013).

Knowledge on mechanical properties of plant tissues is important to understand how they resist physical forces (Read and Stokes 2006), especially in marine organisms such as seagrasses, which are constantly exposed to risk of structural damage due to water motion (Patterson et al. 2001; de los Santos 2011, 2013). Variations in morpho-mechanical properties may result in differential fitness and may be important in seagrass hydrodynamical performance. In addition, the morpho-mechanical properties of plants, and specifically of seagrasses, may entail ecological implications in, for example, plant interaction with herbivores (Read and Stokes 2006; Prado and Heck 2011; Vergés et al. 2011; de los Santos et al. 2012), and hydrodynamics (Bouma et al. 2005), and may thus lead to altered ecosystem functions. For example, plants

that are easy to break, often related to high nutritional status (de los Santos et al. 2012), are preferred by herbivores (Prado and Heck 2011; Vergés et al. 2011) increasing their function as grazing grounds. Stiffer plants have a large capability to trap sediment, relatively to flexible plants, as they can attenuate hydrodynamic to a larger extent (Bouma et al. 2005), thus increasing their ability to modify their habitat as well as contribute to coastal protection.

Considering that tropical areas comprise a large percentage of seagrass species inhabiting the world's coast, a better knowledge on the variability of the inter- and intra-specific mechanical properties will provide a new and interesting perspective to understand their ecology and distribution in the area. Consequently, in the present study, we aimed to enhance our knowledge on the leaf mechanical plasticity of tropical seagrass species, by quantifying their extent of variation *i)* among 2 co-occurring slow-growing climax seagrass species, *ii)* between the tissues within a single seagrass species (i.e., leaf vs. sheath) and *iii)* within the same species when growing in different environmental conditions. Moreover, we asked to which extent mechanical properties depend on plant morphometry. To address these questions, we sampled the 2 dominant tropical species *Enhalus acoroides* and *Thalassia hemprichii* in mixed meadows at 4 Indonesian tropical locations that form a matrix of differences in: *i)* anthropogenic activities (i.e., proxy for nutrient loading ranging from relative pristine vs. heavily anthropogenically impacted) and *ii)* wave exposure (sheltered vs. exposed). We hypothesize that seagrass morphological and mechanical traits *i)* differ between species, the largest and slowest growing species being more resistant which can be a prerequisite for long leaf life-span (*cf.* Duarte 2000), *ii)* differ between tissues, i.e. sheaths being stronger than the leaves because they encompass several leaves and act as protector for the newly developed leaves and more extensible because they contain meristematic tissues and *iii)* differ among sites because abiotic factors has been shown to influence leaf mechanical properties.

4.2 MATERIALS AND METHODS

4.2.1 Study species and sampling sites

Enhalus acoroides and *Thalassia hemprichii* are dominant climax seagrass species in the tropics, and both are commonly found in Indonesian marine waters (Green and Short 2003; Short et al. 2007). They inhabit different types of sediments, from sandy bottoms to coral rubbles, and *E. acoroides* is also found in muddy substrata. The two species share a common leaf morphology (ribbon-like leaves) and structure, possessing leaf blades linked to open sheaths (i.e. split vertically) (Kuo and den Hartog 2006). However, they

differentiate from each other in their dimensions, being *T. hemprichii* smaller than *E. acoroides*, the latter acknowledged as one of the largest seagrass species, with leaves up to 2 m long. In addition, leaf blade margins of *E. acoroides* are thickened, with two thick hair-like fibres and its edges re-curved inwards (Kuo and den Hartog 2006).

Enhalus acoroides and *Thalassia hemprichii* were sampled from the Spermonde archipelago (South Sulawesi, Indonesia) (Fig. 4.1) in a 2-day campaign in mid-September 2010. The Spermonde archipelago is known to have a large variety of local factors influencing the seascape on the islands. In addition to exposure to varying hydrodynamics and human impacts, there is also the nutrient regime related to upwelling at the western edge (Hoeksema 2012). Four locations were selected based on their anthropogenic impact (Anthropogenically impacted with high nutrient versus relatively Pristine) according to population pressure (low population density or uninhabited versus high population density, as a proxy for nutrient loading), and based on their exposure to wind-generated waves (Exposed vs. Sheltered). The four selected locations covered the full combination of the two grouping factors, resulting in location names AS, AE, PS, and PE.

Puntondo bay: an Anthropogenically impacted - Sheltered bay (AS). Puntondo bay is located in Takalar regency, South Sulawesi Province at approximately 60 km from the city of Makassar (Fig. 4.1). There are approximately 646 inhabitants in the bay area and the bay is exploited by aquaculture activities (generally seaweed farming) with mangrove trees on the beaches (Blankenhorn 2008), making it a relatively high nutrient environment. Specifically, sampling was conducted in a seagrass bed in Puntondo village, located 20-50 m from the human populated shore. The bay gathers together three tropical ecosystems: mangrove forests, seagrass meadows, and coral reefs. The most common seagrass species in the bay are: *Cymodocea serrulata*, *E. acoroides*, and *T. hemprichii*; whereas mixed meadows of *C. rotundata*, *Halodule uninervis*, *Halophila ovata*, *H. ovalis*, *H. spinulosa* and *Syringodium isoetifolium* can be occasionally found (Blankenhorn 2008) (Table 4.1). The bay shelters the seagrasses from the wave exposure, creating a low-wave environment which is reflected by the sediment type within the seagrass bed, i.e. very fine sand to coarse sand-type terrigenous sediment. Hence, this site was categorized as anthropogenically impacted and sheltered environment due to the inputs of nutrients from the aquaculture activities and to its low exposure to waves (AS).

Langkai island: an Anthropogenically impacted and hydrodynamically Exposed environment (AE). Langkai island is located in the south-western part of the mainland Makassar (ca. 35.8 km). The island itself covers the area of 27 ha and has a population of ca. 430 persons and it is expected to experience

nutrient enhancement from the inhabitants as well as due to upwelling from the Makassar strait. The island has extensive seagrass meadows consisting predominantly of *E. acoroides* and *T. hemprichii*. Sampling was conducted at 30 – 50 m from the human populated shore. The seagrass meadows are exposed to wind-generated waves, which are to some extent attenuated by the surrounding coral reef and reef flats, especially in the west to south-western part of the island. Accordingly, the sediment within the seagrass meadow consists of carbonated sediment type, with a grain size from sandy to coarse rubble type (Table 4.1). Due to its location, which is highly remote from the mainland and making it un-sheltered, the island is highly impacted by hydrodynamics and this may be worsened during the monsoon season. Hence, this island was categorized as anthropogenically impacted environment due to the anthropogenic inputs of nutrients (in addition to nutrients from upwelling) and hydrodynamically impacted (AE) due to its exposure to waves.

Kodingareng Lompo island: a relatively Pristine and hydrodynamically Sheltered environment (PS). It is situated *ca.* 16 km on the south-west part of the mainland Makassar. Real pristine sheltered locations are not easy to find because sheltered locations are very attractive for people to establish. Even though Kodingareng Lompo is a highly inhabited island (an area of 14 ha with a population of $\pm 4,170$) the sampling site selected is relatively remote from the populated areas (± 500 m) and likely experiences low nutrient loads. The island possesses a sand-spit in the southern part, and the seagrass meadows are evenly distributed along the south-western part of the island and also along the outer and inner part of the sand-spit. *Enhalus acoroides*, *Thalassia hemprichii*, *Cymodocea serrulata*, and *Halodule uninervis* are the most common species (Table 4.1). Being surrounded by coral reefs, the island has carbonated sediment type, with grain size from fine sand to pebbles. The site where we sampled our seagrass were further out from the island and still in the inner part of the sand spit resulting a (relatively) pristine and sheltered area (PS).

Bone Batang island: a relatively Pristine and hydrodynamically Exposed environment (PE). Bone Batang island is a sand bar covering an area of *ca.* 5,000 m², whilst during high tide only *ca.* 1 m² of the island remains emerged. The island is located approximately 27 km from the south-western part of the mainland Makassar.

Being uninhabited, the island experiences no anthropogenic impacts apart from being sand-mined by local islanders from neighbouring islands, which has created considerable erosion in the island. It has large coral reef flats with extensive seagrass meadows including the species: *Thalassia hemprichii*, *Enhalus acoroides*, *Halodule uninervis*, *Cymodocea rotundata*, *C. serrulata*, *Halophila ovalis* and *Syringodium isoetifolium* (Table 4.1). The seagrass meadows near the island are exposed to wind-generated waves, which are

slightly attenuated by the surrounding coral reefs. In line with this exposure, the seagrasses grow on carbonated sediment type, with sediment size from fine sand to pebble. Due to its uninhabited and highly wave-exposed location, it is categorized as (relatively) pristine and exposed site (PE).

4.2.2 Water column parameters

Samples of superficial seawater were collected from each location during low tide (3-5 replicates per site). Seawater samples, both filtered (Whatman no-42) and non-filtered, were transported in 500-mL bottles in cool conditions to the Laboratory of Chemical Oceanography (Hasanuddin University, Makassar, Indonesia). On arrival, non-filtered samples were directly measured for total organic carbon (TOC) and turbidity by spectrophotometry (DREL 2800 HACH, USA). Filtered samples were frozen until further nutrient analysis with a spectrophotometer (DREL 2800 HACH Made in USA). The concentration of phosphate and ammonium in the seawater were analysed following the Indonesian National Standard (SNI) methods No.06-6989.31-2005 and No. 06-6989.30-2005, respectively, as adopted from American Public Health Association 1998 (APHA 1998), whereas nitrate concentration was analysed following APHA (1980). Seawater salinity was measured *in situ* using a hand refractometer.

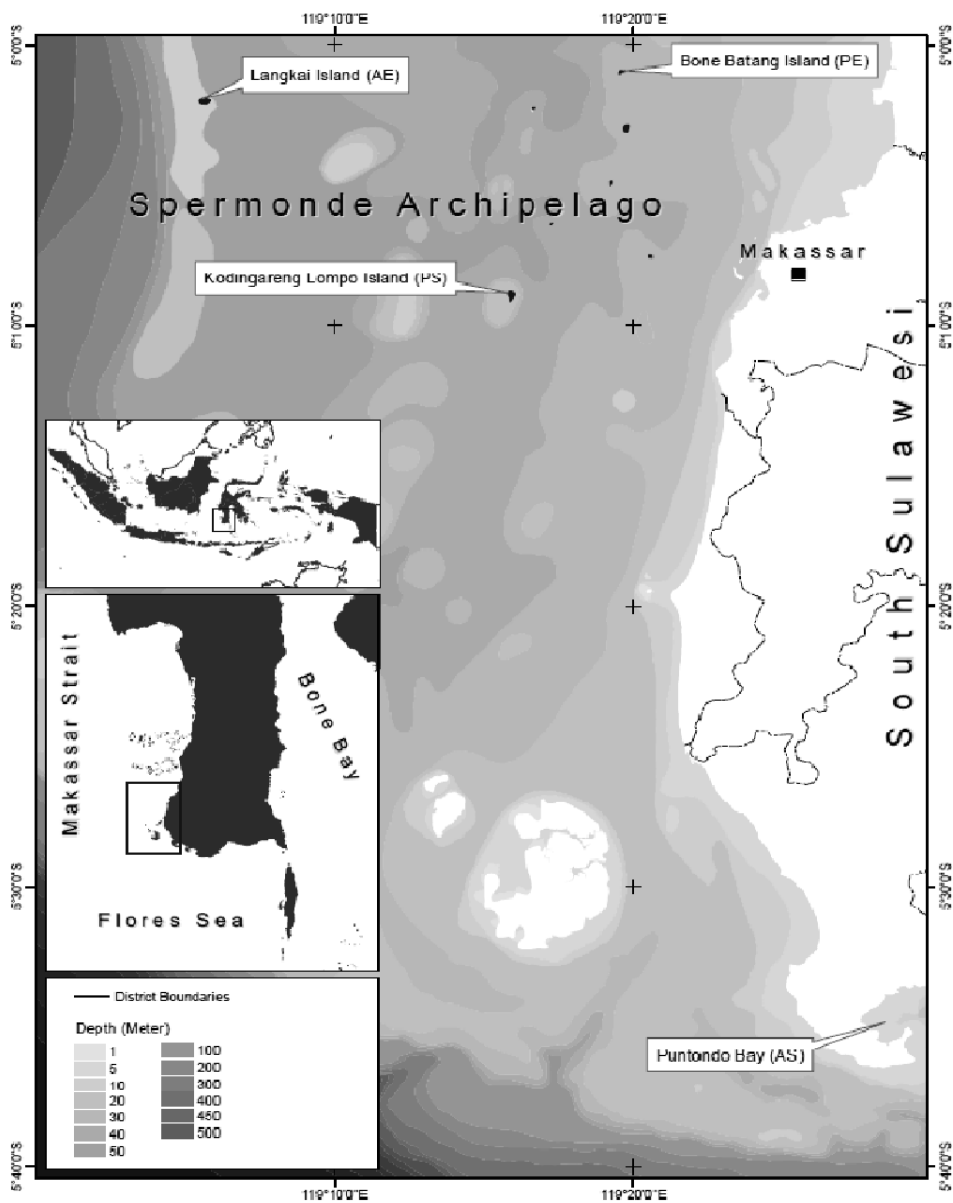


Fig. 4.1 Sampling locations of *Enhalus acoroides* and *Thalassia hemprichii* in Spermonde Archipelago, South Sulawesi, Indonesia. AS = Anthropogenic-Sheltered; AE = Anthropogenic-Exposed; PS = Pristine-Sheltered; PE = Pristine-Exposed

Table 4.1 Sampling sites with descriptions of their environmental conditions. Locations are referred to as anthropogenic impacted versus relatively pristine (A vs. P) and hydrodynamically exposed or sheltered (E vs S), resulting in location names AS (Puntundo Bay), AE (Langkai island), PS (Kodingareng Lompo island), PE (Bone Batang island).

Sampling sites	Coordinates	Anthropogenic/ Pristine - Exposed/ Sheltered	Sediment type at sampling site	Seagrass species
Location AS Puntundo Bay	05°35'20.00" S - 119°29'20.00" E	Anthropogenic – Sheltered	Terrigenous sediment (sandy mud – sand)	<i>Cymodocea serrulata</i> , <i>C. rotundata</i> , <i>Enhalus acoroides</i> , <i>Halodule uninervis</i> , <i>Halophila ovata</i> , <i>H. ovalis</i> , <i>H. spinulosa</i> , <i>Syringodium isoetifolium</i> , <i>Thalassia hemprichii</i> .
Location AE Langkai island	05°02'12.32" S - 119°05'55.41" E	Anthropogenic - Exposed	Carbonated sediment (small – big coral rubble)	<i>H. ovalis</i> , <i>E. acoroides</i> , <i>S. isoetifolium</i> , <i>T. hemprichii</i> .
Location PS Kodingareng Lompo island	05°08'55.30" S - 119°05'39.02" E	Pristine - Sheltered	Mud- carbonated sand	<i>C. serrulata</i> , <i>E. acoroides</i> , <i>H. uninervis</i> , <i>T. hemprichii</i> ,
Location PE Bone Batang island	5°00'47.66" S - 119°19'.35.12" E	Pristine – Exposed	Carbonated sediment (sand – rubble)	<i>C. rotundata</i> , <i>C. serrulata</i> , <i>E. acoroides</i> , <i>Halodule</i> . <i>uninervis</i> , <i>Halophila ovalis</i> , <i>T. hemprichii</i> .

4.2.3 Biological material sampling

Sampling was conducted in shallow sub-tidal parts of all the sites, with water depth ranges from 1 to 2 m. Seagrasses were sampled from the middle

part of the meadows. Entire seagrass shoots (leaves and a portion of rhizome) were collected from the sediment with a spade and then *in situ* cleaned of sediment. Subsequently, samples were carefully allocated in plastic bags into a cool box, and transported to the Hasanuddin University in Makassar. On arrival, plants were pooled in big containers filled with aerated seawater in a controlled temperature room (29°C) under room-light conditions. The following day, plants were wrapped in moist tissues and sent by plane to the University of Cádiz (Spain). Upon arrival, plants were immediately placed into an aquarium with controlled temperature (29°C), salinity (30 ppt), aeration and natural light. Morphological and biomechanical properties were measured the day after arrival. A time difference of 24 or 48 h between collection and testing showed no differences in mechanical properties of macroalgae (Koehl and Wainwright 1985) and seagrasses (Rammsy et al. unpublished). Even though our samples were processed in ± 72 h, the transport and processing was performed with great care, so that plants were still in fresh and healthy condition when measured. In addition, we did a visual inspection of the plants and discarded those that were damaged due to natural causes (herbivory) or due to manipulation, as well as tissues that did not satisfied the methodological premises during the breaking test (explained below). We ended up with 77 samples, i.e., 20 leaves from each species (5 from each location) and 18 sheaths of *E. acoroides* (5 from PS; PE; AS and 3 from AE) and 19 sheaths of *T. hemprichii* (5 from PS; PE; AS and 4 from AE).

4.2.4 Seagrass morphological and biomechanical properties

Among the sampled material, we selected healthy looking seagrass shoots free of herbivore marks and notches. The bulk of epiphytes were carefully removed by gently scraping them off using tissue paper or a razor blade. Samples were kept moist during all the measurement process. For each specimen, seagrass shoot was separated from the rhizome and the second or third outer leaf, so leaf age was similar among replicates, was cut off at the junction of the sheath and the leaf blade (from here up, when referring to the leaf, we mean the leaf blade). We measured mechanical properties of the leaves as well as the sheaths. Leaves and sheaths have different anatomy, hence different function, where sheaths protect the newly developing leaves including the meristems. Subsequently, we measured the leaf and sheath length, width and thickness using a ruler (cm), a digital calliper (mm) and a dial thickness gauge (Mitutoyo®, precision ± 0.01 mm), respectively. Leaf and sheath cross-section area (CA, mm²) and dimension (mm³) were computed from the width, thickness and length, considering a rectangular leaf section approximation.

Following morphological measurements, mechanical properties of leaf parts (leaf and sheath) were measured using a tensometer (Instron® model 3342) by individually clamping them in 5-N (for *T. hemprichii*) and 250-N (for *E. acoroides*) grips (model 2712) with the mountings 10 mm apart (L_0). Once clamped, the leaf parts were stretched at a velocity of 5 mm min⁻¹, while the extension (δ , mm) and the force (F, N) were recorded every 0.1 second until the tissue broke, when the maximum force (F_{MAX} , N) and extension (δ_{MAX} , mm) were directly recorded. From the force-extension curve and the morphology of the specimens, we obtained the specific force-to-tear or strength (breaking stress) (F_{TS} ; equation 1) and the elongation-to-tear or extensibility (breaking strain) (L_T ; equation 2).

$$\text{(equation 1) } F_{TS} = \frac{F_{MAX}}{CA}$$

$$\text{(equation 2) } L_T = \frac{\delta_{MAX}}{L_0} \times 100$$

Specific force-to-tear or strength (F_{TS} , N mm⁻²) refers to the maximum force (F_{MAX} , N) per unit of cross-sectional area (CA, mm²) needed to break the leaf or sheath. This is the equivalent to the property known as ‘tensile’ or ‘breaking stress’.

Elongation-to-tear or extensibility (L_T , mm mm⁻¹ or %) corresponds to the increase in length (δ_{MAX}) from the original specimen length (L_0) that occurs before it breaks as the result of the tensile force applied to it. This property is also called ‘ultimate elongation’ or ‘breaking strain’. Breaking stress (F_{TS}) and breaking strain (L_T) are material properties (size-invariant) and they define the strength and the extensibility (respectively) of a material.

Since the breaking force results from a contribution of morphological characteristics (i.e. cross-sectional area) as well as from breaking stress (force per cross-sectional area) we conducted variance partitioning to find out which component would contribute more to the breaking force (F_{MAX}) of our sample (Legendre and Legendre 1998; Onoda et al. 2011).

4.2.5 Data analysis

All data were log-normally transformed prior to ANOVA. One-way ANOVAs, followed by post-hoc test (Tukey-test), were conducted separately to test differences of mechanical and morphological properties 1) among locations, 2) between species (*Enhalus acoroides* and *Thalassia hemprichii*), and 3) between their tissues (leaves and sheaths). In addition, one-way ANOVA was used to test differences in water variables among locations. Data are shown as averages ($\pm$ SE), and significant differences were set to a critical level of 0.05.

We constructed a correlation matrix with Pearson's coefficient to all pairs of mechanical and morphological properties to assess their inter-correlation.

We assessed the relative contribution of morphological and material properties to breaking force (F_{MAX}) by variance partitioning (Legendre and Legendre 1998; Onoda et al. 2001). F_{MAX} is the result of three components – tissues thickness (T), tissues width (W) and tissues strength (F_{TS}) (see also equation 1):

$$\text{(equation 3) } F_{MAX} = T \times W \times F_{TS}$$

After log-transformation, equation (3) becomes a sum-up equation:

$$\text{(equation 4) } \log(F_{MAX}) = \log(T) + \log(W) + \log(F_{TS})$$

Variance (Var) of $\log(F_{MAX})$ is expressed as covariance (Covar) between $\log(F_{MAX})$ and decomposed components, thus:

$$\text{(equation 5) } \text{Var}(\log(F_{MAX})) = \text{Covar}(\log(F_{MAX}), \log(T)) + \text{Covar}(\log(F_{MAX}), \log(W)) + \text{Covar}(\log(F_{MAX}), \log(F_{TS}))$$

Hence, the relative contribution (Cont) of each decomposed component to F_{MAX} is expressed as Covar/Var, therefore:

$$\text{(equation 6) } \text{Cont}(T) = \text{Covar}(\log(F_{MAX}), \log(T)) / \text{Var}(\log(F_{MAX}))$$

$$\text{(equation 7) } \text{Cont}(W) = \text{Covar}(\log(F_{MAX}), \log(W)) / \text{Var}(\log(F_{MAX}))$$

$$\text{(equation 8) } \text{Cont}(F_{TS}) = \text{Covar}(\log(F_{MAX}), \log(F_{TS})) / \text{Var}(\log(F_{MAX}))$$

The sum up of the relative contribution should be equal to 1, resulting;

$$\text{(equation 9) } \text{Cont}(T) + \text{Cont}(W) + \text{Cont}(F_{TS}) = 1$$

Hence each contribution value is directly translated as the relative importance of each component in F_{MAX} .

4.3 RESULTS

4.3.1 Water column parameters

Irrespective of the location, nitrate concentration was low, followed by phosphate, with the highest values recorded for ammonium (Table 4.2). Location AS had a significantly higher ammonium concentration than locations PS and PE, whereas turbidity was higher in PS than at PE, AS, and AE. Other water variables (TOC and salinity) were not significantly different among the locations ($p > 0.05$).

Table 4.2 Water column properties of the sampling locations. Values are means ($\pm$ SE) from replicates (n ; between brackets). Locations AS = Anthropogenic-Sheltered (Puntondo Bay); AE = Anthropogenic-Exposed (Langkai); PS = Pristine-Sheltered (Kodingareng Lompo); PE = Pristine-Exposed (Bone Batang). NTU = Nephelometric turbidity units. TOC = Total organic carbon. Lettering in brackets indicates homogenous groups after multiple comparison analysis ($\alpha=0.05$).

Variables	Locations			
	AS (5)	AE (3)	PS (3)	PE (3)
NO ₃ ⁻ ($\mu\text{mol l}^{-1}$)	1.72 (0.35)	1.45 (0.145)	1.59 (0.18)	1.22 (0.09)
PO ₃ ⁻ ($\mu\text{mol l}^{-1}$)	3.35 (0.38)	4.70 (0.25)	2.87 (0.65)	4.03 (0.15)
NH ₄ ⁺ ($\mu\text{mol l}^{-1}$)	30.36 (0.62) ^(a)	28.21 (0.40) ^(a,b)	26.63 (0.32) ^(b)	26.67 (0.35) ^(b)
Turbidity (NTU)	0.42 (0.07) ^(a)	0.30 (0.07) ^(a)	1.20 (0.04) ^(b)	0.32 (0.04) ^(a)
TOC (mg L ⁻¹)	15.40 (0.40)	14.33 (0.88)	14.00 (0.00)	16.67 (1.20)
Salinity	33.60 (0.24)	32.00 (0.00)	32.67 (0.33)	31.00 (0.00)

4.3.2 Biomechanical and morphological properties (species and plant parts)

Pooling for the 4 locations, species and plant parts (leaf and sheath) differed in morphology (Table 4.3). Between species, leaves and sheaths of *Enhalus acoroides* supported higher breaking forces (F_{MAX}) than those of *Thalassia hemprichii*. Within species, F_{MAX} was in the same range for sheaths and leaves of *E. acoroides*, whereas *T. hemprichii* leaves were markedly stronger than their sheaths (Table 4.3; Fig. 4.2). Similarly to F_{MAX} , the maximum breaking force per cross sectional area (breaking stress; F_{TS}) of *E. acoroides* leaves and sheaths were relatively higher than plant parts of *T. hemprichii*, especially for the sheaths (Table 4.3). The extensibility (L_{T}) was also highly variable between species and plants parts (Table 4.3; Fig. 4.2).

Sheaths can extend much longer than their leaves, both for *T. hemprichii* and *E. acoroides*, while extensibility was generally higher in *E. acoroides* than in *T. hemprichii* (Fig. 4.2). The mechanical properties F_{MAX} and L_{T} only showed a significant and positive correlation in *E. acoroides* leaves (Table 4.4; Fig. 4.2).

Table 4.3 *Enhalus acoroides* and *Thalassia hemprichii*. Mean values ($\pm$ SE) of the morphological and mechanical properties for both leaf and sheath. Length (L), width (W), thickness (T), breaking force (F_{MAX}), specific force-to-tear or breaking stress (F_{TS}) and elongation-to-tear or breaking strain (L_{T}).

	<i>Enhalus acoroides</i>		<i>Thalassia hemprichii</i>	
	Leaf (n=20)	Sheath (n=18)	Leaf (n=20)	Sheath (n=19)
Morphological properties				
L (cm)	32.88 (1.8)	8.50 (0.5)	7.92 (0.3)	4.17 (0.2)
W (mm)	13.16 (0.4)	14.70 (0.5)	8.67 (0.2)	9.33 (0.3)
T (mm)	0.47 (0.01)	0.46 (0.02)	0.33 (0.02)	0.47 (0.01)
Mechanical properties				
F_{MAX} (N)	21.63 (1.92)	21.31 (1.61)	6.69 (0.43)	2.79 (0.23)
F_{TS} (N mm ⁻²)	3.52 (0.29)	3.18 (0.21)	2.33 (0.1)	0.62 (0.04)
L_{T}	9.87 (0.61)	26.67 (1.58)	7.34 (0.36)	19.17 (1.04)

The breaking force (F_{MAX}) of *E. acoroides* did not correlate strongly to morphometry, only a weak correlation with sheath thickness was found. The tissue breaking stress (breaking force per cross-sectional area, F_{TS}) however was strongly correlated to the breaking force (F_{MAX}). On the contrary, the breaking force of *T. hemprichii* plant parts was strongly correlated to width, thickness, and, in the case of sheaths, also to their length. This indicates that the wider and thicker *T. hemprichii* leaves and sheaths become, the more mechanically resistant they are (Table 4.4, Fig. 4.3). Length, width and thickness in *T. hemprichii* were positively inter-correlated in either sheaths or leaves or both, whereas this pattern was not observed in *E. acoroides* (Table 4.4).

The variance partitioning (Fig. 4.4) show the contribution of the components that underlay the variability of F_{MAX} (maximum force to break or the breaking force), i.e., width, thickness and breaking stress (F_{TS}). The analysis showed that the breaking force of sheaths (both species) was mostly explained

by their material properties (breaking strength, accounting for > 50 %) rather than dimensions (T and W), which contributed evenly (ca. 25% each). Contribution to the breaking force of *T. hemprichii* leaves however, was governed by thickness (> 50%), followed by breaking strength (ca. 25%) and width (ca. 20%), in accordance to the correlation found among morphological and mechanical properties (Table 4.4). In the analysis for *E. acoroides* leaves, breaking stress (F_{TS}) accounted for > 90 % of the variability in breaking force (F_{MAX}), with little contribution of width (ca. 12%). The contribution of thickness of *E. acoroides* leaves to their breaking force(not shown in figure) was negative because explanatory variables (i.e. thickness and breaking stress) have strong and opposite effects to F_{MAX} (Table 4.4) (Legendre and Legendre 1998).

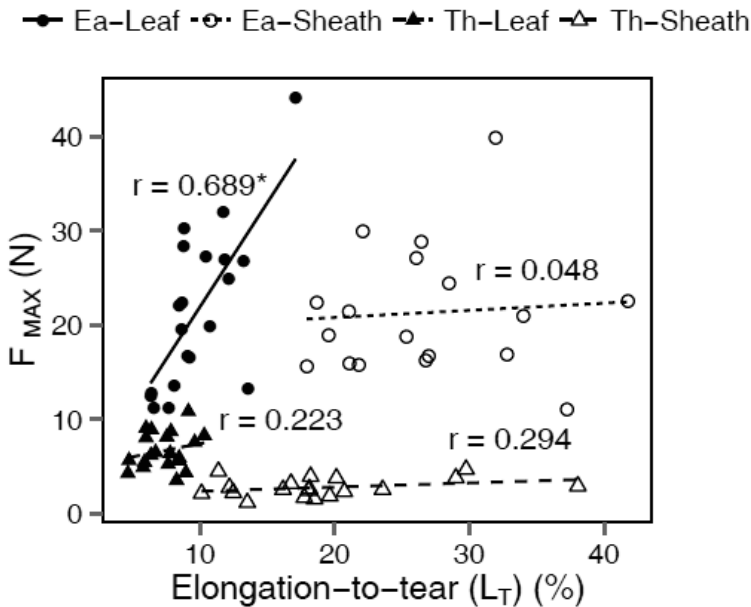


Figure 4.2 Pearson's correlation coefficient between F_{MAX} (breaking force) and L_T (elongation-to-tear) of both leaves and sheaths of *Enhalus acoroides* (Ea) and *Thalassia hemprichii* (Th). * $p < 0.05$

4.3.3 Biomechanical and morphological properties (among locations)

One way ANOVA showed significant differences among locations in the mechanical resistance, both in absolute terms (breaking force, F_{MAX}) and per cross-section area (breaking stress, F_{TS}), for *E. acoroides* leaves (Fig. 4.5), but not for *T. hemprichii* ($p > 0.05$). At locations AE and PS, leaves of *E. acoroides* were significantly weaker (lower F_{MAX} and F_{TS}), compared to those at location

PE (stronger leaves), whereas location AS showed no significant differences from all other locations (Fig. 4.5). The morphological properties of *E. acoroides* were also significantly different among locations, especially in their leaf length and width, and their sheath thickness and width (Fig. 4.6), but again not for *T. hemprichii* ($p>0.05$). At location AE, *E. acoroides* leaves were significantly shorter compared to those in other locations. Location PS had significantly narrower leaves compared to those in AS,whereasthe other two locations showed no significant differences in leaf width when compared to those in AS and PS (Fig. 4.6).

Table 4.4 *Enhalus acoroides* and *Thalassia hemprichii*. Pearson's correlation coefficients between morphological (L=length, W=width and T=thickness) and mechanical properties (F_{MAX} = breaking force, F_{TS} =specific force-to-tear or breaking stress; and L_T =elongation-to-tear or extensibility) for *Enhalus acoroides* and *Thalassia hemprichii* leaves and sheaths. * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$.

	W	T	F_{MAX}	F_{TS}	L_T
<i>Enhalus acoroides</i> leaves (n=20)					
L	-0.219	-0.299	0.313	0.439	0.181
W		-0.158	0.404	0.137	0.295
T			-0.236	-0.356	-0.277
F_{MAX}				0.951**	0.689**
F_{TS}					0.668**
<i>Enhalus acoroides</i> sheaths (n=18)					
L	0.071	-0.462	-0.080	0.114	-0.348
W		0.413	0.454	-0.187	-0.304
T			0.506*	-0.158	0.108
F_{MAX}				0.686**	0.048
F_{TS}					0.145
<i>Thalassia hemprichii</i> leaves (n=20)					
L	0.571**	0.250	0.348	-0.098	-0.016
W		0.406	0.490*	0.314	-0.362
T			0.744**	-0.224	0.177
F_{MAX}				0.380	0.223
F_{TS}					0.401
<i>Thalassia hemprichii</i> sheaths (n=19)					
L	0.448	0.789**	0.689**	0.386	0.135
W		0.418	0.667**	0.192	-0.297
T			0.613**	0.167	0.258
F_{MAX}				0.794**	0.294
F_{TS}					0.484*

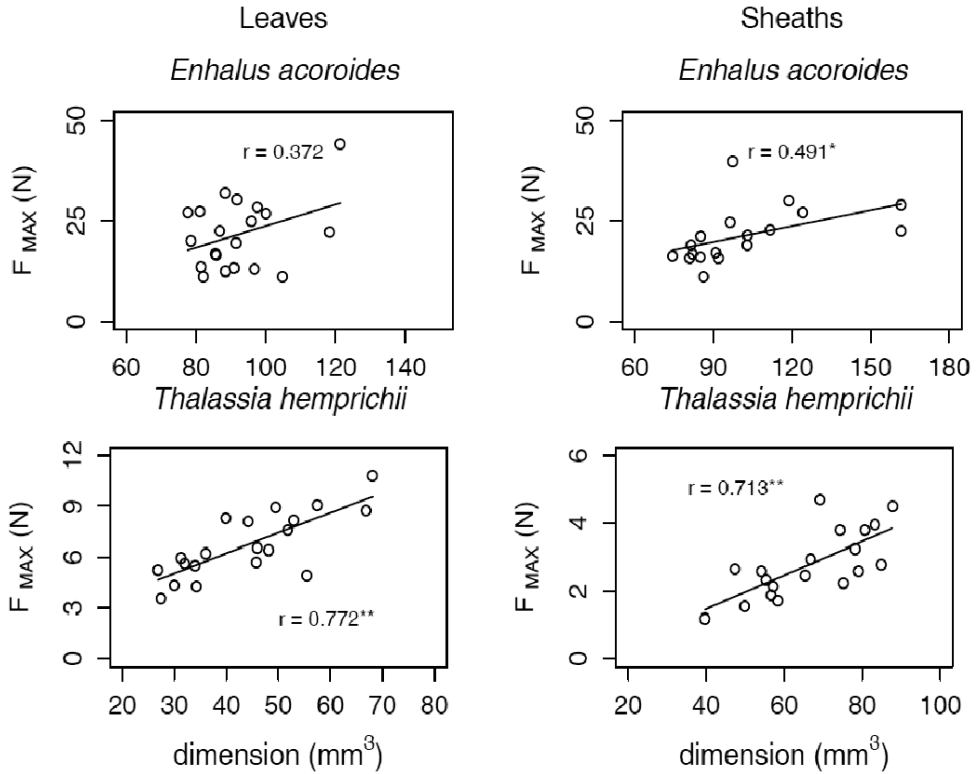


Figure 4.3 Pearson's correlation coefficient between F_{MAX} (breaking force, N) and morphometry (dimension, mm^3) of both leaves and sheaths of *Enhalus acoroides* and *Thalassia hemprichii* (Dimension = thickness * width * length of the specimen used in the tensile test). * $p < 0.05$, ** $p < 0.01$

4.4 DISCUSSION

Biomechanical plasticity of seagrass leaves is important for understanding their resistance to breakage and detachment under environmental heterogeneous conditions (Kopp 1999; La Nafie et al. 2012, 2013; de los Santos et al. 2013). In the present study we enhance the knowledge of mechanical properties of seagrass leaves by quantifying their extent of variation between two tropical species, between plant parts (leaf and sheath in the same species) and, intra-specifically across different environmental conditions. *Enhalus acoroides* was highly plastic, whereas *Thalassia hemprichii* did not show variation in their mechanical and morphological properties among locations. For *T. hemprichii* the mechanical properties were clearly related to their morphological properties (length, width and thickness). To the best of our

knowledge, the present study is innovative in showing inter- and intra-specific differences in the mechanical properties of slow-growing tropical seagrass species over a broad range of environmental conditions, and the analysis of its correlation with morphometry.

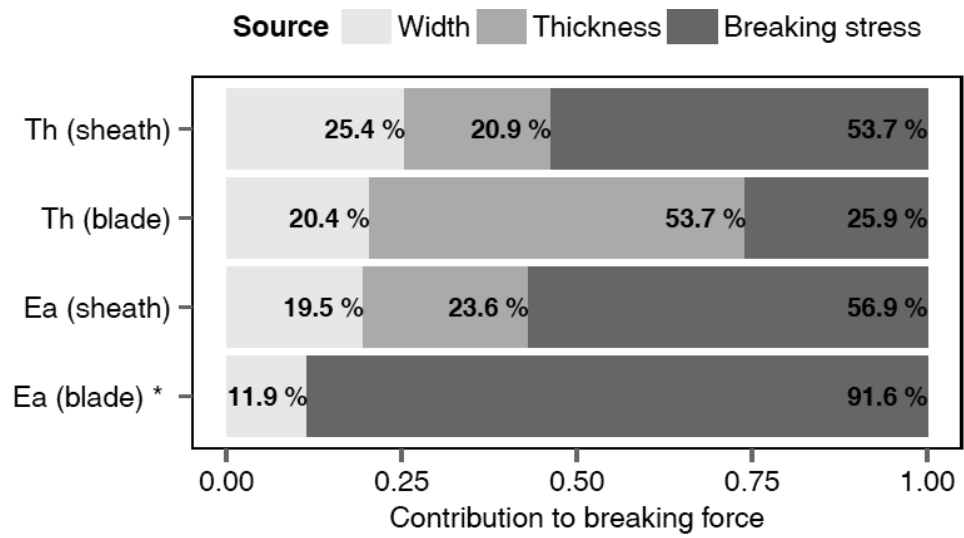


Figure 4.4 Contribution of thickness (T), width (W) and breaking strength (F_{TS}) to the breaking force (F_{MAX}), obtained by variance partitioning in *Enhalus acoroides* (Ea) and *Thalassia hemprichii* (Th)leaves and sheaths, pooled across the study sites. (*) Contribution of thickness was negative as it was strongly but negatively related to breaking strength (F_{TS})

4.4.1 Biomechanical and morphological properties (species and plant parts)

Differences in the morphometry of *E. acoroides* and *T. hemprichii* have been well described in previous studies. *Enhalus acoroides* is the biggest tropical seagrass species (the highest leaf surface and the largest rhizome diameter) with the slowest growth rate (comparable to the temperate species *Posidonia oceanica*) (Duarte 1991; Marbà and Duarte 1998). To date, it is also the seagrass species with the highest leaf resistance or F_{MAX} (21.63 ± 1.92 N) (Table 4.3) recorded for the tropic which is mainly explained by its material properties (i.e., higher F_{TS} when compared to *T. hemprichii*), as probably due to its reinforcement with wiry fibres at the leaf margins. Hence, in terms of

breaking force, the material properties are the most important factor contributing to the high mechanical resistance of *E. acoroides*.

Within species, resistance of seagrass leaves may be mainly explained by their morphometry (width, thickness or both, i.e. cross-sectional area), as found in *Zostera noltii* (La Nafie et al. 2012), *Cymodocea nodosa* (de los Santos et al. 2013), *Halophila ovalis* (La Nafie et al. 2013), and *T. hemprichii* (this study). However, this pattern was not described in *E. acoroides*, seemingly due to its unique leaf design of marginal fibre-reinforcement. Therefore, the leaf traits controlling the resistance of seagrass leaves apparently differ between species (because some mechanical properties are scale-dependent, i.e., resulted from the plant size and material properties, and some are scale-independent, i.e., standardized by material cross-section area or volume) and probably also among environments. However, causal relationships and underlying mechanisms warrant further research.

The high resistance of *E. acoroides* leaves appears to be a necessary adaptation related to its long and wide leaves (i.e. high surface area), what makes the shoots face high drag forces in hydrodynamically active environments (de los Santos 2011). In addition, strong leaves are most likely a prerequisite for long leaves to obtain a long leaf life-span, as is the case for *E. acoroides* (i.e., ~ 100 days, Hemminga et al. 1999). Another possible adaptation is the fibers that are in the edge of the leaves, which provide strength and support to these long leaves, without increasing the second moment of area (a geometrically weighed cross-section area). Hence, in order to be long-lived, the tissues have to be stronger, thicker or, as described for *E. accoroides*, reinforced with fibers at the edge of the leaves.

Thalassia hemprichii leaves were stronger than their sheaths, contrasting to our hypothesis that sheaths would be strongest, as seen in the base of seaweed *Hedophyllum sessile*, which act as a wave absorber (Armstrong 1987). Our result may be due to the fact that the short sheaths are mostly buried within the sediment (Kuo and den Hartog 2006) and thus protected from the imposed hydrodynamic forces. Also younger tissues are weaker than the older ones, as was found in *Zostera marina*, where leaf tissues just above the sheaths (younger tissues) were less strong than near the leaf apex (older tissues) (Kopp 1999). Similarly to *Posidonia oceanica*, where inner younger leaves were weaker than outer older leaves (Rammsy et al. unpublished). In contrast, the sheaths and leaves of *E. acoroides* were equally strong as probably due to the continuity of the marginal fibres from the sheaths to the leaves (pers. observation). For both species, sheaths were more extensible than leaves. This was probably due to the softer meristematic tissues that are part of the sheaths (covered within the sheaths), that still have elastic cell walls; after full elongation or stretching of the cells, the cell wall get fixated (Tyerman 1989).

4.4.2 Biomechanical and morphological properties among locations

The four Indonesian islands studied covered a gradient of anthropogenic activities (relatively pristine versus anthropogenic impacted areas) and wave exposure (sheltered versus exposed). Snapshot water column nutrient conditions showed that ammonium was significantly higher in location AS compared to the other locations. At the PS location water turbidity was high, probably related to the resuspension of the fine sediments. Salinities were comparable among locations.

The seagrass *Enhalus acoroides* was more responsive to local conditions than *T. hemprichii*, both in their morphological and biomechanical properties. At location AE (Anthropogenic impacted and Exposed), *E. acoroides* plants have distinctively different dimensions (i.e., wider and thicker sheaths and shorter leaves) compared to the other 3 locations. Plants were collected in the outer part of the Langkai island (AE), very close to the reef edge and the slope, where seagrass meadows are subjected to strong hydrodynamic conditions (specially waves), which is reflected in the coarse sediment (big rubbles of ca. 3-5 cm diameter). Having short leaves under such conditions, has been previously described as a strategy to cope with physical stresses (cf. La Nafie et al. 2012; de los Santos et al. 2013).

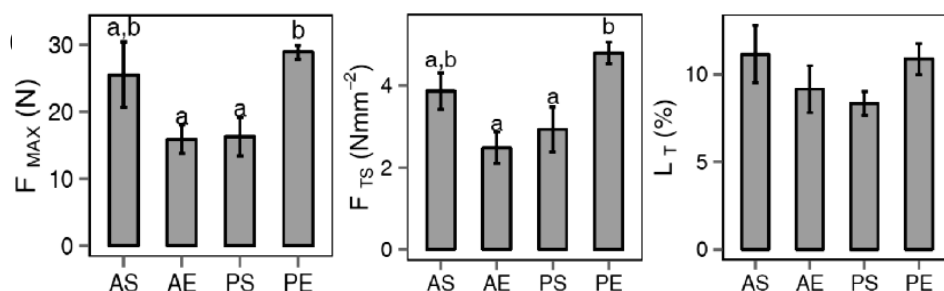


Figure 4.5 Biomechanical properties (F_{MAX} , F_{TS} and L_T) of *Enhalus acoroides* (Ea) leaves in each location. F_{MAX} : breaking force (N). F_{TS} : breaking strength (N mm⁻²). L_T : extensibility (%)

The absolute force needed to break the leaves (F_{MAX}) of *Enhalus acoroides* followed the same pattern to their specific force-to-tear (F_{TS}). Leaves at location PE showed a highly significant F_{MAX} and F_{TS} compared to those at locations AE and PS. Since location PE is uninhabited and being further out from the mainland by not having influence from the mainland run-offs, this location may have much less nutrient concentrations compared to most other locations (Vonk et al. 2008). This low nutrient (but high energy) environment may explain why location PE has stronger *E. acoroides* leaves, in line with

findings of Kopp (1999) and La Nafie et al. (2012), where high nutrient concentrations weaken the plants. It is also in line with findings of de los Santos et al. (2013), where temperate seagrass species exposed to high hydrodynamics showed an increase in breaking stress relative to those from sheltered locations. Plants in location AS did not differ from the other locations in their leaf strength, even though having a high nutrient availability (i.e. from the mangroves, seaweed cultures). However, since location AS has low hydrodynamic activities, this could compensate for the nutrient effect. Unlike *T. hemprichii*, *E. acoroides* showed significant differences in morphometry among locations, suggesting that *E. acoroides* is more plastic than *T. hemprichii*, at least for the studied environmental range. Overall, the significant differences in mechanical properties as well as morphology did not reveal a clear pattern of correlation to anthropogenic influence or exposure across sites. This might be speculated due to a lack of strong systematic contrast between sites on anthropogenic influences, but manipulative experimenting is difficult given the long leaf lifespan of these climax species.

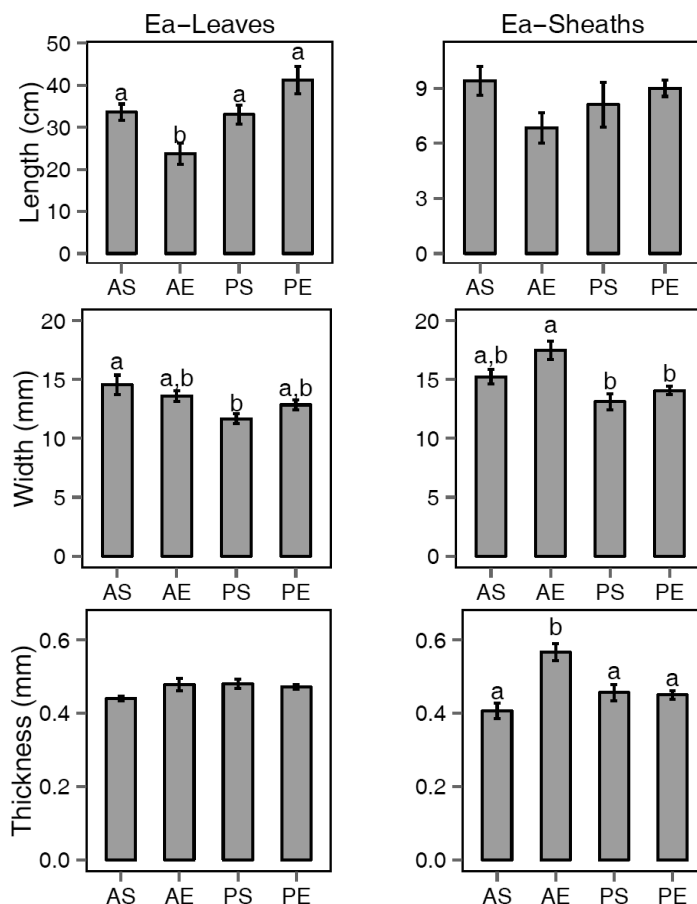


Figure 4.6 Morphological properties (Length, Width and Thickness) of *Enhalus acoroides* (Ea) leaves and sheaths. Lettering indicates homogenous groups after ANOVA and multiple comparisons ($\alpha=0.05$)

4.4.3 Ecological implications

Both *Enhalus acoroides* and *Thalassia hemprichii* are climax species, i.e. large species that can be sensitive to disturbances but may also have high resistance to changes (Hemminga and Duarte 2000). Each species may have different strategies when coping with highly variable environmental conditions. With *E. acoroides* being the biggest (i.e., biggest leaf surface – (100.78 cm²), largest rhizome diameter (15mm)) and the slowest growing seagrass species in the tropics, the leaf appearance rate per year is the lowest among all seagrass species (Duarte 1991; Marba and Duarte 1998) and with a high C/N ratio

(Duarte 1990). Hence, it seems logical that such high carbon investments in mechanically resistant leaves that contain carbon-fibers have benefits by reducing leaf fracture risks due to both abiotic (i.e. hydrodynamic forces; this study) and biotic factors (i.e. grazing; Mariani and Alcoverro 1999; de los Santos et al. 2012). The strategy possessed by *E. acoroides* may be regarded as a tolerance strategy. Through this strategy, plants maximize their resistance to breakage (Puijalon et al. 2011) resulting in a reduced risk of mechanical failure leading to an increased probability of survival.

In this study, we show that even though both *E. acoroides* and *T. hemprichii* are climax species, *E. acoroides* appears to be more plastic in their morphometry than *T. hemprichii*. Such higher responsiveness of *E. acoroides* to different environmental settings is probably needed to enable them to maintain their extremely long-lived leaves (leaf life span for *Thalassia* and *Enhalus* are 35-65 and 75-155 days, respectively; Hemminga 1999). Indeed, in hurricane events, climax species with long-lived leaves are often more resistant than pioneer species (e.g. Fourqurean and Rutten 2004; van Tussenbroek et al. 2008), in line with a previous study showing that climax species are stronger than pioneer species (de los Santos et al. 2012).

Surprisingly, the strong *E. acoroides* plants are more susceptible to sea turtle grazing than *T. hemprichii* (Christianen 2013). The adaptations of *E. acoroides* to reduce the risk of mechanical failure apparently do not protect against grazing. Most likely, *T. hemprichii* may be relatively well protected from grazing by its underground meristems and higher leaf turnover than *E. acoroides*, although *T. hemprichii* remains relatively susceptible to grazing in comparison to other seagrass species (Lal et al. 2010; Christianen 2013). Apparently, megaherbivores such as sea turtles that have specialized-feeding apparatus with a very high (biting) force (i.e. 123 – 303 N; Marshall et al. 2014) do not encounter the mechanical strength of seagrass leaves as a problem. Leaf strength seems therefore most important for resisting hydrodynamic forces and contributing to resisting smaller grazers.

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CHAPTER 5

Waves and high nutrient loads jointly decrease survival and separately affect morphological and biomechanical properties in the seagrass *Zostera noltii*.

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ABSTRACT

*In an 8-week aquarium experiment, we investigated the interactive effects of waves (present vs. absent) and water column nutrient level (high vs. low) on the survival, growth, morphology, and biomechanics of the seagrass, *Zostera noltii*. Survival was reduced when plants were exposed to both waves and high nutrient levels. Wave and nutrient interaction significantly reduced aboveground biomass and leaf lengths, whereas waves independently reduced growth rate, internode abundance, elongation, and appearance rates. Nutrient supply significantly reduced the strength of the leaves. Wave and nutrient interaction was the main driving force affecting survival and morphological properties of seagrass, whereas dynamical characteristics were independently affected by waves, and nutrient supply affected mainly biomechanical properties. In conclusion, this experiment revealed that the combination of exposure to waves and high nutrient levels was detrimental for *Z. noltii*, which indicates that this could be an important unexplored force involved in seagrass declines.*

5.1 INTRODUCTION

Being sessile organisms, seagrasses are prone to facing adverse environmental conditions from which they cannot escape that may lead to their loss, as has been observed worldwide (Waycott et al. 2009). In the current era where human use of coastal areas is intensifying (Small and Nicholls 2003) and global change processes affect the climate, the co-occurrence of adverse environmental conditions in littoral areas may be expected to increase. For example, anthropogenic activities may enhance nutrient loads via fertilizer usage and sewage release (Cabaço et al. 2008) whereas at the same time, global change process may cause increased storm frequency and wave stress (Young et al. 2011). Since these two abiotic factors co-exist in littoral areas and are expected to further increase in the near future (Trenberth et al. 2007); therefore, this study aims to unravel how both abiotic factors interact and may affect the seagrass *Zostera noltii*.

High nutrient levels in coastal environments are mainly due to increased anthropogenic activities (Cabaço et al. 2008). Depending on the environment, an enhanced nutrient load may sometimes have a positive (Perez et al. 1991; Brun et al. 2002) or no effect (Harlin and Thornemiller 1981; Lee and Dunton 2000) on seagrasses. However, nutrient enrichment generally has a negative effect on seagrass growth and survival either directly due to the toxic effect of nitrate (Burkholder et al. 1992) or ammonium (van Katwijk et al. 1997; Brun et al. 2002) or indirectly due to algal overgrowth, which causes light deprivation and enhanced organic matter fluxes to the sediment (Short et al. 1995; Brun et al. 2003). Hydrodynamic conditions from waves and currents are also known to have direct and indirect effects on seagrass ecosystems. For example, increased wave energy can directly reduce survival and number of seagrass shoots (van Katwijk and Hermus 2000), while increased flow velocity can directly affect plant growth and morphometry (Schanz and Asmus 2003; Peralta et al. 2006; de Los Santos et al. 2010). In addition, increased flow velocity and/or waves can have indirect effects on the photosynthetic rates and nutrient uptake by reducing boundary layers (Koch 1994). Last but not least, hydrodynamics can affect seagrasses by affecting the light availability due to self-shading or via turbidity (Koch 2001).

Although the individual effects of nutrient levels and hydrodynamic on seagrasses have both been studied in detail, their interactive effect may be difficult to predict as plant responses to individual stressors are diverse and, in some cases, opposite. For example, leaf size of *Zostera marina* was increased in response to nutrient enrichment (Short 1983), whereas *Zostera noltii* reduced leaf size under high flow velocities (Peralta et al. 2006). Hence, the aim of the

present study is to assess in an aquarium experiment the interactive effects of wave forces and nutrient enrichment on the survival, morphodynamical, and biomechanical properties of seagrass *Zostera noltii*, a fast growing species with an extensive morphological plasticity (Peralta et al. 2005). In most seagrass studies, morphodynamical responses are typically included by measuring variables such as length, appearance and elongation rates of the different modules of the plant (i.e., shoots, rhizomes, and roots [Short et al. 1995; Lee and Dunton 2000; Peralta et al. 2005]). Biomechanical properties such as leaf strength, and stiffness are however, poorly studied on seagrasses (but see Patterson et al. 2001), even though they have been identified as important acclimation traits to the mechanical drag that imposes stress in several marine organisms (Koehl 2000) as well as terrestrial plants (Anten et al. 2005). Waves form a major mechanical stress, so we included biomechanical properties in our study. We address the following questions: Are plants responding separately to both stressors? or is there any combined effect of both stressors?

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

In an experimental setup, *Zostera noltii* plants were exposed during 46 d to waves (present vs. absent) combined with water column nutrient level (high vs. low) in a full factorial design: (1) wave-high nutrient (W-HN); (2) no wave-high nutrient (NW-HN); (3) wave-low nutrient (W-LN); and, (4) no wave-low nutrient (NW-LN). Two replicate tanks per treatment were installed, giving a total of eight tanks which were all filled with a 30 cm water column. Four big tanks (120 x 100 x 74 cm³; 346 liters) were equipped with a hydraulic wave generator (operated 24 h daily), and two big tanks were split into two independent compartments to obtain four replicates without waves. Waves in the experiment were generated using a wave paddle that precisely fitted the width of the tank; the paddle was moved via a hydraulic piston from which the outward and inward movement could be controlled separately. To simulate natural waves in a small experiment is difficult because wave reflections will typically result in a standing wave. To prevent occurrence of such standing wave, we set the system to give approximately every 50 s a quick (10 s) push followed by a slow (40 s) retreat of the wave paddle. This resulted in a large wave, followed by a series of attenuating reflecting waves (Fig. 5.1). The reflecting waves were small enough to prevent a standing wave from forming. The resulting chaotic wave pattern does not reflect a natural system, but rather mimics a hydrodynamic stress in that the water movement causes the leaves to flap back and forth. The height of the pots was 11 cm and the water height was

~ 30 cm. The maximum wave height was ~ 4 cm (Fig. 5.1; Druck PTX1830 pressure sensor). Each of the tanks contained 12 transparent pots (17.5 x 11 x 11 cm³) with one experimental plant unit (EPU) consisting of a piece of a ramet composed of an apical and the first lateral shoot, plus two internodes in each pot (in total of 96 EPUs). The pots were surrounded by bricks acting as weight to prevent the pots from moving with the wave action. The sediment was composed of clean natural pebble (2-4 mm grain size) and sand in a 2:1 volume ratio. To minimize heterogeneity, the sediment was homogenized with a cement mixer. After filling the pots with the mixture, some more pebbles were placed on the surface of all the pots to avoid erosion due to wave action.

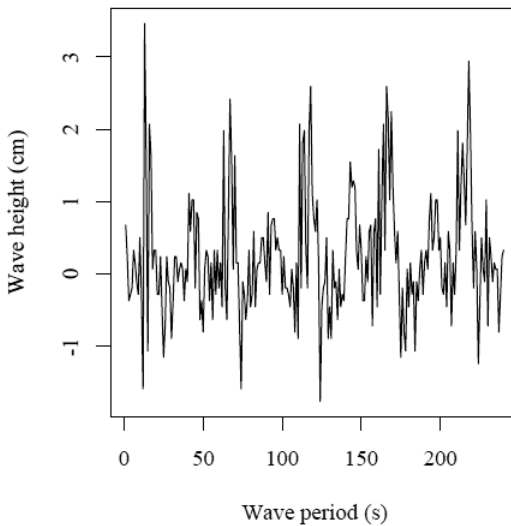


Figure 5.1 Wave height in the tank, showing wave period approximately 50 seconds, as a result of a quick (10 s) push followed by a slow (40 s) retreat of the wave paddle.

All tanks were filled with 0.2 μm filtered seawater from the eastern Scheld (southwest Netherlands) and the water of all the tanks was renewed twice per week. The ambient nutrient concentration of this seawater was used as low-nutrient treatment- (i.e., W-LN and NW-LN). For the high-nutrient treatments (i.e., W-HN and NW-HN), the water column in our treatments was enriched with $\text{Ca}(\text{NO}_3)_2$ and $(\text{NH}_4)_2\text{HPO}_3$ to a final desired average concentration of ~ 55 $\mu\text{mol L}^{-1}$ nitrate, 10 $\mu\text{mol L}^{-1}$ ammonium, and 10 $\mu\text{mol L}^{-1}$ phosphate. These nutrient concentrations corresponded to a moderately high eutrophication level (Burkholder et al. 1992; Valiela and Cole 2002). In addition to that, most of the cultural eutrophication (urban wastewater, agricultural run-off discharges) that affect seagrass habitats is mainly composed of nitrogen in the form of nitrate and ammonium and phosphorous in the form of phosphate (Burkholder et al. 2007; Cabaço et al. 2008). Continuous aeration was supplied

to assure complete mixing of the water column. Every time the water was refreshed (twice per week), the position of the aeration systems and the pots (including plants) were changed randomly. During water refreshments, accumulated algae were removed by cleaning the pots, tanks and leaves carefully. Amounts of algae were small and no large differences between treatments were observed. Observed dead plants and floating dead leaves were also taken out of the tanks. In addition, light irradiance was measured before water refreshment four times during the experiment with a Li-Cor LI-1000. At the same time, the chemical properties of the water column (i.e., salinity, temperature, and pH) were also measured by using an YSI multimeter sonde (Model 556).

Light was supplied by 14 Son-T lamps (400 Watt each) and the irradiance did not vary among and within treatments during the experiment. The average surface irradiance was $273 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (ranging from 248 to $294 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Photoperiod was set at 18:6 light:dark period. The resulting daily-integrated photon irradiance of $17.7 \text{ mol photons m}^{-2} \text{d}^{-1}$ is known to facilitate the clonal growth in this species (Peralta et al. 2002). Experiment room temperature was kept constant throughout the entire experimental period (19°C). Prior to refreshment, water samples were collected to check whether nutrient uptake had occurred. At the beginning of the experiment, water samples were collected to check the applied concentrations. Ammonium, nitrate, and phosphate concentrations in water column were measured colorimetrically on an autoanalysing system (QUAatro).

5.2.2 Biological material and measurement

Zostera noltii Horneman plants were collected from an intertidal bed in Cádiz Bay Natural Park (Southern Spain, $36^{\circ}29'19.79''\text{N}$; $6^{\circ}15'53.05''\text{E}$) in May 2009, 7 days before starting the experiment. Some plants were randomly separated for initial measurements at the University of Cádiz (Cádiz, Spain). The rest of the plants were packed in moist, dark, and cool conditions to be transported the same day of collection to the Netherlands Institute for Sea Research (Yerseke, The Netherlands), where the experiment was conducted. Upon arrival at the Netherlands Institute for Sea Research (within 2 d), plants were kept in a reservoir with filtered natural seawater, aeration and light in a mesocosm room (controlled temperature 19°C) for an acclimation period, before starting the experiment. Among the pool of plants, 96 EPU were randomly taken.

Table 5.1 Morphological, dynamical, and biomechanical properties of the experimental plant units (EPU) measured and/or calculated, at initial (t_0) and final conditions (t_f). Subscript $i = 1, 2, 3, \dots$; t = experimental time (d). F (N) force applied to the specimen; δ (mm) displacement of the specimen at the breaking point; L_0 (mm) initial length of the specimen.

Variables	Units	Description
Survival (S)	%	$S = \frac{\text{Live EPU}}{\text{Initial EPU}} \times 100$
Growth rate (GR)	g fresh wt d ⁻¹ EPU ⁻¹	$GR = \frac{\text{Biomass}_f - \text{Biomass}_0}{t_f - t_0}$
Aboveground biomass (AG)	g dry wt EPU ⁻¹	leaf biomass
Belowground biomass (BG)	g dry wt EPU ⁻¹	rhizome and root biomasses
Morphological properties		
Leaf length (LL)	cm	mean leaf length in an EPU
Internode length (IL)	cm	mean internode length in an EPU
Root length (RL)	cm	mean root length in an EPU
Internode abundance (IA)	internodes EPU ⁻¹	mean no. of internodes in each EPU
Root abundance (RA)	roots EPU ⁻¹	mean no. of roots in each EPU
Cross-section area (CA)	mm ²	CA = leaf width × leaf thickness
Morphodynamical properties		
Internode appearance rate (IAR)	internodes EPU ⁻¹ d ⁻¹	$IAR = \frac{IA_f - IA_0}{t_f - t_0}$
Internode elongation rate (IER)	mm EPU ⁻¹ d ⁻¹	$IER = \frac{\sum (IL_{(i,f)} - IL_{(i,0)})}{t_f - t_0}$
Biomechanical properties		
Specific force-to-tear (F_{TS})	N mm ⁻²	$F_{TS} = \frac{F_{MAX}}{CA}$
Young's modulus of elasticity for tension (E_T)	N mm ⁻²	$E_T = \frac{L_0 \times F}{CA \times \delta}$

At the beginning of the experiment, we randomly separated 16 EPU from the pool, and measured their morphological characteristics (leaf length, width and thickness; internode length and abundance; and root length and abundance; Table 1). Prior to being transplanted, each randomly taken EPU was weighed (fresh weight [fresh wt]), and each rhizome was individually number-tagged in the youngest internode. EPUs were randomly assigned to experimental pots, and pots randomly distributed among the treatments. During water refreshments (twice per week, i.e., 13 times), we quantified plant survival.

At the end of the experiment, all plant material was carefully harvested to keep rhizomes and roots intact. We conducted morphometric measurements on all of them, which allowed us to estimate the dynamic properties of the plants following Peralta et al. (2005; Table 5.1). Furthermore, living EPUs (tagged at the beginning) were individually weighed (fresh wt) to calculate the net growth rate (GR; mg fresh wt EPU⁻¹ d⁻¹) using a linear growth model (Table 5.1). Finally, EPUs were divided into modules (leaves, roots and rhizomes), freeze-dried and weighed (dry weight; dry wt) to calculate the aboveground-belowground biomass ratio (AG:BG ratio).

5.2.3 Biomechanical properties

Mechanical properties of leaves were measured with a tensometer (Instron® model 3342) - at the University of Cádiz. We measured the strength and stiffness of the leaves (Patterson et al. 2001) which are scale-invariant material properties as they are standardized by leaf volume or cross-sectional area. These properties illustrate how leaves respond under the mechanical load resulting from wave action. Tearing (or tensile) tests - which measures the breaking force required to tear a tissue fragment - were conducted on a pool of 16 plants at the end of the experiment, by taking living shoots randomly from the harvested plants. The tests were always performed on the third leaf from the apical shoot of the EPUs, which was carefully cleaned of epiphytes and kept moist all the time. The chosen leaves were cut off at the junction between the sheath and the blade. Prior to the test, the width and thickness of the leaves were measured with calliper and dial thickness gauge (Mitutoyo®, precision ± 0.01 mm) respectively, to calculate the cross-section area (CA, mm²) (Table 5.1). Then, the leaf blades were individually clamped into the grips of the tensometer with the mountings 10 mm apart. Once clamped in the 5-Newton (N) grips (model 2712), the leaf blades were stretched at a velocity of 10 mm min⁻¹, while the extension (δ , mm) and the force (F, N) were recorded every 0.1 s until the leaf blades broke, at which point the maximum force (F_{MAX}, N) and extension (δ_{MAX} , mm) were recorded. As a result, we obtained the two biomechanical properties: (1) specific force-to-tear or strength (F_{TS}, N mm⁻²) calculated as the

the maximum force (F_{MAX} , N) per cross-sectional area (CA, mm^2), and (2) the modulus of elasticity for tension or stiffness (E_T , N mm^{-2}), which define the resistance to deformation (Niklas 1992) and is calculated by taking the initial, computer-fitted slope from the force against extension graph (F per δ), as well as considering the initial length (L_0) and the cross-sectional area of the specimen (CA) (Table 5.1).

5.2.3 Statistical analysis

In the factorial design, the experimental plots (independent tanks) were replicated twice. To demonstrate that there were no significant differences among the replicated tanks, a two-factor nested (ANOVA) test was conducted - where the tanks (the random factor) were nested within the treatments (the fixed factor). No significant differences were found between replicated tanks, so each plant in each pot was considered as an independent replicate when conducting further statistical tests. Plant survival was examined by Kaplan-Meier survival analysis (nonparametric) and the effects of the treatments were tested by log rank (Mantel-Cox) test with sequential Bonferoni-corrected α levels. We set the α values as highly significant, significant and marginally significant when $\alpha = 0.01$, 0.05 , and 0.1 , respectively. Survival data are presented as percentage of living (survived) plants. To test the effect of waves (W), nutrients (N), and their interaction (W x N) on two categories of response variables, (i.e., morphodynamical and biomechanical properties of the plants), we used a multivariate analysis of variance (MANOVA). The morphodynamical properties included growth rate; above- and belowground biomass; leaf, root, and internode length; internode and root abundance; internode appearance and internode elongation rate. The biomechanical properties included strength and stiffness. The p-values of the univariate responses were used to indicate which of these separate morphodynamical variables gave the strongest response within group of morphodynamical variables. This was also applied to the group of biomechanical variables (i.e., toughness and stiffness). Those data that deviated from normality (Kolmogorov-Smirnov's test) or homoscedasticity (Levene's test) were transformed prior to analyses to meet MANOVA assumptions. Data for these variables are presented as mean $\pm$ 1 standard error. Differences in nutrient concentrations among treatments were tested by a two-sample Student's t-test for independent samples.

Table 5.2 Averaged nutrient concentrations before and after water refreshment for each nutrient treatment. Results of the two-sample Student's *t*-test for independent samples, showing the differences in nutrient concentration among treatments. Significantly different when $p < 0.05$; superscript letters indicate significant differences between HN and LN (ns=nonsignificant).

	NH_4^+ ($\mu\text{mol L}^{-1}$)	NO_3^- ($\mu\text{mol L}^{-1}$)	PO_4^- ($\mu\text{mol L}^{-1}$)
After refreshment (nutrient $p < 0.05$)			
HN	9.6 ± 0.3	55.4 ± 3.2	11.0 ± 0.5
LN	0.7 ± 0.1	19.3 ± 0.5	1.2 ± 0.04
Before refreshment (inserted letters indicate significant differences between HN and LN)			
HN	$0.81 \pm 0.13^{\text{ns}}$	$6.87 \pm 3.37^{\text{ns}}$	$5.05 \pm 0.80^{\text{a}}$
LN	$0.84 \pm 0.11^{\text{ns}}$	$1.89 \pm 0.84^{\text{ns}}$	$0.21 \pm 0.07^{\text{b}}$

5.3 RESULTS

5.3.1 Water parameters

Throughout the experiment, water temperature (21-22°C), salinity (30-31‰), pH (8.0-8.2), and dissolved oxygen (85-92%) remained fairly constant. The initial nutrient concentration after water refreshments statistically differed between HN and LN treatments (Table 5.2, two-sample Student's *t*-test, $df = 3$, $p < 0.05$), which demonstrates that the nutrient treatment was correctly applied. The nutrient concentrations in the water before water refreshment were lower than the applied ones, indicating that there was nutrient uptake in all the treatments (Table 5.2). Although this uptake may have been partly due to uptake by algae, we expected that this 'sink' was limited due to (1) the high refreshment rate, (2) the frequent removal of algae, and (3) the observation that the amount of removed algae was small (not quantified).

5.3.2 Biological measurements

All plants survived during the first 4 weeks (eighth observation) independent of the treatment (Fig. 5.2A). It was not until the fifth week (i.e., ninth observation; Fig. 5.2A) when some of them started to die in the W treatments (both HN and LN), where in many dead rhizomes were buried without aboveground biomass. Only 42% and 62% of the initial plants were

alive at the end of the experiment in the W-HN and W-LN treatment, respectively.

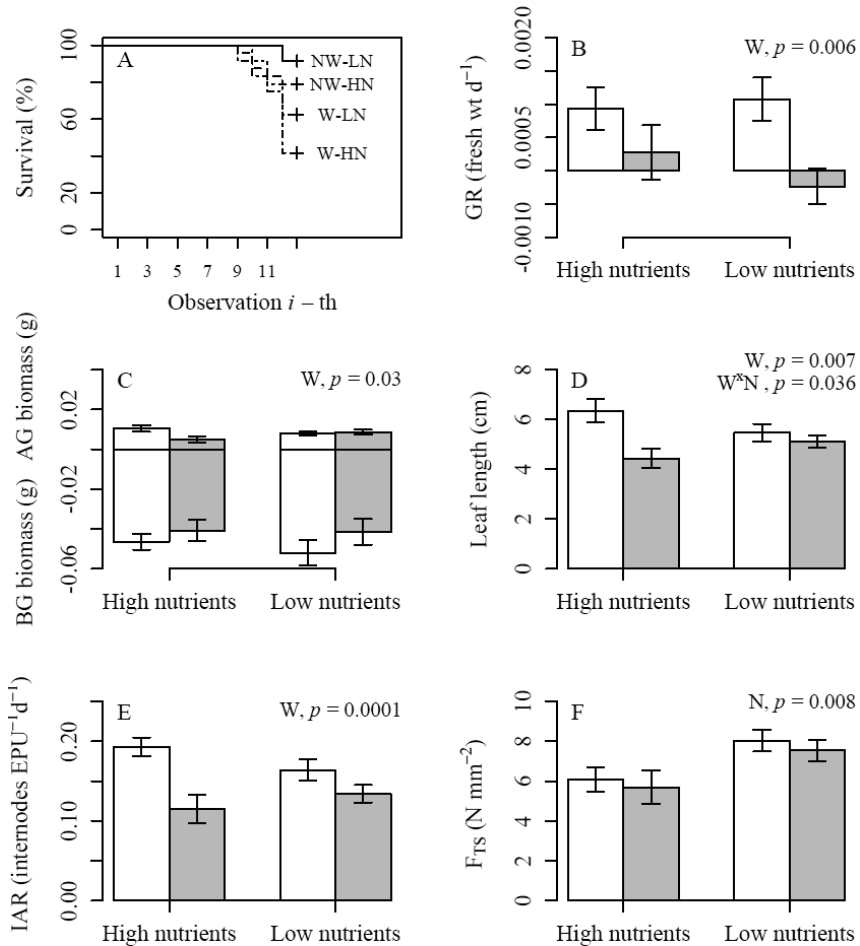


Figure 5.2 (A) Kaplan-Meier survival function for each treatment, showing the percentage survival and number of observations (significances *see* Table 5.2). (B-E) show the univariate responses that are most related to the significant multivariate response of morphodynamic properties to waves and the interaction of waves and nutrients, i.e., (B) growth rate, (C) aboveground biomass (belowground biomass also depicted), (D) leaf length, and (E) internode abundance rate. (F) shows the univariate response of stress (or specific force-to-tear - F_{TS}) that is most related to the significant multivariate response of the biomechanical properties to nutrients. The p -values give an indication of the strength of the univariate response (Table 5.4). Error bars represent mean $\pm$ 1 SE. All blank bars correspond to NW-no wave- treatment while grey bars correspond to W-wave. The letter W indicating waves, and N indicating nutrients.

Contrastingly, plants in NW treatments remained alive until sixth and seventh week for HN and LN, showing a final survival percentage of 79% and 92%, respectively. Results of the Kaplan – Meier survival analysis (Table 5.3; Fig. 5.2A) showed significantly lower survival under wave treatments as compared with non-wave treatments, both when comparing the high nutrient treatments and comparing the low nutrient treatments ($\alpha = 0.1$; Table 5.3). In addition, results of the two-way MANOVA showed a negative influence of waves on morphodynamic properties. The strongest negative univariate responses to waves were on internode abundance and appearance rate, growth rate and leaf length (Table 5.4, Fig. 5.2B, D, E). Moreover, strong interactive effects were shown for survival and morphodynamical properties. The combination of wave treatment and high nutrients showed a significantly reduced survival as compared with the non-wave, low-nutrient treatment ($\alpha = 0.01$; Table 5.3). Morphodynamical properties were synergistically negatively affected by the combination of waves and nutrients, with dominant univariate effects on aboveground biomass and leaf length (Table 5.4; Fig. 5.2C-D). 5.3.3 Biomechanical Properties

The biomechanical properties (the combination of strength or specific force-to-tear, F_{TS} and stiffness or modulus of elasticity for tension, E_T) were significantly negatively influenced by nutrient additions, with the strength having the strongest response (MANOVA, Table 5.4, Fig. 5.2E). The biomechanical properties were not influenced by wave presence (Table 5.4).

5.4 DISCUSSION

Increasing (anthropogenic) nutrient loads and storm frequency (due to climate change) in coastal areas may globally affect seagrass habitats. To assess the seagrass responses to such multi-stress conditions, we quantified the combined effects of the presence vs. absence of waves and high vs. low nutrient load on the seagrass *Zostera noltii*. To our best knowledge, we show for the first time how waves and high nutrient loads interact and affect the survival and morphodynamical properties of a seagrass species. Furthermore, our study showed that waves mainly had a negative effect on the morphodynamical properties, whereas nutrient enrichment affected biomechanical properties on the seagrass *Zostera noltii*. However, the effect of combined stresses (waves and nutrient enrichment) was much larger than expected from assessing both factors separately.

In our experiment, survival was negatively affected by waves and by the interaction of waves and nutrient enrichment. The highest survival percentage was found in NW-LN (92%), followed by NW-HN treatment (79%), whereas the lowest survival was by far recorded in W-HN (42%), followed by W-LN (63%;

Fig. 5.2A). Significant (and/or marginally significant) differences resulted among W and NW treatments, which indicated that waves predominated over nutrient effects regarding plant survival. It may be speculated that toxic effects of ammonium (van Katwijk et al. 1997; Brun et al. 2002) might, in part, explain the lowest survival in W-HN followed by W-LN treated EPU's, because wave treated EPU's (both HN and LN) are likely to have lower diffusive boundary layers (Koch 1994; Morris et al. 2008) and, hence, could have a higher ammonium uptake (Britto and Kronzucker 2002). Although the ammonium levels used in this experiment ($\approx 10 \mu\text{M}$) are close to toxic levels (van Katwijk et al. 1997; Brun et al. 2002) we lack the data to substantiate the hydrodynamic influence on ammonium toxicity in the present study, and this should be studied in the future.

Table 5.3 Kaplan – Meier survival analysis (non-parametric) showing results of log – rank (Mantel – Cox) test comparing survival of EPU's among treatments (n = 6).

Treatment	Log-rank (Mantel-Cox)		
	Chi square	Df	<i>p</i>
W-HN – NW-HN	5.634	1	0.018*
W-HN – W-LN	1.777	1	0.182
W-HN – NW-LN	13.906	1	0.0001***
NW-HN – W-LN	1.208	1	0.272
NW-HN – NW-LN	1.685	1	0.194
W-LN – NW-LN	6.108	1	0.013*

* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$.

We found shorter leaves as well as lower aboveground biomass under high wave conditions (especially in W-HN treatment) compared with those under no-wave treatments. Differences in hydrodynamic treatments are such that present results on waves cannot be directly compared with previous studies using unidirectional flow. However, the observed shorter leaves seems in line with previous studies describing the effect of waves (van Katwijk and Hermus 2000), flow velocities (Puijalon et al. 2007) and nutrient enrichment (Short et al. 1995) on leaf length (LL) in seagrass as well as in freshwater plants. This

morphological acclimation appears to be functional in reducing drag forces (Bouma et al. 2005) and thereby lowering the risk of uprooting or mechanical damage (Schutten and Davy 2000).

We observed a general negative effect of waves on the belowground dynamics, because internode abundance (IA), internode appearance rate (IAR), and root appearance rate (RAR) were lower in W treatments than in NW. Previous studies on *Z. noltii* responses to enhanced current velocity have reported contrasting effects, varying from enhanced belowground growth in order to enhance seagrass anchorage (Peralta et al. 2006; de Los Santos et al. 2010), versus findings in agreement with present results (i.e., Schanz and Asmus [2003] for *Z. noltii* and Puijalón et al. [2006] for freshwater plants). The lack of consistent results emphasizes the need for direct comparative studies on waves and flow, and a great emphasis on the effects of hydrodynamics on plant adaptation in general. In general terms, it may be speculated that low investment in belowground biomass in high energetic environments, may threaten the sustainability of *Z. noltii*.

Sessile organisms (such as seagrasses) experience drag and deformation due to hydrodynamic forces. The strength of this effect depends on the biomechanical properties and the exposed surface area of the tissue (Koehl et al. 2001). Our measurements on both the material properties and size of *Zostera noltii* leaves showed that predominantly nutrient enrichment weakened the leaves of *Zostera noltii* by reducing their strength. This agrees with previous observations by Burkholder et al. (1992) that *Zostera marina* leaves under moderate (but chronic) nitrate enrichment seemed more brittle and broke easily. The strongest leaves were found in the treatment with the highest survival (i.e., NW-LN [Fig. 5.2A, E]). This suggests that having strong leaves, contributes to a higher survival chance, and that biomechanical properties are an important factor to be studied in seagrass ecology.

In summary, this study shows for the first time that, when assayed together waves and nutrient enrichment had a negative effect on *Zostera noltii* survival, aboveground biomass and leaf length. When assayed separately, waves negatively affected survival and morphodynamical properties of the plants, whereas nutrient enrichment negatively affected biomechanical properties. These specific responses may be expected to induce negative feedbacks that may eventually lead to meadow collapses. For example, a decrease in aboveground biomass and leaf length due to waves will reduce the wave attenuation capacity of the bed, thereby resulting in more stress and, thus, a further reduction of aboveground biomass and leaf length. This negative feedback may be accelerated by an increasing risk of seagrass loss by uprooting, due to the decreased belowground growth under wave and high nutrient conditions. Another example of a potential negative feedback may originate

from the reduced strength of the seagrasses leaves under nutrient loading. The latter may lead to increased plant losses under even relatively mild hydrodynamic regimes. Obviously, in combination, these negative feedbacks enforce each other even further, accelerating the potential collapse. Though the threshold concentration of nutrients as well as hydrodynamics may vary with geographic region, our study shows that restoration and conservation managers should be well aware of the increased risk of collapse when two such omnipresent stressors as high level of nutrients and waves are interacting.

Table 5.4 Results of two-way MANOVA based on categorical data, showing the effects of nutrients and waves on the morphodynamical and biomechanical properties of the EPU's. The morphodynamical properties include growth rate; above- and belowground biomass; leaf, root, and internode length; internode and root abundance; internode appearance; and internode elongation rate. The biomechanical properties included strength and stiffness. Univariate responses are shown per treatment ($\pm$ SE) with (mean(SE) n). To show an indication of the strength of the response, p -values are included in the table. Data transformation is specified when conducted.

Variables	Treatments				Data transformation	p - value	
	NW-HN	W-HN	NW-LN	W-LN		W	N
							W ^x N
Morphodynamical							
Biomechanical							
GR	0.0009 (0.0003)19	0.0003(0.0004)10	0.001 (0.0003)22	0.0002 (0.0003)15	-	0.006	0.597
AG	0.010 (0.002)19	0.005 (0.001)10	0.008 (0.001)22	0.009 (0.001)15	$^{10}(\log x + 3)$	0.03	0.188
BG	0.046 (0.004)19	0.041 (0.005)10	0.052 (0.006)22	0.042 (0.006)15	$(\sqrt{x})^{-1}$	0.179	0.914
AG:BG	0.22 (0.022)19	0.13 (0.031)10	0.16 (0.013)22	0.22 (0.03)15	-	-	-
Morphological properties							
LL	6.36 (0.46)19	4.44 (0.38)10	5.47 (0.35)22	5.11 (0.25)15	$^{10}\log x$	0.007	0.942
IL	6.12 (0.50)19	7.98 (1.5)10	6.77 (0.65)22	7.06 (0.55)15	$(x+1)^{-1}$	0.168	0.524
RL	2.51 (0.18)19	2.70 (0.24)10	2.94 (0.18)22	2.61 (0.20)15	-	0.750	0.413
IA	10.89 (0.53)19	7.30 (0.83)10	9.55 (0.63)22	8.13 (0.52)15	$\sqrt{x}$	0.0001	0.866
RA	24.42 (2.91)19	17.40 (3.39)10	21.33 (2.04)22	17.40 (1.58)15	$^{10}\log x$	0.020	0.998
Morphodynamical properties							
IAR	0.19 (0.012)19	0.12 (0.018)10	0.17 (0.014)22	0.13 (0.011)15	$^{10}(\log x + 1)$	0.0001	0.760
IER	1.18 (0.12)19	0.77 (0.09)10	1.02 (0.07)22	0.91 (0.08)15	-	0.012	0.947
							0.149

Table 5.4 (continued)

Biomechanical properties							
F _{TS}	6.09 (0.61)6	5.70 (0.84)6	8.04 (0.54)6	7.55 (0.54)6	-	0.501	0.008
E _T	137.79 (10.66)6	134.87 (15.31)6	159.17 (7.71)6	152.07 (6.20)6	-	0.640	0.083

* $p < 0.05$; ** $p < 0.01$.

† Variables measured are growth rate (GR; g fresh wt EPU⁻¹ d⁻¹), aboveground biomass (AG; g dry wt EPU⁻¹), belowground biomass (BG; g dry wt EPU⁻¹), leaf length (LL; cm), internode length (IL; cm), root length (RL; cm), internode abundance (IA; internodes EPU⁻¹), root abundance (RA; roots EPU⁻¹), internode appearance rate (IAR; internodes EPU⁻¹ d⁻¹), internode elongation rate (IER; mm EPU⁻¹ d⁻¹), specific force-to-tear (F_{TS}; N mm⁻²); and Young's modulus of elasticity for tension (E_T; N mm⁻²)

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CHAPTER 6

General Discussion



6.1. GENERAL BACKGROUND

Despite their high ecological, biological, physical and economical values, we have lost seagrasses in a catastrophic amount over the last decades (Orth et al. 2006, Short et al. 2007; Waycott et al. 2009). Thousands of hectares of the world seagrass have diminished due to environmental stresses which commonly resulted from anthropogenic activities rather than natural events. Unfortunately, these stresses are exacerbated by the continuously increasing population size in coastal areas and an (expected) increasing in extreme weather phenomenon such as increasing in storm frequencies due to the climate change. Hence we are urged to increase our knowledge on how seagrass can cope with interacting environmental stresses, to enable coastal management with knowledge on how to avoid further loss.

This thesis offers new insights on how seagrass respond physiologically, morphologically and biomechanically to environmental stresses. All studies looked at nutrient stresses, either being too low (**chapter 2**) or too high (**chapters 3-5**) and originating from the water column or sediment. Other stresses were as well studied, such as light deprivation (**chapter 3**), human population and hydrodynamic stresses (**chapters 4 and 5**). Analyzing the mechanical responses of seagrass to stresses (**chapters 3-5**) represents an important contribution of this thesis to seagrass ecology, in addition to the physiological and morphological responses of seagrass to environmental stresses (**Table 6.1**). This chapter presents an overview from experimental approaches in the laboratory (**chapter 2 and 5**) and in the field (**chapter 3 and 4**). It discusses the most important findings, how different growth strategies in seagrass have different ways to cope with stresses, provides ecological implications of our studies and some insights for future studies.

6.2. SEAGRASS RESPONSES TO ENVIRONMENTAL STRESSES

6.2.1 Biomechanical response: varieties in properties

Having to live in a highly hydrodynamic environment, seagrasses are prone to break- off (either tissues that get ripped off or whole shoots that get uprooted). Other disturbances may also trigger the breaking of a seagrass. Using a biomechanical approach -by applying simple mechanical engineering principles- we can measure the capability of an organism in coping with physical stresses (Koehl and Wainwright 1985). Biomechanical properties had been mainly studied on marine macroalgae (Koehl and Wainwright 1977, Koehl

2000, Armstrong 1987, Demes et al. 2013), corals (e.g. Chamberlain 1978), followed by terrestrial plants (Onoda et al. 2008, 2011, Anten et al. 2005), and freshwater plants (Puijalon et al. 2007, 2008), but remain poorly studied on seagrass (but see Kopp 1999; Patterson et al 2001; de los Santos et al 2012, 2013). Patterson et al. (2001) and de los Santos et al. (2012, 2013) conducted correlative field studies to see differences in mechanical traits of seagrass reproductive and vegetative shoots (Patterson et al. 2001), the mechanical properties of seagrass leaves in relation to leaf nutrient content that may imply herbivore relationship (de los Santos et al. 2012) and how seagrass properties varied spatially and seasonally (de los Santos et al. 2013). Kopp (1999) studied how nitrate affects *Zostera marina* mechanical properties (mesocosm and *in situ*). In this thesis, we show how nutrients negatively affect the mechanical properties of seagrass, which is inline with Kopp (1999), and adds a new broader view on how biomechanical properties of (temperate, tropical, short-lived, long-lived) seagrass respond to various environmental stresses (i.e. water-column and sediment nutrients; shading; population density; interacting stresses of nutrients and hydrodynamics).

Table 6.1. Overview of seagrass types (i.e., fast vs. slow growing species) for which the physiological, morphological and/or biomechanical response was measured in response to a range of environmental stress factors. Nutr=nutrient addition (NO_3^- , NH_4^+ and PO_4^{3-})

Stress	nutrient	Water	response		
			physiology	morphology	biomechanical
		Water	<i>fast (ch 2)</i>		
		Water		2 x slow (ch 4)	2 x slow (ch 4)
		sediment		2 x fast (ch 3)	2 x fast (ch 3)
	light	Shading		2 x fast (ch3)	2 x fast (ch 3)
	Hydrodynamically exposed	anthropogenic pressure		2 x slow (ch 4)	2 x slow (ch 4)
	nutr. X waves	mesocosms		<i>fast (ch 5)</i>	<i>fast (ch 5)</i>

In this thesis, we found weakened *Halophila ovalis* and *Zostera noltii* leaves due to nutrient stress (**chapters 3 and 5**), by having lower breaking stress (F_{TS}) compared to those in low nutrient concentration. These two species also had lower stiffness (Young's modulus of elasticity, E_T) under nutrient enrichment. However, under similar treatment (sediment enrichment), *Halodule uninervis* leaves showed no significant changes in their mechanical properties (**chapter 3**). This is similar to *Thalassia hemprichii*, which showed no mechanical differences among 4 sites with (a matrix of) differences in

anthropogenic activities and wave exposure (**chapter 4**). For *Enhalus acoroides* leaves, we again did find higher breaking stress (F_{MAX}) and breaking force (F_{TS}) in uninhabited (considered as low nutrient) -but exposed- (PE) site (**chapter 4**). This implied that in high nutrient conditions, *E. acoroides* would have lower breaking force and breaking stress as the others had experienced (apart from *Halodule uninervis*). Overall, our results showed that nutrient enrichment tend to make leaves weaker and easy to break under high nutrient supply (as inline with Kopp, 1999 for seagrass *Z. marina*; Lamberti-Raverot and Puijalon, 2012 for freshwater plants). In addition, nutrient enrichment also reduced seagrass stiffness as was also observed in freshwater plants (Lamberti-Raverot and Puijalon 2012). Similar to nutrient enrichment, *H. ovalis* responded negatively to light reduction by having lower breaking stress (F_{TS}) and lower stiffness (E_T) compared to those in control treatment (chapter 3). In contrast, *Halodule uninervis* had an opposite biomechanical response as *H. ovalis*, by having a higher extensibility under light deprivation (**chapter 3**).

Having lower stiffness due to environmental stresses (as established in *H. ovalis* and *Z. noltii*), directly showed that these species had increased in deformation capability, which may also be considered as a strategy to counteract the weakened leaves. Overall, the studies in this thesis show that depending on the environmental stresses present, seagrasses can alter their mechanical properties in a range of ways (i.e. species and/or a site-specific) (de los Santos et al. 2013). In addition to that, variation in mechanical properties also occurred intra-specifically. Compared to their leaves, sheaths of *Enhalus acoroides* (containing the meristems) endured higher mechanical force and showed more extensibility before they broke implying that these lower parts of the plant needed to be strong as to act as wave absorber and protector for the younger leaves. In contrast, sheaths of *Thalassia hemprichii* were less strong than their leaves, probably due to being short and (most of the time) buried in the sediment, avoiding the need for having a higher breaking stress.

Eutrophic conditions have been well-documented as a trigger for blooms of non-rooted algae, causing loss of rooted seagrass plants by poor competition for light. My thesis shows that this negative effect of eutrophication can become exacerbated, since high nutrient and low light condition reduce seagrass strength and stiffness of the seagrass leaves. Under low light conditions, seagrass invest more in aboveground tissues so as to efficiently conduct photosynthesis, which may lead to an increase in drag force. However, both nutrient enrichment and shading caused seagrass leaves to become less stiff (**chapter 3**), meaning that they have high deformation capability and thus experience less drag. The latter may compensate for the weakened leaves thereby promoting seagrass survival. Thus, habitat collapse may be reduced. In a contrasting situation, our interacting multi-stress (nutrient enrichment vs

hydrodynamic) experiment (**chapter 5**), showed that *Z. noltii* under the condition of no wave and low nutrient, had the highest survival being strong plants by having highest breaking stress (F_{TS}). This would suggest that a non-stressful environmental condition would benefit the plants' survival and its habitat function by creating habitat for other organisms.

6.2.2 Morphological response: does size matter?

Seagrasses are well acknowledged for their morphological plasticity (Peralta et al. 2007, 2008) in response to nutrient limitation (Agawin et al. 1996) and enrichment (Short 1983, Lee and Dunton 2000), to light levels (Lee and Dunton 1997, Dalla Via et al. 1998, Gordon et al. 1994) and to hydrodynamics (van Katwijk and Hermus 2000, Schanz and Asmus 2001, Peralta et al. 2007, 2008, de los Santos et al. 2010). In response to the latter, seagrasses –like other aquatic plants- develop a variety of strategies such as “avoidance strategy” (by minimizing the drag forces they experienced) or “tolerance strategy” (by maximizing the drag forces that the organism can bear before a mechanical failure) (Denny et al. 1997, Puijalon et al. 2005, 2008, 2011).

In this thesis, we showed that leaf morphology of *Z. noltii* under the interaction of wave and nutrient stress were predominantly affected by wave stress (**chapter 5**). Due to that, they pose an “avoidance strategy” by having shorter leaves, thus reducing the area exposed to the fluid (Bouma et al. 2005, Peralta et al. 2005, 2006, de los Santos et al. 2010, van Katwijk and Hermus 2000, Schanz and Asmus 2003). Similar response was as well observed in *Enhalus acoroides* leaves growing at an anthropogenic-exposed (AE) site (**chapter 4**). Following nutrient enrichment, *Halophila ovalis* showed the opposite response for their morphological reconfiguration. Their dimensions had prominent changes (thicker, longer and wider leaves) (**chapter 3**) inline with Lee and Dunton (1997, 2000), Short et al. (1983). In *Halodule uninervis* however, nutrient enrichment only gave a significant positive effect to the leaf length, which may indicate that our experimental site was strictly nutrient limited.

Due to light deprivation, changes in the architectural features of a leaf and/or leaf dimensions may be a bit diverse. Light deprivation may often enhances leaf length (Longstaff and Dennison 1999) and width (e.g. in *Thalassia testudinum*, Lee and Dunton 1997) enabling plants to collect more light. However, sometimes the opposite effect may occur (Gordon et al. 1994) or no changes were observed (Gordon et al. 1994; Ochieng et al. 2010). In this thesis (**chapter 3**), we showed that *Halophila ovalis* under low light responded by increasing their leaf thickness, whereas *Halodule uninervis* did not respond at all. The increasing thickness of *H. ovalis* was probably related to light

absorption efficiency (Enriquez 2005), whilst the unresponsiveness of *H. uninervis* in their morphology may be due to the high tolerance of *H. uninervis*. This shows that different species respond differently to environmental stresses they encounter in their habitat and that morphological reconfiguration is important for seagrass survival especially in a stressful environment (table 6.2).

6.2.3 Physiological response: mighty in a tiny nitrogen-compound environment

Seagrass capacity in taking up single (dissolved inorganic) nitrogen compound has been studied by many scholars (e.g. Short and McRoy 1984, Stapel et al 1996, 2001, Cornelisen and Thomas 2004). Recently, studies showed that dissolved organic matter (DOM) also facilitates the seagrasses as a nitrogen source which enables the seagrass to shortcut N cycling (Evrard et al. 2005, Barron et al. 2006, Vonk et al. 2008). Like terrestrial plants (Harrison et al. 2007), seagrass have different uptake rates for different organic substrates which seem to be related to the substrate's bio-availability, molecular complexity and/or chemical stability of the molecules (Van Engeland et al. 2011, 2013, Vonk et al. 2008). Seagrasses in nature, indeed, encountered a compound pool of low concentration of inorganic and organic nitrogen-containing substances with bioavailability variances. However, seagrass research on nitrogen uptakes are dominated by single nitrogen substrates at a time. We lacked information if there are any nutrient-nutrient interactions in the uptake dynamics of inorganic and organic nitrogen, which was hence studied in this thesis (**chapter 2**).

This thesis (**chapter 2**) showed that even in low (i.e. all substrates were 1 μM) but realistic concentration of dissolved inorganic and organic nitrogen (DIN and DON), seagrass uptake rates for ammonium was higher than nitrate (e.g. Alexandre et al. 2010, Touchette and Burkholder 2000, Van Engeland et al. 2011) and organic N-source (Van Engeland et al. 2011, 2013, Vonk et al 2008). This indicated a “constitutive preference” of ammonium over the other substrates as was also shown by Alexandre et al. (2010). However, an “induced preference” of ammonium by seagrass *Z. noltii* was not observed in our study as it was by Alexandre et al. (2010), probably due to the lower nutritional conditions used in this study. There was no down- or up-regulation that favoured one nitrogen source over the others which stressed the non-dependency of seagrass *Z. noltii* to the availability of background nutrients, indicating a difference in uptake capacity rather than substrate preference. This may imply that in lower nutritional concentration seagrass *Z. noltii* takes up whatever is available, making them mighty in a tiny (nitrogen) condition. Yet, indeed, the eventual contribution of different sources in the overall nitrogen

uptake may further depend on the relative concentrations of the different sources.

When organic nitrogen substances are available, they neither showed influence on the uptake of any nitrogen sources, nor was their uptake rate affected by the presence of another substrate. Since the uptake rates of aboveground tissues showed a similar pattern of ammonium preference as those found by Van Engeland et al. (2011), this may reflect a “constitutive preference” order from ammonium to urea, nitrate and glycine as least preferred (though the differences with glycine were not statistically significant in our study). Hence, we are still left with unanswered question of whether an “inducible preference” mechanism exists in *Z. noltii* when organic nitrogen substances are available. Since the amino acid concentrations used in our study is very low (i.e. 1 μ M), which is at the upper limit (or beyond) of the observed range for seagrass ecosystem (Hansen et al. 2000), it may imply that the chance of not detecting an existing role for amino acids in the down-regulations of the uptake of some nitrogen sources is smaller than the chance that such a role actually exists.

The capacity of seagrass in taking up nutrients from low-nutrient concentration is beneficial to seagrass plants. They can take whatever nutrient is available and in such nutrient-poor environments, they do not need to compete with micro- and/or macro-algae for light and space as is the case in more eutrophic areas. Even though low nutrient is a stress for seagrass, because it may limit their productivity and growth, seagrass may thrive with less nutrients.

6.2.4 Morphology vs biomechanics vs physiology

The studies in this thesis (**chapters 3, 4 and 5**) revealed several situations concerning morpho-mechanical properties of seagrass in response to environmental heterogeneity and are described as follows:

- i) under high nutrient stress and light stress for *Halophila ovalis*: no effect on the breaking force (F_{MAX}) (a size-dependent material property), a decrease in breaking strain (F_{TS}) and stiffness (E_T), with increased cross-sectional area (ca) (**chapter 3**),
- ii) under light deprivation stress for *Halodule uninervis*: increased inextensibility (L_T), where F_{MAX} , F_{TS} and cross-sectional area (ca) remained the same (**chapter 3**),
- iii) under wave-exposure and antropogenic influence in *Enhalus acoroides* a : a decrease in breaking force (F_{MAX}) and breaking stress (F_{TS}), but no effect on cross- sectional area, whereas no affect on *Thalassia hemprichii* (**chapter 4**)

- iv) under high nutrient stress: a decrease in breaking force (F_{MAX}), breaking stress (F_{TS}) and stiffness (E_T), but no effect on the cross sectional area (ca) (**chapter 5** and Lamberti-Raverot and Puijalon, 2012)

Under interacting stress of high hydrodynamics and nutrient enrichment, the morphological reconfiguration of seagrass *Zostera noltii* was highly influenced by the hydrodynamics rather than the nutrients (**chapter 5**). Nutrient stress, however, negatively affected the seagrass mechanical properties prominently (**chapters 3-5**). The four situations explored above, showed that under high nutrient condition, the breaking force (F_{MAX}) -a size-dependent material property- was not responsible for the differences in the breaking stress (strength; F_{TS}) of the plants, but rather their morphological features. As the cross sectional area increased, this resulted in a reduced F_{TS} and E_T (situation i), since breaking stress is derived from the division of the breaking force (F_{MAX}) by the material cross-sectional area (i.e. thickness and width). This may imply that changes in leaf-morphology due to any environmental changes may result in varieties of the leaf mechanical properties. Due to nutrient enrichment, *H. ovalis* leaves were significantly wider, longer and thicker than the control treatment. Hence, even though nutrient enrichment made the tissues weaker (lower F_{TS}), the breaking force F_{MAX} was not altered, due to the increased cross-sectional area (ca) of the leaf tissues. The same occurred in *H. ovalis* leaves under light deprivation (**chapter 3**). Despite the lower breaking stress (F_{TS}) of *H. ovalis* compared to the control, F_{MAX} was not affected, due to the significant thickening of the leaves (probably to compensate with light absorption; Enriquez 2005), causing an increased cross-sectional leaf area (situation i). In *Halodule uninervis*, shading did not affect leaves morphology neither their breaking stress (F_{TS}) nor breaking force (F_{MAX}). However, their extensibility (L_T) was higher under shading compare to the plants in control treatment (situation ii). The mechanism that triggered this condition is still unclear and requires further exploration.

In situation (iii), environmental heterogeneity caused changes in breaking force (F_{MAX}) and breaking stress (F_{TS}), while the cross-sectional area stayed the same for both *Enhalus acoroides* and *Thalassia hemprichii* (**chapter 4**). Even though the cross-sectional area did not influence both F_{MAX} and F_{TS} , these two mechanical properties were closely related in that both responded in a similar way to environmental heterogeneity. That is, whenever the mechanical force (F_{MAX}) is high, the F_{TS} followed by having a high breaking stress, and vice versa.

Situation (iv) was observed under high nutrient stress, where *Zostera noltii* leaves broke after applying a low mechanical force (i.e., lower F_{MAX}), hence had lower breaking stress (low F_{TS}) (weaker) and was also less stiff

(lower E_T). However, the cross-sectional area was not affected (**chapter 5**). This condition of nutrient stress clearly imposed negative effects on the mechanical properties of *Z. noltii*.

Seagrasses seem well adapted to growing in low nutrient environments. Firstly, they can take up nutrients in any form available (**chapter 2**). Secondly, they may develop tissues with higher breaking force (F_{MAX}) and breaking stress (F_{TS}). This implies that under low nutrient conditions, seagrasses are less prone to breakage, which may help to reduce leaf losses (**chapter 3, 5**). However, responses to nutrient enrichment vary among species (see also table 6.2). For example, *Halophila ovalis* (**chapter 3**), did not show differences in absolute breakability (F_{MAX}), but dimensions increased. In general, differences between species responses can be related to their growth strategies, as will be elaborated below.

6.3 GROWTH STRATEGIES

Plant growth strategies (i.e., being fast vs. slow growing species) revealed to be a more important factor in explaining the mechanical properties of seagrass (**chapter 4**) than abiotic stresses such as e.g., nutrients, waves and light (**chapter 3 and 5**). There seems to be a general trend where slow-growing seagrasses (except *Thalassia hemprichii*) have a higher leaf resistance (F_{MAX}) and are stronger (i.e., higher breaking stress, F_{TS}) compared to fast-growing ones (*Halodule uninervis*, *Halophila ovalis* and *Zostera noltii*) (**chapters 3-5**) (Fig. 6.1). *Enhalus acoroides* in the tropics has the highest leaf mechanical resistance (but lower than *Posidonia australis* in temperate region) (Fig. 6.1). These slow-growing species compensate their leaf turn-overrate by having longer leaf life span. To be long-lived, it may be a prerequisite to have strong leaves in order to cope with environmental stresses (abiotic and biotic, see **chapter 4**). There was no specific pattern between temperate and tropical seagrass species; both followed the trend where slow-growing species have lower leaf resistance (Fig. 6.1) representing a trade-off of being easily and fastly produced versus producing strong long-lived leaves.

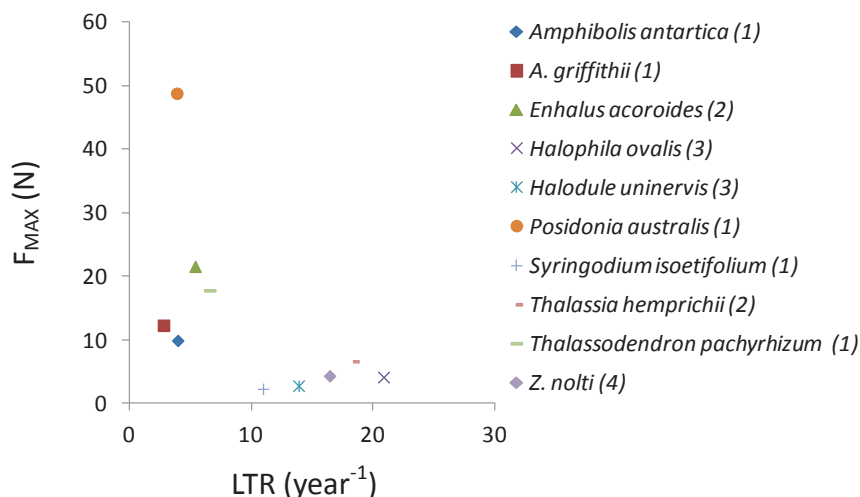


Figure 6.1. Relationship of seagrass leaf resistance (mechanical force, F_{MAX}) and leaf turnover rate (year^{-1}). Numbers after species names indicate data origin: (1) de los Santos et al. 2013; (2) chapter 4; (3) chapter 3 (La Nafie et al. 2013); (4) chapter 5 (La Nafie et al. 2012). Leaf turn-over rate was taken from Duarte 1991.

6.4 STRESSES AND ECOLOGICAL CONSEQUENCES

Increasing stresses due to anthropogenic activities rather than the natural events are causing a global decline in seagrass ecosystems. Due to the climate change that may increase extreme weather events, this decline may be worsened. Storm frequency may increase, resulting in increased nutrient runoffs, hydrodynamic forces and turbidity in coastal areas that are likely to negatively affect seagrass ecosystems. When waves and high nutrient co-occurred, they may reduce seagrass survival as shown for *Z. noltii* (chapter 5). Waves may prominently reduce seagrass belowground biomass and leaf length, whereas high nutrients reduce leaf strength. These specific responses may induce meadow collapse by reducing wave attenuation capacity of the bed due to the reduced aboveground biomass and shorter leaves, hence resulting in more stress and a further reduction of aboveground biomass and leaf length. This negative feedback may be exacerbated and increase the risk of seagrass loss by uprooting due to the decreased belowground growth under wave and high-nutrient condition. Another potential negative feedback may occur due to high nutrient loading that weakens the seagrass, leading to increased plant losses under even relatively mild hydrodynamic regimes. Due to high storm frequencies that may increase hydrodynamic activities and turbidity resulting in light deprivation in coastal areas, loss of seagrass is inevitable. Seagrass

develop weak leaves not merely due to high nutrient stress but also to light deprivation (chapter 3). This may also create a shift in species composition to species that are adapted to lower light condition. Such shift can be used as a seagrass health indicator (Fig. 6.2). Obviously, these combinations of abiotic stresses may worsen seagrass conditions even further, eventually accelerating potential collapse.

6.5 LESSONS LEARNT

Seagrasses are decreasing in an alarming rate due to environmental stresses that may lead to their decline, unless they can acclimate. Seagrasses respond differently to environmental stresses. They may possess plasticity in their morphological as well as mechanical traits. Studies on mechanical properties in seagrasses are only starting to emerge compared to marine macroalgae, terrestrial plants and some freshwater plants. This thesis showed how environmental stresses can reduce seagrass mechanical resistance, which thereby may influence seagrass ecological functions. However, like other plants, seagrass tissues are more complex than most engineered material. Hence, their mechanical properties are often difficult to measure, interpret and predict (Read and Stokes 2006). This thesis aimed to increase our understanding of the complexity of seagrass mechanical traits in response to environmental stresses and aimed to contribute to our knowledge on the function and ecological significance of biomechanical properties.

6.6 FUTURE PERSPECTIVES

There is still so much to learn about how seagrass can cope with environmental stress and to gain more knowledge on how to manage our seagrass ecosystem to prevent further loss. The present thesis pioneered the study of how our understanding of biomechanical properties can be used as a tool to find out how seagrass can cope with environmental stresses, which evidently has been well-acknowledged in marine macroalgae (Koehl and Wainwright 1977, Koehl 2000), terrestrial plants (Onoda et al 2011), freshwater plants (Puijalon et al. 2007, 2008, Lamberti-Ravero and Puijalon 2012), but much less attention to seagrass (except Kopp 1999, Pattersen et al. 2001).

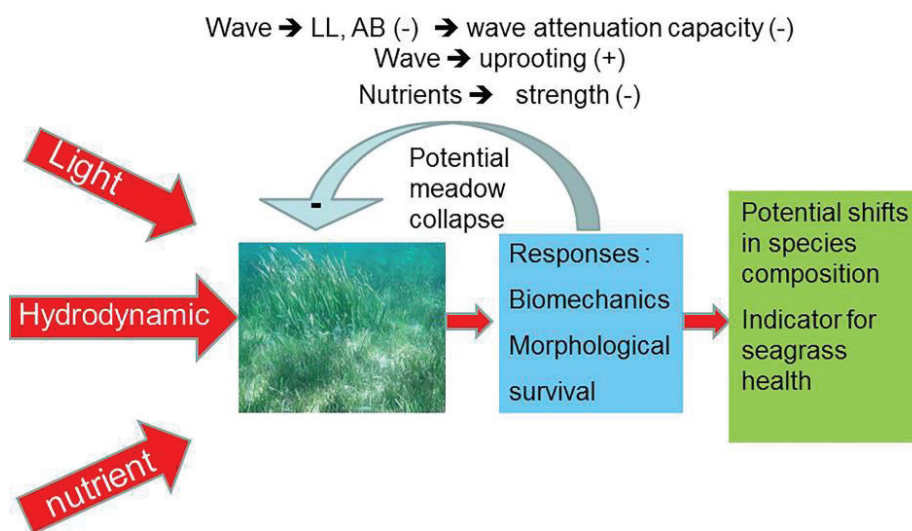


Figure 6.2. Schematic representation of how seagrass respond to environmental stress (high nutrients, hydrodynamics and lights) and how this affects the seagrass ecosystem. (LL = leaf length; AB = aboveground Biomass; (-) = reduced; (+) = increased)

To extend the knowledge on seagrass responses to the increasingly (high) frequencies and intensities of environmental stresses, further studies should be conducted, such as : 1) conducting nutrient-nutrient interactions (DIN-DON) with several levels of nutrient concentrations to observe if “inductive preference” exist (**chapter 2**); 2) combining hydrodynamics (Cornelisen and Thomas 2004) and (low) concentration of DIN and DON (**chapter 2**); 3) using multiple levels of lights to several seagrass species (**chapter 3**); 4) comparing climax (**chapter 4**) and pioneer seagrass species with specific site characteristic; and 5) conducting direct comparative studies on waves and flow (**chapter 5**). In addition to that, monitoring of environmental changes to identify responses and early warning signs of seagrass decline, is a necessary prerequisite. In this activity, it would be important to involve local community and government (as in seagrasswatch.org) for studying the local condition, where eventually the information from monitoring can contribute to the seagrass global knowledge (as applied in seagrassnet.org). Specifically to seagrass biomechanical response, which is just starting to emerge, the effect of raising temperature (due to climate change) also would be an interesting subject to study as it is still an unexplored issue. Overall, a modeling work on how seagrasses respond to multiple stresses concerning climate change, would be an important and interesting study to

conduct in order to assess future developments in seagrass beds under climate change.

6.7 SUMMARY

The work presented in this thesis showed that environmental stress (low and high nutrient, high hydrodynamics and light deprivation) caused seagrass prone to breakage. Due to that, seagrass performed several strategies to counter balance the stresses they are experiencing by having morphological plasticity as well as mechanical. They also exhibit inter- and intra-specificities, and species-specificities. It also showed, dominantly, that nutrients weakened the seagrass. The ecological functions (eventually the economical values) of seagrass can be decreased due to these environmental stresses. In Table 6.2, the summary of the main questions addressed in **chapters 2-5** and their answers are provided.

Table 6.2. Summary of questions and answers in this thesis

Questions	Answers	Chapter
How does temperate seagrass <i>Zostera noltii</i> respond to (low) nitrogen compound pools	- Seagrass <i>Z. noltii</i> performed a “constitutive preference” for ammonium over the other substrate - Seagrass <i>Z. noltii</i> can use inorganic as well as organic nitrogen sources and do not differentiate between substrates.	2
How do light and (sediment) nutrients <i>independently</i> affect morphological and mechanical properties of slow-growing tropical seagrass?	- Seagrass performed species-specific response to decreasing light and nutrient enrichment. - <i>Halophila ovalis</i> were weaker, less stiff and had an increased in leaf dimensions under nutrient stress and shading. - <i>Halodule uninervis</i> did not show any changes, except that under nutrients they had an increased length and under shading their leaves were more extensible. - When comparing the two species: (1) <i>H. ovalis</i> were less stiff than <i>H. uninervis</i> under nutrient enrichment and (2) <i>H. ovalis</i> were weaker and less extensible than <i>H. uninervis</i> under shading.	3
How plastic are 2 tropical climax species?	- <i>E. acoroides</i> has higher mechanical resistance than <i>T. hemprichii</i> ; probably a prerequisite to have longer leaf life span.	4
Have inter- and intra-specific plasticity a similar magnitude?	- <i>Enhalus acoroides</i> sheaths exhibit a higher mechanical resistance than their leaves as they probably act as wave absorber and younger-leaf protector. In contrast, <i>T. hemprichii</i> sheaths have lower mechanical resistance which maybe due to their short sheath that is (most of the time) buried in the sediment.	
How do site differences influence plasticity of both climax species?	- Morphological plasticity due to site differences were shown by <i>E. acoroides</i> because they were more responsive to local condition than <i>T. hemprichii</i> both in their morphological as well as mechanical traits	
How does high nutrient stress <i>interacting</i> with waves affect seagrass?	- The interacting effect of waves and high nutrient loads caused a lower seagrass survival compared to those with the non-wave, low-nutrient treatment. - Nutrient enrichment negatively influenced the strength and stiffness of <i>Zostera noltii</i> , - Leaf morphology (i.e. the length) was influenced negatively by waves.	5

NOTE: Please view **Table 1.1** for the hypotheses of these questions and view the indicated chapters for the more complete description of the questions and answers.

CHAPTER 7

REFERENCES

SUMMARY

SAMENVATTING

RINGKASAN

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CURRICULUM VITAE



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SUMMARY

Seagrasses are coastal and marine flowering plants that inhabit the tropical and temperate coastal and marine areas around the globe. They have many important functions and values, physically, ecologically and economically. Physically, seagrass contribute to coastal protection as they attenuate wave energy and stabilizing sediments. Through this, neighboring ecosystem may benefit because water movement is reduced and suspended sediment to settle, thereby decreasing water turbidity. Moreover, this results in a positive feedback to the seagrass, as it sustains light intensity for plants' photosynthetic activity. Ecologically, seagrass can function as habitats for various marine organisms as their feeding, nursery and/ or as their breeding ground. This way, seagrass meadows sustain both biodiversity as well as commercially valuable species. However, regrettably, our seagrass ecosystems are declining around the globe. Anthropogenic activities are the major cause for this decline, rather than natural events. Yet, due to climate change, extreme weather events (such as increasing storm frequencies and intensities) are expected to increase, which may worsen the current condition. We have still much to learn about how seagrass can cope with these environmental stresses, in order to better manage our seagrass ecosystems and to reduce their loss. Hence, we studied how seagrass respond physiologically, morphologically and biomechanically to a range of environmental stresses: being exposed to low and high nutrients, high hydrodynamic and reduced light.

In nature, seagrasses encounter a mixture of inorganic and organic nitrogen containing substances with varying bioavailability in low concentration. Yet, seagrass research had mainly focused on nitrogen uptake on a single nitrogen substrate at a time. By using a combination of one of ^{15}N -labeled substrate and one ^{14}N -unlabelled background substrate we demonstrated seagrass "constitutive preference" for ammonium uptake over other substrates (i.e., nitrate as the dissolved inorganic nitrogen and urea or glycine as the dissolved organic nitrogen). However, substrate uptake was always independent from the background nutrient (which occurred both in above and belowground plant parts), implying that there was no down- or up-regulation that favoured one nitrogen source over the other (induced preference). This was probably due to the low (but realistic) concentration used in the experiment as compared to other studies that showed both "constitutive" and "induced" preferences of ammonium on nitrate uptake. For the dual labeled (^{15}N and ^{13}C) urea and glycine, a strong relationship existed between nitrogen and carbon uptake, but with deviations from expectations under complete uptake of the molecules. In summary, we may conclude that at realistically low (ambient) nutrient concentrations, seagrasses can use inorganic as well as organic nitrogen sources

and do not differentiate between substrates. In other words, seagrasses are capable to take up whatever is available (**chapter 2**), yet the eventual contribution of different sources in the overall nitrogen uptake may further depend on the relative concentrations of the different sources. Indeed, having the capability to take up whatever is available may serve as an advantage for seagrass exposed to low nutrient environment.

Apart from the benefit to be able to take up whatever is available in low nutrient environments, seagrass mechanical performance also benefits from low nutrient conditions. That is, under low nutrient conditions, seagrasses are strong (having higher specific-force-to-tear, F_{TS}) as shown for *Halophila ovalis* (**chapter 3**), *Zostera noltii* (**chapter 5**) and *Enhalus acoroides* (**chapter 4**). In contrast, under high nutrient concentrations, seagrass can easily be broken. Light deprivation is another environmental stress that can cause seagrass to easily break (**chapter 3**). Both high nutrient and light deprivation caused seagrass (*Halophila ovalis*) leaves to be weakened by having lower breaking stress (F_{TS}) even though the absolute breaking force (F_{MAX} -a size-dependent material property) stayed the same. Under nutrient enrichment, however, seagrass *Zostera noltii* had lower breaking stress (F_{TS}) as well as lower breaking force (F_{MAX}). This may imply that leaf mechanical resistance may result from acclimation to any environmental heterogeneity by both morphological and mechanical changes (**chapter 3**). Seagrass *Halodule uninervis* however, did not show any prominent changes in their morphology and mechanical performance due to environmental changes. Thus, mechanical responses are species-specific which was also observed in seagrass *Enhalus acoroides* and *Thalassia hemprichii* (**chapter 4**). Apart from being mechanically species-specific, seagrass also possessed intra-species specificity in terms of their morphologies and mechanical properties. Leaf and sheath tissues have obvious differences in their morphology. Similar differences exist for the mechanical properties. For example, sheaths of *Enhalus acoroides* exhibit higher extensibility (by being able to extend more before they break) than their leaves. This is probably due to the softer meristematic tissues that form the sheaths that still bear elastic cell walls. Yet, in contrast, sheaths of *Thalassia hemprichii* had a lower breaking force than their leaves. Because the sheaths are short and (most of the time) are in the sediment, hence having stronger leaves for *Thalassia hemprichii* is probably more necessary than having stronger sheaths.

Apart from seagrass mechanical and morphological responses to (single) environmental stresses as mentioned above, in this thesis, we also revealed exciting new findings on the effects of interacting wave and nutrient stress (**chapter 5**). Wave and high nutrient stress decrease survival of seagrass *Zostera noltii*. However, waves independently reduce seagrass length and belowground biomass, whereas high nutrient concentrations reduce seagrass strength and

stiffness. The latter was in agreement with our observations for *Halophila ovalis* (**chapter 3**). These specific responses of seagrass *Zostera noltii* to interacting stress of waves and high nutrient conditions may be expected to induce negative feedbacks that may eventually result in meadow collapse. For example, a decrease in leaf length and aboveground biomass due to waves can reduce wave attenuation capacity of the bed hence can increase stress to seagrass by a further reduction of aboveground biomass and leaf length. This condition, however, can be exacerbated by an increasing loss by uprooting. In addition, high nutrient stress can result another potential negative feedback for weakening the plants which eventually can increase plant losses. Obviously, in combination, these negative feedbacks enforce each other even further, accelerating potential collapse.

With respect to mechanical properties, some important generalizations arise from comparing all chapters. There seem to be a general trend where slow-growing seagrass have a higher leaf resistance (F_{MAX}) and are stronger (i.e., higher breaking stress, F_{TS}) compared to fast-growing ones. The slow-growing species apparently compensate their low leaf turn-overrate by having stronger leaves that allow for a longer leaf life span. To be long-lived, it may be a prerequisite to have strong leaves in order to cope with environmental stresses (abiotic and biotic).

Understanding the mechanical properties for living materials such as seagrass tissue, is more complex than obtaining such understanding for most engineered material. This thesis aimed at increasing our understanding of the complexity of seagrass mechanical traits in response to environmental stresses and to contribute to our knowledge on the function and ecological significance of biomechanical properties.

SAMENVATTING

Zeegrassen zijn bloeiende onderwaterplanten die voorkomen in tropische en gematigde kust- en zeegebieden over de hele wereld. Ze hebben veel belangrijke functies en waarden, zowel fysiek, ecologisch als economisch. Fysiek draagt zeegras bij aan bescherming van de kust omdat het golven dempt en sedimenten stabiliseert. Hier kunnen naburige ecosystemen van profiteren omdat door de remming van de water beweging sediment bezinkt, waardoor de troebelheid van het water afneemt. Deze verbetering van de waterkwaliteit leidt tot een positieve terugkoppeling naar het zeegras. Door de grotere hoeveelheid licht die de planten bereikt, neemt de fotosynthetische activiteit toe. Zeegras fungeert als habitat voor diverse mariene organismen door hen van fourageergebied en een kraamkamer te voorzien. Op deze manier ondersteunen zeegrasvelden zowel de biodiversiteit als commercieel waardevolle soorten. Helaas nemen zeegras ecosystemen overal ter wereld af. Antropogene activiteiten zijn de belangrijkste oorzaak voor deze daling. De huidige toestand zal waarschijnlijk verder verergeren als gevolg van klimaat verandering, door dat extreme weer somstandigheden zoals stormen zullen toenemen in frequentie en intensiteit. Er is nog veel te leren over hoe zeegrassen omgaan met deze stressfactoren, en hoe de zeegras-ecosystemen beter beheerd kunnen worden om verdere achteruitgang zo veel mogelijk te beperken. Vandaar dat we in dit proefschrift hebben onderzocht hoe zeegrassen fysiologisch, morfologisch en biomechanisch reageren op een reeks van milieu-invloeden: blootstelling aan lage en (te) hoge concentraties voedingsstoffen, sterke hydrodynamische krachten en afgenomen licht hoeveelheid.

In onverstoorde natuur gebieden worden zeegrassen blootgesteld aan een mengsel van anorganische en organische stikstof bevattende stoffen, met wisselende biologische beschikbaarheid, en veelal bij lage concentratie. In eerdere studies is het onderzoek vooral gericht op stikstofopname van één stikstofsubstraat per keer. Door het aanbieden van een mengsel van een ^{15}N gemerkt substraat met een ^{14}N ongemerkt achtergrondsubstraat lieten we zien dat zeegrassen voorkeur heeft voor ammoniumopname boven andere N-bevattende substraten (bijvoorbeeld, nitraat als opgeloste anorganische stikstof of ureum of glycine als opgeloste organische stikstof). Substraatopname bleek altijd onafhankelijk van het als achtergrond aangeboden nutriënt, waarschijnlijk omdat er met realistisch lage concentraties is gewerkt. Dit staat in tegenstelling tot andere studies die zowel "constitutieve" en "geïnduceerde" voorkeuren voor ammoniumopname toonden. Voor dubbel (d.w.z. met zowel ^{15}N en ^{13}C) gelabeld ureum en glycine bestond er een sterk verband tussen stikstof- en koolstofopname. De ratio waarin de ^{15}N en ^{13}C werd opgenomen week echter sterk af van de ratio waarin ^{15}N en ^{13}C in deze moleculen aanwezig zijn.

Samenvattend kunnen we concluderen dat, bij realistisch lage (heersende) concentraties van voedingsstoffen, zeegrassen zowel anorganische als organische stikstofbronnen gebruiken. Zeegrassen zijn dus in staat op te nemen wat beschikbaar is (**hoofdstuk 2**), waarbij de uiteindelijke bijdrage van verschillende stikstofbronnen in de totale stikstof opname afhangt van de relatieve concentraties waarin de verschillende bronnen aanwezig zijn. De capaciteit van zeegrassen om zoveel verschillende stikstofbronnen te kunnen gebruiken biedt een mogelijk voordeel aan zeegrassen die leven in een nutriëntarme omgeving.

Naast het voordeel alle beschikbare nutriënt en bronnen te kunnen gebruiken in een nutriëntenarme omgeving, profiteert zeegras ook van betere mechanische eigenschappen onder lage nutriënt condities. Onder lage nutriëntconcentraties zijn zeegrassen relatief sterk (met een hogere specifieke - force - to- tear, F_{TS}), zoals aangetoond voor *Halophila ovalis* (**hoofdstuk 3**), *Zostera noltii* (**hoofdstuk 5**) en *Enhalus acoroides* (**hoofdstuk 4**). Daarentegen kunnen zeegrassen gemakkelijk breken onder hoge nutriënten concentraties. Dat is ook het geval als ze te weinig licht krijgen (**hoofdstuk 3**). Zowel hoge concentraties van voedingsstoffen en lichtgebrek kunnen dus leiden tot verzwakking van zeegrasbladeren (aangetoond voor *Halophila ovalis*), zichtbaar in een lagere breukspanning (F_{TS}). De absolute breeksterkte (F_{MAX} ; een grootte-afhankelijke materiële eigenschap) bleef echter gelijk bij deze soort. Wanneer nutriënten concentraties werden verhoogd, nam voor *Zostera noltii* zowel de breukspanning (F_{TS}), alsde breeksterkte (F_{MAX}) af. Dit kan betekenen dat de mechanische weerstand van het blad zich aanpast aan veranderingen in omgevingsfactoren door aanpassingen van zowel morfologische als mechanische eigenschappen (**hoofdstuk 3**). De zeegrass oort *Halodule uninervis* liet echter geen prominente veranderingen in morfologie en mechanische eigenschappen zien bij veranderingen in de omgeving. Hieruit kunnen we opmaken dat mechanische reacties sterk soortspecifiek zijn, zoals ook werd waargenomen in *Enhalus acoroides* en *Thalassia hemprichii* (**hoofdstuk 4**). Behalve soortspecifieke mechanische aanpassingen, zijn er ook verschillen in morfologische en mechanische eigenschappen binnen dezelfde soortwaargenomen. Weefsels van blad en bladschede hebben duidelijke verschillen in hun morfologie. Vergelijkbare verschillen bestaan er voor de mechanische eigenschappen. De scheden van *Enhalus acoroides* vertonen bijvoorbeeld hogere uitrekbaarheid (d.w.z., de mogelijkheid om meer te verlengen voordat ze breken) dan hun bladeren. Dit komt waarschijnlijk door het zachtere meristeemweefsel van de bladscheden, die nog elastische celwanden hebben. Daarentegen hebben debladscheden van *Thalassia hemprichii* een lagere breeksterkte dan hun bladeren. De scheden zijn kort en

bevinden zich meestal in het sediment. Wellicht is het voor *Thalassia hemprichii* belangrijker om sterke bladeren te hebben dan sterke bladscheden.

Afgezien van de hierboven besproken mechanische en morfologische aanpassingen van zeegras aan enkelvoudige milieu-invloeden, is er in dit proefschrift ook aangetoond dat de interactie tussen golven en voedingsstoffen spannende nieuwe interactieve effecten veroorzaken (**hoofdstuk 5**). Golven en hoge concentraties nutriënten veroorzaakten gezamenlijk een afnemende overleving van het zeegras *Zostera noltii*. Alleen de golfstress veroorzaakte een afname van zeegras lengte en ondergrondse biomassa, terwijl de hoge concentraties van nutriënten de afname in zeegrassterkte en –stijfheid veroorzaakte. Dezelaatste bevinding was in overeenstemming met onze waarnemingen voor *Halophila ovalis* (**hoofdstuk 3**). Het valt te verwachten dat deze specifieke reacties van het zeegras *Zostera noltii* op de combinatie van golven en hoge voedingsstoffen leiden tot een negatieve feedback, en daarmee tot het verdwijnen van een zeegrasveld. Door de afname in bladlengte en bovengrondse biomassa onder invloed van golven, zal de golf-uitdempende werking van het zeegrasbed afnemen. Hierdoor kan de stress in het zeegrasveld toenemen, wat resulteert in een verdere afname van bovengrondse biomassa en bladlengte. Dit effect kan verder worden versterkt door het verlies van zeegrasplanten ten gevolge van erosie. Daarnaast kan hoge nutriënt belasting de planten ook nog verzwakken en daarmee ook leiden tot een potentiële negatieve feedback die resulteert in het verlies van zeegrasplanten. Gecombineerd kunnen deze twee negatieve feedbacks elkaar nog verder versterken, en potentiële instorting van het zeegras versnellen.

Door alle hoofdstukken te vergelijken zijn er een aantal belangrijke generalisaties te maken met betrekking tot de mechanische eigenschappen. Er lijkt een algemene tendens te zijn dat traaggroeiende zeegrassoorten een hogere bladweerstand hebben (F_{MAX}) en sterker zijn (d.w.z. hogere “breaking-stress”, FTS), in vergelijking met de snelgroeiende soorten. De langzaamgroeiende soorten compenseren blijkbaar de lage turnover van hun blad door het hebben van sterkere bladeren die een langere bladlevensduur mogelijk maken. Het lijkt voor lang levende soorten dus een vereiste te zijn om sterke bladeren te hebben, om daarmee wisselende (abiotische en biotische) omgevingsstress te kunnen weerstaan.

Een goed begrip van de mechanische eigenschappen van levende materialen zoals zeegrasweefsel, is complexer dan het verkrijgen van een dergelijk begrip voor de meeste niet-natuurlijke materialen. Dit proefschrift draagt bij aan ons begrip van de complexiteit van de reactie van de mechanische eigenschappen van zeegras op milieu-invloeden en aan onze kennis over de functie en de ecologische betekenis van deze biomechanische eigenschappen.

RINGKASAN

Lamun adalah tumbuhan berbunga yang hidup di perairan laut dan pantai wilayah tropik dan ughari. Lamun memiliki berbagai fungsi dan nilai, baik fisik, ekologis dan ekonomis. Secara fisik, lamun berfungsi sebagai pelindung pantai karena mereka mampu mengurangi energi gelombang dan menstabilkan sedimen. Dengan demikian, ekosistem sekitarnya dapat diuntungkan karena pergerakan air menjadi berkurang sehingga sedimen-sedimen terlarut dapat terendap, yang menyebabkan berkurangnya kekeruhan air. Ditambahkan lagi bahwa hal tersebut dapat memberikan sifat timbal balik yang positif terhadap lamun oleh adanya intensitas cahaya untuk fotosintesis lamun. Secara ekologis, lamun berfungsi sebagai habitat bagi berbagai organisme laut, yaitu sebagai daerah makan, asuhan dan/atau perkembangbiakan. Dengan demikian, lamun dapat menjaga keanekaragaman serta nilai komersil jenis-jenis organisme penting. Namun sayangnya, kondisi ekosistem lamun telah mengalami penurunan, dan hal tersebut terjadi di seluruh dunia. Berbagai kegiatan manusia merupakan penyebab utama penurunan kondisi lamun, dan bukan karena faktor alam. Tetapi, dengan ditambahkan lagi dengan perubahan iklim, diprediksikan bahwa kejadian cuaca ekstrim (misalnya dengan meningkatnya frekuensi dan intensitas terjadinya badai) akan meningkat, yang akan memperburuk kondisi lamun yang ada sekarang. Untuk dapat mengelola ekosistem lamun dengan lebih baik lagi, dan mengurangi kecepatan pengrusakannya, perlu mencari tahu dan mempelajari bagaimana lamun merespon stres lingkungan seperti yang tersebut di atas. Berdasarkan hal tersebut, kami telah meneliti bagaimana respon fisiologis, morfologis dan biomekanik lamun terhadap berbagai stress lingkungan; yaitu lamun yang terekspose pada nutrien rendah maupun tinggi, hidrodinamika tinggi dan cahaya rendah.

Di alam, lamun mengalami kondisi dimana bahan-bahan yang mengandung nitrogen organik maupun inorganik dengan berbagai bio-availabilitasnya terdapat dalam konsentrasi rendah. Namun, penelitian lamun umumnya hanya fokus pada *uptake* nitrogen dengan substrat yang mengandung satu jenis (*single*) nitrogen saja. Dengan menggunakan kombinasi dari satu substrat terlabel ^{15}N dan satu substrat *background* tanpa label, ^{14}N , kami menemukan bahwa, lamun memiliki kesukaan *constitutive* terhadap *uptake* ammonium dibandingkan dengan substrat lainnya (yaitu ammonium ataupun nitrat sebagai nitrogen inorganik terlarut; dan urea ataupun glisin sebagai nitrogen organik terlarut). Namun, *uptake* substrat selalu memperlihatkan ketidaktergantungannya dengan *background* nutrien (baik tumbuhan bagian atas maupun bawah). Hal ini menunjukkan bahwa tidak terdapat *regulasi* atas ataupun bawah yang menyebabkan lamun menyukai satu *source* nitrogen tertentu dibanding *source* nitrogen lainnya. Hal ini kemungkinan disebabkan

oleh rendahnya konsentrasi nutrisi (namun realistis) yang digunakan didalam eksperimen ini dibanding studi-studi lainnya yang menunjukkan kesukaan *constitutive* maupun *induced* terhadap ammonium dibandingkan *uptake* nitrat. Dengan menggunakan *label* dobel (^{15}N dan ^{13}C) urea dan glisin, terdapat hubungan yang erat antara *uptake* nitrogen dan karbon, namun saat terjadi *uptake* molekul secara keseluruhan, terjadi sedikit penyimpangan dari yang semestinya. Ringkasnya, dapat kami simpulkan bahwa pada konsentrasi nutrisi rendah (namun realistis) (yang terjadi), lamun dapat menggunakan inorganik maupun organik sebagai sumber nutrisinya dan tidak membedakan antara *substrat* yang ada. Dengan kata lain, lamun mampu meng-*uptake* apapun yang tersedia (**Bab 2**), namun keseluruhan *uptake* nitrogen (yang terdiri dari kontribusi *sources* yang berbeda) dapat pula bergantung pada konsentrasi relatif dari *sources* yang berbeda-beda. Tentunya, kemampuan meng-*uptake* apapun yang tersedia dapat merupakan keuntungan bagi lamun yang terekspos di lingkungan bernutrien rendah.

Keuntungan dari kemampuan lamun meng-*uptake* nutrisi apapun di lingkungan bernutrien rendah, ternyata juga memberi keuntungan bagi performa mekaniknya. Artinya, di saat kondisi nutrisi rendah, lamun menjadi kuat (memiliki *specific-force-to-tear*, F_{TS} (kekuatan spesifik untuk merobek/memutus) yang tinggi) seperti pada *Halophila ovalis* (**bab 3**), *Enhalus acoroides* (**bab 4**) dan *Zostera noltii* (**bab 5**). Sebaliknya, dengan konsentrasi nutrisi tinggi, lamun patah dengan mudahnya. Selain itu, kekurangan cahaya juga merupakan stres lingkungan yang dapat menyebabkan lamun mudah patah (**bab 3**). Baik nutrisi tinggi maupun cahaya yang kurang dapat menyebabkan daun lamun (*Halophila ovalis*) menjadi lemah dengan memiliki *breaking stres* (F_{TS}) yang rendah walaupun *absolute breaking force*nya (F_{MAX} – properti material yang bergantung pada ukuran) tetap sama. Akan tetapi, dengan konsentrasi nutrisi tinggi, lamun *Z. noltii* memiliki *breaking stres* (F_{TS}) juga *breaking force* (F_{MAX}) yang rendah. Hal tersebut menunjukkan bahwa resistansi mekanik daun dihasilkan dari aklimasi tumbuhan terhadap kondisi lingkungan yang beragam dengan menghasilkan perubahan morfologi maupun mekanik (**bab 3**). Tetapi, lamun *Halodule uninervis* tidak memperlihatkan adanya perubahan besar pada properti morfologi maupun mekaniknya di lingkungan yang telah mengalami perubahan. Dengan demikian, dapat dikatakan bahwa respons mekanik bersifat *species specific* (tergantung dari jenisnya), yang ternyata juga ditemukan pada lamun *Enhalus acoroides* dan *Thalassia hemprichii* (**bab 4**). Selain daripada sifat mekaniknya yang *species specific* (tergantung jenis), lamun juga memiliki sifat *intra-species* (hubungan sifat antar bagian tumbuhan yang sejenis) yang spesifik, utamanya pada properti morfologi dan mekanik. Jaringan daun dan seludang memiliki perbedaan morfologi maupun mekanik yang mencolok. Misalnya, dibandingkan daun, seludang

Enhalus acoroides memiliki ekstensibilitas yang lebih tinggi (mampu memanjangkan seludangnya lebih jauh sebelum putus/patah). Hal tersebut kemungkinan karena jaringan meristem yang lebih lembut yang membentuk seludang yang masih memiliki dinding sel yang elastis. Namun sebaliknya, seludang *Thalassia hemprichii* memiliki *breaking force* (kekuatan untuk patah/putus) yang lebih rendah dibandingkan daunnya. Hal tersebut kemungkinan disebabkan oleh karena seludang pendek dan (lebih sering) terendap di dalam sedimen, sehingga akan lebih bermanfaat apabila *T. hemprichii* memiliki daun yang lebih kuat dari seludang.

Selain mengungkapkan respon mekanik dan morfologi lamun terhadap salah satu (single) stres lingkungan seperti tersebut diatas, thesis ini juga mengungkapkan temuan baru yang menarik yaitu pengaruh dari stres yang saling berinteraksi yaitu gelombang dan nutrisi (**bab 5**). Interaksi stress gelombang dan nutrisi tinggi dapat menurunkan tingkat kelangsungan hidup *Zostera noltii*. Akan tetapi, bila terjadi stress gelombang saja, maka terjadi pengurangan panjang dan biomassa lamun bawah, dan jika terjadi stress nutrisi saja, lamun akan lemah dan kaku. Kondisi terakhir sejalan dengan hasil pengamatan kami pada *Halophila ovalis* (**bab 3**). Response spesifik dari lamun *Zostera noltii* terhadap interaksi stress gelombang dan nutrisi tinggi diduga dapat menyebabkan timbal-balik negatif, yang pada akhirnya dapat merusak padang lamun secara keseluruhan. Misalnya, pendeknya daun lamun dan biomassa atas oleh karena gelombang, dapat mengurangi kapasitas lamun sebagai peredam gelombang, sehingga dapat meningkatkan stress pada lamun dengan berlanjutnya pengurangan biomassa atas dan panjang daun. Kondisi ini, dapat diperburuk dengan semakin banyaknya lamun yang rusak akibat akar mudah terlepas dari substratnya. Sebagai tambahan, stress nutrisi yang tinggi dapat menghasilkan satu lagi efek timbal-balik negatif yaitu, karena lamun melemah, sehingga pada akhirnya dapat menambah kerusakan tanaman lamun. Sangat jelas bahwa dengan kombinasi timbal-balik negatif tersebut akan saling memperbesar dampak satu sama lain, yang pada akhirnya menyebabkan semakin besarnya potensi kerusakan lamun.

Berdasarkan pada properti mekanik, dapat diambil beberapa hal penting yang bersifat umum dari hasil perbandingan semua bab dalam thesis ini. Nampak bahwa ada tren umum dimana lamun dengan tingkat pertumbuhan rendah memiliki daun dengan resistansi (F_{MAX}) tinggi dan lebih kuat (*breaking stress*- F_{TS} yang tinggi; kekuatan spesifik untuk merobek/ memutus) dibandingkan dengan yang memiliki pertumbuhan cepat. Jenis yang bertumbuh lambat mengimbangi tingkat pergantian daun yang rendah dengan memiliki daun yang lebih kuat agar daun dapat hidup lebih lama. Yang menjadi persyaratan untuk dapat hidup lebih lama, kemungkinan adalah memiliki daun yang kuat agar dapat mengatasi stress lingkungan (abiotik maupun biotik).

Memahami properti mekanik semua jaringan hidup, seperti jaringan pada tumbuhan lamun, merupakan hal yang sangat kompleks dibandingkan dengan bahan-bahan buatan (manusia). Thesis ini bertujuan untuk meningkatkan pengetahuan dan pemahaman kita mengenai betapa kompleksnya properti mekanik lamun dalam merespon stres lingkungan dan untuk menyumbangkan pengetahuan kami terhadap pentingnya fungsi ekologis dari properti mekanik.

CURRICULUM VITAE

Yayu La Nafie was born and grew up in Makassar (previously called Ujung Pandang), the capital city of South Sulawesi Province, Indonesia. Living in coastal areas made her love the beach and the surrounding environment and made her eager to learn more about marine and coastal environment. She then opted to study Marine science for her bachelor degree at the Department of Marine Science, Hasanuddin University, in her hometown. For the last 2 years of her study, she received a scholarship from the Ministry of Education and Culture for her achievement during the studies. At her last year of bachelor, she assisted junior students working in the field and lab, for introduction to marine science and malacology. She was also interested in the effect of pollutants on marine organisms hence she conducted a study on “Toxicity of endosulfan pesticide to stomatopode *Gonodactylellus viridis*” for her final project of her bachelor degree. She was also involved in a research of stomatopode distribution in South Sulawesi by Dr. Mark Erdman (who was a PhD student during that time) of University of California Los Angeles (UCLA).

After graduation, she joined a local environmental Non Government Organization (NGO) (environmental partner institute) that focused on the protection of the coastal environment. While working, she applied for a scholarship to pursue her master degree and was awarded an Aus-AID scholarship (Australian Agency for International Development). She studied at University of Technology Sydney, undertaking masters in coastal resource management, which offered her a variety of topics of interest. She was involved in a small project on Damselfish in Heron Island and studied the “Distribution, abundance and feeding habits of damselfish *Pomacentrus flavicauda* in Heron Island, Great Barrier Reef, Australia”. After achieving her masters degree she went back to her hometown and applied for a teaching staff position at their previous university. She started as a junior staff member at the Department of Marine Science, Hasanuddin University, South Sulawesi, Makassar, in 2002. Seagrass had not been much studied, but they are important organisms yet they are declining, so she turned her wheel of interest to seagrass studies. In 2005 she joined a training and workshop of Seagrass conducted by University of New Hampshire (UNH), USA, collaborating with Queensland Department of Primary Industry (QDPI), and was funded by the Lucille Packard Foundation. She is now studying how seagrass respond to abiotic stresses for her PhD studies in the Netherlands Institute for Sea Research and The Radboud University, Nijmegen, funded by the Minister of National Education, Republic of Indonesia in the expectance to be able to bring home and share the knowledge gained.

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