New Zealand seagrass (*Zostera muelleri)* response to acute sedimentation: Linking non-structural carbohydrate reserves to resilience

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Abstract

Seagrass meadows form healthy near-coastal marine environments that provide a wide range of ecosystem services in New Zealand and Australia. However, seagrass habitats are declining regionally and globally. It is therefore essential that research fills the current knowledge gaps associated with seagrass disturbance-response regimes and develops standardised methods to measure seagrass health and resilience. Sedimentation associated with increased human activity is a major environmental stressor to seagrass; yet little is known about how sedimentation affects New Zealand's only seagrass species, *Zostera muelleri*. This thesis, therefore explores how *Z. muelleri* responds to catastrophic burial (acute) events in Tauranga Harbour, New Zealand.

Non-structural carbohydrates (NSC) are synthesised and stored in seagrass tissues when photosynthetic outputs exceed metabolic demands and, in turn, are mobilised when photosynthesis is unable to meet metabolic demands. Hence, NSC reserves can be used to measure the impact of a disturbance and/or the resilience of seagrass. A literature review in Chapter 2 illustrates that multiple methods are used to estimate NSC reserves. Experimental comparisons of a selection of these methods demonstrate that NSC estimates by different analytical methods cannot be compared. The outcome of Chapter 2 is the development of a 5-step standard analytical protocol for the quantitation of NSC reserves in seagrass (specifically *Z. muelleri*). The implementation of a standardised protocol will enable researchers to compare and synthesise results and, thus, increase the application of NSC as a seagrass health measure.

Chapter 3 investigates the effects of acute sedimentation (i.e. burial) on *Z. muelleri*. *In situ* manipulative experiments across three sites in Tauranga Harbour, New Zealand were used to assess the spatial variation in resilience (i.e. resistance and recovery) to burial. Resistance and recovery following burial varied significantly between the three distinct morphotypes that existed in the three sites; a large, a small and an intermediate morphotype that overlapped with the small and the large morphotypes. These morphometric differences were believed to be phenotypic responses to site-specific environmental conditions. Having a large morphotype maximises the potential to capture light and store reserves and presumably an acclimation of *Z. muelleri* existing in a chronically impacted environment (high

sediment mud/organic matter (SOM) and low sediment grain size). The largest seagrass displayed high resistance to both single and repeated burial events of 2 cm (no significant effects). In contrast, the smaller seagrass had low resistance and the slowest ability to recover following burial (up to 251 days), whereas, the intermediate morphotype displayed a faster ability to recover (up to 168 days). Results, therefore, suggest that the resilience to burial events increases in populations that have acclimated to a chronically impacted environment. It is, however, noted that a degraded environment will eventually reach a limiting threshold from which recovery is not plausible and results should therefore be interpreted with some caution.

While rhizome NSC reserves were not affected by burial treatments, these varied significantly throughout a full growing year across the three locations. NSC reserves were at the lowest levels in winter and spring, consistent with seasonal trends. Sucrose levels at the peak of the growing season (February) were significantly related to relative shoot cover the following winter ($r^2 = 0.54$, $P < .001$) and spring ($r^2 = 0.66$, $P < .001$). As such, chapter 3 also documented a link between seagrass cover and sucrose reserves.

Estimates of NSC reserves from the literature vary greatly, presumably due to the wide variety of analytical methods used. Chapter 4 explores the variability of *Z. muelleri* NSC reserves and the specific partitioning of carbohydrate groups (sucrose and starch) across different spatial scales (within meadow, between sites, between regions), including temperate (Port Phillip Bay) and tropical populations (Townville and Magnetic Island) in Australia. When measured in spring, the total NSC reserves were similar across all spatial scales (193.59 ± 10.88 mg g^{-1} DW); however, the proportional contents of sucrose and starch varied significantly at different spatial scales. Sucrose contents varied at site level and were best explained by a combination of sediment pH and aboveground to belowground biomass ratio ($r^2 = 0.49$, $P < 0.001$). Starch contents varied between regions, with seagrass in the tropical Townsville containing significantly higher starch levels and as such lower sucrose to starch ratios. The best model for starch included only one variable; sediment surface temperature ($r^2 = 0.33$, $P < 0.001$) and results therefore suggest that starch is influenced by large-scale processes linked to climatic processes. The adaptive strategies of seagrass to moderate NSC allocation appear to be of particular importance to seagrass' ability to acclimate to a diverse range of environments.

Chapter 5 synthesises the results of the three research chapters and provides recommendations for best practices. Of particular importance, is the method that was developed to ensure accurate measurements of NSC reserves in seagrasses, allowing this measure to be used as a monitoring tool of potential resilience and health of seagrass meadows. This thesis clearly illustrates that the partitioning of NSC groups (sucrose/starch) vary spatially and temporally and that these are influenced by distinct processes. Furthermore, it demonstrates that the phenotypic plasticity of *Z. muelleri* in New Zealand enhances their resilience to acute sedimentation events (i.e. burial). It is therefore suggested that the timing and location of human-induced disturbances (i.e. catchment developments, dredging of harbours and ports) should match optimum seagrass resilience (e.g., high sucrose reserves and consideration of phenotypic expression), as this is likely to increase the survival rates of the impacted seagrass meadows. The new insights gained from this research provides crucial information to ensure sustainable management of *Z. muelleri* into the future.

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Table of Contents

List of Figures

List of Tables

Glossary

ABR	Above to belowground biomass ratio
amu	Atomic mass unit
APB	Absolute percentage burial
CV	Coefficient of variance
DW	Dry weight
F_v/F_m	Quantum yield of electron transfer
GC	Gas-liquid chromatography
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HMF	5-hydroxymethylfurfuraldehyde
HPLC	High-performance liquid chromatography
KOH	Potassium hydroxide
LC-MS	Liquid chromatography-mass spectrometry
MBTH	3-Methyl-2-benzothiazolinonehydrazone
NaOH	Sodium hydroxide
NSC	Non-structural carbohydrates
RID	Refractive index detector
SOM	Sediment organic matter
SST	Sea surface temperatures
tNSC	Total non-structural carbohydrates
UDP	Uridine diphosphate
v/v	Volume/volume

Chapter 1
General Introduction

Seagrass (*Zostera muelleri*) meadow in Tauranga Harbour, New Zealand. Photo: Sørensen, S.T.

1.1 Introduction to seagrass biology

Seagrasses are marine angiosperms, and their unique adaptation to submerged marine life has significantly influenced their morphology and anatomy (Kuo & Hartog, 2006). They form an ecological group rather than a taxonomic group (Brasier, 1975) as they evolved on at least three independent occasions from common terrestrial ancestors (McRoy & Helfferich, 1977). Fossil evidence suggests that seagrasses have existed since the late Cretaceous, but the evidence is limited to only a few geographical locations. The limited geographical extent of fossil evidence is probably due to the difficulty involved in identifying plant fossils without the preservation of the reproductive parts, and the fact that seagrasses lack phytoliths, and their pollen grains lack an exine, which limits the preservation of either (Brasier, 1975). Currently, 72 seagrass species are recognised worldwide and are found in all but polar climates (Short *et al.*, 2011).

Seagrasses are often found in estuaries, lagoons, or near coastal habitats, and exist in saline conditions where concentrations change regularly due to tidal movements (Touchette, 2007). Existing in these environments requires adaptations of an organism to avoid cellular desiccation due to the concentration of salts in the aqueous surroundings and exposure during low tide in the intertidal zone. To prevent water loss, the anatomy of seagrass leaves differs from terrestrial plants: they lack stomata, and instead have a thin cuticle layer and generally have fewer water-conducting elements (Kuo & Hartog, 2006). Furthermore, seagrasses are active osmoregulators and have invaginated plasmalemma-mitochondrial transport systems (Jagels, 1973, 1983) with salt-tolerant H^+ ATPase that inhibits sodium (Na^+) entering the cytoplasm by generating an electrochemical membrane potential (Kuo & Hartog, 2006).

Other adaptations to saline environments include the ability to produce sulphated polysaccharides, which are commonly found in marine organisms such as marine algae and invertebrates (Aquino *et al.*, 2004). The genes coding for carbohydrate sulfotransferases and sulfatases appear to have been lost in terrestrial and aquatic plants (Olsen *et al.*, 2016). Sulfation of carbohydrates supports osmotic equilibrium of seagrasses by facilitating ion and water retention in the cell and this is further enhanced by the presence of organic osmolytes such as sucrose, trehalose and proline (Olsen *et al.*, 2016). Although starch is generally the preferred energy reserve of plants (Smith & Zeeman, 2006), a comparison of the *Zostera marina*

genome (marine) and an aquatic (freshwater) monocot (*Spirodela polyrhiza*) revealed that genes coding for starch metabolism are significantly reduced in *Z. marina*, whereas those coding for sucrose synthase (SS) and sucrose transport are significantly expanded (Olsen *et al.*, 2016). This greater reliance on sucrose rather than starch for energy reserves appear to be an adaptation of seagrass to the marine environment. Since osmotic pressure is a colligative property, relying on the number of molecules present small molecules such as sucrose are much more effective osmolytes than large polymeric molecules such as starch.

Primary production is limited by carbon dioxide (CO_2) availability (Portis, 2012), but, in marine waters, dissolved inorganic carbon is more readily available in the form of bicarbonate (HCO_3^-) than CO_2 (Raven *et al.*, 2005). The majority of tested seagrass species have therefore developed mechanisms to use HCO_3^- as an additional source of carbon to accumulate higher levels of CO_2 around Rubisco active sites similarly to algae (Touchette & Burkholder, 2000; Olsen *et al.*, 2018). However, sourcing carbon from HCO_3^- requires more energy compared to CO_2 and seagrasses are not as efficient as algae in using carbon from HCO_3^-, thus, experimental exposure of seagrasses to elevated levels of CO_2 significantly increases photosynthetic rates (e.g., Beer & Koch, 1996; Invers *et al.*, 2001; Egea *et al.*, 2018).

Approximately two percent of all angiosperms (~300,000 species) are aquatic, but only 130 angiosperms are hydrophiles, meaning that pollination occurs during submersion (Cox, 1988; Les, 1988). Underwater pollination appears to be an essential transitional evolutionary step back to marine life, as all seagrass species, except for *Enhalus acoroides,* are hydrophilic, (Les *et al.*, 1997). Seagrasses include bisexual, monoecious, and dioecious species (Les, 1988; Les *et al.*, 1997). However, the majority of seagrass are dioecious or monoecious with diclinous flowers, which is believed to aid outcrossing rates and, thus, limit inbreeding depression due to self-pollination (Sherman *et al.*, 2018). Pollination is facilitated by passive water-dispersal (Ackerman, 2000) and biotically by the movement of marine invertebrates (van Tussenbroek *et al.*, 2012; van Tussenbroek *et al.*, 2016). The transition to underwater pollination has caused adaptations of the floral structures and the pollen (Sherman *et al.*, 2018).

Seagrasses, like many terrestrial plants, display a range of sexual and asexual reproductive strategies. Generally, sexual propagules increase the dispersal range

and genetic diversity of a population, thereby, increasing the probabilities of survival in an unpredictable environment (Williams, 1975; Smith, 1978). In contrast, asexual reproduction is commonly believed to be favourable in stable environments where a parent genotype has been successful (Williams, 1975; Smith, 1978). Consistent with these theories, environmental disturbances influence sexual reproductive efforts in seagrass in 72% of documented cases (Cabaço & Santos, 2012). In contrast, the spread of perennial seagrass in more stable environments appear to occur more frequently by rhizome elongation, resulting in clonal plants and low genetic diversity within meadows (Tomlinson, 1974; Marba & Duarte, 1998; Kuo & Hartog, 2006).

Some seagrass species form seedbanks in sediments of existing seagrass meadows and nearby sand/mudflats, and the genetic diversity may increase as a result, thereby enhancing the ability of the seagrasses to adapt to environmental change (Ehlers *et al.,* 2008). Seedbanks may also facilitate recovery or recolonisation following severe disturbances (Duarte & Sand-Jensen, 1990; Hemminga & Duarte, 2000). Annual seagrass populations generally rely on seedbanks for regrowth in spring, following winter senescence (Harrison, 1982).

Although sexual reproduction may promote long-distance dispersal and asexual reproduction short-distance dispersal in terrestrial plants (Williams, 1975; Smith, 1978), these reproductive strategies do not transfer directly to seagrasses (Sherman *et al.,* 2018). Instead, long-distance dispersal can occur by seagrass fragments rather than sexual propagules, as these may remain buoyant for more extended periods than seeds (e.g., Campbell, 2003; Stafford-Bell *et al.,* 2015; Thomson *et al.,* 2015; Smith *et al.,* 2016; Weatherall *et al.,* 2016). Examples of major long-distance dispersal events involve the spread of *Heterozostera nigricaulis* propagules from Australia across the Pacific Ocean to Chile (approximately 14,000 km), which appears to have occurred on two separate occasions (Smith *et al.,* 2018). These extreme dispersal events (pulse-events) have resulted in two distinct clonal populations of *H. nigricaulis,* forming some of the most prominent clones in the world today (Smith *et al.,* 2018).

The production of flowers and seeds requires a significant allocation of energy resources; thus seagrass species with larger rhizomes (i.e., potentially greater quantities of energy reserves) may be more likely to meet the metabolic demand associated with sexual reproduction (Cabaço & Santos, 2012). Seagrasses, like

other primary producers, synthesise and store carbohydrates through photosynthetic activity, as light-driven reactions cause plants to convert CO_2 and water (H_2O) into carbohydrates and oxygen (O_2). Regulation of CO_2 uptake relates to the inherent photosynthetic capacity of leaves and the availability of CO_2 (Portis, 2012). When photosynthetic productivity exceeds respiration, a plant has a positive carbon balance, which enables it to store carbohydrates that can be used at a later stage, or to facilitate growth and to produce flowers and seeds. Thus, reduction in seagrass photosynthesis generally results in the mobilisation of stored non-structural carbohydrate (NSC) reserves (e.g., Pirc, 1989; Burke *et al.*, 1996; Lee & Dunton, 1996; Alcoverro *et al.*, 1999; Alcoverro *et al.*, 2001)

The speed of seagrass meadow propagation through horizontal rhizome elongation varies significantly between seagrass species and across bioregions. It can range from 1.2 to 574 cm $year^{-1}$, with shoot addition every 1.1 to 7.5 cm of rhizome extension (Marba & Duarte, 1998). Smaller species are generally faster growing and shorter-lived (often referred to as *r*-selected species), whereas larger species are slower growing but longer-lived (K-selected) (MacArthur & Wilson, 1967; Harrison, 1979). Trade-offs in specific strengths relate to the two types of life history selection traits, with *r*-strategists investing more in reproductive outputs (sexual/asexual) making them fast colonisers, and K-strategist investing more in biomass making them stronger competitors (Bohn *et al.*, 2014). As such, *r*-strategists generally have a lower disturbance-threshold than K-strategist, but faster recovery rates (MacArthur & Wilson, 1967). Organisms usually exist on a spectrum somewhere between the *r*- and K-strategies (Pianka, 1970) and external interactions may cause disturbance-response paradigms to be unpredictable.

The *r*-K selection model developed by MacArthur and Wilson (1967) groups organisms by their size. Pianka (1970) described the *r*-K model as a continuum between two extremes; the *r*-endpoint as an ecological vacuum with no competition and the K-endpoint as an ecological climax with maximal densities and saturated organisms. A relatively recent life-history model for seagrasses was described by Kilminster *et al*. (2015), which considers growth forms as well as reproductive strategies and categorises seagrass into three groups; *colonising*, *opportunistic* or *persistent* (Figure 1.1).

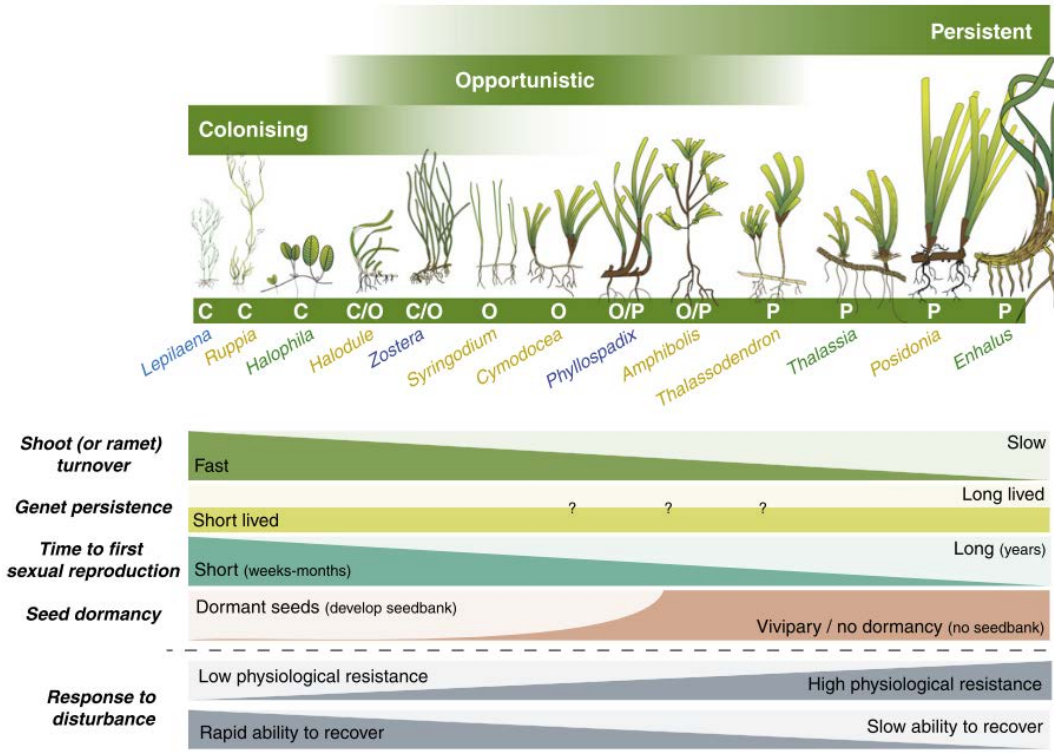

Figure 1.1. Conceptual diagram of the main life-history traits of colonising, opportunistic and persistent seagrass species (source: Kilminster *et al.*, 2015 with permission from Elsevier).

Similar to *r*-strategists, colonising species are fast-growing, have short turn-over times and high seed production, and tend to form seedbanks. Persistent species are similar to K-strategist as they are slow-growing and long-lived, and although they may produce seeds, they tend not to have seedbanks. Opportunistic species can combine life-history strategies and, as such, have both colonising (*r*-strategists) and persistent traits (K-strategists) (Kilminster *et al.*, 2015). A comparison of annual and perennial populations of *Z. marina* in the United States of America (USA) revealed that these populations were genetically similar (Gagnon *et al.*, 1980). Life-history traits, therefore, appear to be a function of environment and disturbances rather than genetically determined.

1.2 **Ecological importance of seagrass**

Seagrasses are habitat modifiers and, therefore, act as ecosystem engineers (Bos *et al.*, 2007; van Katwijk *et al.*, 2010; Reynolds *et al.*, 2016). The presence of submerged vegetation increases bed roughness and drag forces which consequently extends the boundary layer into the water column. This, in turn, alters the above seagrass/substrate free-stream current (Ghisalberti & Nepf, 2002; Bryan *et al.*, 2007). As a result, the current velocity is often reduced, and waves are attenuated,

which influences sediment transport dynamics (Fonseca *et al.*, 1982; Ward *et al.*, 1984; Gambi *et al.*, 1990; Wu *et al.*, 2001; Lefebvre *et al.*, 2010; van Katwijk *et al.*, 2010). The baffling effect of seagrass increases with blade size and surface area (Fonseca *et al.*, 1982) and larger seagrasses generally have a more significant effect on water movement. Meadows of larger seagrass species can therefore filter out larger quantities of sediments (Heiss *et al*., 2000).

Seagrass may increase the light penetration of the water column above the seagrass bed because of increased sediment deposition and reduced sediment resuspension (Newell & Koch, 2004). Sediment silt content (Bos *et al.*, 2007), as well as the contents of inorganic and organic matters (Nepf, 2009), are therefore generally elevated in seagrass meadows compared to adjacent unvegetated substrates. It was recently demonstrated that seagrass meadows might also act as bacterial filters and, therefore, increase the health of nearby ecosystems (Lamb *et al.*, 2017). In addition to the modifying effects of seagrass blades, the extensive belowground networks of rhizomes and roots increase seabed stability and, thereby, reduce the risk of coastal erosion in near shore habitats (Gacia & Duarte, 2001; de Boer, 2007).

The seagrass belowground biomass anchors them in the substratum and facilitates the uptake of nutrients from the sediment porewater through the roots in addition to diffusion across leaves from the water column (Short & McRoy, 1984). The ability of seagrass to obtain nutrients from both the water column and sediment porewater increases their capacity to survive in oligotrophic waters (Hemminga, 1998). In contrast, other macrophytes (such as algae) that are attached to the substratum by a holdfast, rely solely on the availability of nutrients in the water column. Despite this ability, seagrass may still experience nutrient limitations, with seagrasses in terrigenous substrata more likely to be nitrogen (N) limited and phosphorus (P) limited in carbonate substrates (Short, 1987).

Rates of nitrogen (N-) fixation in the rhizosphere (the shallow region of sediment that is influenced by root secretions and associated with the root microbiome) influence seagrass productivity (Welsh, 2000), and in turn, N-fixation by microbes in the rhizosphere, is fuelled by oxygen diffusion from seagrass roots into sediments (Welsh *et al.*, 2000). As such, a feedback mechanism exists between seagrass productivity and nitrogen cycling. Elevated quantities of remineralised nitrogen are common in seagrass substrates compared to unvegetated substrates (Kemp *et al.*, 1982; Kenworthy *et al.*, 1982; Hemminga, 1998). Despite this, oxygen is generally

limited to a shallow top layer of the marine sediments. Thus, anaerobic bacteria (e.g., sulphate reducing bacteria) may contribute to most of the organic matter decomposition in the rhizosphere (Jørgensen, 1982).

Hydrogen sulphide accumulation may increase in the rhizosphere and can have a negative effect on the photosynthetic potential of seagrass (Goodman *et al.*, 1995). To alleviate the effects of sulphide toxicity, seagrass may create benthic oxic-shields by transporting oxygen from their photosynthetic tissue via the aerenchyma into belowground tissues from where it diffuses from young roots into the sediments (Brodersen *et al.*, 2015; Campbell *et al.*, 2018; Martin *et al.*, 2019). These oxic-shields play a vital role in enabling vulnerable young rhizomes in the growing edge to colonise into sulphide-rich substrates (Martin *et al.*, 2019). Additionally, a three-stage symbiosis between seagrass, lucinid bivalves and their sulphide-oxidising gill-bacteria may also reduce sulphide toxicity (van der Heide *et al.*, 2012). Elevated levels of organic matter and radial oxygen release from seagrass roots benefit lucinid bivalves, which can reduce sulphide levels in the rhizosphere via their oxidising gill bacteria, consequently increasing the biomass of seagrass (van der Heide *et al.*, 2012).

It is well established that coastal vegetation supports diverse benthic macroinvertebrate communities (Heck & Wetstone, 1977; Alfaro, 2006; Lundquist *et al.*, 2018). However, seagrass meadows may be able to support a particularly high density and diversity of benthic invertebrates (compared to, for example, mangrove stands and their associated pneumatophore zones (Alfaro, 2006)). The 3-dimensional bio-structural component of seagrass meadows makes them essential ecosystems for fishes and invertebrates by providing shelter, protection from predation, and food (Thayer *et al.*, 1984; Bell & Pollard, 1989; Morrison & Francis, 2001; Nakamura & Tsuchiya, 2008; Lee *et al.*, 2016; Parsons *et al.*, 2016; Sato *et al.*, 2016). Even, large megafauna (such as dugongs, manatees, turtles, and herbivorous waterfowl) rely on seagrass as a source of food (Heck & Valentine, 2006; Dos Santos, 2011; Scott *et al.*, 2018). As a result, seagrasses form central habitats for a wide range of marine and nearshore organisms.

The term “blue carbon” refers to the carbon stock stored in marine ecosystems (e.g., Mcleod *et al.*, 2011; Macreadie & Hardy, 2018). Coastal vegetation such as seagrasses, mangroves, and saltmarshes, capture and sequester CO_2 from the atmosphere into oxygen-poor marine sediments (Fourqurean *et al.*, 2012;

Macreadie *et al.*, 2014b). Here, the period of carbon storage is significantly longer than that of terrestrial forests due to the anaerobic conditions of marine sediments compared to terrestrial soils (Mcleod *et al.*, 2011). Seagrass ecosystems are highly efficient carbon sinks due to their high productivity, as well as their ability to capture suspended particles and organic carbon (Marbà *et al.*, 2015b). They account for approximately 15% of the total ocean carbon storage, which is a disproportionally large component, considering that seagrass cover only 0.1-0.2% of the ocean floor (Duarte, 2002).

The total organic carbon (C_{org}) content in seagrass sediments is derived directly from seagrass biomass in the form of detritus and belowground tissues, or from allochthonous sources that are filtered by seagrass leaves (Gacia *et al.*, 2002; Kennedy *et al.*, 2010). The allochthonous proportion is affected by environmental conditions, because the ability of seagrass meadows to trap allochthonous C_{org} depends on the extent of the influence of aboveground biomass on the ambient hydrodynamic conditions (Fonseca *et al.*, 1982; Heiss *et al.*, 2000; Tanaya *et al.*, 2018) and the availability of C_{org} in the water column (Marbà *et al.*, 2015b). Seagrass tissue contributes a large proportion (22 – 50%) of the total C_{org} in surface sediments (Kennedy *et al.*, 2010; Tanaya *et al.*, 2018). However, the fraction of blue carbon that derives from seagrass biomass directly relates to seagrass carbon reserves and the ability to maintain their biomass. Seagrass aboveground biomass decomposes faster than rhizomes and roots due to the chemical composition of their tissues coupled with higher oxygen exposure (Enriquez *et al.*, 1993) and is also more likely to be exported from the system by currents, waves, or herbivory (Duarte & Cebrian, 1996). Therefore, leaves usually provide short-term carbon storage, compared to longer-term storage provided by belowground biomass (Fourqurean *et al.*, 2012).

The ability of a meadow to sequester carbon is highly variable and linked to habitat characteristics that may change spatially and temporally (Mazarrasa *et al.*, 2018). Environmental conditions that reduce photosynthetic activity inevitably influence the blue carbon potential of a meadow. Increased anthropogenic activities in coastal regions have resulted in severe global seagrass losses (Walker & McComb, 1992; Short & Wyllie-Echeverria, 1996; Duarte, 2002; Orth *et al.*, 2006; Waycott *et al.*, 2009). As meadows are lost, carbon stores are released back into the atmosphere,

and the status of a former seagrass meadow may change from carbon sink to carbon source (Macreadie *et al.*, 2015; Marbà *et al.*, 2015b; Lovelock *et al.*, 2017).

The Paris Agreement, which was signed by 196 state parties within the United Nations Framework Convention on Climate Change (UNFCCC) in 2016, is an agreement to limit the global temperature increase to below 2°C (United Nations Climate Change, 2019). A key aim of this agreement is to limit greenhouse gas emissions to mitigate climate change. New Zealand recently made history by being the first nation to pass legislation that commits future governments to the Paris Agreement by setting a target to be carbon neutral by 2050 (Ministry for the Environment, 2019). Thus, conservation and restoration of blue carbon ecosystems, such as seagrass meadows, are of crucial importance to climate change mitigation (Mcleod *et al.*, 2011; Fourqurean *et al.*, 2012; Greiner *et al.*, 2013; Marbà *et al.*, 2015a; Macreadie & Hardy, 2018; Stankovic *et al.*, 2018).

1.3 Thesis rationale

1.3.1 Introduction to case study species: *Zostera muelleri*

This thesis is focused on the seagrass *Zostera muelleri* (Irmisch ex Ascherson, 1867), which is the only seagrass species found in New Zealand. Meadows of *Z. muelleri* are found in sheltered waters such as bays and estuaries in New Zealand, and Australia, where it spans temperate, subtropical and tropical regions (Figure 1.2). Previously, *Z. muelleri* was classified as four separate species: *Z. capricorni, Z. novazelandica, Z. muelleri, and Z. mucronata* (Jones *et al.*, 2008), however, due to a lack of unique morphometric and molecular traits, these four taxa are now synonymised to one taxon (Les *et al.*, 2002; Jacobs *et al.*, 2006). A molecular study suggests that populations of *Z. muelleri* in New Zealand may be a result of a long-distance dispersal event from the east coast of Australia (Ticli, 2014).

Figure 1.2. Southern Hemisphere distribution of *Zostera muelleri* (Source: Waycott *et al*. 2014 with permission from CSIRO Publishing).

A recent study described flowering shoots in *Z. muelleri* meadows on the north island of New Zealand (Dos Santos & Matheson, 2016). Before this study, sexual reproduction in New Zealand *Z. muelleri* was described only once in a study from the South Island (Ramage & Schiel, 1998). This led to a common belief that sexual reproduction in New Zealand *Z. muelleri* meadows is rare (Dos Santos & Matheson, 2016). The recent descriptions of flowering from multiple North Island meadows infer that this may not be as uncommon as previously believed. However, densities of flowers in the North Island (max 72 shoots m^{-2}) are generally lower than South Island populations (max 550 shoots m^{-2}) and Australian populations (max 2,904 shoots m^{-2}) (Dos Santos & Matheson, 2016).

Efforts to identify seeds in New Zealand *Z. muelleri* meadows have so far been unsuccessful (Cade, 2016; Dos Santos & Matheson, 2016), which may be a reflection of unsuccessful pollination of flowers, high abortion rates in fruit, predation in the seedbank, or disease within the seedbank. Similarly, it was recently discovered that the germination success of *Z. marina* seeds is reduced up to 6-fold by the common spread of the pathogens *Phytophthora* and *Halophytophthora* across meadows in the northern Atlantic and the Mediterranean (Govers *et al.*, 2016). Instead, the intra-meadow genetic diversity seen in New Zealand *Z. muelleri* meadows may be related to the natural dispersal of fragments rather than seeds (Cade, 2016), as these remain buoyant and viable for at least five weeks in temperate conditions (in Australia) (Stafford-Bell *et al.*, 2015; Weatherall *et al.*, 2016).

Genetic similarities of *Z. muelleri* populations in New Zealand are higher within meadows than between meadows, which infers a low degree of gene flow between populations (Jones *et al.*, 2008). Jones *et al*. (2008) identified correlations between ocean currents and genetic diversity of *Z. muelleri*, with apparent genetic differences between North and South island New Zealand populations, and between the east coast and west coast populations. Australian *Z. muelleri* seagrasses have a high level of unique genotypes between meadows, with little genetic diversity at a fine scale (< 1 m), but a higher level of diversity at site scale (tens of metres) (Sherman *et al.*, 2016). These studies from both Australia and New Zealand suggest that this species typically displays restricted gene flow and connectivity between meadows.

1.3.2 Threats to seagrass ecosystems

It is estimated that global seagrass coverage has declined by 29% since 1879, at a rate of 110 km^2 yr^{-1} since 1980 (Waycott *et al.*, 2009). In a New Zealand context, 34% of total seagrass cover in Tauranga Harbour was lost between 1959 and 1996, with the subtidal meadows affected most significantly (90% lost) (Park, 1999). If seagrass decline in Tauranga Harbour represents the general trend in New Zealand, then national seagrass loss may be significantly greater than the global average presented by Waycott *et al.* (2009). *Zostera muelleri* is currently listed on the World Conservation Unions (IUCN) Red List as being a species of "least concern" (IUCN, 2015). The IUCN Red Listing is based on criteria presented by Short *et al*. (2011) where *Z. muelleri* is reported not to have experienced significant declines. However, Matheson *et al.* (2011) argue that the IUCN listing is biased towards Australian studies, as New Zealand *Z. muelleri* has suffered severe losses of especially subtidal plants (e.g., Park 1999). The occurrence of such a bias is not surprising, given the lower rates of seagrass research and subsequent publication on seagrasses in New Zealand (66 publications) compared to Australia (378 publications; as per Scopus database search, keyword: *Zostera,* August 2019).

Anthropogenic pressures on coastal environments worldwide have increased significantly with more than half of the global population now living less than 100 km from the coast, and this figure is expected to increase by 25% within the next 20 years (Roca *et al.*, 2015a). In New Zealand, 97% of the population lives less than 50 km from the coast (Statistics NZ, 2017). Increasing and ongoing development of land (i.e., urbanisation) and coastal habitats (reclamation of land/port development) may affect seagrass via increased sedimentation (Cabaço & Santos, 2007), nutrients (van Katwijk *et al.*, 1997; Brun *et al.*, 2002), organic matter (Delgado *et al.*, 1999; Pérez *et al.*, 2007), herbicides (Devault & Pascaline, 2013) and heavy metals (Ralph & Burchett, 1998; Zheng *et al.*, 2018) entering coastal waters.

Nutrient enrichment may lead to eutrophication (Burkholder *et al.*, 2007), which has adverse effects on seagrasses via reduced light regimes due to microalgae blooms, as well as shading and smothering by macroalgae mats (Drouin *et al.*, 2012), and/or epiphytic growth (Cambridge & Hocking, 1997; Lee *et al.*, 2007). Physiological damage may occur due to anaerobic bacterial respiration causing elevated levels of ammonium (van Katwijk *et al.*, 1997; Brun *et al.*, 2002) and

sulphide ions (García *et al.*, 2012; García-Marín *et al.*, 2013) in the rhizosphere. Furthermore, the influx of suspended sediments attenuates light, thereby decreasing the photosynthetic capabilities of seagrasses (Hessing-Lewis *et al.*, 2014), and when suspended sediment loads become high, deposits may smother or even bury seagrass (e.g., Cabaço & Santos, 2007; Benham *et al.*, 2019; Browning *et al.*, 2019).

Terrestrial sediments naturally erode into rivers and estuaries, but, in New Zealand erosion rates are particularly high due to the geomorphic conditions (steep slopes, tectonic activity, volcanism), and high frequency and intensity of rainfall (Hicks *et al.*, 2011). Additionally, the intensified removal of terrestrial vegetation to make way for agricultural, urban and industrial development has further increased the sedimentation regimes in New Zealand in modern times (Hicks *et al.*, 2000; Thrush *et al.*, 2004). Furthermore, severe weather events exacerbate the amount of terrestrial run-off. For example, three cyclones reached the shores of Coromandel, New Zealand within 32 days in 2017, causing partial burial of local seagrass meadows (Martin, R., pers.comm; Campbell, M. L., pers.obsv). Similarly, three major hurricanes hit the northeastern Caribbean in 2017 and caused what equated to between one and three centuries of sediment deposition (Browning *et al.*, 2019). These severe weather events are expected to intensify and occur more frequently due to climate change (Young *et al.*, 2011). Thus, sediment influxes to coastal waters may continue to increase in New Zealand. Sedimentation is predicted to be the largest threat to coastal ecosystems in New Zealand (Thrush *et al.*, 2004; Turner & Schwarz, 2006a) and may therefore affect the resilience of local seagrass meadows.

1.3.3 Resilience of seagrass

The term resilience was first introduced by Holling (1973) and is now ubiquitous in the ecological literature. Holling (1973) described resilience as the amount of disturbance that an ecosystem can sustain, with persistent internal relationships, before causing an ecosystem shift into a permanent alternative state (Holling, 1973). Another popular definition of resilience was defined by Pimm (1984) as the ability of a system to recover to its pre-disturbance state. These two definitions are now commonly referred to as ecological resilience (Holling 1973) and engineering resilience (Pimm 1984), however, the terms are not used consistently used in the literature (Standish *et al.*, 2014). A more recent application of the term resilience combines the two definitions thereby describing two central mechanisms; the

resistance to change and; the ability to recover (Levin & Lubchenco, 2008; Côté & Darling, 2010; McClanahan *et al.*, 2012) and it is recommended that both be considered simultaneously when assessing resilience (Hodgson *et al.*, 2015). Assessments of a systems' threshold to a disturbance (i.e. tipping point) at which stage a permanent shift occurs, as well as, the predictions of recovery timeframes are important concepts in the context of ecosystem management (Côté & Darling, 2010; Standish *et al.*, 2014).

The dynamics of these two mechanisms of resilience are often linked to life-strategies (reproductive strategies and growth forms) at the level of the individual species (Figure 1.1). As such, colonising species (~r-strategists) often have low physiological resistance to disturbance but display fast recovery, whereas, persistent species (~K-strategist) generally have higher physiological resistance but are slower to recover (Kilminster *et al.*, 2015; O'Brien *et al.*, 2018). However, local conditions play a crucial part in these dynamics as a system/population that is exposed to chronic anthropogenic disturbances may either 1) become degraded and express a lowered level of resistance i.e. a threshold is reached sooner or; 2) become more resistance to disturbances as less tolerant species/individual are removed from a system (Côté & Darling, 2010).

A recent study of *Z. muelleri* in Australia demonstrated that seagrass in chronically disturbed environments had significantly lower genotypic diversity but displayed a higher level of resilience to an extreme flooding event (Connolly *et al.*, 2018b). As such, the differences in seagrass resilience appear to occur at genotypic or phenotypic levels (Ehlers *et al.*, 2008; Maxwell *et al.*, 2014; Salo *et al.*, 2015; Connolly *et al.*, 2018a). In seagrass ecosystems, alternative states generally include comparatively less productive unvegetated, or algae-dominated mudflats (Kendrick *et al.*, 2002; Unsworth *et al.*, 2015) and degradation may become recalcitrant due to the loss of seagrass feedback systems coupled with the loss of seagrass recruits (i.e., seedbanks, propagule, fragments) (Kilminster *et al.*, 2015; O'Brien *et al.*, 2018).

The empirical assessment of seagrass resistance and recovery to disturbances relies on measurements of changes in response to numerous morphometric, biochemical and physiological variables. Yet, the wide variety of methods used to measure many of these variables are often not directly comparable, reducing the ability of researchers to combine and compare data across studies (e.g., Quentin *et al.*, 2015).

Furthermore, the large number of variables used to measure the health of seagrass may also hinder the production of robust estimates of seagrass ecosystem resilience at large scales (Marbà *et al.*, 2013). Morphometric parameters are most commonly used to detect disturbance responses in seagrasses and include rhizome elongation, total biomass, seagrass shoot density, and new leaf production (Duarte *et al.*, 1997; Cruz-Palacios & Van Tussenbroek, 2005; Craig *et al.*, 2008; Marbà *et al.*, 2013; McMahon *et al.*, 2013). However, morphometric measures are not always sensitive enough to detect early responses; instead, biochemical indicators may provide a better measure of seagrass resilience (Govers *et al.*, 2015; Roca *et al.*, 2015b).

In a meta-analysis including several seagrass species, McMahon *et al.* (2013) demonstrated that rhizome sucrose levels, shoot carbon to nitrogen ratios (C:N), leaf growth, and the number of leaves per shoot, were the most robust indicators of light stress at the individual level. At a meadow scale, however, shoot density and above-ground biomass were the most robust bioindicators (McMahon *et al.*, 2013). The development and implementation of relevant bioindicators are necessary to identify seagrass habitat resilience across species, bioregions and hence, enable improved management of seagrasses.

1.3.4 Research scope

In New Zealand, there is a shortage of empirical knowledge about how *Z. muelleri* plants and meadows respond to environmental changes, particularly sedimentation, despite sedimentation being recognised as a major environmental stressor to New Zealand coastal environments. In an attempt to address these knowledge gaps, this thesis examines the spatial variation in the resilience of *Z. muelleri* to chronic and acute sedimentation that occurs *in situ*, at the meadow and plant scale. The outcomes of both observational and manipulative studies provide outcomes to develop a robust method of measuring seagrass resilience.

1.4 Thesis overview and objectives

In the previous sections, I have outlined how seagrasses provide many essential ecosystem services. However, they have experienced both global (Orth *et al.*, 2006; Waycott *et al.*, 2009) and local (Park, 1999; Inglis, 2003) declines due to increasing pressures linked to expanding anthropogenic effects. Rhizome sucrose content is one of the most robust bioindicators of sublethal change at a plant level (McMahon *et al.*, 2013). Sucrose is an NSC reserve used by plants to subsidise metabolic

demands when photosynthetic outputs are unable to meet demand, thus providing resilience to seagrass (Drew, 1978b; Burke *et al.*, 1996; Alcoverro *et al.*, 1999; Alcoverro *et al.*, 2001; Brun *et al.*, 2003). Any impact that affects seagrass photosynthetic and respiratory activity can, therefore, be measured as a change in NSC reserves. As such, it appears that measures of NSC reserves may provide a robust indication of sublethal changes. However, different analytical methods for measuring NSC are currently used and each may produce significantly different results (Quentin *et al.*, 2015). To ensure accurate and comparable NSC estimates the methods need to be standardised. Therefore, Chapter 2 consists of a literature review of the existing analytical methods for seagrass NSC quantitation, followed by an experimental comparison of the most commonly used methods. Based on the experimental work in Chapter 2, a standard protocol is presented as a shared tool to enhance global research efforts, especially concerning how seagrasses respond to environmental stressors such as sedimentation.

The effects of sediment smothering, or burial, on *Z. muelleri*, have not been explored in a New Zealand context. Yet, the species is at risk and sedimentation is an acknowledged major stressor (e.g., Thrush *et al.*, 2004; Young *et al.*, 2011). Chapter 3 investigates the effects of single and repeated burial events on *in situ Z. muelleri* meadows in New Zealand. As an initial step, the response of *Z. muelleri* to this stressor is measured along an environmental gradient. The new method developed in Chapter 2 is used to explore *Z. muelleri* capacity to adapt to sedimentation and burial events. Rhizome NSC reserves are measured to investigate their relationship with resilience along the environmental gradient. This chapter aims to provide an understanding of the spatial variability of seagrass resilience to burial to improve management of *Z. muelleri* in New Zealand.

Numerous feedback systems exist between seagrass and their environment (Folmer *et al.*, 2012; Maxwell *et al.*, 2017). Climate influences the synthesis and metabolism of NSCs, with previous studies suggesting that high latitude seagrasses store more substantial quantities of NSC compared to lower latitude seagrasses (Drew, 1978a; Soissons *et al.*, 2018b). *Zostera muelleri* is distributed along a broad latitudinal gradient including both temperate and tropical regions (see Figure 1.2) and to ensure the robustness of NSC as a bioindicator of resilience across latitudes, it is relevant to understand whether carbon reserves are comparable between different climatic regions. Chapter 4 therefore investigates and compares the content and

proportional allocation of NSC in *Z. muelleri* of temperate and tropical populations, for the first time in the literature. This research took place in Australia (Port Phillip Bay, Victoria, and Townsville, Queensland), to ensure that an appropriate latitudinal gradient was available. This thesis, therefore, presents a number of novel insights focussed on the detection of *Z. muelleri* resilience based upon carbohydrate stores. The main objectives of the thesis are summarised as follows:

- To review the analytical methods used for NSC quantitation in the published literature (*Chapter 2*);
- To compare experimentally appropriate analytical methods from the literature (*Chapter 2*);
- To develop a standard protocol for the quantitation of NSC in seagrasses (*Chapter 2*);
- To explore *Z. muelleri* resilience (i.e. resistance and recovery) to burial events within a New Zealand estuary (*Chapter 3*);
- To investigate the partitioning of NSC reserves of *Z. muelleri* at different spatial scales and (*Chapter 4*), and;
- To explore the relationships between NSC groups and environmental and morphological variables (*Chapter 4*).

Each of the research chapters (Chapters 2 to 4) are constructed as individual studies and are linked by this introductory chapter (Chapter 1) and the final synthesis of results and general conclusion (Chapter 5).

Chapter 2 has been published in *Aquatic Botany* vol 151 (2018) under the title: "A standard, analytical protocol for the quantitation of non-structural carbohydrates in seagrasses that permits inter-laboratory comparison" by Sørensen, S.T., Campbell, M. L., Duke, E., & Manley-Harris, M. (doi: 10.1016/j.aquabot.2018.08.006). I conceived the ideas and designed the methodology for this study with input from Campbell, M. L. and Manley-Harris, M. I ran the analytical experiments and collected the data with assistance from Duke, E. Finally, I analysed the data and wrote of the manuscript with critical contributions and final approval from Campbell, M. L., Duke, E., and Manley-Harris.

Chapter 3 has been prepared to be submitted for peer review to *New Phytologist* under the title "The spatial variability in the resilience of *Z. muelleri* to burial events in New Zealand" by Sørensen, S.T., Manley-Harris, M,, Sherman, C.D.H., Miller,

S. & Campbell, M. L. Again, I conceived the ideas and designed the methodology for this study with input from Campbell, M. L. and Manley-Harris, M. I organised and executed the *in situ* experiments and performed the sample analysis. I analysed the data and wrote the manuscript with critical contributions and final approval from Campbell, M. L., Miller, S., Sherman, C.D.H. and Manley-Harris, M.

Chapter 4 has been prepared to be submitted for peer review to *Global Ecology and Conservation*, under the title "A tentative study of non-structural carbohydrate partitioning of *Zostera muelleri* at different spatial scales: Exploring the relationships between environmental and morphometric variables and carbohydrate partitioning" by Sørensen, S.T., Manley-Harris, M., Sherman, C., Long, B. & Campbell, M. L. The ideas and design of the methodology for this study were mine, with input from Campbell, M. L. I organised and executed sample collections and performed sample analysis with assistance from Long, B. I analysed the data and wrote the manuscript with critical contributions and final approval from Campbell, M. L., Sherman, C.D.H., Long, B. and Manley-Harris, M.

Chapter 2

A standard, analytical protocol for the quantitation of non-structural carbohydrates in seagrasses that permits inter-laboratory comparison

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Intertidal *Zostera muelleri* during tidal emersion. Photo: Sørensen, S.T.

2.1 Introduction

Clear definitions of seagrass ecosystem health status and consistent global monitoring programs that enable comparison across natural and restored ecosystems are required for successful conservation and restoration of seagrasses (Duarte, 2002; Orth *et al.*, 2006; Duarte *et al.*, 2015). Currently, various seagrass indicators (e.g., 49 different indicators in 42 European monitoring programs) are reported in the literature, which complicates the determination of overall seagrass health (Marbà *et al.*, 2013). There are at least 56 published indicators used to measure seagrass responses to reduced light (McMahon *et al*., 2013). Of these, 21 are robust in the detection of light reduction. Among the robust indicators are rhizome sugar (soluble non-structural carbohydrates), which is noted as one of the most effective bio-indicators of early detection of sublethal plant-scale changes (McMahon *et al.*, 2013).

Plant NSC reserves provide resilience as these are metabolised during times of photosynthetic reduction/inhibition, and thereby act as important buffers against disturbances (Maguire & Kobe, 2015). Consistently, reductions of seagrass NSC content have been reported in response to environmental stress (e.g., Alcoverro *et al.*, 1999; Brun *et al.*, 2002; Cabaço & Santos, 2007; Brun *et al.*, 2008; Salo *et al.*, 2015). Furthermore, seagrasses depend on sufficient carbon reserves to ensure winter survival (Burke *et al.*, 1996; Vermaat & Verhagen, 1996; Alcoverro *et al.*, 2001; Touchette & Burkholder, 2002a; Govers *et al.*, 2015; Soissons *et al.*, 2018b). Monitoring of NSC in seagrasses may therefore provide an early indication of declining health and aid the prediction of resilience in natural and restored seagrass ecosystems.

This study provides background on the biochemistry of NSC, then presents a review of the analytical methodologies used for NSC quantitation that are published in the seagrass literature. The review highlights the lack of a standard protocol for seagrass NSC quantitation, despite the apparent robustness of carbon reserves as an important bio-indicator. The second part of the study experimentally compares appropriate analytical methods from the seagrass literature. Specifically, the accuracy and precision of various analytical assays were tested and soluble NSC and starch yields following different sample preparation procedures were compared.

We discuss the implications of using different analytical methods and finally present a 5-step standard protocol for the quantitation of NSC in seagrasses.

2.1.1 Non-structural carbohydrates (NSC)

Non-structural carbohydrates generally refer to mono- or small oligosaccharides (e.g., sucrose, glucose and fructose; collectively termed sugars) and starches. Sugars are readily water-soluble and are therefore termed soluble NSC. Starches usually exist in semi-crystalline form and are insoluble in cold water (Zobel, 1984). Structural carbohydrates include components of the plant cell wall such as cellulose and hemicelluloses.

Starch is a complex polysaccharide made up of D-glucopyranose moieties joined by α-(1-4) linkages (amylose) and branch points of α-(1-6) linkages (amylopectin) and is the most common form of energy storage in plants (Smith & Zeeman, 2006). When the plant metabolic budget is not met by the rate of photosynthesis, specific enzymes breakdown the non-reducing ends of stored polymer chains, liberating glucose monomers as substrates for plant respiration (Zeeman *et al.*, 2010). Branched amylopectins have more non-reducing ends for enzyme action compared to linear amylose and thus, amylopectins allow for rapid release of glucose. The amylose/amylopectin ratio of starch granules varies and amylose content generally ranges from 15% to 30% (Zobel, 1984).

Starch or other polysaccharides such as inulin are the most common form of energy storage in plants, however, seagrasses tend to store the majority of their carbohydrates in the form of the disaccharide sucrose (Touchette & Burkholder, 2000). Sucrose is a non-reducing disaccharide containing α-D-glucopyranose and β-D-fructofuranose moieties linked by their anomeric carbons (Berg *et al.*, 2002). The smaller molecular size, and hence solubility of sucrose compared to starch, makes it easier to transport over long distances (Lemoine, 2000), and a more efficient organic osmolyte than starch. Seagrass is found to accumulate sucrose to attain osmotic adjustments in response to salinity changes (Touchette, 2007) and storing sucrose appears to be one of the few important evolutionary adaptations that have enabled seagrasses to inhabit dynamic coastal environments (Olsen *et al.*, 2016).

Sucrose is the main transport form of photo-assimilated carbon from the photosynthetic tissues to the plant sink organs, that are unable to photosynthesize (Lemoine, 2000). In the sink organs, sucrose can be used as a substrate for both structural and non-structural carbohydrate synthesis. An average of 45% of total NSC is stored in seagrass rhizomes (average across species) (Touchette & Burkholder, 2000).

Olsen *et al.* (2016) sequenced the genome of *Zostera marina* and demonstrated that genes coding for starch synthase and transport are reduced whilst those coding for sucrose synthase and transport are greatly expanded when compared to the aquatic monocot duckweed (*Spirodela polyrhiza*), from which *Zosteraceae* diverged between 135 to 107 million years ago.

2.1.2 Review of methods used for seagrass NSC quantitation

The methods used for quantitation of NSC in seagrasses were identified through a search of the Web of Science literature database (May 2018), using two sets of keyword searches. The first set consisted of "seagrass" and "eelgrass", whilst the second set consisted of "non-structural carbohydrates", "carbohydrates" and "sugars". Studies that undertook NSC quantitation and contained an adequate amount of detail pertaining to these methods (79 of 227 studies) were compiled and examined to determine the common analytical procedures in use (Appendix A; Tables A.1 to A.3).

The reported estimates of NSC are highly variable in the existing literature. For example, reported mean soluble NSC content of *Z. marina* rhizomes range from 9 to 32 mg g^{-1} dry weight (DW) in one study (Touchette and Burkholder, 2002) and from 200 to 500 mg g^{-1} DW in another (Eriander, 2017) (Table A.1). Similarly, reported mean starch content in *Z. noltii* range from 2 to 16 mg g^{-1} DW rhizome in one study (Brun *et al.*, 2003) and from 200 to 620 mg g^{-1} DW in another study (Cabaço & Santos, 2007) (Table A.2). A range of assays is described in the literature, however, the vast majority of studies use colorimetric assays to quantitate soluble NSC and starch (Table 2.1). The colorimetric assays used include the anthrone assay (Yemm & Willis, 1954), the phenol-sulfuric acid assay (Dubois *et al.*, 1956), the resorcinol assay (Huber & Israel, 1982), and the 3-Methyl-2-benzothiazolinonehydrazone (MBTH) assay (Pakulski & Benner, 1992). An

integral part of colorimetric assays is the rapid addition of a strong acid (30% hydrochloric acid (HCl) or concentrated sulfuric acid (H_2SO_4)) to samples dissolved in water, thereby generating heat and low pH. This results in total hydrolysis of any poly- and oligosaccharides to monosaccharides.

Table 2.1. Frequencies of reporting of the analytical assays (anthrone, phenol-sulfuric, resorcinol, 3-methyl-2-benzothiazolinonehydrazone (MBTH), high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GC)) used for quantitative estimation of non-structural carbohydrates appearing in the existing literature (79 studies, Appendix A, Tables A.1 - A.3).

	Frequency (%)	
Assays	**Soluble NSC**	**Starch**
Colorimetric	**85.5**	**98.2**
Resorcinol	30.9	-
Anthrone	25.5	76.8
Phenol-sulfuric	25.5	14.3
MBTH	1.8	3.6
Enzymatic	1.8	3.6
Chromatographic	**14.5**	**1.8**
HPLC	7.3	1.8
GC	7.2	-
Total	**100.0**	**100.0**

The acid further converts pentoses (5-carbon sugars) to furaldehyde, and hexoses (6-carbon compounds) to 5-hydroxymethylfurfuraldehyde (HMF), which combine with a reagent (e.g., anthrone, phenol, or resorcinol) to produce a color that can be detected by ultraviolet/visible spectrophotometry at λ-max (Shallenberger & Mattick, 1983; Brummer & Cui, 2005; Nielsen, 2010). The assay specific λ-max values suggested in the original referenced methods were 490 nm for phenol (Dubois *et al.*, 1956), 630 nm for anthrone (Yemm & Willis, 1954), 520 nm for resorcinol (Huber & Israel, 1982) and 635 nm for MBTH (Pakulski & Benner, 1992). The absorbance produced is linearly correlated to the composition of the sugar content in the sample matrix. Sugars are completely consumed as part of the colorimetric assays and hence, the identification of the individual carbohydrates in

samples is not possible. Instead, the dominant sugar of a sample matrix is used to create standard curves for calibration (Nielsen, 2010).

Fewer of the identified studies (Table 2.1) used chromatographic assays (gas-liquid chromatography (GC) and high-performance liquid chromatography (HPLC)) for the quantitation of soluble NSC, and only one study used HPLC to quantitate starch as glucose equivalents following enzymatic digestion of starch (Vermaat & Verhagen, 1996). HPLC is generally preferred over GC for carbohydrate analyses because GC requires a sample to be volatile, thus non-volatile sugars require derivatisation prior to GC analysis. Both instruments allow for the identification and quantitation of individual carbohydrates in a sample matrix using standard calibration curves. Furthermore, HPLC and GC allow for the quantitation of sugar alcohols (e.g., inositol, sorbitol and mannitol) and other organic compounds (e.g., soluble proteins and free amino acids). Chromatographic assays are more expensive than colorimetric assays since they require access to analytical instrumentation; nevertheless, HPLC instrumentation is commonly found in chemistry laboratories in research institutions.

The methodological variability identified in the literature existed not only because of the specific assays used for quantitation but also because of sample processing procedures (Table 2.2). The first analytical procedure commonly described, includes the exhaustive extraction of soluble NSC from the examined seagrass tissue. The majority of the studies (~89%) used a hot water/ethanol mixture to extract the soluble NSC fraction (Table 2.2). Ethanol is commonly used for this purpose as it lowers the polarity of water (Macedo, 2005; Galvão *et al.*, 2016), making the starch granules less likely to solubilise during the heating process.

Table 2.2. Frequency (%) at which solvents have been used to extract soluble non-structural carbohydrates and for solubilising starch (Hydrochloric acid (HCl), potassium hydroxide (KOH), sodium hydroxide (NaOH), perchloric acid (HClO4), amyloglucosidase and ***α***-amylase (enzymatic), ethanol/water (*v*/v), methanol/water (*v*/v), water and anthrone) from seagrass tissue, as published in the existing literature (79 studies, Appendix A Tables A.1-A.3).

	Concentration	**Duration**	**Extractions**	**Frequency (%)**
Soluble NSC extraction				
Ethanol/water (*v/v*)	70 – 96%	5 - 20 min	1-5	89.0
Water (*v/v*)	100%			5.5
HCl	0.2 M	3 - 22 h	1	4.1
Methanol/water (*v*/v)	90%	15 min	3	1.4
Total				**100.0**
Starch solubilisation				
KOH	0.1 – 1 M	12 - 24 h		34.4
NaOH	0.1 – 1 M	12 - 48 h		26.2
$HClO_4$	30 – 70%	15 - 20 min		16.4
HCl	0.1 – 2 M	15 min - 22 h		9.8
Enzymatic				9.8
Anthrone Assay (Conc. H_2SO_4)				1.6
Total				**100.0**

Other solvents used to extract soluble NSC in the literature included hot water, methanol and hydrochloric acid (HCl) (Table 2.2). Despite the prevailing use of the binary liquid mixture of ethanol/water as the soluble NSC extraction solvent, large inconsistencies existed between studies including; ethanol concentration (ranging from 70% to 96% volume/volume (*v/v*)), duration of extraction (5 to 20 min), temperature (30°C to 80°C) and the number of sequential extractions (one to five). Sugar solubility varies with solvent, duration, temperature and pressure (Macedo, 2005; Montañés *et al.*, 2007). Galvão *et al.* (2016) demonstrated that temperature influences sucrose solubility more than the dielectric constant of the solvent; however, for each temperature the solubility was directly proportional to the dielectric constant of the solvent. It is therefore highly likely that this variability in soluble NSC extraction procedures influenced the final yields measured.

The next analytical step in NSC quantitation involves solubilising starch from the solid fraction after extraction of soluble NSC. The process of solubilising starch is also termed gelatinisation and involves the disruption of the semi-crystalline granule structure which causes swelling, hydration and solubility of starch (Zobel, 1984). The solvents used to solubilise starch from seagrass included potassium hydroxide (KOH), sodium hydroxide (NaOH), hydrochloric acid (HCl) and perchloric acid ($HClO_4$) (Table 2.2). Alkaline solvents (NaOH and KOH) were consistently used at room temperatures, however, solvent concentrations ranged from 0.1M to 1M and treatment durations from 12 h to 48 h. Acid modifications (HCl and $HClO_4$) of starch were most commonly conducted under heating (80°C to 100°C), but at inconsistent concentrations (0.1 M to 12 M). Most studies performed short acid modifications (15 min to 45 min), yet, a few studies used mild acid dilutions (0.2 M) for periods of 20 h to 22 h (Lee & Dunton, 1996; Lee & Dunton, 1997; Campbell & Fourqurean, 2013).

Enzymatic digestions (by amyloglucosidase and/or α-amylase) of starches are less commonly used (9.84% of studies; Table 2.2). The analytical processes involved in enzymatic digestions are relatively complex and are affected by a range of factors such as starch branching structure, molecular size and weight distribution as well as sample preparation methods (Dona *et al.*, 2010). Enzyme catalysed starch hydrolyses are typically used to identify starch structural composition in plant tissue

(i.e. amylopectin-amylose ratio), although these techniques have been restricted to starch quantitation in the seagrass literature.

It is hypothesised that the analytical inconsistencies identified in the seagrass literature have had significant effects on NSC estimations. Thus, this study aims to test and compare:

1. **The precision and accuracy of sugar recoveries by colorimetric assay and HPLC assay;**
2. **Methods for soluble NSC extractions; and**
3. **Methods for starch solubility and hydrolysis.**

We discuss the implications of the high variability of analytical methods used for NSC quantitation in the seagrass literature based on our results and finally present an optimised standard protocol for the accurate and efficient quantitation of NSC from seagrasses.

2.2 Materials and Methods

2.2.1 Plant samples

Zostera muelleri sprigs were collected during low tide from an intertidal seagrass meadow in Tauranga Harbour, New Zealand (latitude 37°67'S, longitude 176°16'E) in August 2017 (austral winter). Seagrasses here existed in continuous meadows in fine sand substrates. Apical meristems including four to six shoots were gently dislodged from the sediment, cut from the main plant, and collected. Collected sprigs were placed into a dark, cold container for transport to the laboratory. Upon return to the laboratory, the roots and blades were removed from the sprigs leaving only rhizomes. The samples were then rinsed with deionised water to remove epibionts and salts, as these may have an unforeseen effect on the solubility of sugars (Macedo, 2005). Rhizome samples were freeze-dried for five hours to obtain constant weight (water content 86.50% ± SE 0.36), homogenised (<1 mm) and pooled before being frozen (<-20°C) until further analysis. Triplicate subsamples of the homogenised stock matrix were used to test each method.

2.2.2 Colorimetric assay

Determinations of fructose, glucose and sucrose were performed using the phenol-sulfuric method modified from DuBois *et al.* (1956) as a proxy for all colorimetric

assays. All colorimetric assays are very sensitive to operational discrepancies, however, the phenol-sulfuric assay was selected over other colorimetric assays because it may be less sensitive to traces of residual solvent that may compromise the validity of other tests (Dubois *et al.*, 1956). Furthermore, the phenol-sulfuric acid assay is an inexpensive alternative that produces permanent colour change compared to the anthrone and resorcinol assays.

A subsample (1 mL) of a standard solution with known concentrations of sucrose (0.256 mg mL^{-1}), glucose (0.248 mg mL^{-1}),) and fructose (0.246 mg mL^{-1}) (99% standards from Sigma Aldrich in deionized water) was mixed with aqueous phenol (1 mL, 5% *v/v*) in a glass test tube under a fume hood. H_2SO_4 (95-97%, 5 mL) was added to the test tube in a steady, rapid stream after which the sample was mixed (2 min) using a vortex mixer (VELP Scientifica). An aliquot of the final sample was placed in a plastic cuvette (Greiner Bio-One) and absorbance read at 490 nm (Thermo Scientific; Genesis 10s UV-vis). The blank used was water that had been subjected to the assay. Soluble NSC content was calculated as sucrose equivalent using sucrose calibration curves (Standard sucrose 99%, from Sigma Aldrich). This process was replicated 10 times (using subsamples from the same standard solution) to investigate the precision and accuracy of the assay.

2.2.3 HPLC assay

High-performance liquid chromatography (HPLC) was carried out using a Waters 515 pump, a Waters 414 refractive index detector (RID), a column oven, a Rheodyne 7725i injector fitted with a 20-µL loop and an Alltech Elite degassing system. Separation was achieved with a Shodex SUGAR KS-801 column eluted isocratically with water (0.4 mL/min, 50°C). The system was controlled using Waters Empower™ 2 Chromatography software. In this system, sucrose eluted at 16.10 min (n = 36, SE ± 0.03 min), glucose at 19.40 min (n = 36, SE ± 0.02 min) and fructose at 21.60 min (n = 36, SE ± 0.03) (Fig. 2.1). The contents of sucrose, glucose and fructose were calculated using calibration curves derived from standard sugars (Sigma Aldrich). Ten subsamples of a standard solution with known concentrations of sucrose (0.256 mg mL^{-1}), glucose (0.248 mg mL^{-1}) and fructose (0.246 mg mL^{-1}) (99% standards from Sigma Aldrich in deionised water) were assayed in order to investigate precision and accuracy of assay and to compare with the phenol-sulfuric assay.

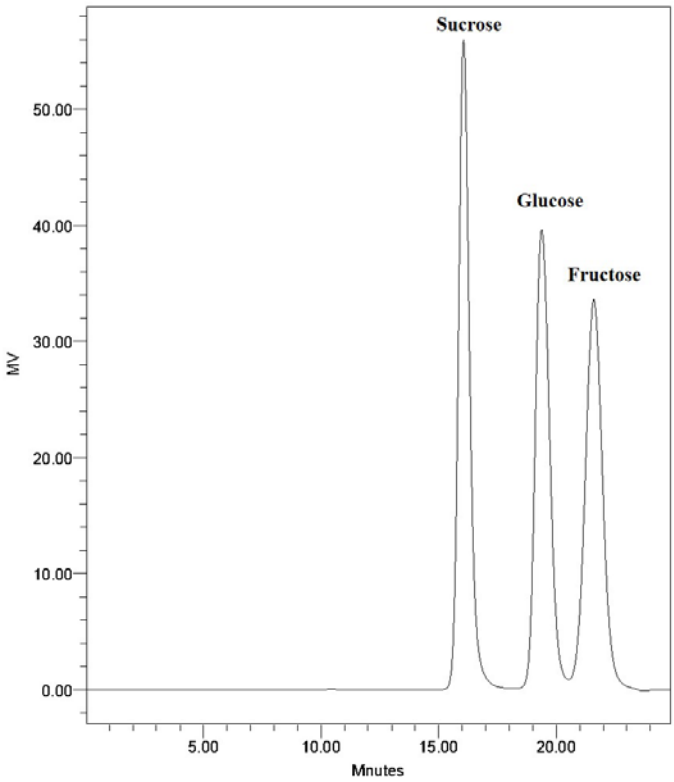

Figure 2.1. High-performance liquid performance (HPLC) analysis of a standard mixture containing pure; sucrose (retention time 16.1 min), glucose (retention time 19.4 min) and fructose (retention time 21.6 min). Sugars were separated by Shodex SUGAR KS-801 column, eluded isocratically with deionised water at 0.4 mL min-1 and detected by refractive index detector (RID)

2.2.4 Extraction of soluble NSC

Subsamples of homogenised rhizomes (50 mg of rhizome fractions < 1 mm in length) were suspended in solvent (5 mL - ethanol/water (80% *v/v*); methanol/water (80% *v/v*); and water (100%)) and soluble NSC were extracted by stirring (80°C, 15 min). Samples were centrifuged (4400 rpm, 10 min); the supernatant was filtered (0.45 µm, Whatman). Binary liquids were evaporated to dryness under nitrogen (N_2) gas at room temperature after which the extracted residue was rehydrated with water for quantitative assay. Solid residues were extracted the requisite number of times (one to five times in additional aliquots of 5 mL of solvent) and the supernatants combined. Extracts were stored (4°C) until quantitation, which occurred within 2 days.

2.2.5 Starch solubility

Semi-crystalline starch must be solubilised for quantitation to occur. Two solvents were compared for this purpose, the base NaOH (1M) and the acid HCl (1M). The solid residue after extraction of soluble NSC was rinsed with deionised water and

the residual solvent evaporated under a stream of N_2 gas at room temperature. The alkaline treatments occurred by placing the solid residue in 5 mL NaOH 1M (~ 10 mg mL^{-1}) at room temperature for three different durations: 18 h; 24 h; and 48 h. The acid treatments were effected by heating with stirring (100°C) the solid residue in 5 mL HCl 1M (~ 10 mg mL^{-1}) for 15 min, 30 min, and 45 min durations. Samples were then centrifuged (4400 rpm, 10 min) and the supernatant filtered (0.45 µm, Whatman) in order to remove solid residue and thus avoid hemicelluloses being cleaved during the ensuing hydrolysis treatment. Finally, the treatments were terminated by neutralising the pH with an equal volume of HCl 1M/NaOH 1M and adjusting as required using a pH-meter.

2.2.6 Starch hydrolysis

Starch molecules are excluded by the KS-801 SUGAR column (exclusion limit 1,000 or ~ 7 glucopyranosyl moieties) because of their size and must, therefore, be cleaved to free glucose monomers for quantitation by HPLC. Acid hydrolysis of starch was conducted with HCl (1M) under stirring and heating (100°C). Solubilised starch (~10 mg^{-1} mL) was suspended in HCl (1M) and the hydrolysis was terminated by neutralising with equimolar amounts of NaOH. Complete recovery of starch was confirmed using a pure soluble starch standard (Sigma Aldrich).

2.2.7 Calibration curves

Triplicate standard solutions were made with pure sucrose (99+%), D-(-)-fructose (99+%), D-glucose (99+%) solubilised in deionised water. Six solutions of known concentrations ranging from 0.001 mg mL^{-1} for each analyte to 1.000 mg mL^{-1} were analysed both by HPLC and the phenol-sulfuric assay. The peak area, or absorbance at λ-max (490 nm), for each of the sugars, were plotted against known concentrations in separate scatterplots for each of the quantitation methods, to create a series of external standard curves. The drift of the HPLC equipment was tested by analysing a standard solution every 10 runs.

The concentration of a particular carbohydrate was calculated using Equation 2.1. The total mass of sugar in the sample was calculated by multiplying the concentration by the total volume of the given sample.

Equation 2.1:

Carbohydrate concentration = $\frac{\textbf{Peak Area or Absorbance at } \boldsymbol{\lambda}\textbf{−max (490 } \boldsymbol{nm}\textbf{)}}{\textbf{Slope of external standard curve}}$ mg mL^{-1}

2.2.8 Carbohydrate conversions

When monomers are cleaved from an oligo- or polysaccharide during acid hydrolysis, a water molecule (18 amu) is added to each liberated anhydrous monomer (162 amu). The expected glucose mass is defined by Equation 2.2, in which 180 g mol^{-1} is the molecular mass of a glucose monomer and 162 g mol^{-1} is the molecular mass of an anhydroglucose moiety in starch:

Equation 2.2:

Glucose mass = Original starch mass x $\frac{\textbf{180 g mol}^{\textbf{-1}}}{\textbf{162 g mol}^{\textbf{-1}}}$

Thus, 1.00 g of starch upon complete hydrolysis will yield 1.11 g of glucose monomer. This calculation assumes that the chain length is such that the mass contribution from hydrogen (H) and hydroxide (OH) at reducing and non-reducing ends is negligible. When calculating theoretical soluble sugar recoveries from the phenol-sulfuric assay, the sucrose estimations were adjusted using Equation 2.3, to account for the molecular mass of a sucrose disaccharide. After application of this equation complete recovery was signified by 1000 mg g^{-1} original sugar content for both phenol-sulfuric and HPLC assays.

Equation 2.3:

Monosaccharide mass = Original sucrose mass x $\frac{\mathbf{360\ g\ mol^{-1}}}{\mathbf{342\ g\ mol^{-1}}}$

2.2.9 Statistics

The data were tested for normality in histograms and homogeneity of variance using Levene's tests. Significant results were identified at $P < 0.05$. Parametric data were analysed using paired t-tests (two groups), one-way ANOVA tests (multiple groups) and Tukey HSD post-hoc tests. Non-parametric data was analysed using Mann-Whitney U-tests (two groups), Kruskal-Wallis ANOVA (multiple groups) and multiple comparisons of mean ranks post-hoc tests. Statistical tests were performed in Statistica (Version 13) and data was reported as means ± standard errors (SE).

2.3 Results

2.3.1 Phenol-sulfuric colorimetric assay compared to HPLC assay

Ten sub-samples of a standard mix containing sucrose (0.26 mg mL^{-1}), glucose (0.25 mg mL^{-1}) and fructose (0.25 mg mL^{-1}) were analysed with both HPLC and the phenol-sulfuric assay. The mean yield of detected NSC using the phenol-sulfuric assay (1157.93 ± SE 46.56 mg g^{-1} NSC) was significantly higher ($U_{[10]}$ = 20.00, P = 0.03) and had a larger standard error than that of the HPLC assay (1010.99 ± SE 2.81 mg g^{-1} NSC). Thus, the mean of the detected soluble NSC content fell within ± 1.10% of the predicted result when using the HPLC assay, whereas, the phenol-sulfuric method yielded a mean that differed by 15.79% from the predicted outcome (Table 2.3). The two methods yielded estimates with highly significant heterogeneity of variance ($F_{[1,19]}$ = 101.01, $P < 0.001$) and the percentage coefficient of variance (CV%) was 12.72% for the phenol-sulfuric assay and 0.88% for the HPLC assay indicating greater precision in the HPLC assay (Table 2.3). These results clearly highlight the HPLC assay as the most precise and accurate of the two assays.

2.3.2 Extraction of soluble NSC

The number of sequential extractions used to exhaust soluble sugars from seagrass tissues has varied from one to five in the literature. Thus, we tested the number of sequential extractions needed to exhaust soluble NSC from rhizome tissue. After three sequential extractions, the mean cumulative percentage of NSC extracted was

99.93% (95% CI ± 0.10) for all three solvents tested (ethanol (80% *v/v*), methanol (80%) and water (100%) (Fig. 2.2). Thus, three sequential extractions are required for complete exhaustion of soluble NSC.

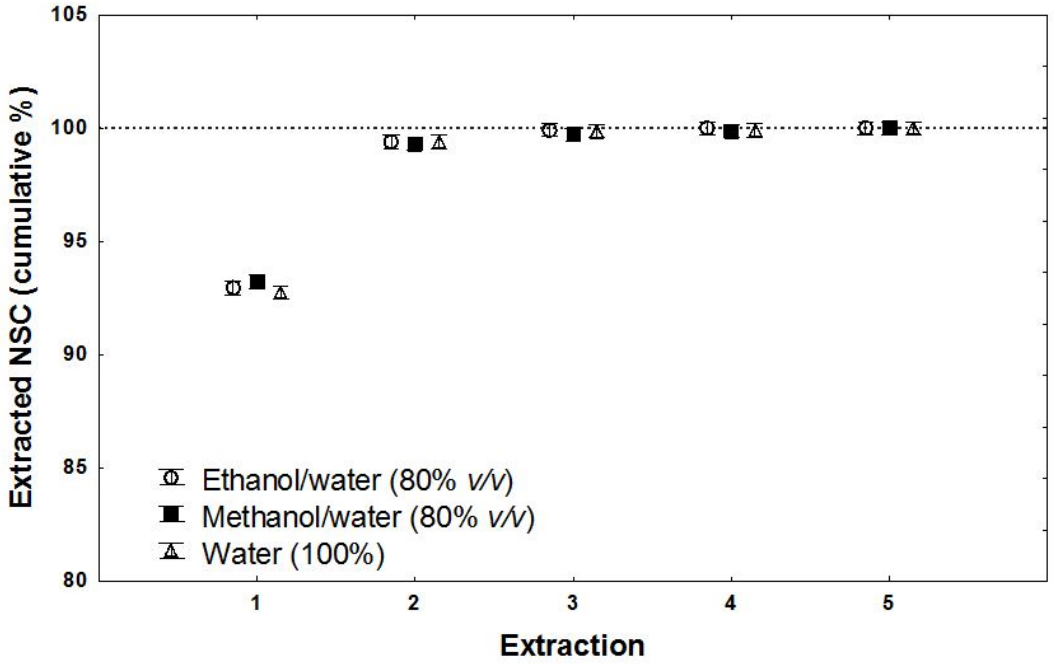

Figure 2.2. Cumulative percentage of total non-structural carbohydrates (NSC) extracted from *Zostera muelleri* rhizomes after five sequential extractions using three solvents; ○ ethanol/water (80% *v/v*), ■ methanol/water (80% *v/v*) and ∆ deionised water (100%). Error bars denote 95% confidence interval and dashed line indicates complete non-structural carbohydrates (NSC) exhaustion (100% extraction)

Table 2.3. Statistical summary of the measured content of total non-structural carbohydrates (NSC) in 10 subsamples of a standard solution by phenol-sulfuric- and high-performance liquid chromatography (HPLC) assays. Coefficient of determination (CV), Deviation of estimate from expected recovery (deviation). *Significant $P < 0.05$.

		Total sugar recovery (mg g^{-1} original NSC content)		**CV**	**Deviation**	**Mann-Whitney U-test**	
Assay	n	Mean	(±SE)	%	%	U-stats	P-value
Phenol-sulfuric	10	1157.93	(46.56)	12.72	15.79	20.00	***0.030**
HPLC	10	1010.99	(2.81)	0.88	1.10	-	-

The solvents used to extract soluble NSC had a significant effect on final yield ($F_{[2,6]}$ = 26.37, P = 0.001, Fig. 2.3). Water 100% resulted in a significantly higher mean soluble NSC content (192.79 ± 4.10 mg g^{-1} rhizome DW) than either ethanol/water 80% (*v*/v) (165.60 ± 2.13 mg g^{-1} rhizome DW, P = 0.02) and methanol/water 80% (*v*/v) (175.53 ± 0.47 mg g^{-1} rhizome DW, P = 0.009).

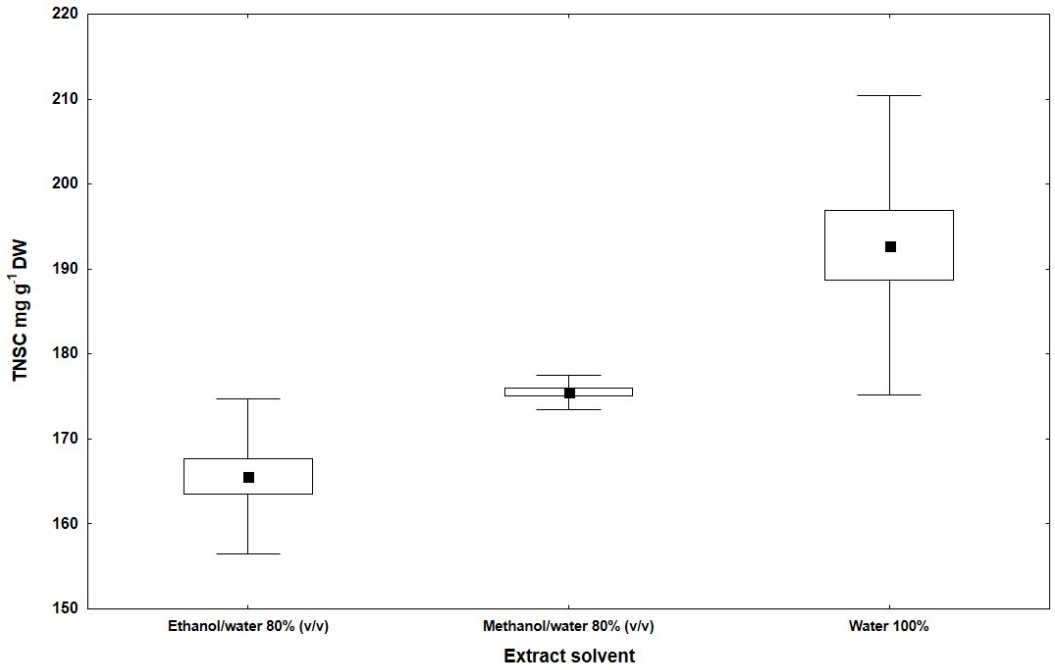

Figure 2.3. Mean total non-structural carbohydrate (TNSC) content (■) of triplicate subsamples of *Zostera muelleri* rhizome tissue, after soluble non-structural carbohydrates extraction by different solvents: Ethanol/water 80% (*v*/v), methanol/water 80% (*v*/v) and water 100%. Box represents standard error and whiskers 95% confidence intervals.

Table 2.4 displays the statistical summaries of each of the identified sugars and starch in triplicate rhizome samples. The solvents had a significant effect on the extracted sucrose and fructose content ($F_{[2,6]}$= 19.44, *P* = 0.002; $F_{[2.6]}$= 31.78, P < 0.001 respectively). Water was a more efficient solvent of sucrose (158.85 ± 2.55 mg g^{-1} rhizome DW) than either ethanol/water 80% (*v*/v) (142.46 ± 1.99 mg g^{-1} rhizome DW, P = 0.002), and methanol/water 80% (*v*/v) (148.09 ± 0.47 mg g^{-1} rhizome DW, P = 0.016). Similarly, water 100% also resulted in significantly higher fructose estimation (33.73 ± 1.57 mg g^{-1} rhizome DW) compared with ethanol/water 80% (*v*/v) (22.48 ± 0.72 mg g^{-1} rhizome DW, P = 0.002) and methanol/water 80% (*v*/v) (27.19 ± 0.12 mg g^{-1} rhizome DW, P = 0.009). The overall glucose content was very low compared to fructose and there was no significant difference (P = 0.60) in the mean content when extracting with the

different solvents (0.37 ± 0.18 mg g^{-1} rhizome DW). Starch was solubilised and hydrolysed as outlined in the final protocol (see section 5). Extracting soluble NSC by ethanol/water 80% (*v*/v), methanol/water 80% (*v*/v) and water 100% had no significant effect on final starch estimates ($F_{[2,6]} = 2.91$, $P > 0.05$, Table 2.4).

Table 2.4. Results of one-way ANOVA testing the statistical differences between measured non-structural carbohydrate content (soluble non-structural carbohydrates (soluble NSC), sucrose, glucose, fructose and starch) from *Zostera muelleri* when soluble NSC was extracted with three different solvents; ethanol/water (80% *v*/v), methanol/water (80% *v*/v) and water (100%). Significant $P < 0.05$ are denoted in **bold.** Groups with same letters (^{a,b}) are not significantly different from each other as a result of post-hoc testing (Tukey HDS)

	Soluble NSC extraction solvent treatment groups (mg g^{-1} rhizome DW)								
		Ethanol/water (a) (80% *v*/v)		**Methanol/water (b)** (80% *v*/v)		**Water (c)** (100%)		**One-way ANOVA**	
	N	Mean	(±SE)	Mean	(±SE)	Mean	(±SE)	df	F-stats	P-value
Soluble NSC	3	165.60	(2.13)^{a}	175.53	(0.47)^{a}	192.79	(4.10)^{b}	2	26.37	**0.001**
Sucrose	3	142.46	(1.99)^{a}	148.09	(0.47)^{a}	158.85	(2.55)^{b}	2	19.44	**0.002**
Glucose	3	0.65	(0.53)	0.25	(0.12)	0.21	(0.19)	2	0.55	0.602
Fructose	3	22.48	(0.72)^{a}	27.19	(0.12)^{b}	33.73	(1.57)^{c}	2	31.78	**0.001**
Starch	3	28.96	(3.12)	30.89	(0.26)	35.02	(0.33)	2	2.91	0.131

2.3.1 Starch solubility and hydrolysis

2.3.1.1 Starch solubility

Solvents and durations of starch solubility treatments are highly variable in the literature (Table 2.2). Starch solubility treatments by HCl commonly range from 15 min to 45 min in the literature, whereas NaOH treatments commonly range from 18 h to 48 h. We compared mean starch yields of triplicate homogenised rhizome subsamples when performed by HCl and NaOH solubility treatments (Table 2.5). The duration of the HCl treatment had significant effect ($F_{[2,6]} = 22.20$, $P = 0.002$) on the mean estimated starch content (means ranging from 28.45 ± 1.07 to 39.92 ± 1.42 mg g^{-1} rhizome DW). Similarly, the duration of the NaOH treatments also had significant effects ($H_{[2,9]} = 7.20$, $P = 0.027$) on starch estimates (means ranging from 3.75 ± 0.12 to 12.59 ± 0.57 mg g^{-1} rhizome DW). Generally, solubility treatments by HCl resulted in significantly higher starch yields ($t_{[16]} = 16.56$, $P < 0.0001$) than NaOH treatments. Overall, the most efficient solubility treatment (1M HCl for 30-min) yielded a mean starch estimate that was almost 10-fold greater than the least efficient treatment (1M NaOH for 18 h) (Table 2.5).

In addition to glucose, other unidentified compounds were apparent in the HPLC chromatograms of the starch hydrolysates. In order to identify glucose, a sample was spiked with a pure glucose standard (+99%) which increased the peak area of the compound eluted at 19.40 min. Additionally, a starch hydrolysate sample was derivatised (per-O-trimethylsilylated) and analyzed by gas chromatography-mass spectrometry (GC-MS), which confirmed the presence of other sugars (besides α- and β-glucopyranose). Amongst these were possibly fructose in α- and β- pyranose and furanose forms, and other aldohexopyranoses.

Table 2.5. Results of One-way ANOVA (F-stats) and Kruskal-Wallis test (H-stats), investigating the effect of treatment duration using hydrochloric acid at 100°C (HCl: a = 15 min, b = 30 min, and c = 45 min) and sodium hydroxide at room temperature (NaOH: a = 18 hours, b = 24 hours and c = 48 hours) to solubilise starch from *Zostera muelleri* rhizomes. Significant P-value < 0.05 denoted in **bold**. Groups with same letters (^{a,b}) are not significantly different from each other as a result of Tukey HDS post-hoc test.

		Treatment duration groups (starch mg g^{-1} rhizome DW)						**Statistical analysis**			
		a		**b**		**c**					
Solubilising agent	N	Mean	(±SE)	Mean	(±SE)	Mean	(±SE)	df	F-stats	H-stats	P-value
HCl	3	28.45	(1.07) ^{a}	39.92	(1.42) ^{b}	31.34	(1.29) ^{a}	2	22.20		0.002
NaOH	3	3.75	(0.12) ^{a}	5.03	(0.10) ^{ab}	12.59	(0.57) ^{b}	2		7.20	0.027

Starch hydrolysis

Hydrolysis is not an integral part of the HPLC assay, thus complete starch hydrolysis must occur in a separate step prior to quantitation of liberated glucose. In order to find the point of complete recovery, acid hydrolyses (HCl 1M) of triplicate pure soluble starch standards (starch concentration ~ 120 μg^{-1} mL) were conducted by heating (100°C) under stirring for durations from 1 min to 210 min (Fig. 2.4). After 180 min of hydrolysis, the 95% confidence interval of the mean glucose recovery (1154.13 ± 54.18 mg g^{-1} original starch content) encompassed the expected complete recovery, proving an efficient duration for hydrolysis.

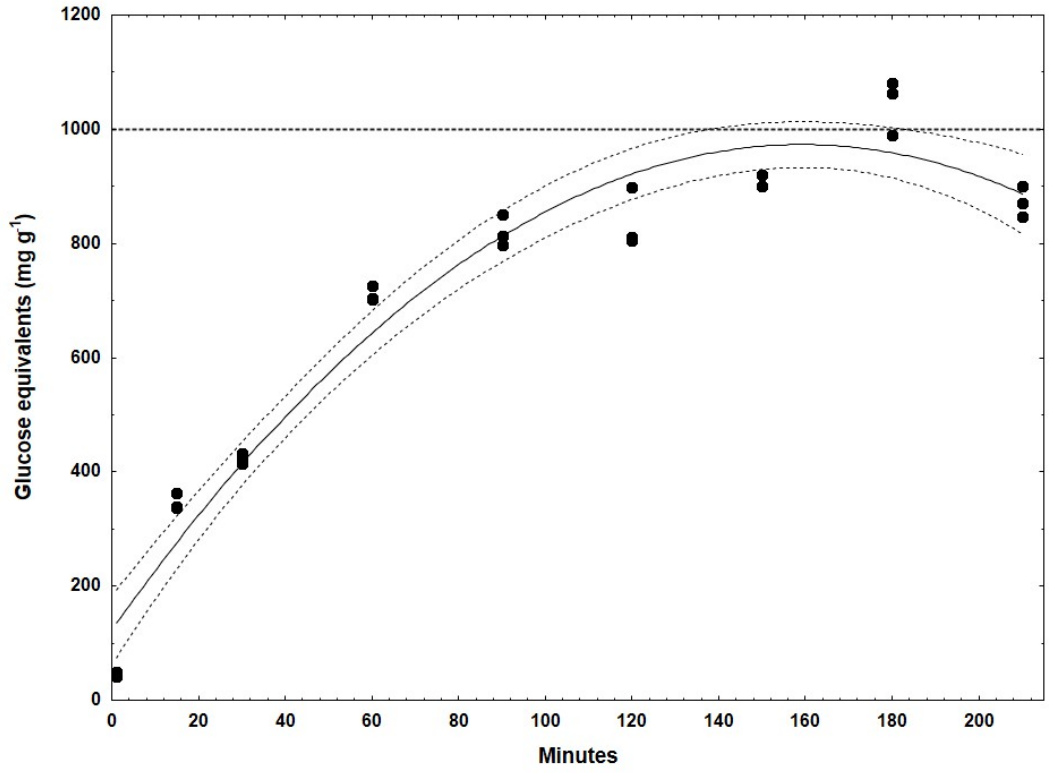

Figure 2.4. Glucose (mg g^{-1}) estimations (●) after hydrolysis treatment of starch standards (n=3) by hydrochloric acid (1M at 100°C) at durations of zero to 210 min. Standard curve with 95% confidence intervals (dashed lines) and reference line indicates complete recovery (1000 mg glucose g^{-1} original starch content).

2.4 Discussion

Non-structural carbohydrates can provide researchers with a quantitative measure of stress and recovery in seagrasses. However, we identified high variability of NSC quantitative methods utilised in the existing literature, and when select methods were tested experimentally the results were non-comparable. Similarly, a recent publication emphasised the non-comparability of NSC estimates from

woody plants when performed by different laboratories using different protocols (Quentin *et al*., 2015). Thus, different analytical procedures may result in non-comparable NSC estimations for a broad range of tissues. After examining the existing literature, we identified highly variable NSC estimations (see Appendix A Tables A.1 to A.3). This has implications for how we manage seagrasses in stress environments and under future climate change conditions. Seagrass was the focus of this study. However, the NSC analyses compared and contrasted may be of interest to other researchers working on different plants, and the recommended standard protocol may be adapted to suit sample material of other botanical origins.

2.4.1 Phenol-sulfuric colorimetric assay compared to HPLC assay

Seagrass researchers have routinely used colorimetric assays to measure NSC content (Table 2.1). However, this study indicates that the phenol-sulfuric assay is both less accurate and less precise than the HPLC assay (Table 2.3). Consequently, there may be under/over-estimation of carbohydrates in studies that have used phenol-sulfuric assays. Colorimetric assays are highly sensitive to interference, relatively non-selective, and require considerable practice by the operator to reduce the variation of the assay. Besides offering more accurate and precise estimates of NSC samples, HPLC assays are robust and easy to use (Dong, 2013). The development of HPLC also confers two additional advantages over colorimetric methods: 1) It enables quantitation of the individual sugars and other organic compounds (e.g., cytolitols; see Drew (1983)) so that trends in plant activity can be more clearly understood; and 2) it removes the possibility of other sugars, originating from other sources (e.g., from hemicellulose) interfering with the assay since these sugar types would be identified by their different retention times.

The peaks of unknown sugar compounds identified in chromatographs of starch hydrolysate may be monosaccharides derived from hemicelluloses (possibly fructan) solubilised during the starch gelatinising processes. Sucrose was not the origin of the unknown peaks as sucrose was exhaustively extracted prior to starch extraction. Starch hydrolysis procedures can destroy acid-labile fructose monomers, thus making it difficult to quantify these without further investigation. In contrast to colorimetric assays, quantitation of starch in HPLC chromatograms is based upon the integration of the glucose peak and therefore is independent of contribution

from hemicelluloses or fructans (unless there is a significant presence of, for example, glucomannan). Solubilised carbohydrates from structural polysaccharides are very likely to affect all colorimetric assays (including the anthrone, resorcinol, MBTH and phenol-sulfuric acid assays) leading to starch overestimation. The relatively higher levels of monosaccharide fructose compared to glucose in the NSC fraction imply the possibility of fructan as a storage carbohydrate; there is a single reference to this possibility in the literature (Pollard, 1982). Despite copious attempts in this study to use mild acid hydrolysis (acetic acid) to reveal the presence of fructan, we were unsuccessful.

Characterisation and quantitation of the hemicelluloses and possible fructans fell outside the scope of this study and were thus not pursued further. We note that HPLC assays are more expensive than colorimetric assays and require access to instrumentation, which may be a significant limitation to some studies. However, expenses associated with the purchase of HPLC instrumentation are decreasing due to the development of ultra-performance liquid chromatographs (UPLC) and consequent replacement of HPLC in many analytical laboratories.

2.4.2 Extraction of soluble NSC

In this study, we determined that 100% water extracted significantly higher yield of soluble NSC than the binary liquids ethanol/water 80% (*v*/v) and methanol 80% (*v*/v) (Table 2.4). Nevertheless, binary liquids of ethanol/water (70% to 96% *v*/v) have been the preferred solvents for soluble NSC in previous seagrass studies (Table 2.2). Galvão *et al*. (2016) found that as the mole-fraction of alcohol (methanol or ethanol) increased the mass fraction of dissolved sucrose decreased. Thus, the different ratios of ethanol to water previously used (Table 2.2) have undoubtedly affected the sugar solubility in the binary-liquid solvents. In turn, this has potentially affected the total sugar yields rendering comparison between studies ineffectual. Ethanol is commonly mixed with water when extracting soluble NSC (Table 2.2) to prevent the water-soluble fraction of starch being solubilised in the same process. However, we detected no significant difference in the starch content of the solid residue when extraction of soluble NSC had occurred by either water 100%, ethanol/water 80% (*v*/v), or methanol/water 80% (*v*/v) (Table 2.5).

Amylopectin, which generally constitutes the major fraction of starch, is water-soluble because of its branched nature, with the solubility increasing with the degree of branching (Zobel, 1984). The other starch component, amylose is a linear α-(1-4)-glucan, and is nearly water insoluble (Mukerjee & Robyt, 2010). Thus, the failure to extract the starch into hot water in this study may be a reflection on the degree of starch branching in *Z. muelleri*. We recommend comparing NSC yields after sugar extraction by water 100% and binary liquids for other seagrass species, as the degree of starch branching may be species-specific. However, in the case of *Z. muelleri*, results in Table 2.4 indicate that water should be used to extract the soluble NSC as it results in the highest yield of soluble NSC and did not affect starch estimates. Using water simplifies the analytical process, as it removes the requirement to evaporate the alcohol and re-dissolve in water prior to HPLC on the stationary phase used in this study.

In the existing literature, soluble NSC extractions have occurred in one to five sequential extractions (Table 2.2); however, we showed that three sequential extractions are necessary to ensure exhaustion of soluble NSC (Figure 2.2). If only one extraction is used, more than 7% of the total soluble NSC may remain in the plant tissue, thus, resulting in underestimation of total soluble NSC. Furthermore, any residual soluble NSC would compromise the quantitation of starch, especially in a colorimetric assay, which does not distinguish the different contributory sugars.

2.4.3 Starch solubility and hydrolysis

Historically, starch from seagrass has been solubilised using a range of solvents at various concentrations and treatment durations, and those most frequently used were alkaline solvents (Table 2.2). Our results show that both solvent and treatment durations have significant effects on the measured starch content and that starch contents by various methods are non-comparable, with starch yields ranging from 3.75 mg g^{-1} DW to 39.92 mg g^{-1} DW (Table 2.5). While alkaline solvents are used most frequently in the literature, we showed that the acid HCl was significantly more efficient in solubilising starch from rhizome samples (yields almost 10-fold greater). Only one study (Vermaat & Verhagen, 1996) has previously quantitated starch from seagrass by HPLC. Vermaat and Verhagen (1996) used enzymatic digestion; however, we demonstrated that a simple 180 min acid hydrolysis (HCl 1M), resulted in complete recovery of starch. Performing acid hydrolysis is

considerably simpler than enzymatic digestion; however, acid hydrolysis does not offer insight to starch structure (e.g., amylose to amylopectin ratios).

Highly variable levels of NSC are reported for seagrasses (intra- and inter-species) with soluble NSC estimates ranging from 5 to 550 mg g^{-1} DW rhizome (Table A.1) and starch estimates ranging from 2 to 620 mg g^{-1} DW rhizome (Table A.2). In the current study, *Z. muelleri* mean soluble NSC content was 192.79 mg g^{-1} DW rhizome and starch content 35.02 mg g^{-1} DW rhizome; however, we note that the samples were collected in winter. Carbohydrate reserves are affected by season (Pirc, 1989; Burke *et al.*, 1996), latitudinal gradient (Soissons *et al.*, 2018b) and other environmental variables such as nutrients (e.g., Touchette & Burkholder, 2007) and salinity (e.g., Sandoval-Gil *et al.*, 2012).

The natural variation in NSC contents coupled with the analytical inconsistencies that we highlight in this study makes it difficult for researchers to get an idea of expected baseline carbohydrate reserves. Internal mobilisation of carbon reserves may occur during short-term disturbances such as shading (e.g., Ruiz & Romero, 2001; Jiang *et al.*, 2013a) and burial (e.g., Cabaço & Santos, 2007; Munkes *et al.*, 2015) but without standardised measurements, seagrass resilience thresholds to such stressors remain unclear. The creation and adoption of a single methodology for the estimation of NSC in seagrasses will enable spatial and temporal comparisons of results as it facilitates standardised measurements. This will allow researchers to develop efficient tools to assess the impact of stressors upon seagrasses that may aid in the identification of resilience thresholds and enable a consistent approach to large-scale seagrass monitoring, and developing biodiversity offsets. This will improve seagrass ecosystem restoration and prediction of seagrass resilience to future disturbances.

2.5 Conclusions – a standardised protocol

In conclusion, we present the following optimised procedure for the quantitation of non-structural carbohydrates in seagrass. Use of this optimised 5-step method will improve the detection and reporting of carbohydrates within seagrasses. We recommend performing a preliminary test when first performed on species other than *Z. muelleri*: Use all three solvents listed in Methods (Section 2.4) on subsamples to extract soluble NSC as outlined in “Step 1”. Follow the protocol

systematically and finally compare soluble NSC and starch yields in the subsamples. If starch granules have a higher degree of branching in other species (compared to *Z. muelleri*), some starch may be solubilised in the first step, resulting in significantly different starch yields of subsamples. In this case, we recommend substituting water 100% with the most appropriate binary liquid to extract soluble NSC.

2.5.1 Sample treatment

1. Extract soluble sugars from freeze-dried tissue in deionised water (~50 mg in 5 mL) under stirring and heating (80°C) in three repeated extractions. Combine supernatants for soluble NSC quantitation (final rhizome concentration ~3.33 mg mL^{-1});
2. Rinse solid residue with deionised water remove excess water. Solubilise starch from solid residue by suspending in 5 mL HCl 1M under stirring and heating (100°C) for 30 min. Centrifuge sample (4400 rpm, 10 min) after solubilising starch and filter supernatant (0.45 µm) to remove solid residue.
3. Hydrolyse starch in the supernatant. As samples remain suspended in HCl 1M solution, simply treat the supernatant by heating (100°C) and stirring for an additional 180 min. Terminate treatment by adding an equal volume of NaOH 1M to neutralise the sample matrix (final rhizome concentration ~ 5.00 mg mL^{-1}) prior to chromatography, use a pH meter to adjust pH.

2.5.2 Quantitation

4. Quantitate soluble sugar content using HPLC equipped with Shodex SUGAR KS-801 column (full description in Methods Section 2.3). Calibration of NSC content must occur using external standards of the appropriate individual sugars (sucrose, glucose, and fructose).
5. Quantitate glucose content post hydrolysis of starch using HPLC equipped with Shodex SUGAR KS-801 column. Calibrate starch content using glucose standard and adjust using Equation 2 (Section 2.8).

The utilisation of a standard protocol will result in inter-laboratory comparable estimations that will produce outcomes that are far more confident. Furthermore, the creation of a standardised method enables improved management of seagrass ecosystems, specifically how we determine seagrass health.

Chapter 3

The spatial variability in the resilience of *Z. muelleri* to burial events in New Zealand

Burial treatments of *Zostera muelleri* at Te Puna Estuary, Tauranga, New Zealand. Photo: Sørensen, S.T.

3.1 Introduction

It is predicted that the intensity, frequency and spatial scale of sediment influx to coastal waters will increase dramatically in the years to come (e.g., Thrush *et al.*, 2004; Whitehead *et al.*, 2009). Human activities on land such as urban development, agriculture, and deforestation and in coastal regions (e.g. harbour construction and channel dredging) coupled with predictions of increased storm frequencies due to climate change are the main drivers of the increasing sediment regimes (Thrush *et al.*, 2004; Orth *et al.*, 2006; Waycott *et al.*, 2009). Direct and indirect effects of sedimentation, such as increased turbidity (Cabello-Pasini *et al.*, 2002), eutrophication (Burkholder *et al.*, 2007), and sulphur reduction (Raven & Scrimgeour, 1997) have adverse effects on seagrasses. In severe weather events, sedimentation may smother or even bury seagrass (Cabaço *et al.*, 2008; Browning *et al.*, 2019), and is known to have adverse effects on seagrass seedlings and adult plants (e.g., Cabaço *et al.*, 2008; Campbell, 2016; Benham *et al.*, 2019). Despite the increased risk of sedimentation, relatively few studies have focused on seagrass resilience to the direct effects of sediment deposition.

In a review of the effects of burial on seagrass (various species), sediment burial thresholds causing 50% mortality were identified when burial occurred to a depth of 2 to 19.5 cm (Cabaço *et al.*, 2008). Yet, the effects are species-specific and profoundly influenced by allometric relationships, with evidence of greater resilience to increasing burial levels by larger species (e.g., *Posidonia australis*, *P. oceanica*, *P. sinuosa*) (Cabaço *et al.*, 2008). This is not surprising as a fixed burial level will cover a higher proportion of a small plant than a larger plant. Also, larger rhizomes have a greater capacity to store non-structural carbohydrates (NSC) that can be mobilised during adverse conditions (Alcoverro *et al.*, 2001; Erftemeijer & Robin, 2006; Vermaat, 2009). Hence, smaller plants may be less tolerant to burial, however, their recovery rates are generally faster than larger plants due to faster growth rates (Duarte, 1991; Duarte *et al.*, 1997). Despite species-specific responses to burial, increasing burial depths and burial durations significantly exaggerate impacts (e.g., decreases in survival, shoot densities, and NSC stores) across multiple seagrass species (Duarte *et al.*, 1997; Manzanera *et al.*, 1998b; Campbell, 2000; Munkes *et al.*, 2015).

Since the review by Cabaço *et al*., (2008), at least an additional 11 peer-reviewed studies have investigated seagrass responses to experimental burial treatments (Table 3.1). However, only a few of these studies have investigated the effects of cyclic or repeated burial events on seagrass (Campbell, 2000; Han *et al.*, 2012; Campbell, 2016) which appear to differ between species and life stages.

Table 3.1. List of seagrass species and locations where responses to experimental burial are described in the literature (based upon a Web of Science database search, September 2018).

Species	Place	Latitude	Climate	Study type	Reference
Cymodocea nodosa	Italy	43°19'N	Temperate	*In situ*	Balestri and Lardicci (2014)
	Italy	43°19'N	Temperate	*In situ*	Balestri and Lardicci (2014)
	Spain	27°44'N	Subtropical	*In situ*	Tuya *et al.* (2013)
	Spain	40°60'N	Temperate	Mesocosms	Marba and Duarte (1994)
Cymodocea rotundata	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Cymodocea serrulata	Malaysia	2°30'N	Tropical	*In situ*	Ooi *et al.* (2011)
	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Enhalus acoroides	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Halodule uninervis	Malaysia	2°30'N	Tropical	*In situ*	Ooi *et al.* (2011)
	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Halodule wrightii	Mexico	20°50'N	Tropical	*In situ*	Cruz-Palacios and Van Tussenbroek (2005)
Halophila ovalis	Malaysia	2°30'N	Tropical	*In situ*	Ooi *et al.* (2011)
	Australia	23°50'S	Tropical	Mesocosms	Benham *et al.* (2016)
	Australia	23°50'S	Tropical	*In situ*, mesocosms	Benham *et al.* (2019)
	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Posidonia australis	Australia	32°05'S	Subtropical	Mesocosms	Campbell (2016)

Table 3.1 continued.

Species	Place	Latitude	Climate	Study type	Reference
	Australia	32°05'S	Subtropical	Mesocosms	Campbell (2000)
Posidonia oceanica	Italy	40°34'N	Temperate	*In situ*	Ceccherelli *et al.* (2018)
	Spain	42°30'N	Temperate	*In situ*	Manzanera *et al.* (1998b)
Syringodium filiforme	Mexico	20°50'N	Tropical	*In situ*	Cruz-Palacios and Van Tussenbroek (2005)
Syringodium isoetifolium	Malaysia	2°30'N	Tropical	*In situ*	Ooi *et al.* (2011)
	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Thalassia hemprichii	Philippines	16°26'N	Tropical	*In situ*	Duarte *et al.* (1997)
Thalassia testudinum	Mexico	20°50'N	Tropical	*In situ*	Cruz-Palacios & van Tussenbroek (2005)
Zostera marina	Germany	54°24'N	Temperate	*In situ*	Munkes *et al.* (2015)
	USA	35°42'N	Subtropical	*In situ*	Mills and Fonseca (2003)
Zostera muelleri	Australia	23°50'S	Tropical	Mesocosms	Benham *et al.* (2016)
	Australia	23°50'S	Tropical	*In situ*, mesocosms	Benham *et al.* (2019)
Zostera nigricaulis	Australia	38°21'S	Temperate	*In situ*	Hirst *et al.* (2017)
Zostera noltii	Netherlands	51°N	Temperate	*In situ*, mesocosms	Han *et al.* (2012)
	Portugal	37°N	Temperate	*In situ*, mesocosms	Cabaço and Santos (2007)

The adverse effects of seagrass caused by burial events become more severe when the intensity (i.e., burial depth and duration) of the disturbance increases, however, the effects of increased burial frequencies may generate different responses (Han et al. 2012; Campbell 2016). For example, cyclic burial events increased the horizontal rhizome elongation rates of *P. australis* compared to a prolonged (higher intensity) burial event (Campbell 2016). Whereas, *Z. noltii* responded to small-scale cyclic burial events by vertically migrating, thereby, relocated its' horizontal rhizomes to a suitable depth (Han et al. 2012). However, the increased frequencies of natural burial events do not automatically parallel lower intensities. In the autumn of 2017, three cyclones landed on the shores of the north island of New Zealand within a 3-week period (personal comm. Martin, R). In fact, these high intensity cyclic burial events are likely to be a more realistic model than one-off burial events in many coastal and estuarine. For example in the north-eastern Caribbean in 2017, three major hurricanes caused what equated to between one and three centuries of sediment deposition in two weeks (Browning *et al.*, 2019).

The effects of burial are commonly tested on seagrasses in isolation from clonal integration (i.e., cut rhizomes or transplant units) (Duarte *et al.*, 1997; Manzanera *et al.*, 1998b; Cabaço & Santos, 2007; Munkes *et al.*, 2015). However, clonal integration allows for resource transfer between ramets, which may alleviate adverse conditions for a genet in a heterogenic environment, or when affected by localised small-scale stressors (Liu *et al.*, 2016). A study from Moreton Bay, Australia demonstrated that small-scale burial treatment that caused significant effects on *Z. muelleri* in a controlled mesocosm experiment, were insignificant when replicated *in situ* (Benham et al., 2019). This result may partially be due to resource transfer from unaffected ramets (Benham *et al.*, 2019).

The translocation of resources between clonal seagrass ramets is linked to seagrass size (Marbà *et al.*, 2006) and it appears that smaller seagrass species such as *Halophila ovalis* and *Halodule uninervis* do not benefit from clonal integration (Ooi *et al.*, 2011; Tuya *et al.*, 2013). Smaller seagrass species typically have faster rhizome elongation rates but are generally shorter-lived, whereas, larger seagrass species are long lived and have relatively slow rhizome elongation rates (Duarte, 1991; Vermaat, 2009). Larger seagrass species may benefit more from sharing resources to buffer disturbances as they invest more in the individual ramet(s). To

scale-up individual-level resilience to a population-level, impact assessments should, therefore, consider species-specific dependence on resource transfer to sustain stressors.

Processes that influence seagrass recovery following burial events may be independent from those that provide resistance i.e., burial threshold (Cabaço *et al.*, 2008). Seagrass recovery occurs through recolonisation via both sexual (seed production) and asexual (rhizome elongation/fragmentation) pathways (see Chapter 1, section 1.1). The relative importance of the different reproductive strategies are influenced by intensity, frequency and scale of a disturbance, but it also varies between species, genotypes, geographical location and environmental conditioning (Billingham *et al.*, 2003; Orth *et al.*, 2006). When a meadow becomes isolated, or fragmented, the ability to recover from disturbances via rhizome elongation is potentially hindered by the lack of availability of asexual fragments and recovery may, therefore, be dependent on the presence of a seedbank or connectivity to other meadows.

Studies on seagrass recolonisation show different results depending on reproductive strategies. For example, following a severe anoxic crisis, *Z. marina* is able to recolonise from seedbank and reach pre-crisis biomass levels within nine months due to rapid rhizome elongation rates (Plus *et al.*, 2003). In contrast, recovery via rhizome elongation is a lengthy process for larger and slower growing species such as *P. australis* with growth rates of 9.8 to 37.0 cm $year^{-1}$ (Duarte, 1991; Campbell, 2000; Meehan & West, 2000; Campbell, 2003). Recovery of *Z. muelleri* after localised disturbances appears to occur through rhizome elongation and may take from 2 weeks to 12 months depending on location (Rasheed, 1999; Macreadie *et al.*, 2014a). Despite no evidence of recovery from seeds in disturbed plots of *Z. muelleri* in Lake Macquarie, Australia (~33°S), the genetic diversity within the meadows indicates that both sexual and asexual reproduction play active parts in meadow maintenance (Macreadie *et al.*, 2014a).

Spatial variation in seagrass morphometrics is linked to environmental conditions (e.g., Peralta *et al.*, 2005; Maxwell *et al.*, 2014; Soissons *et al.*, 2018a). In New Zealand, *Z. muelleri* acclimates to high terrigenous impacts (increased mud and porewater phosphate content) with increased investment in aboveground biomass as well as high blade photosynthetic pigment content (Kohlmeier *et al.*, 2014). As

such, it appears that increased investment in the photosynthetic apparatus acts as an adaptation to sedimentation. In contrast, research indicates that *C. serrulata* in India increases its investment in belowground tissues and vertical elongation rates along a sedimentation gradient (Gangal *et al.*, 2012). This acclimation process may allow for greater accumulation of NSC reserves to withstand metabolic deficits associated with a more degraded environment. Prior acclimation to light stress can result in enhanced photosynthetic yields and elevated starch reserves of *Z. muelleri* meadows in the turbid environments of Moreton Bay, Queensland, Australia, which in turn increased meadow resilience to disturbances associated with flooding events (Maxwell *et al.*, 2014).

Despite sedimentation being a significant stressor in New Zealand estuaries, it is unknown how *Z. muelleri* responds to direct effects of sediment smothering. Recent studies based in subtropical Australia described *Z. muelleri* as being highly reactive to even low burial levels (≥ 7.5 mm burial depths) (Benham *et al.*, 2016; Benham *et al.*, 2019), which is consistent with other Zosteraceae, such as *Z. marina* (Mills & Fonseca, 2003) and *Z. noltii* (Cabaço & Santos, 2007). New Zealand *Z. muelleri* exist in temperate climates and their resilience to burial may differ from their subtropical/tropical counterparts, as high latitude seagrasses exhibit seasonal vulnerability due to climatic forcing (Soissons *et al.*, 2017, 2018). Furthermore, gradual acclimation to environmental changes may affect seagrass disturbance-response patterns (Maxwell *et al.*, 2014) and responses may differ between single and cyclic burial events (Campbell, 2000; Han *et al.*, 2012; Campbell, 2016). This chapter sets out to explore *Z. muelleri* resilience (i.e. resistance and recovery) to burial events within a New Zealand estuary.

3.2 Materials and methods

3.2.1 Field site

This experiment occurred in Tauranga Harbour, which is 201 km^2 in area and is the largest estuary in the Bay of Plenty region, New Zealand (Figure 3.1). Two-thirds of the harbour area is intertidal (Park, 2014). The harbour is divided into two basins: the northern and southern basins, which are considered to be two distinct bodies of water due to the bathymetry and tidal divide (De Lange & Healy, 1990). Tauranga city is located in the catchment of the southern basin and is home to the Port of Tauranga, which is one of the busiest ports in New Zealand. Tauranga Harbour lost

1679 ha of seagrass between 1959 and 2011 (Park, 1999, 2016). Nearly all subtidal seagrass was lost by 1996 (90%) and the urbanised southern harbour suffered greater losses than the rural northern harbour (Park, 1999, 2016).

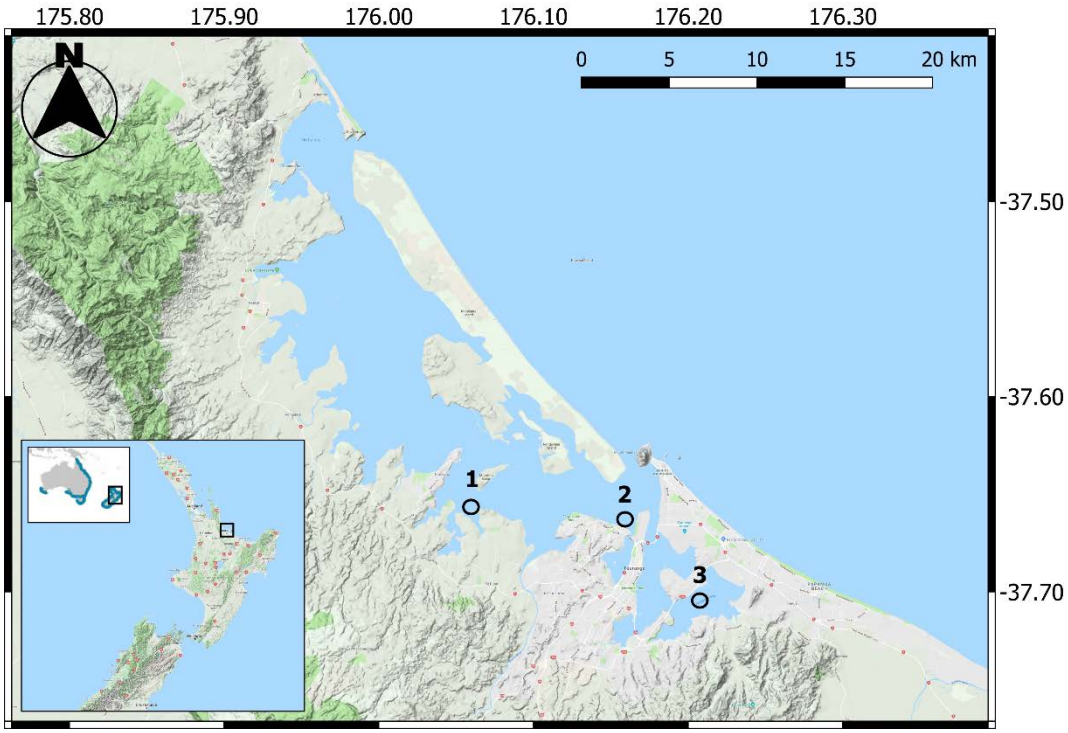

Figure 3.1. The three sample sites for the research in Southern Tauranga Harbour, New Zealand: Site 1, Te Puna Estuary; Site 2, Otumoetai; and Site 3, Rangataua Bay. The small map inset illustrates the distribution of *Zostera muelleri* (source: Waycott *et al.* (2014)).

The experiment was replicated in three intertidal seagrass meadows in southern Tauranga Harbour; **Site 1)** Te Puna Estuary (37°69'07" S, 176°17'99" E); **Site 2)** Otumoetai (37°66'70" S, 176°16'12" E); and **Site 3)** in Rangataua Bay (37°70'04" S, 176°20'61" E). Site selection aimed at including different environmental conditions and was based on personal observation and estimates from the existing literature (Ellis *et al.*, 2013; Tay *et al.*, 2013). Additional site criteria included accessibility, similar seagrass emersion times as well as meadow extents.

3.2.2 Sample design

The experiment ran for a full growing season (12 months), starting in February 2016 (austral summer). Summer usually coincides with annual peaks of *Z. muelleri* biomass and vegetative growth rates (Larkum *et al.*, 1984; Kerr & Strother, 1990; McKenzie, 1994; Turner & Schwarz, 2006a). At each site, a 10 m x 10 m sample station was installed in the seaward fringe of the intertidal seagrass meadow. Within

each station, nine 120 cm x 120 cm quadrats (referred to as 'plots') were permanently installed 200 cm apart using plastic stakes to mark each corner of the quadrat, which ensured that clonal integration was maintained (i.e., the seagrass rhizomes were left undisturbed). Within the quadrats, the experimental area measured 100 cm x 100 cm, allowing for a 10 cm buffer around the edge of the quadrat. Each plot was numbered, and treatments were randomly assigned to the plots. Two burial treatments and one control treatment occurred in each station (Figure 3.2) with each treatment triplicated. These treatments were:

1. Single burial treatments (see section 3.2.2.1);
2. Repeated burial treatments (see section 3.2.2.2); and
3. Control treatments (see section 3.2.2.3).

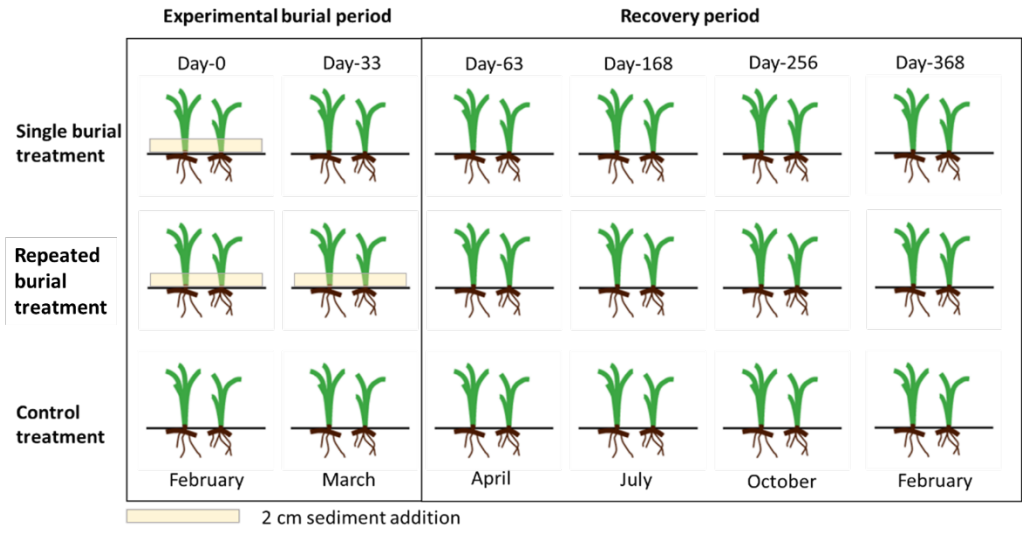

Figure 3.2. Conceptual infographic of the experimental design to test burial regime impacts upon *Zostera muelleri*. Time points (k=6) represent "seagrass response variable" sampling events.

3.2.2.1 Single burial treatment

On day 0, triplicate single burial treatments were started by evenly covering randomly allocated experimental plots with 20 L of sand (fine washed sand purchased from a local landscape retailer) within a 100 cm x 100 cm frame (Figure 3.3). The frame was removed after sediment deposition to allow for natural movement of sediment. The washed landscaping sand was used to control for confounding effects between the sites that would arise if local (site-specific) sediments were used. The sediment burial depth aimed to be 20 mm deep but

averaged 18.4 mm ± 0.5 mm (SE) across all sites. This burial level was chosen as *Zosteraceae* generally display low resilience to burial. For example, Benham *et al.* (2016) described that burial of >7.5 mm had significant adverse effects on *Z. muelleri* in mesocosm treatments, however, not in *in situ* experiments. Furthermore, Mills & Fonseca (2003) reported that an absolute percentage burial (APB) of *Z. marina* of more than 20% significantly reduced survival and productivity, whereas, Cabaco & Santos (2007) found that the threshold for total shoot loss was somewhere between 40 to 80 mm of *Z. noltii* measuring between 175 mm and 260 mm (APB between 15 and 46%).

Experimental sediment levels were not maintained and natural remobilisation of the added sediment was documented, by measuring sediment levels following burial (see section 3.2.3). Seagrass metrics (see section 3.2.5) were sampled on day 0 (pre-burial), and days 33, 63, 168, 250 and 369. These sample days were chosen to assess the effects of burial 1-month post first burial (day 33), 1-month post second burial (day 63), and during winter (day 168), spring (day 250) and summer (day 369) following the burial events.

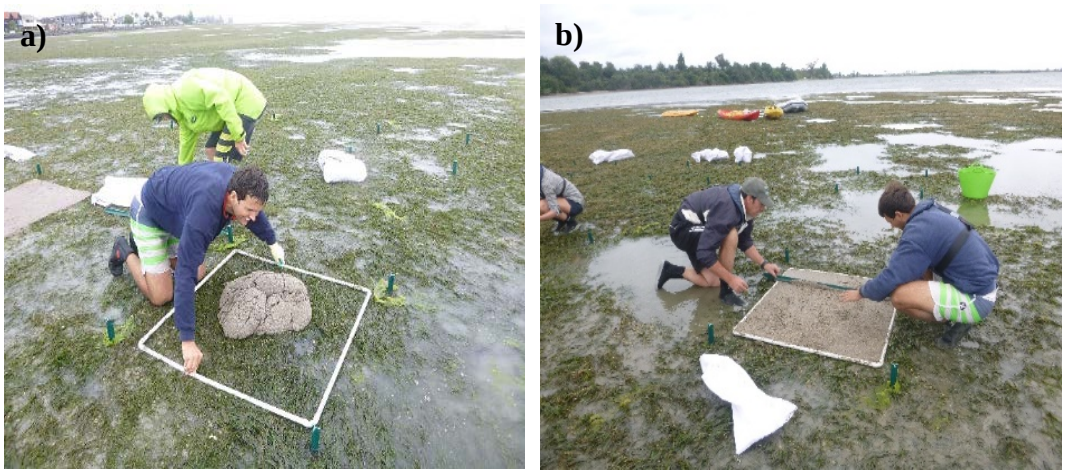

Figure 3.3a) Sand deposition (20 L) on sample quadrat (100 cm x 100 cm) and; **b)** smoothing of sand to ensure relatively even coverage.

3.2.2.2 Repeated burial treatments

Triplicate repeated burial treatments were achieved in each site identically to the single burial treatments outlined above, with the addition of more sand to the original material to simulate a second burial event on day 33 (Figure 3.2). At the time of first burial (day 0), the burial depth across the quadrats averaged 18.40 mm ± 0.50 mm (SE) across all sites and the second burial event yielded an average depth

of 19.10 mm ± 0.60 mm (SE). The sampling protocol for these treatments was identical to that described above for the single burial treatments, with second burial event sand added to the plots and hence any residual sand from the initial burial treatment potentially still present.

3.2.2.3 Control treatment

Triplicate control treatments were installed at each site on day 0 and were marked by plastic stakes in a similar fashion to the burial treatments. These plots were left unburied. The sampling protocol for the control treatments was identical to that described above for the single and repeated burial treatments (Figure 3.2).

3.2.3 Sedimentation level and sedimentation rate

Four plastic stakes were installed to mark the corner of each sample plot. These stakes also served as a reference point for measuring the sediment level (Figure 3.4). Each plastic stake measured 40 cm in length and was buried to a depth of 28 cm. These were placed 10 cm away from each 100 cm^2 quadrat corner (within the buffer of the quadrat) to reduce scouring effects within the experimental quadrat area. A hole was drilled into each plastic stake 2 cm from the protruding end of the stake before the stakes were installed *in situ*. Once installed, the hole in the protruding end of the stake marked 10 cm above the sediment surface. The holes enabled a flexible cord (nylon-cased rubber cord) to be attached to the stakes (at the 10 cm mark) and extend diagonally between the stakes, providing a static reference point above the sediment where the height from the cord to the sediment could be measured (Figure 3.4).

Treatments were replicated in an adjacent unvegetated patch in each site, to measure sedimentation without the influence of seagrass. The literature suggests that seagrasses baffle/attenuate (e.g., El Allaoui *et al.*, 2016) and trap sedimentation (e.g., Gacia *et al.*, 1999) and hence, it was anticipated that unvegetated plots would lose the added sediments at a relatively faster rate than plots that contained seagrasses. As with the treatment plots in the seagrass meadows, each plot was covered with 20 L of sand resulting in an average burial depth across each sample plot of 15.58 ± 0.97 mm. The added sediments caused a lesser increase in sediment level, as the absence of seagrass aboveground biomass in the unvegetated sites resulted in more compact sediment addition.

Sediment level measurements were measured six times one month following burial treatments (days 0, 1, 3, 7, 14 and 33). Sediment measurements were discontinued at day 33 due to a member of public protesting the presence of protruding objects on mudflat in Site 2. At this time, the plastic stakes were levelled with the sediment surface and marked with a bright yellow cap to ensure easy relocation of experimental units, thus, ensuring the continued monitoring of seagrass responses.

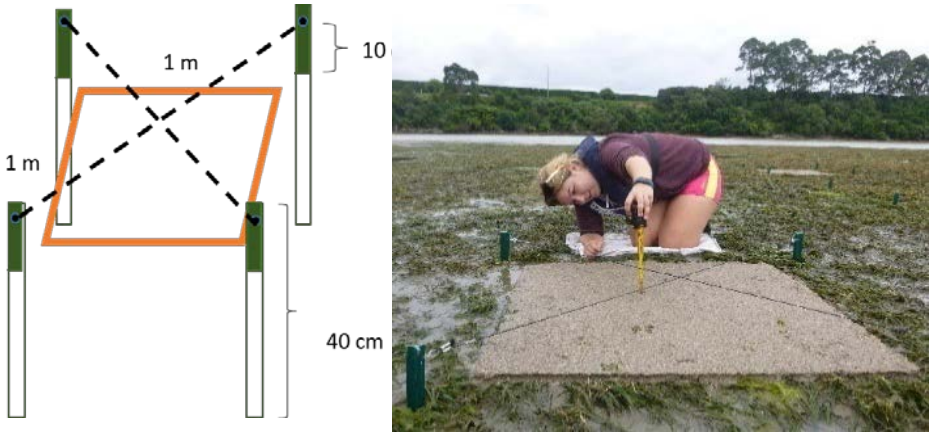

Figure 3.4. Cords extended diagonally between static plastic stakes. Each cord was marked at ten equidistant points, resulting in 20 sediment level measurements per sample.

Preliminary measurements revealed a high variance in sediment levels within each plot due to the highly undulating sediment topography. Subsequently, to increase the power of the later statistical analysis, each of the two cords was marked with ten static measurement points (evenly placed along cord), resulting in 20 sediment level measurements per quadrat per sampling event (Figure 3.4). These measurements were used to obtain an average sediment level to track sediment resuspension and mobilisation rates over 33 days (five sample times).

3.2.4 Environmental variables

3.2.4.1 Light levels

Light levels were measured at each site using data logging sensors (HOBO Pendant Temperature/Light Data Loggers 64k, Onset Computer Corporation). Three loggers were installed immediately above the seagrass canopy with sensors facing upwards and pointing north. Data was logged at 30 min intervals from the 3rd February 2016 until 6th March 2016 (late summer to early autumn). Data logging sensors recorded

light intensity in Lux, with data calibrated to relevant PAR values (µmol m^{-2} s^{-1}) and used to calculate daily irradiance (I_d in mol photons m^{-2} d^{-1}).

3.2.4.2 Sediment grain size and sediment organic matter

At each site, sediment samples (n = 5) were collected at the start of the experiment (day 0) to compare the sediment grain size and sediment organic matter (SOM) content between the sample sites. Five core samples (2.4 cm diameter, 2 cm depth) of sediment were collected at random positions within each sample station. Upon collection, samples were placed in a dark container and chilled immediately. On return to the laboratory, these samples were frozen (≤ 20°C) until laboratory analyses were conducted.

Sediments were defrosted and filtered through a 2 mm filter before grain size composition, and median sediment particle size was determined using the Master Sizers 2000 (Malvern Instruments Ltd.). The Wentworth Scale was used to classify the substrate types based on sediment grain sizes (Wentworth, 1922). Sediment organic matter (SOM) samples were defrosted and dried in an oven for 24 h at 110° C and weighed (dry weight) before combustion in a muffle furnace (5.5 h at 550°C). After combustion, the samples were reweighed (ash-free dry weight) to provide a difference in sample weight. The SOM was measured as the percentage difference between the dry weight and the ash-free dry weight as shown in Equation 3.1:

Equation 3.1:

$$\text{SOM} = \frac{\text{sediment ash-free (DW)}}{\text{sediment (DW)}} \text{ x } 100$$

3.2.4.3 Sediment porewater nutrient content

Five sediment porewater samples were randomly collected within each of the sample stations immediately after tidal emersion at the onset of the experiment (day 0). The top 5 cm of sediment was collected with a 2.5 cm diameter syringe. These samples were placed in a dark container and chilled immediately. Upon return to the laboratory, the content from the sediment porewater samples was centrifuged at 4000 rpm for 20 min. Samples were filtered through 0.45 µm syringe filters (Whatman FP) and frozen (≤ 20°C) until analysis occurred. Concentrations of nitrate and nitrite (NO_x^-) ions and phosphate (PO_4^{3-}) ions were detected spectrophotometrically, and ammonium ion (NH_4^+) was detected fluorometrically

using a continuous flow injection analysing system (Skalar SAN^{++} system) (Grasshoff *et al.*, 1983).

3.2.4.4 Apparent redox potential discontinuity (aRPD)

The depth of the apparent redox potential discontinuity (aRPD) was measured visually to assess the level of oxygen penetration into the sediment. At each sampling station, five core samples (5 cm diameter, 20 cm deep) were randomly collected, from which the depth of aRPD was measured to the nearest millimetre. The aRPD is detectable as a marked colour change in the vertical layers of the sediment. The colour change is caused by microbes that reduce iron and sulphur in the absence of oxygen, turning the sediments grey-black (Diaz & Trefry, 2006). Thus, a shallow aRPD is related to reduced sediment oxygen contents associated with eutrophic conditions (Gerwing *et al.*, 2018).

3.2.4.5 Other environmental variables

Triplicate measurements of porewater salinity (ppt), pH and dissolved oxygen (DO%) were measured at each sampling station using a digital multi-probe (Aquaprobe AP-2000, Aquaread Ltd.).

Average long-term environmental conditions

Average monthly sea-surface temperature (°C) and air temperatures (°C) were supplied from Mount Maunganui Meteorological Station by the Bay of Plenty Regional Council with available long-term data spanning from December 2005 to December 2017 (Bay of Plenty Regional Council, 2019). Average monthly rainfall (mm) and daylight hours from Tauranga were sourced from the National Institute of Water and Atmospheric Research (NIWA) with available long-term data ranging from January 1990 to December 2010 (National Institute of Water and Atmospheric Research, 2019).

3.2.5 Seagrass characteristics

Seagrass samples collected at the onset of the experiment (day 0) before manipulative treatments were affected, were used to compare seagrass characteristics between the three sites.

3.2.5.1 Rhizome growth rates

Seagrass productivity (rhizome elongation) was measured at each sampling site by randomly tagging ten rhizomes at the growing edge of the seagrass meadow. Cable-ties were attached between the second and third shoot behind the apical meristem on day 0 of the experiment. Subsequently, the rhizome length was measured after the initial first shoot (from the apical meristem) at day 63. These measurements were used to calculate a yearly seagrass rhizome growth rate for each site.

3.2.5.2 Seagrass morphometry

Digital imagery analysis (Acrobat Reader, DC) was used to measure rhizome width, blade width, blade length and internode distance between the apical and the second shoot. Measurements were made to the closest 0.1 mm.

3.2.5.3 Absolute percentage burial

The absolute percentage burials (APB) were calculated (Equation 3.2) by comparing the depths of the sediment addition with the average blade length in each experimental plot:

Equation 3.2:

$$\text{APB (\%)} = \left(\frac{\text{sediment addition (mm)}}{\text{Blade length (mm)}}\right) \times 100$$

3.2.5.4 Shoot cover

Sample plots were photographed six times throughout the experimental period. The photographs were captured during tidal emersion (at various times throughout daylight hours) directly above (~130 cm above sediment) the treatment plot which was framed temporarily by a 100 cm x 100 cm quadrat. The frame allowed for later digital recognition of the experimental area and these were analysed using Coral Point Count with Excel^{TM} extensions (CPCe). The software was programmed to randomly allocate 100 points across the quadrat, from which seagrass presence or absence was manually determined.

3.2.5.5 Photosynthetic properties

The quantum yields of electron transfer (F_v/F_m) in seagrass blades (three blades from each plot) were measured in the laboratory immediately upon return from the field. Sampling used a pulse-amplitude modulated (mini-PAM) fluorometer (Walz, Effeltrich, Germany). Measurements were made on the second youngest blade on the apical meristem, 1-2 cm above the blade sheath. Any visible detrital and epiphytic matter on the blade was gently removed from the blade before it was placed in a blade clip and dark-adapted for 5 min (Beer *et al.*, 2001). The fibre-optic cable was held 5 mm from the blade surface when conducting measurements.

3.2.6 Seagrass response variables

Seagrass response variables were measured on six occasions throughout the experimental period (369-days, Figure 3.2) to track the immediate response to burial and recovery. The selected variables described in this section have been suggested as robust indicators of early detection of severe reduction in seagrass health at plant-scale (non-structural carbohydrates) and long-term changes at meadow-scale (shoot cover) (McMahon *et al.*, 2013).

3.2.6.1 Relative shoot cover

Relative shoot cover describes the percentage change in shoot cover by comparing percentage shoot cover at relevant sample period against the initial percentage shoot cover as described in equation 3.3:

Equation 3.3:

$$\text{Relative shoot cover} = \left(\frac{S_x}{S_i}\right) \times 100$$

Where S_i is initial shoot cover (%) at day 0 and S_x is the seagrass cover (%) at time x.

3.2.6.2 Non-structural carbohydrates

Triplicate rhizome samples (piece of rhizome with >4 shoots) were collected from each experimental unit at each sampling event (Figure 3.2) for NSC quantitative analysis. The analysis was conducted using high-performance liquid chromatography (HPLC) with refractive index (RI) detection as outlined in Chapter 2. Rhizomes were rinsed in deionised water and freeze-dried to constant weight. Soluble NSC was extracted in deionised water in three sequential extractions (15

min at 80°C) and the supernatant pooled before analysis by HPLC using a Shodex SUGAR KS-801 column. The content of sucrose, glucose, and fructose was calculated using external calibration curves derived from standard sucrose, glucose and fructose (Aldrich). Starch was solubilised from the rinsed solid residue in hydrochloric acid (HCl 1M) for 30 min at 100°C, centrifuged and the resultant supernatant hydrolysed in HCl (1M) for a further 180 min at 100°C. Treatments were terminated by neutralising the sample with sodium hydroxide (NaOH 1M) and quantitated as glucose equivalent by HPLC. Sucrose, glucose, and fructose represent soluble sugars, and total non-structural carbohydrates (tNSC) are soluble sugars and starch combined.

3.2.7 Statistical analyses

Environmental variables (replicated within sites) and seagrass variables (experimental plots before burial) collected at day 0 were used to describe sites conditions and seagrass characteristics at onset of the experiment. Data were tested for normal distribution using a Kolmogorov-Smirnov test and for homogeneity of variance using a Levene's test. Both environmental and seagrass variables were tested for significant site-effects using a series of one-way ANOVA and Tukey HSD post-hoc tests or Chi-squared tests, Kruskal-Wallis ANOVA by rank and Duncan's new multiple range post hoc tests depending on the outcome of model assumption testing. Principal component analyses (PCA) were undertaken in R-Studio™ using the package "factoextra" to identify the variables that explained the majority of variation in seagrass metrics between the three sites. Variables were standardised by log-transformation (log(x)+1) prior to PCA.

Three-way repeated measures ANOVA were used to test the effects of the within-subject factor (time = 5 or 6 levels) and between-subject fixed factors (Site = 3 levels, Treatment = 3 levels) on the dependent response variables (shoot cover, sucrose, starch and total NSC). Significant interactions between-subject factors were explored in 2-way ANOVA models and further assessed using Tukey's post hoc test. Before analysis, normality was tested for each combination of factor levels using Shapiro-Wilks test and in a series of quantile-quantile (QQ) probability plots (see Appendix B, Figure B.1-B.4). Finally, linear regression analyses were used to investigate the relationship between sucrose/starch contents and relative shoot cover across all site and treatments throughout time. The significance level of all statistical

tests was α=0.5 and statistical analyses were performed in TIBCO Statistica™ (version 13.3) and R-Studio™ (version 1.1.442)

3.3 Results

3.3.1 Site conditions

3.3.1.1 Sedimentation and sediment resuspension

The average sedimentation rates measured in control plots in seagrass meadows were 0.02 ± 07 mm day^{-1} in Site 1, 0.03 ± 05 mm day^{-1} in Site 2 and 0.19 ± 31 mm day^{-1} in Site 3. In the adjacent unvegetated plots the sedimentation rates were -0.21 ± 0.04 mm day^{-1} in Site 1, 0.16 ± 0.42 mm day^{-1}, 0.19 ± 0.29 mm day^{-1}. However, there was no significant effect of sample station ($F_{[1]}$=0.08 p = 0.78), site ($F_{[2]}$=2.00, p = 0.18) or interaction of station and site (F[2,12]= 0.79, p = 0.477) on the change in sediment levels in control plots over the measured period (Figure 3.5).

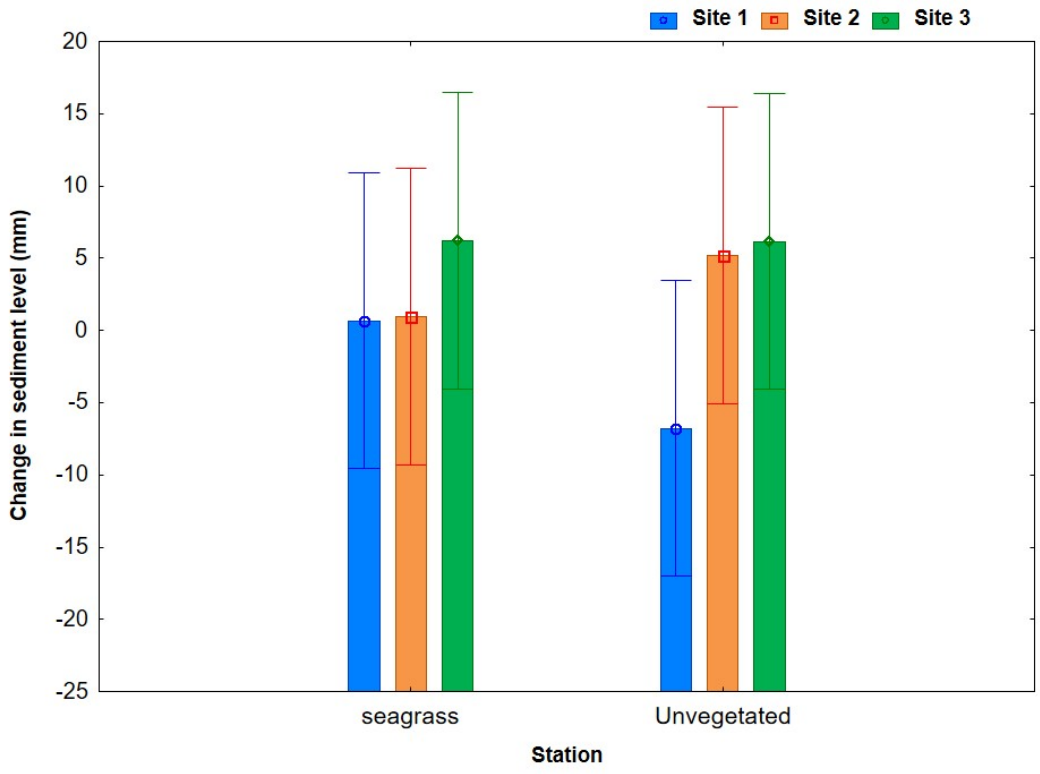

Figure 3.5. Change in sediment levels (over 33 days) in seagrass and unvegetated sampling stations in three sites in Tauranga Harbour, New Zealand.

The average sediment levels in burial treatments in Site 1, (-0.53 ± 7.05 mm) was reduced to pre-burial levels after 14 days, however, after only three days, the average sediment levels of burial and control treatments ($U_{[6]}$ = 1.00, p = 0.053,

Figure 3.6). In Site 2, mean sediment levels remained elevated after 33 days (11.22 ± 6.33 mm), however, sediment levels of control and burial treatment were statistically similar three days after the first sediment addition ($U_{[6]} = 7.00$, $p = 0.69$). In Site 3, sediment levels of burial treatments also remained elevated (19.40 ± 0.64 mm) and significantly higher than control treatments throughout the measured period ($U_{[6]} = 0.00$, $p = 0.028$) (Figure 3.6).

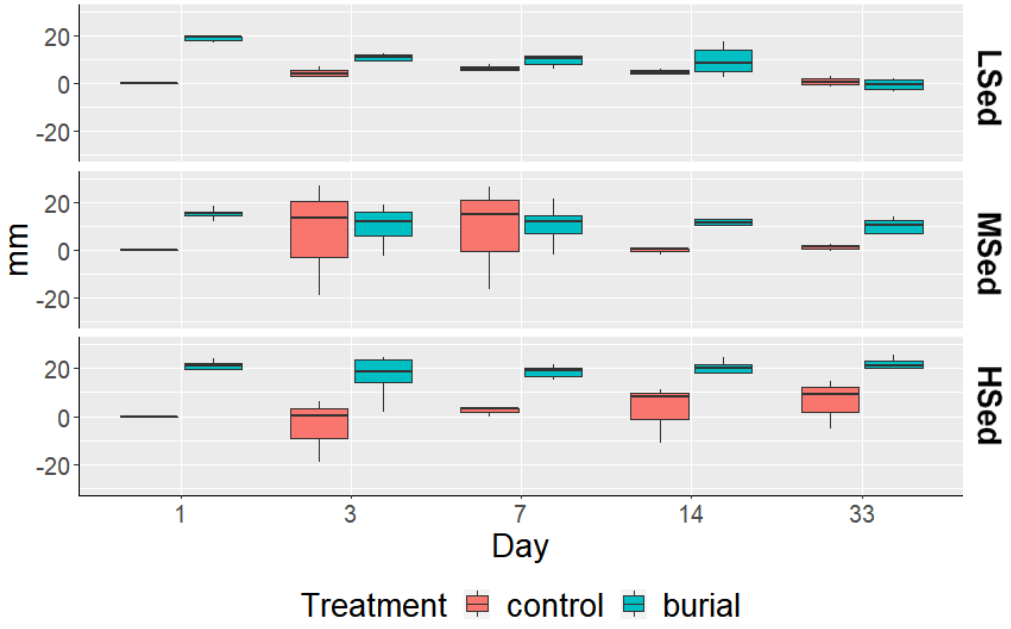

Figure 3.6 Median sediment levels (mm) in control and burial treatments of *Zostera muelleri* measured over 33 days from onset of the experiment in three sites in Tauranga Harbour, New Zealand. Boxes indicate 25th and 75th percentiles and whiskers min/max values.

3.3.1.2.Sediment classification

The mean mud and silt fractions of sediment samples from Site 1 (10.11±0.70%) and Site 2 (8.58±3.28%) appeared to be lower than Site 3 (20.92±7.2%) (Figure 3.7). Medium (33.70%) and coarse sand (33.36%) accounted for the majority of particles in sediment samples from Site 1, whereas sediment samples from Site 2 had a higher proportion of fine sand (47.55%) and medium sand (26.11%). Furthermore, sediment samples from Site 3 had the greatest content of smaller particles including very fine (23.74 ±7.26%) and fine sand (34.33 ± 6.19%). As such, the median particle size in Site 3 was 180.18 µm, 205.71 µm in Site 2 and 438.95 µm in Site 1. Regrettably, samples from Site 2 and Site 3 were lost in transport/storage and, thus, statistical inferences on sediment particles distributions can, therefore, not be made.

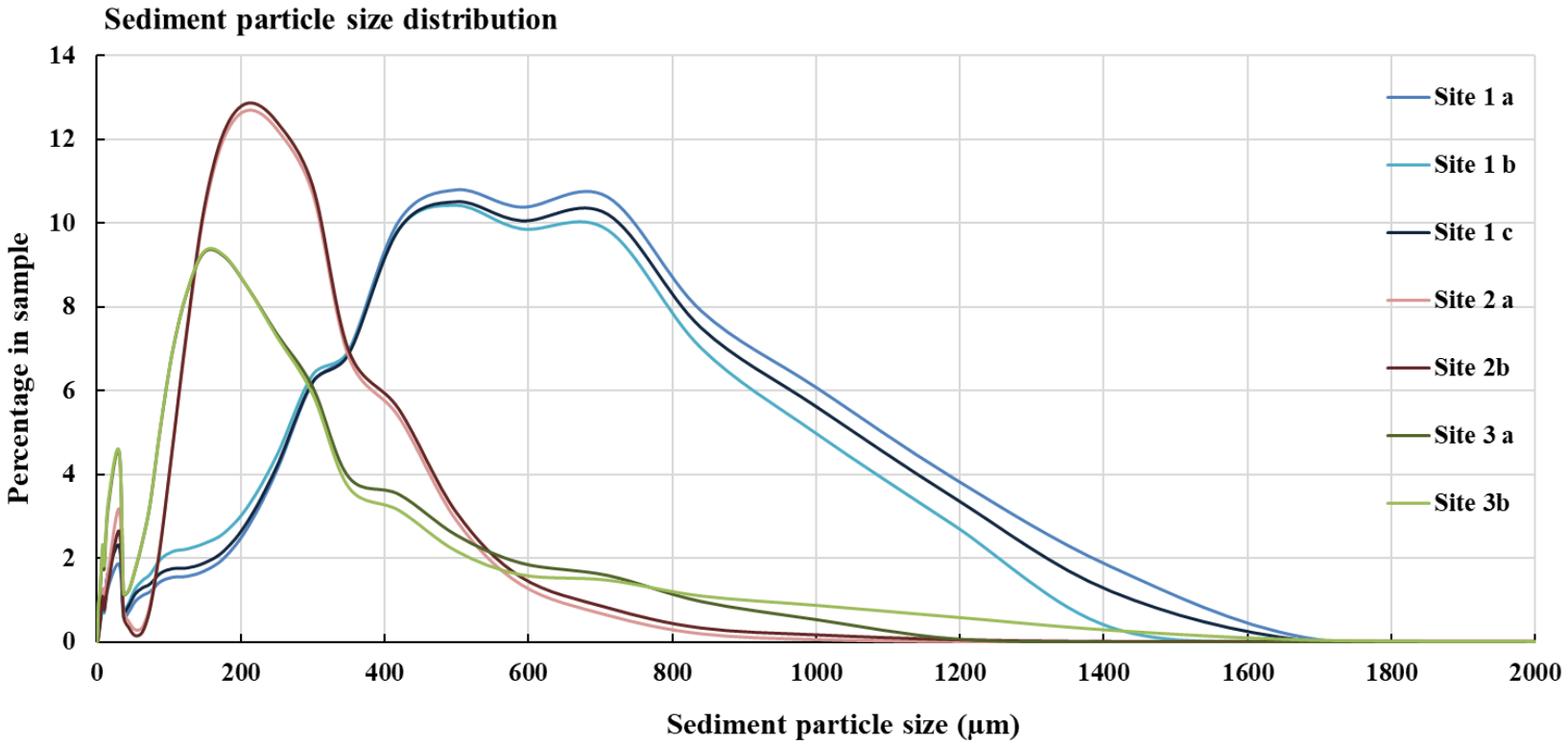

Figure 3.7 Sediment particle size distribution of replicated sediment samples from three sites in Tauranga Harbour, New Zealand.

3.3.1.3. Other environmental variables

The SOM content varied between the three sites ($F_{[2, 6]} = 262.19$, $p < 0.001$), with Site 1 (2.70 ± 0.06%) and Site 2 (2.70 ± 0.03%) having similar content whilst SOM was significantly higher in Site 3 (4.49 ± 0.09%, Table 3.2). Site 1 had significantly higher porewater salinity (19.37 ± 1.64 ppt) compared with Sites 2 (11.76 ± 0.72 ppt, $p = 0.005$) and 3 (12.25 ± 0.13 ppt, $p = 0.007$) (Table 3.2). The total porewater nutrient content was highest in Site 2 (1.05 mg L^{-1}), being 2.7-fold that of Site 1 (0.39 mg L^{-1}) and 1.5-fold that of Site 3 (0.71 mg L^{-1}). Porewater in Site 1 contained the lowest amount of PO_4^{3-} (0.08 mg L^{-1}), and Site 2 (0.28 mg L^{-1}) contained more PO_4^{3-} than Site 3 (0.21 mg L^{-1}). Site 1 also contained lower porewater NH_4^+ content (0.29 mg L^{-1}) than Site 2 (0.75 mg L^{-1}) and Site 3 (0.48 mg L^{-1}).

A statistically significant difference in porewater DO (%) existed between the three sites ($F_{[2,6]} = 37.07$, $p < 0.001$), with Site 1 having approximately an order of magnitude higher DO (%) than Site 2 and Site 3 (Table 3.2). The much higher DO measurements in Site 1 may be due to the much coarser sediment coupled with higher exposure, as more porous sediments naturally would allow for greater interstitial flux between porewater and the water column. The pH levels varied significantly between the three sites ($F_{[2,6]} = 12.51$, $p = 0.007$) with pH in Site 1 being significantly higher than in Site 3 ($p = 0.006$). There were no statistically significant differences in aRDP between sites ($p = 0.06$; Table 3.2)

Table 3.2 Summary of one-way ANOVA and Chi-square tests between environmental characteristics of the three sites at the onset of the experiment. Significant p-values ($p < 0.05$) are reported in **bold.** Variables with same letters (^{a,b}) are not significantly different from each other as a result of post-hoc testing (Tukey HDS). Acronyms include: N = nitrogen, P = phosphorous, SOM = sediment organic matter, DO = dissolved oxygen, Sal = salinity, aRDP = apparent redox potential discontinuity, I_d = daily irradiance.

Variables		**Site 1 (a)**		**Site 2 (b)**		**Site 3 (c)**		**Chi-square test / one-way ANOVA**		
	n	Average	(+SE)	Average	(±SE)	Average	(±SE)	χ^2	F-stats	p
NH_4^+ (mg L^{-1})	3	0.29	*	0.75	*	0.482	*			
PO_4^- (mg L^{-1})	3	0.08	*	0.28	*	0.21	*			
Total N+P (mg L^{-1})	3	0.39	*	1.05	*	0.71	*			
N:P ratio	3	3.68	*	2.71	*	2.34	*			
SOM (%)	9	2.70	$(0.06)^a$	2.70	$(0.03)^a$	4.49	$(0.09)^b$		262.19	<0.001
DO (%)	9	9.70	$(1.22)^a$	1.07	$(0.63)^b$	0.93	$(0.38)^b$		37.07	<0.001
Sal (ppt)	9	19.37	$(1.64)^a$	11.76	$(0.72)^b$	12.25	$(0.13)^b$		16.90	0.003
pH	9	7.51	$(0.04)^a$	7.37	$(0.01)^{ab}$	7.23	$(0.06)^b$		12.51	0.007
aRDP (mm)	9	21.80	(0.87)	32.53	(0.75)	33.53	(3.66)	5.6		0.061
I_d (mol m^{-2} d^{-1})	297	30.09	(1.76)	32.02	(1.95)	27.27	(1.57)	0.69		0.710
Photon flux (µmol m^{-2} s^{-1})	297	348.22	(20.35)	370.55	(22.52)	315.58	(18.14)	2.43		0.300

*Insufficient porewater sample volume to perform replicated nutrient analysis.

3.3.2 Investigation of the seagrass characteristics between the study sites before experimental treatments.

Figure 3.8 displays the statistical summaries of the seagrass parameters in each site at the onset of the experiment (day 0). The number of blades in the apical meristem shoot was significantly different between sites ($F_{[2,81]} = 6.77$, $p = 0.002$) with Site 3, having on average more blades than Sites 1 and 2 (Figure 3.8a). The blade width ($F_{[2,81]} = 18.10$, $p < 0.001$) and blade length ($\chi^2_{[2,81]} = 98.99$, $p < 0.001$) were also significantly affected by site. Seagrass in Site 3 had significantly wider blades than seagrass in Sites 1 and 2 (Figure 3.8b). The average length of blades was significantly shorter in Site 1 compared to Sites 2 and 3 (Figure 3.8b). The significant differences in blade length *in situ* meant that the experimental burial treatments resulted in significantly different levels of absolute percentage burial (APB) of the photosynthetic tissue ($F_{[2,15]} = 21.98$, $p < 0.001$). As such, the APB on seagrasses at Site 1 was significantly higher (30.22 ± 1.52%) than Site 2 (16.13 ± 0.77%) and Site 3 (19.35 ± 2.13%).

Rhizome diameter was influenced by site effects ($F_{[2,81]} = 45.71$, $p < 0.001$), with Site 3 having almost 50% broader rhizome than Sites 1 and 2 (Figure 3.8d). The rhizome elongation rates (Figure 3.8e) were also significantly different between the three sites ($H_{[2,30]} = 11.69$, $p < 0.01$). Specifically, growth rates were significantly slower in Site 3 (73.00 ± 11.31 cm $year^{-1}$) than in Sites 1 (276.67 ± 31.39 cm $year^{-1}$) and 2 (308.06 ± 83.22 cm $year^{-1}$). The percentage surface cover differed statistically between sites ($F_{[2,81]} = 9.34$, $p = 0.001$, Figure 3.8f), with a higher percentage shoot cover in Site 3 (83.22 ± 3.28%) compared to Site 1 (60.78 ± 4.34%).

The mean F_v/F_m was similar between sites (Figure 3.8g) and averaged 0.80 ± 0.01 across all sites on day 0. Mean values of 0.83 are consistent with unstressed higher plants (Björkman & Demmig, 1987), whereas the average F_v/F_m of unstressed *Zosteraceas* is 0.80 (calculated from mean values in Table 6 in the review by Touchette & Burkholder, 2000). Thus, none of the sites appeared to experience light stress at the onset of the experiment.

There was a significant effect of site on sucrose content at the start of the experiment ($F_{[2,27]} = 11.19$, $p < 0.001$, Figure 3.8h). The mean sucrose content in Site 2 (180.33

±16.55 mg g^{-1} DW) was almost 4-fold higher than Site 1 (47.73 ± 15.36 mg g^{-1} DW) and more than 2-fold higher than that of Site 3 (81.78 ± 4.59 mg g^{-1} DW). However, rhizome starch levels were similar ($F_{[2,27]} = 0.98$, $p = 0.98$, Figure 3.8i) and averaged 162.21 ± 11.88 mg g^{-1} DW across all sites. The significant differences in sucrose resulted in a significant site effect on tNSC at the onset of the experiment ($F_{[2,27]} = 5.05$, $p = 0.01$, Figure 3.8j). Specifically, seagrass in Site 1 stored significantly lower average tNSC (241.02 ± 27.41 mg g DW^{-1}) than seagrasses in both Site 2 (328.40 ± 18.59 mg g DW^{-1}) and Site 3 (275.58 ± 13.00 mg g DW^{-1}).

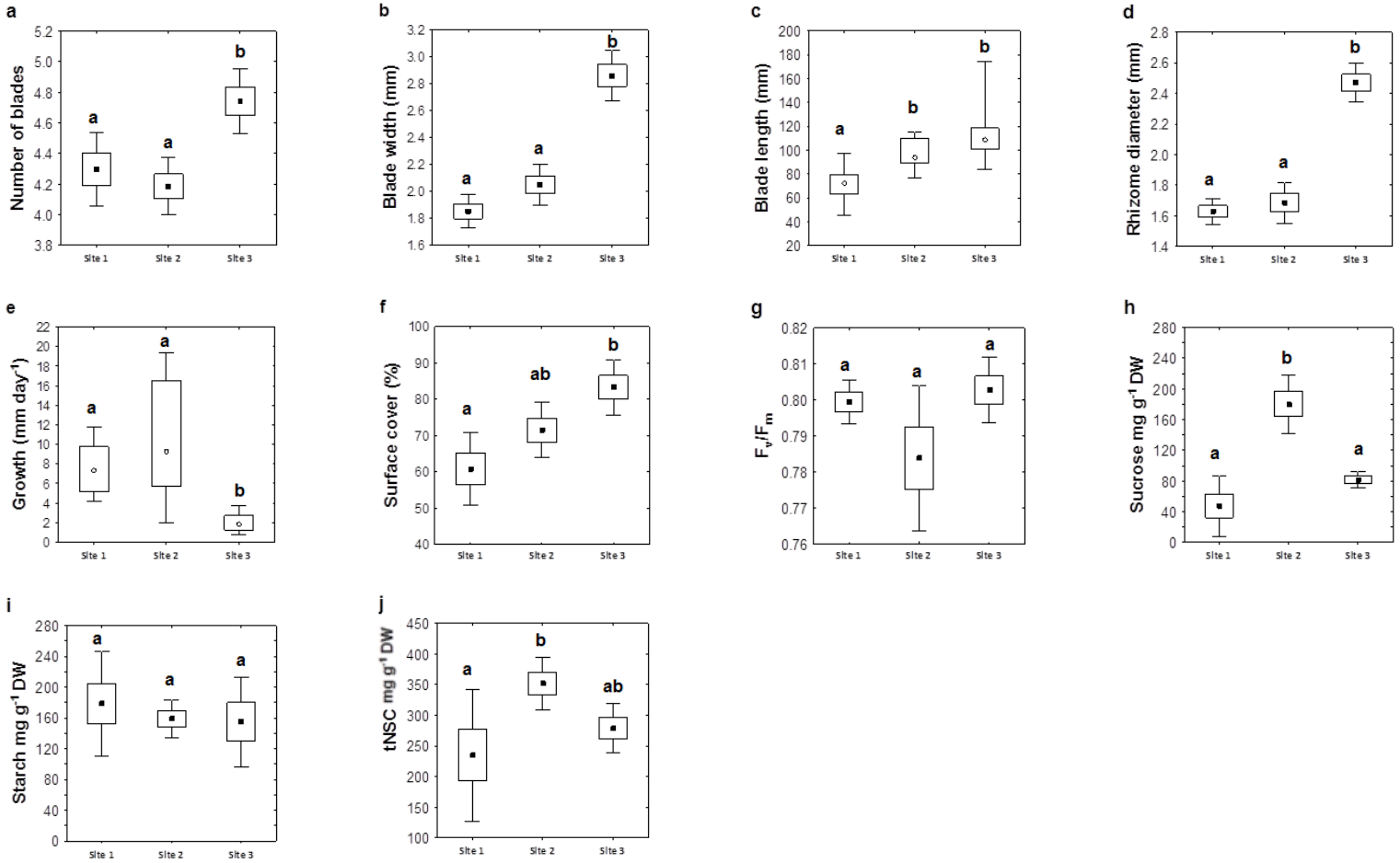

Figure 3.8 Differences in seagrass metrics between Site 1, Site 2 and Site 3 in Tauranga Harbour. Variation in: **(a)** blades in apical meristem; **(b)** blade width; **(c)** blade length; **(d)** rhizome diameter; **(e)** rhizome elongation rate; **(f)** percentage surface cover; **(g)** Fv/Fm; **(h)** rhizome sucrose content; **(i)** rhizomes starch content; and **(j)** rhizomes total non-structural carbohydrate (tNSC) content. Mean values = ■ (box = standard error, and whisker = 95% confidence interval) and median values = ○ (box = 25 percentiles, and whisker = min/max).

A PCA determined that three main components explained 69.45% of the variation of the measured seagrass parameters. The first component (PC1) accounted for 34.75% of the variation (Figure 3.9) and was correlated to blade width (29.94%), rhizome diameter (27.52%), blade number at the apical meristem (21.43%) and blade length (17.29%). The second component (PC2) accounted for 21.25% of the total variation (Figure 3.9) and was mainly correlated to rhizome sucrose content (38.6%), Fv/Fm (22.3%) and above- below ground ratio (ABR = 9.8%). The third component explained 13.45% of the total variation and was mostly correlated to rhizome starch content (68.9%). The 95% confidence ellipses plotted around the site means, revealed that Site 1 and Site 3 are significantly different from each other along the PC1 axis, whereas Site 2 is placed in-between the two, but deviating more from Site 1 along the PC2 axis (Figure 3.9).

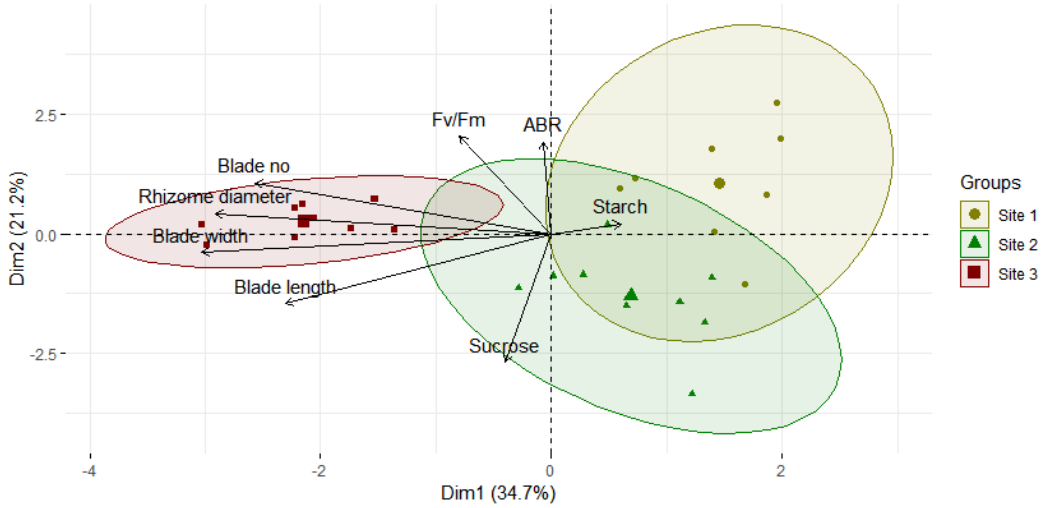

Figure 3.9. Principal component analysis (PCA) of the seagrass parameters with 95% confidence ellipses grouping points by the sites. The first principal component (PC1) explains 34.7% and the second principal component (PC2) explains 21.2% of the variation of the seagrass parameters across the sites.

3.3.3 Effects of burial treatments on *Z. muelleri*

A three-way repeated measures ANOVA found that the relative shoot cover was significantly influenced by between subject interactions of "site x treatment" ($F_{[4,18]}$=4.17, p = 0.01), as well as within subject interactions of "time x site" ($F_{[8,72]}$= 6.40, p < 0.001) and "time x treatment" ($F_{[8,72]}$=3.78, p < 0.001) (see Table 3.3).

Table 3.3 Three-way repeated measures ANOVA models testing the interactions of the fixed factors "Site" and "Treatment" over "Time" on seagrass response variables (dependent variables = relative shoot cover, sucrose, starch, total non-structural carbohydrates (tNSC). Significant effects ($p < 0.05$) are denoted in ***bold***.

Effect	SS	df	MS	F	*p*	Partial η^2
Relative shoot cover						
Between subjects						
Intercept	561898.4	1	561898.4	1012.229	***0.000***	0.983
Site	17170.0	2	8585.0	15.465	***0.000***	0.632
Treatment	12006.1	2	6003.0	10.814	***0.001***	0.546
Site*Treatment	9255.7	4	2313.9	4.168	***0.015***	0.481
Error	9992.0	18	555.1			
With-in subjects						
Time	9832.3	4	2458.1	13.331	***<0.0001***	0.425
Time*Site	9440.9	8	1180.1	6.400	***<0.0001***	0.416
Time*Treatment	5579.2	8	697.4	3.782	***0.001***	0.296
Time*Site*Treatment	3388.1	16	211.8	1.148	0.330	0.203
Error	13275.8	72	184.4			
Sucrose						
Between subjects						
Intercept	1196607	1	1196607	603.327	***<0.0001***	0.971
Site	101151	2	50575	25.500	***<0.0001***	0.739
Treatment	212	2	106	0.053	0.948	0.006
Site*Treatment	7248	4	1812	0.914	0.477	0.169
Error	35700	18	1983			
With-in subjects						
Time	117695	5	23539	28.737	***<0.0001***	0.615
Time*Site	57771	10	5777	7.053	***<0.0001***	0.439
Time*Treatment	9577	10	958	1.169	0.322	0.115
Time*Site*Treatment	16726	20	836	1.021	0.447	0.185
Error	73721	90	819			
Starch						
Between subjects						
Intercept	4307240	1	4307240	911.992	***<0.0001***	0.981
Site	7242	2	3621	0.767	0.479	0.078
Treatment	5275	2	2638	0.558	0.582	0.058
Site*Treatment	29671	4	7418	1.571	0.225	0.259
Error	85012	18	4723			
With-in subjects						
Time	239806	5	47961	16.597	***<0.0001***	0.480
Time*Site	106031	10	10603	3.669	***<0.0001***	0.290
Time*Treatment	12722	10	1272	0.440	0.923	0.047
Time*Site*Treatment	36692	20	1835	0.635	0.876	0.124
Error	260084	90	2890			
tNSC						
Between subjects						
Intercept	11784556	1	11784556	2274.516	***<0.0001***	0.992
Site	59226	2	29613	5.716	***0.012***	0.388
Treatment	6474	2	3237	0.625	0.547	0.065
Site*Treatment	12099	4	3025	0.584	0.678	0.115
Error	93260	18	5181			
With-in subjects						
Time	583482	5	116696	31.571	***<0.0001***	0.637
Time*Site	179940	10	17994	4.868	***<0.0001***	0.351
Time*Treatment	29594	10	2959	0.801	0.628	0.082
Time*Site*Treatment	35355	20	1768	0.478	0.969	0.096
Error	332670	90	3696			

To assess the significant interactions of the between-subject and within-subject factors on relative shoot cover, a series of two-way ANOVA models (fixed factors = site and treatments) were produced for each sampling event (Table 3.4). At day 33, a significant treatment effect affected relative shoot cover ($F_{[2,18]}$=5.07, p = 0.02). Post-hoc tests identified that relative shoot cover was significantly reduced in single (p = 0.01) and repeated burial treatments (0.04) compared to control treatment on day 33 across all sites. A significant interaction of "site x treatment" influenced relative shoot cover at day 63 ($F_{[4,18]}$ = 4.94, p < 0.001) and day 168 ($F_{[4,18]}$=5.34, p = 0.005, Table 3.4). Relative shoot cover was no longer significantly affected by burial treatments from day 251 onwards (Table 3.4).

Table 3.4 Two-way ANOVA models exploring the effects of "site" and "treatment" on relative shoot cover at 5 sampling events (day 33, day 63, day 168, day 251 and day 369). Significant effects ($p < 0.05$) are denoted in ***bold***

Effect	SS	df	MS	F	p	Partial η^2
Day 33						
Intercept	152433.5	1	152433.5	527.040	***8.77E-15***	0.967
Site	65.3	2	32.6	0.113	0.894	0.012
Treatment	2931.4	2	1465.7	5.068	***0.0180***	0.360
Site*Treatment	926.9	4	231.7	0.801	0.540	0.151
Error	5206.1	18	289.2			
Day 63						
Intercept	89547.61	1	89547.61	438.405	***4.36E-14***	0.961
Site	5202.34	2	2601.17	12.735	***3.58E-04***	0.586
Treatment	9051.30	2	4525.65	22.157	***1.40E-05***	0.711
Site*Treatment	4038.56	4	1009.64	4.943	***0.007***	0.523
Error	3676.64	18	204.26			
Day 168						
Intercept	97783.63	1	97783.63	414.135	***7.139E-14***	0.958
Site	12656.46	2	6328.23	26.801	***4.009E-06***	0.749
Treatment	2835.71	2	1417.85	6.005	***0.010***	0.400
Site*Treatment	5053.02	4	1263.25	5.350	***0.005***	0.543
Error	4250.07	18	236.12			
Day 251						
Intercept	82369.14	1	82369.14	413.732	***7.194E-14***	0.958
Site	8183.36	2	4091.68	20.552	***2.254E-05***	0.695
Treatment	1295.86	2	647.93	3.254	0.062	0.266
Site*Treatment	1817.91	4	454.48	2.283	0.100	0.337
Error	3583.59	18	199.09			
Day 369						
Intercept	149596.8	1	149596.8	411.014	***7.610E-14***	0.958
Site	503.4	2	251.7	0.692	0.514	0.071
Treatment	1471.0	2	735.5	2.021	0.162	0.183
Site*Treatment	807.4	4	201.9	0.555	0.698	0.110
Error	6551.5	18	364.0			

In Site 1, shoot cover was significantly reduced in single and repeated burial treatments at day 33, day 63 ($p = 0.03$ and $p = 0.004$) and day 168 ($p = 0.02$ and $p < 0.001$) (Figure 3.10). The effects of single and repeated burial treatments were statistically similar in Site 1. In Site 2, the effect of single burial treatments on relative shoot cover was significant at day 33 but could not be detected from 63-days onwards ($p > 0.05$). However, the effects of repeated burial treatment remained significant 63-days post first burial event ($p < 0.001$). From day-168 onwards, treatment effects were not significant in Site 2 ($p > 0.05$). Post-hoc testing found no significant effects of burial treatments throughout the 369-day sampling (Fig. 3.10).

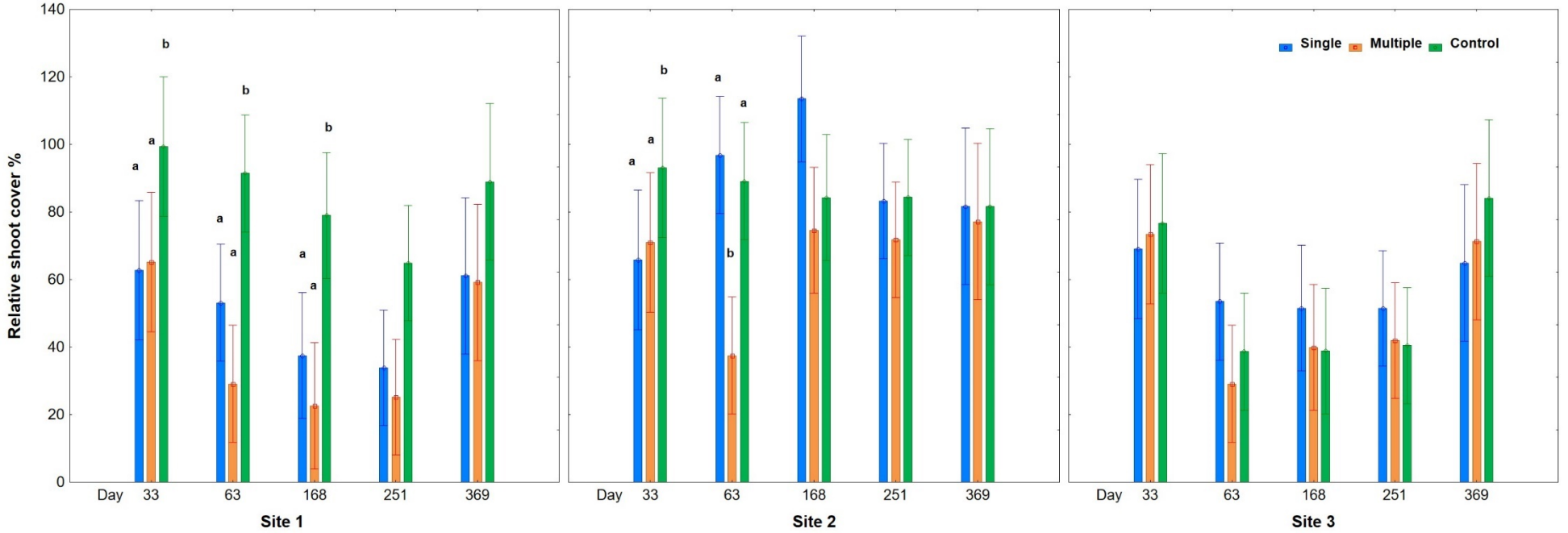

Figure 3.10 Mean relative shoot cover in single burial, repeated burial and control treatments in three sites in Tauranga Harbour, New Zealand in a 369-day period. Error bars denote 95% confidence intervals and different small cap letters (a,b) denote significantly different values of treatment groups at given sampling event (lack of denotation at sampling event = no significant differences between treatments) as a result of Tukey HSD post-hoc testing.

No significant treatment effects or interactions between "treatment x site" "treatment x time" influenced sucrose, starch or tNSC reserves (Table 3.3). However, there were significant interactions of "time x site" affected sucrose ($F_{[10,90]}$ = 7.05, $p < 0.001$), starch ($F_{[10,90]}$ =3.67, $p < 0.001$) and tNSC reserves ($F_{[10,90]}$ = 4.87, $p < 0.001$). As such, seagrass in Site 2 contained greater sucrose reserves than Site 1 on day 0 ($p < 0.001$), day 63 ($p < 0.001$) and 251 ($p < 0.001$), as well as Site 3 on day 0 ($p < 0.001$, Fig. 3.11).

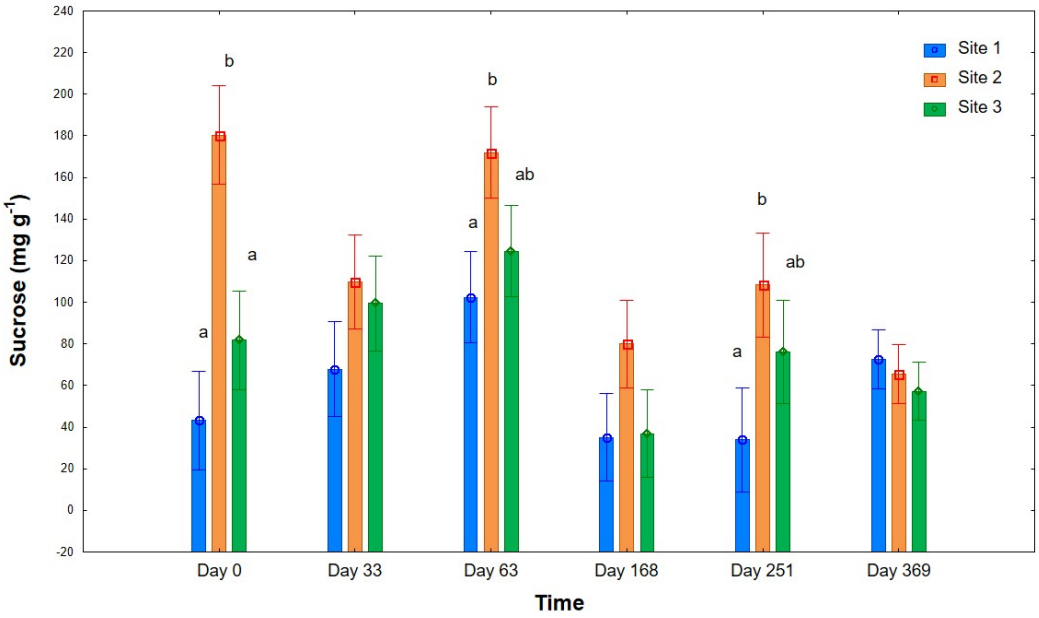

Figure 3.11 Mean sucrose content (mg g^{-1} DW rhizome) of *Zostera muelleri* in three sites in Tauranga Harbour, New Zealand measured 6-times over a 369-day period. Error bars denote 95% confidence intervals and different small cap letters (a,b) denote significantly different values of treatment groups at given sampling event (lack of denotation at sampling event = no significant differences between treatments) as a result of Tukey HSD post-hoc testing.

Starch reserves of seagrass in Site 2 were significantly higher than in Site 3 on day 251 ($p = 0.02$, Figure 3.12). Furthermore, tNSC reserves of seagrass in Site 2 were significantly higher compared to Site 1 on day 0 ($p < 0.001$) and day 251 ($p < 0.001$) as well as Site 3 on day 251 ($p < 0.001$, Figure 3.13).

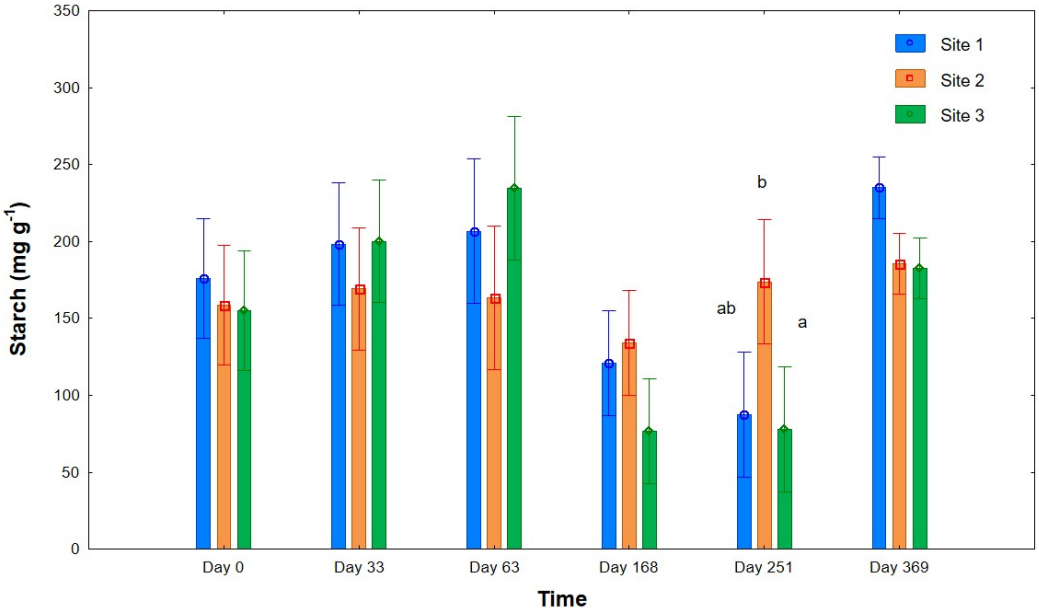

Figure 3.12 Mean starch contents (mg g^{-1} DW rhizome) of *Zostera muelleri* in three sites in Tauranga Harbour, New Zealand measured 6-times over a 369-day period. Error bars denote 95% confidence intervals and different small cap letters (a,b) denote significantly different values of treatment groups at given sampling event (lack of denotation at sampling event = no significant differences between treatments) as a result of Tukey HSD post-hoc testing.

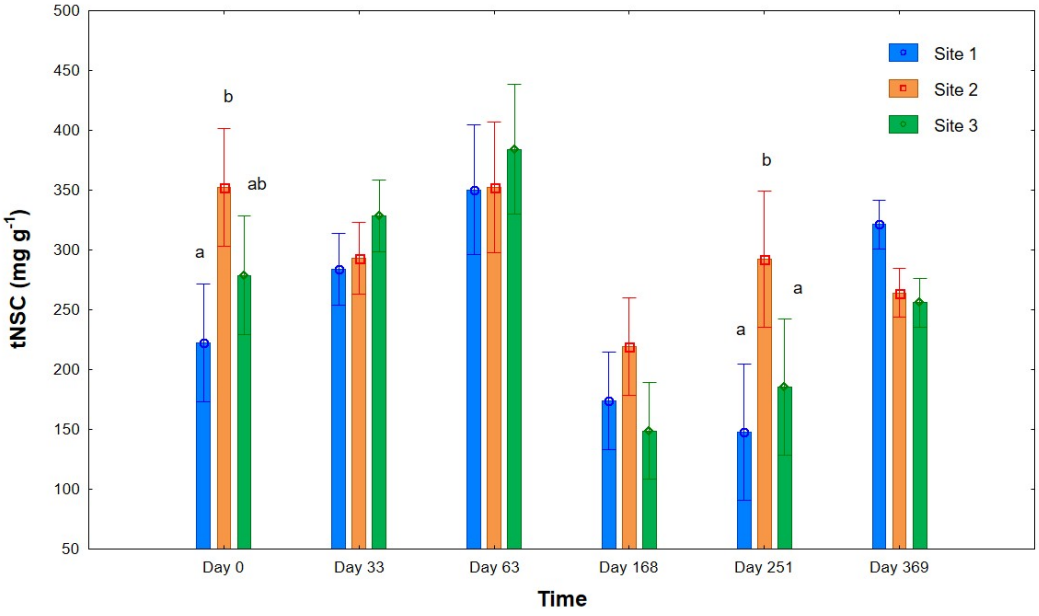

Figure 3.13 Mean total non-structural carbohydrate (tNSC) contents (mg g^{-1} DW rhizome) of *Zostera muelleri* in three sites in Tauranga Harbour, New Zealand measured 6-times over a 369-day period. Error bars denote 95% confidence intervals and different small cap letters (a,b) denote significantly different values of treatment groups at given sampling event (lack of denotation at sampling event = no significant differences between treatments) as a result of Tukey HSD post-hoc testing.

3.3.4 Predicting survival by rhizome non-structural carbohydrate contents

The correlations between rhizome NSC content and relative shoot cover at different stages throughout the recovery period (Figure 3.2) were explored in a correlation matrix (Table 3.5). There was a significant correlation ($r = 0.77$, $P < 0.001$) between initial rhizome sucrose content measured in summer 2016 (day 0) and relative shoot cover in winter ($P < 0.001$) and spring ($P < 0.001$, Table 3.5). No significant relationships existed between starch content at the height of the growing season and relative shoot cover at any stage throughout the year (Table 3.5).

Table 3.5 Correlations between non-structural carbohydrates (sucrose, starch and tNSC) in summer 2016 (February) and relative shoot cover in winter (July 2016), spring (October 2016) and the following summer (February 2017). Bold font indicates a significant Pearson correlation coefficient (r) at P-values < 0.05. Abbreviations: non-structural carbohydrates (NSC) and total non-structural carbohydrates (tNSC).

NSC reserves	**Relative shoot cover**					
Day 0 (Summer)	**Day168 (winter)**		**Day 251 (spring)**		**Day 369 (winter)**	
	r	P-value	r	P-value	r	P-value
Sucrose	0.74	**<0.001**	0.81	**<0.001**	-0.04	0.843
Starch	-0.20	0.367	0.01	0.976	-0.02	0.941

Significant relationships were then explored in linear regression analysis (Figure 3.14). Summer sucrose reserves explained 54% of the variation of relative shoot cover on day 168 (winter) and 66% on day 251 (spring).

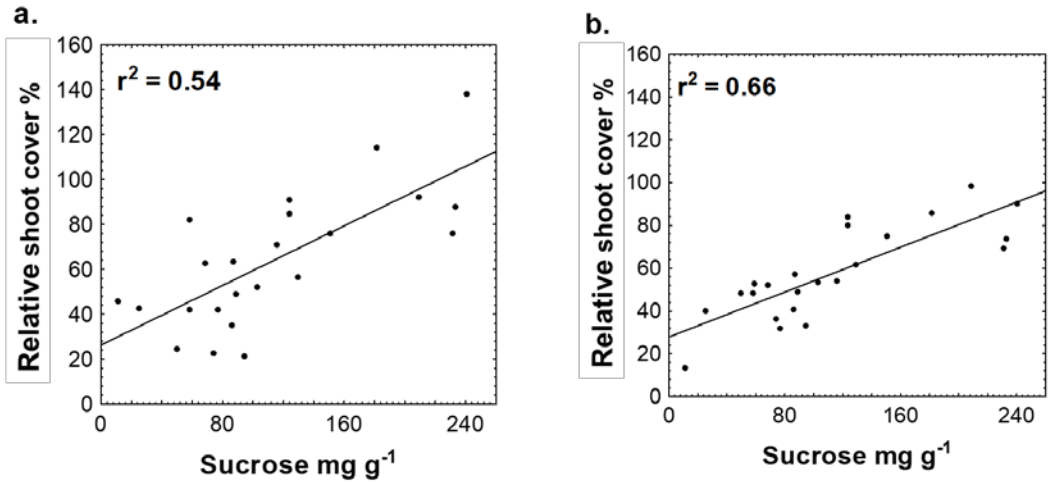

Figure 3.14 Relationships between *Zostera muelleri* rhizome sucrose content in summer and: **(a)** relative shoot cover the following winter; and **(b)** the following spring in Tauranga Harbour, New Zealand. Survival is measured as the percentage of initial surface cover.

3.4 Discussion

3.4.1 Investigation of site-specific seagrass characteristics before experimental treatments.

This study demonstrated that *Z. muelleri* in Tauranga Harbour experience significant spatial variation in morphometric and biochemical characteristics (Fig. 3.8) likely as a response to local conditions. Morphometric characteristics including blade width, blade length, surface cover, number of blades in the apical meristem and rhizome diameter suggested a significantly larger seagrass morphotype in Site 3 compared to Site 1 (Figure 3.8). Increased investment in aboveground biomass enhances the photosynthetic capacity of a plant and is a common acclimation of seagrass to sedimentation and chronic low light conditions (Bulthuis & Woelkerling, 1983; Lee & Dunton, 1997; Terrados *et al.*, 1998; Maxwell *et al.*, 2014). Thus, the large morphotype of seagrass in Site 3, likely reflects a growth morphology that maximises the potential to capture light and store reserves. In accordance, sediment grain size analysis (Figure 3.7) as well as environmental parameters such as SOM, sediment pH, DO and porewater nutrient contents (Table 3.2) support the hypothesis that Site 3 receives a higher degree of terrestrial influx compared to Site 1 and Site 2. Seagrass at Site 2 displayed a mix of morphological characteristics (Fig. 3.8), having narrow but long blades and intermediate surface cover, thus displaying some acclimation of the photosynthetic apparatus to enhance productivity.

This described morphometric variability of *Z. muelleri* exposed to different environmental conditions is likely a result of phenotypic plasticity rather than a genetic adaptation. Genetic adaptation involves the mutation of at least one gene within a population or a species that provides a fitness advantage in a particular environment or habitat (e.g., Orr, 2005) and is highly unlikely given the spatial scale of this study. Even small amounts of gene flow rapidly breakdown genetic adaptation, and as the three sites in this study are located in the same estuary, gene flow is expected to occur between the meadows (e.g., Jones *et al.*, 2008; Sherman *et al.*, 2016). Phenotypic plasticity is common among seagrass species and allows seagrass to survive in a wide range of environments (Peralta *et al.*, 2000; Peralta *et al.*, 2005; Maxwell *et al.*, 2014; McDonald *et al.*, 2016; Soissons *et al.*, 2018a).

The greater investment in aboveground tissues may in addition to potentially lower light availability, also be due to the elevated demand for gas exchange between above and belowground tissues as muddy environments become increasingly anoxic (Table 3.2). This gas exchange is crucial for seagrass roots to create and maintain oxygen shields, especially around vulnerable new roots, that protect from phytotoxins (such as hydrogen sulphides) in the rhizosphere (Brodersen *et al.*, 2015; Campbell *et al.*, 2018; Martin *et al.*, 2019). Short (1987) demonstrated that *Z. marina* grown in muddy substrates had significantly larger blades (leaf biomass, leaf weight and shoot height) than those grown in sandy substrates. These adaptations are consistent with the *Z. muelleri* characteristics measured in this study and previous studies (Kohlmeier *et al.*, 2014; Ferguson *et al.*, 2016). However, as the sediment mud content increases, so does the metabolic demands of the oxygen-pump and when a tolerance threshold is reached, seagrass communities experience adverse effects such as a rapid reduction in species richness and community leaf biomass (Terrados *et al.*, 1998). Therefore, it appears that *Z. muelleri* in Site 3 has not yet reached its tolerance threshold for sediment mud-content, as the plants here have the largest biomass of the three sites sampled.

Seagrass in Site 2 had almost 4-fold more rhizome sucrose content than Site 1, more than 2-fold that of Site 3, and faster rhizome elongation rates (Fig. 3.8). The high energy reserves in Site 2 may be linked to the higher porewater nutrient concentrations (Fig. 3.2), indicating that the three meadows were likely to be nutrient-limited. Addition of fertilisers to the rhizosphere of nutrient-limited meadows is known to increase seagrass growth and biomass (Balestri & Lardicci, 2014; Jackson *et al.*, 2017). However, the average horizontal rhizome growth rate in Site 1 was similar to Site 2 (Figure 3.8), despite Site 1 appearing to have lower porewater nutrient contents than Site 2 (Fig. 3.2). In contrast, Site 3 had significantly slower rhizome growth rates than Site 2 (73.00 ± 11.31 cm $year^{-1}$), which may be a reflection of the higher investment in aboveground biomass to compensate for low light levels and/or high sediment mud-content (i.e. Short, 1983, 1987; Peralta *et al.*, 2002) rather than porewater nutrients.

3.4.2 Effects of burial treatments on *Z. muelleri*

Evidence presented in this study suggests significant spatial and temporal variabilities in the effects of burial events. The large seagrass morphotype in Site 3,

displayed a high level of resistance to burial as no treatment effects were detected following both single and repeated burial events. In contrast, the smaller morphotypes in Site 1 and Site 2 expressed lower resistance to the burial disturbances but recovered at different rates. As such, relative shoot cover of the small seagrass morphotype in Site 1 recovered within 251 days following both single and repeated burial events, whereas, single burial treatments recovered within 63 days and repeated burial treatments within 168 days in site 2. This increased ability to recover following a disturbance reflects an increased resilience of seagrass in Site 2, which may be linked to the significantly higher sucrose reserves of seagrass in this site.

Rhizome NSC reserves at the three sites were not influenced by the single or repeated burial treatments. A mesocosm study has since demonstrated that severely stressed *Z. muelleri* sprigs, mobilise all rhizome sucrose stores within ten days of severe light reduction (Appendix C). Similarly, sucrose stores of *C. nodosa* are depleted in just over six days of light-starvation in a laboratory trial (Drew, 1978b). It is therefore likely that the sampling regime in this study (NSC levels measured one month post each sediment addition) was unable to capture the potential mobilisation and recovery of NSC stores of seagrass *in situ*. Furthermore, the lack of treatment effect on NSC stores in this current study may also reflect successful resource transfer from unaffected ramets to the experimental units via the maintained clonal integration (Vermaat, 2009; Ooi *et al.*, 2011; Tuya *et al.*, 2013; Benham *et al.*, 2019). Resource transfers may, therefore, moderate the effects of burial on *Z. muelleri*. It is important to note that the translocation of resources within a genet may not be possible if a large-scale burial caused a meadow to become isolated or fragmented, in which case clonal integration may be irrelevant and the effects of burial would be more severe than observed in this study.

This study demonstrates a significant relationship between rhizome sucrose content and seagrass survival, with summer sucrose content explaining 54% of winter survival and 66% of spring survival (Fig. 9). Declining daylight hours and temperatures coupled with increases in storm frequency and precipitation occur seasonally throughout autumn and winter in Tauranga Harbour (see Appendix A, Fig. A.1) which may influence seagrass photosynthetic rates and may result in blade senescence and loss of belowground biomass (e.g., Vermaat *et al.*, 1987; Lee *et al.*,

2007). Seagrass in Site 3 experienced significantly reduced relative shoot cover in winter (day 168, Figure 3.10), consistent with previously described seasonal patterns for temperate seagrasses (e.g., Dawes & Lawrence, 1980; Bulthuis & Woelkerling, 1983; Vermaat *et al.*, 1987; Kerr & Strother, 1990; Vermaat & Verhagen, 1996; Ramage & Schiel, 1999; Mascaró *et al.*, 2014). Sedimentation influx can lead to higher mud contents (Burkholder *et al.*, 2007), anoxic conditions and elevated concentrations of porewater ammonium (van Katwijk *et al.*, 1997) and sulphides (Brodersen *et al.*, 2015; Martin *et al.*, 2019). This consequently increases the respiration of belowground tissues and overall higher metabolic demands, making seagrass more vulnerable in winter conditions. Burial treatments were instigated in summer (February) when rhizome NSC contents are at an annual high (Figure 3.11-3.13). It is, therefore, reasonable to expect that seagrasses have lower resilience to burial in winter (July) when the mean level of rhizome sucrose contents constitutes only 46% of the energy reserves, which are available in summer when production is high.

The different hydrodynamic forcing in the three study sites meant that sediment levels returned to control levels at different rates (Figure 3.6). Despite Site 1 experiencing the fastest remobilisation of sediments and, thus, the shortest burial duration, seagrasses here experienced the most long-term effects of both burial treatments (up to 251 days recovery). The burial treatments in this study simulated a major burial event that might occur during a severe storm (e.g., Browning *et al.*, 2019), as 100% of the blades were covered at the onset of experiment (Figure 3.3). In Site 3, the sediment levels were still significantly elevated after one-month, however, seagrass remained unaffected by burial treatments (Figure 3.10-3.13). This infers that either the leaves bounced through the deposited sediment or the horizontal rhizomes migrated vertically towards the sediment surface. In either case, the potential for a plant to achieve uncovering may be linked to the APB. Hence, the morphometric acclimation of *Z. muelleri* to local conditions including increased blade length, appear to mediate the effects of burial as these ultimately lower the APB of a given burial event. Resilience to burial is known to be species-specific (Cabaço & Santos, 2007), however, this study reveals that it is crucial to evaluate the phenotypic expression of seagrass in a specific environment to obtain an accurate estimate of its resilience to burial.

3.5 Conclusions

The outcomes of the research presented in this chapter demonstrate that intra-specific variations in seagrass morphometric and biochemical characteristics significantly influence the resilience of *Z. muelleri* to burial. In conclusion, recovery trajectories following burial events are highly site-specific, and it is therefore important that resilience-assessments of *Z. muelleri* occur at appropriate spatial scales. Similarly, activities that may cause sediment impact need to be assessed on a site by site scale to determine if seagrasses may be unduly impacted. Furthermore, the significance of rhizome sucrose stores for seagrass survival, suggests that resilience varies not only spatially but also temporally as energy stores varied seasonally. The timing of a disturbance is therefore likely to be a determining factor of seagrass survival and resilience is expected to be lowest in winter due to significantly reduced sucrose stores.

Chapter 4

A tentative study of non-structural carbohydrate partitioning of *Zostera muelleri* at different spatial scales: Exploring the relationships between environmental and morphometric variables and carbohydrate partitioning

Top photo: Intertidal seagrass meadow in Avalon Shore, Port Phillips Bay, Victoria, Australia; **Bottom photo**: Intertidal seagrass meadow in Cockle Bay, Magnetic Island, Queensland, Australia. Photos: Sørensen, S. T.

4.1 Introduction

Climate plays an integral part in the distribution of seagrass (Lee *et al.*, 2007) and species-specific minimum light requirements and optimum temperature ranges generally dictate the environment in which a species can be found (Dennison *et al.*, 1993; Zimmerman *et al.*, 1995; Collier *et al.*, 2017). Photosynthesis and respiration rates of seagrasses increase with temperatures until a thermal threshold is reached after which photosynthetic activities begin to decline and respiration may exceed photosynthetic rates (Touchette & Burkholder, 2000). At this stage non-structural carbohydrate (NSC) reserves are mobilised to meet the metabolic deficit, thus resulting in a negative carbon balance and eventually mortality (Touchette & Burkholder, 2000; Campbell *et al.*, 2006; Collier *et al.*, 2011; York *et al.*, 2013; Collier *et al.*, 2017). Carbon reserves, therefore, reflect the imbalances in supply and demand on various temporal-scales (incl. diel, seasonal and decadal scales) and provide a quantifiable measure of plants' current health and resilience (Hartmann & Trumbore, 2016; Martínez-Vilalta *et al.*, 2016; Sørensen *et al.*, 2018).

Recent studies revealed that estimations of NSC concentrations in plant tissues are non-comparable when performed by different analytical protocols (Quentin *et al.*, 2015; Sørensen *et al.*, 2018). As such, an assessment of previous research efforts does not immediately clarify the specific roles of the main energy reserves in seagrass (Sørensen *et al.*, 2018) and studies investigating the driving forces of NSC allocation of seagrasses in heterogeneous environments are currently lacking. *Zostera muelleri* is distributed along a broad latitudinal gradient including both temperate and tropical regions, thereby, providing an opportunity to investigate the distribution trade-offs of seagrass NSC reserves across climatic regions. Ultimately, this knowledge may assist in the prediction of *Z. muelleri's* ability to cope with future climate changes.

The disaccharide sucrose (composed of the monosaccharides glucose and fructose) is smaller than the polysaccharide starch and is, therefore, usually the preferred mode of transport in the phloem and is more readily broken-down into its reducing components. As a result, soluble sugars are rapidly available to subsidise metabolism (short-term storage) but also perform transitional functions such as osmoregulation and cryoprotection whereas, starch act as long-term energy storage

(Touchette & Burkholder, 2000; Hartmann & Trumbore, 2016; Martínez-Vilalta *et al.*, 2016). A recent study investigating the driving forces behind NSC allocation in the reed *Phragmites australis* in China found that while the total NSC content (rhizomes) was similar along an environmental gradient from wetland to desert, the soluble sugar content increased and the starch content decreased (Jiao *et al.*, 2020). This indicated that as the environment becomes less than optimal (i.e. more arid), the ratio of soluble sugars to starch increased as starch is broken down to increase soluble sugar levels to maintain osmotic balance and support increased metabolic demands (Jiao *et al.*, 2020). In contrast, two pine tree species (*Pinus sylvestris* and *Picea abies*) in Russia (Ivanov *et al.*, 2019) as well as the laurel *Laurus nobilis* in Italy (Trifilò *et al.*, 2017) responded to drought/water stress with increasing starch levels but decreasing levels of sugars. As such, it appears that the distribution trade-offs in NSC reserves may differ between plant species in terrestrial systems.

In subtropical Moreton Bay, Australia (latitude 27°S), *Z. muelleri* that are impacted by freshwater and terrestrial run-off contain higher levels of starch reserves but lower levels of sucrose reserves compared to less impacted meadows (Maxwell *et al.*, 2014). This trade-off may reflect an adaptation to facilitate survival in a chronically degraded environment. Regardless of prior acclimation, soluble NSC reserves acted as short-term energy reserves as these were mobilised immediately following a flood disturbance, while, starch reserves were mobilised more gradually (Maxwell *et al.*, 2014).

Seagrass meadows in high latitudes often experience greater seasonal variations in temperature, light availability and hydrodynamic conditions compared to meadows in lower latitudes (Duarte, 1989; Mascaró *et al.*, 2014). Climatic conditions influence plant phenology and cause leaf senescence when seasonal conditions become unfavourable, allowing the transition from an active to a dormant stage (Estiarte & Peñuelas, 2015). As such, the perennial seagrass *Z. noltii* experiences winter senescence in latitudes above 44°N along the west coast of continental Europe but exist as evergreen meadows in latitudes below 44°N (Soissons *et al.*, 2018b). Ideally, leaf senescence allows deciduous plants to reabsorb nutrients from leaves into storage tissues and these energy reserves become crucial to support respiration throughout winter and regrowth in spring (Estiarte & Peñuelas, 2015). In accordance, it appears that winter survival of *Z. noltii* in the Netherlands (latitude

51°N) is positively related to starch reserves at the end of the previous growing season (Govers *et al.*, 2015). Soissons *et al.* (2018) demonstrated a positive relationship between latitude and starch reserves of *Z. noltii* when measured at the end of the growing season, suggesting that starch accumulation is important to temperate populations especially. A study by Drew (1980) suggested that sucrose reserves also increase in seagrass blades with increasing latitudes, although estimates were from various seagrass species and various seasons. Evidence, therefore, suggests that both sucrose and starch increase with latitude, presumably due to a higher dependence on energy reserves for winter survival and regrowth in spring/summer in high-latitude seagrasses. However, there is a paucity of information about the distributional trade-off between sucrose and starch for seagrass across large-spatial scales.

Non-structural carbohydrate contents of seagrass may alter at different life-stages. When young plant tissues undergo fast development and the carbon demand is high which can be measured as an increase in sucrose synthase activity (Sturm & Tang, 1999). The enzyme sucrose synthase (EC 2.4.1.13) cleaves sucrose into fructose and uridine diphosphate (UDP-) glucose, which may allow plants to meet their carbon demand. As development rates slow down with tissue age, sucrose synthase activity appears to decrease in the congener *Z. marina* root tissues (Kraemer & Alberte, 1993). Concurrently, the activity of the enzyme sucrose-P synthase, which is involved in sucrose synthesis, increases as a tissue matures, and tissues may shift from being a carbon sink to a carbon source (Touchette & Burkholder, 2000). As meadows tend to spread from a central point, a higher proportion of older ramets may exist in the mid-meadow compared to the edge in an expanding meadow (Duarte & Sand-Jensen, 1990; Duarte *et al.*, 1994), potential influencing the spatial variability of NSC within meadows. Furthermore, conditions may also vary at these small scales as edge plants tend to expand into more anoxic environments, thereby experiencing higher metabolic rates associated with the creation of oxygen-shields to protect new roots from phototoxins like hydrogen sulphides (Brodersen *et al.*, 2015; Martin *et al.*, 2019)

The ecological and physiological roles of sucrose and starch in seagrasses remain unclear. Environmental processes vary at different spatial scales and understanding how this influence the specific roles of sucrose and starch will allow for these bio-indicators to be included as a critical component in seagrass models to predict resilience with higher accuracy. This chapter acts as a tentative investigation of NSC partitioning across different spatial scales (within meadow, between sites, between regions) and an exploration of the drivers behind this. The main objectives of this study are: **1)** To investigate the partitioning of NSC reserves of *Z. muelleri* at different spatial scales and; **2)** To explore the relationships between NSC groups and environmental and morphological variables.

4.2 Materials and methods

4.2.1 Sample sites and sample design

To test the effects of large-scale processes on NSC partitioning, *Z. muelleri* tissues were collected in a tropical region in Townsville (TWN) and a temperate region in Port Phillip Bay (PPB) in Australia (Figure 4.1). In each region, samples were collected across two sites in a one-off sampling event in austral spring (Oct – Nov 2017), when seagrass productivity is high resulting in the replenishment of NSC stores due to increasing daylight hours and temperatures (e.g., Pirc, 1989; Kerr & Strother, 1990; Burke *et al.*, 1996; Soissons *et al.*, 2018b). The daylight duration in the tropical TWN locations (12 hours 54 min) was similar to that of temperate PPB location (13 hours 17 min) at the time of sampling. Sampling occurred at neap tides upon the first low tide of the day (Table 4.1).

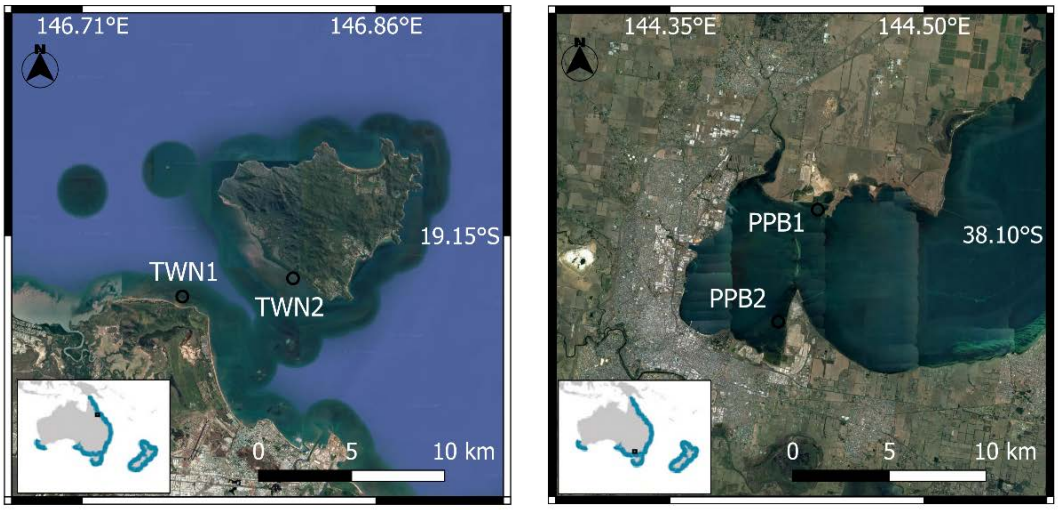

Figure 4.1. Locations of the four sample sites in the two different climatic regions in Townsville (TWN1 and TWN2) and Port Phillip Bay (PPB1 and PPB2). Small map inserts show the global distribution of *Zostera muelleri*, adapted from Waycott *et al.* (2014).

Table 4.1 Location of sites and time of sampling.

Site	Latitude	Longitude	Sample date	Low tide
TWN1	19°18`415 S	146°76`174 E	22/10/2017	15:59hrs
				1.36 m
TWN2	19°17`853 S	146°83`138 E	30/10/2017	11:58hrs
				1.16 m
PPB1	38°08`490 S	144°43`290 E	7/11/2017	12:40hrs
				0.44 m
PPB2	38°14`120 S	144°41`770 E	8/11/2017	13:27hrs
				0.44 m

Edge plants and mid-meadow plants were sampled in each site to assess small-scale processes with-in a meadow that may affect NSC partitioning (Duarte & Sand-Jensen, 1990; Duarte *et al.*, 1994; Alcoverro *et al.*, 2001). Triplicate core samples (15 cm diameter, 10 cm deep) were collected along the meadow edge (edge plants, n = 3), and in the mid-meadow, at least one meter from the edge (mid-meadow, n = 3). Core samples were sieved in ambient seawater on-site to rinse sediments from the seagrass tissue before each sample was placed in separately labelled bags and placed in a cooler box. Upon return from the field, seagrass tissues were rinsed to

remove epiphytes and attached fauna, photographed, labelled and frozen (≤ -10°C) until sample analysis could occur (see section 4.2.3).

4.2.2 Environmental conditions

4.2.2.1 Long-term climate averages

Monthly mean (min/max) air temperatures, sea surface temperatures (SST) and solar radiation values were acquired from the Australian Bureau of Meteorology (http://bom.gov.au) for Townsville (station Townsville, QLD) and Port Phillip Bay (station Geelong North, VIC). Available long-term data ranged from 1998 to 2017.

4.2.2.2 Sediment temperature and pH

Triplicate readings of temperature and pH were collected from both the edge and the mid-meadow in each site. Sediment temperature profiles were measured at the sediment surface and in-sediments using a portable waterproof digital thermometer ('Pocket temp' IP65 Digital Probe; HLP Controls, South Windsor, NSW, Australia) with a ± 0.1°C accuracy. The thermometer was gently placed on the sediment surface or pushed 5 cm into the substratum (in-sediment) and allowed to stabilise before the temperature was recorded. Sediment pH was measured using a portable electronic pH meter (McGregor's). The metallic probe was gently pushed 5 cm into the sediment and pH recorded directly once the display reading had stabilised.

4.2.2.3 Sediment particle composition and sediment organic matter analysis

For each seagrass core sample collected, a sediment core sample (2.4 cm diameter, 5 cm depth) was collected immediately adjacent to the seagrass core, thus triplicate sediment samples from the edge and the mid-meadow were collected from each site. These samples were immediately placed in a dark container and upon return from the field were frozen (≤ -10°C) until laboratory analyses were conducted. Sediment samples were defrosted and dried in an oven for 72 h at 60°C, after which these were homogenised and subsampled for sediment particle analysis and sediment organic matter (SOM) analysis.

Sediment particle size analysis was conducted using a modified pipette method (Gee & Bauder, 1986). Samples for SOM analysis were filtered through a 2 mm sieve and combusted in a muffle furnace for 5.5 h at 550°C. The organic matter

content was measured as the percentage weight loss of dry sediment sample from the dry weight to the ash-free dry weight, as shown in Equation 4.1:

Equation 4.1:

$$\text{SOM} = \frac{\text{sediment ash-free weight (DW)}}{\text{sediment weight (DW)}} \text{ x } 100$$

4.2.2.4 Porewater nutrient content

In each site, three porewater samples were collected at the edge (n = 3) and mid-meadow (n = 3) immediately adjacent to seagrass core samples (total n = 6 porewater samples). The top 5 cm of sediment was collected with a 2.5 cm diameter syringe. Porewater samples were placed into a dark container, stored (≤ -10°C) until laboratory analyses were conducted. Before analysis, porewater was defrosted and filtered through a 0.45 µm syringe filter (Whatman). Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) ions were determined using flow injection analysis based on automated colorimetric methods (Greenberg *et al.*, 2005b, 2005a).

4.2.3 Seagrass metrics

4.2.3.1 Morphometric characteristics

Triplicate subsamples of seagrass shoots were randomly selected from each core sample and then photographed before samples were frozen for later analysis of rhizome NSC content. The photographs enabled digital image analysis using Adobe Acrobat Reader DC (version 2018.009.20044) for measurements of rhizome diameter, root length, blade width, blade length, and internode distance between the apical meristem and the second shoot (Figure 4.2).

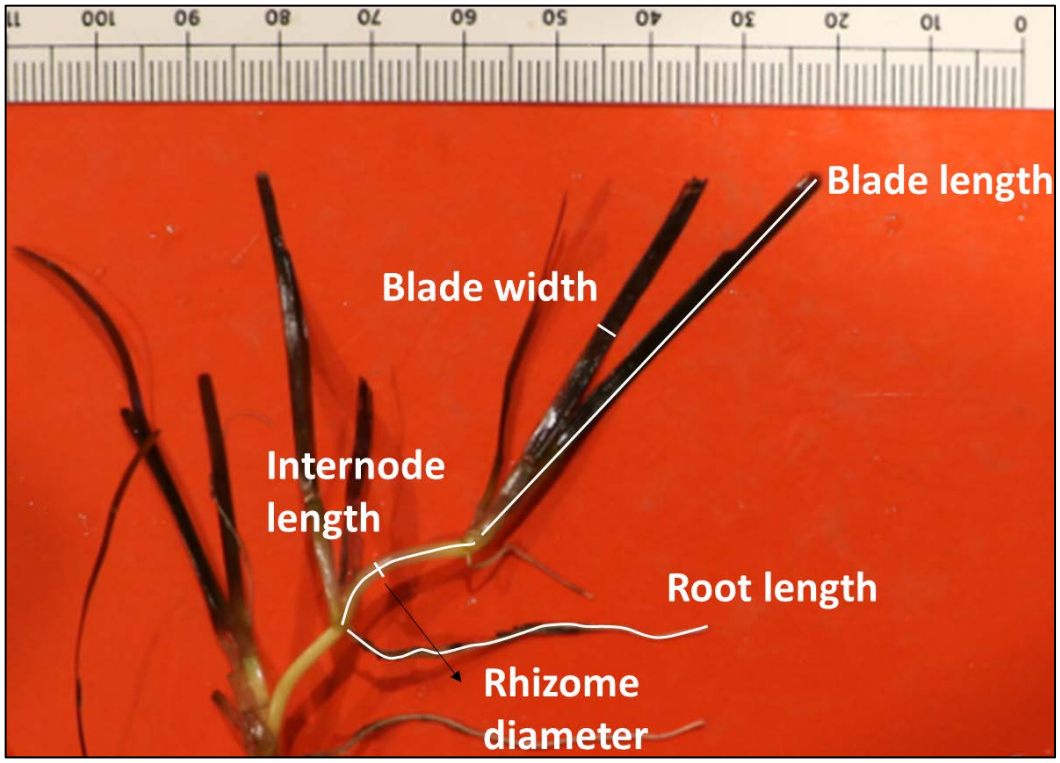

Figure 4.2. Diagram indicating the morphometric variables measured using digital image analysis.

Dry weight (DW) of aboveground biomass (blades and shoots) and belowground biomass (rhizomes and roots) were measured after freeze-drying (-40°C) the samples to constant weight (minimum 5 hr). The above and belowground biomass values were used to calculate the above to belowground biomass ratio (ABR) using Equation 4.2:

Equation 4.2:

$$\text{ABR} = \frac{\text{aboveground biomass (g DW)}}{\text{belowground biomass (g DW)}}$$

4.2.3.2 Non-structural carbohydrate quantification

Rhizome samples were analysed for NSC content using the methodology outlined in Chapter 2. However, liquid chromatography-mass spectrometry (LC-MS) on a Shimadzu LCMS 8030 was used for the analysis of samples as it was the available instrumentation at the laboratory at Federation University, Australia, where sample analysis was conducted. The separation of components in samples was achieved with a Shodex SUGAR KS-801 column eluted with water (0.4 mL/min, 50°C) and detection was accomplished in positive mode with the interface voltage set at 4.5 kV.

Rhizomes were rinsed in deionised water and then freeze-dried (-40°C) to constant weight (24 h). Soluble NSC was extracted in deionised water in three sequential extractions (15 min at 80°C) and the supernatant pooled before being analysed by single ion monitoring on the LC-MS system. The content of sucrose, glucose, and fructose was calculated using external calibration curves derived from standard sucrose, glucose and fructose (Aldrich).

Starch was solubilised from the rinsed solid residue in hydrochloric acid (HCl 1M) for 30 min at 100°C, centrifuged and the resultant supernatant hydrolysed in HCl (1M) for a further 180 min at 100°C. Treatments were terminated by neutralising the sample with sodium hydroxide (NaOH 1M) and quantitated as glucose equivalents by LC-MS. Sucrose, glucose, and fructose represent soluble sugars, and total non-structural carbohydrates (tNSC) are soluble sugars and starch combined. Using a different detector, in this case, the MS-detector, did not affect the accuracy of the method described in Chapter 2 as the higher sensitivity of the MS-detector compared to the RI-detector was adjusted for by diluting the samples and standards 20-fold.

4.2.4 Statistical analysis

Environmental variables were compared by one-way ANOVA models and Tukey HDS post-hoc tests. Multilevel regression models were produced in R-studio, Inc. using the "lme4" package with maximum likelihood estimations. Models were used to test for significant relationships between the dependent variables (sucrose, starch, tNSC and Su:St) and the fixed factors "region" (two levels = TWN/PPB) and "meadow" (two levels= mid/edge). For each dependent variable, a null model was fitted using "Site" (four levels) as a random factor to account for spatial autocorrelation. Before fitting models, residuals were assessed for normal distribution in probability plots (QQ plots). Further models were produced by adding one fixed factor at the time, all of which were compared to the null model. The best performing model was selected using the Bayesian Information Criterion (BIC), with lower BIC values being better supported by the data (Raftery, 1995). Tukey HDS post-hoc tests were used to evaluate the significant effects identified in the multilevel models.

Correlation analyses were used to investigate correlations between environmental variables and NSC reserves as well as between morphometric variables and NSC reserves. Stepwise linear regression (SLR) models with a backwards selection were then used to evaluate spatial variables (categorical variable), environmental variables and morphometric variables as predictors of sucrose and starch content. Predictor variables that were correlated to the dependent variable, were first screened for autocorrelation and deselected if $r > 0.7$. All remaining predictor variables were then added to the model and the least significant (lowest r^2) variables removed one at the time until no none significant variables were included in the model.

4.3 Results

4.3.1 Environmental conditions

4.3.1.1 Long-term averages

In Townsville, the highest monthly average air temperature was 29.2°C (min/max: 25.3 - 31.2°C) in December, while the lowest average was 21.8°C (min/max: 17.2 - 23.8°C) in June (Figure 4.3a). In Port Phillip Bay, the highest monthly mean air temperature was 26.3°C (min/max: 24.0 – 28.4°C) in January, one month later than annual average maxima in Townsville. The lowest mean air temperature was 14.1°C (min/max: 12.2 – 15.7°C) in July (Figure 4.3a). The difference in average air temperature between Townsville and Port Phillip Bay range from 1.90°C in February to 8.10°C September. In Townsville, the average monthly sea surface temperature (SST) peaks at 29.2°C (min/max: 28.1 – 30.2°C) in February and is lowest at 22.8°C (min/max: 21.2 -24.3°C) in July and August (Figure D.4.3b). In Port Phillip Bay the highest average SST 18.25°C (min/max: 16.9 - 19.6°C) also occurs in February, while the annual low SST is 13.8°C (min/max: 13.1 - 14.5°C) and occurs in August and September.

In Northern Queensland, Australia the wet season runs from November to April and cloud cover, therefore, increases throughout the summer, causing highest solar radiation in Townsville to occur in spring in November (mean: 26 MJ m^{-2}, min/max: 19 - 28 MJ m^{-2}, Figure 4.3c). Whereas, in temperate Port Phillip Bay, highest monthly average solar radiation (mean 25 MJ $m^{-2,}$ min/max: 22 - 30 MJ m^{-2}) occurs

mid-summer in January (Figure 4.3c). Meanwhile, the lowest monthly averages occur, as expected, in winter (June) in both Townsville (15 MJ m^{-2}, min/max: 11 – 17 MJ m^{-2}) and Port Phillip Bay (6 MJ m^{-2}, min/max: 6 - 7 MJ m^{-2}, Figure 4.3c).

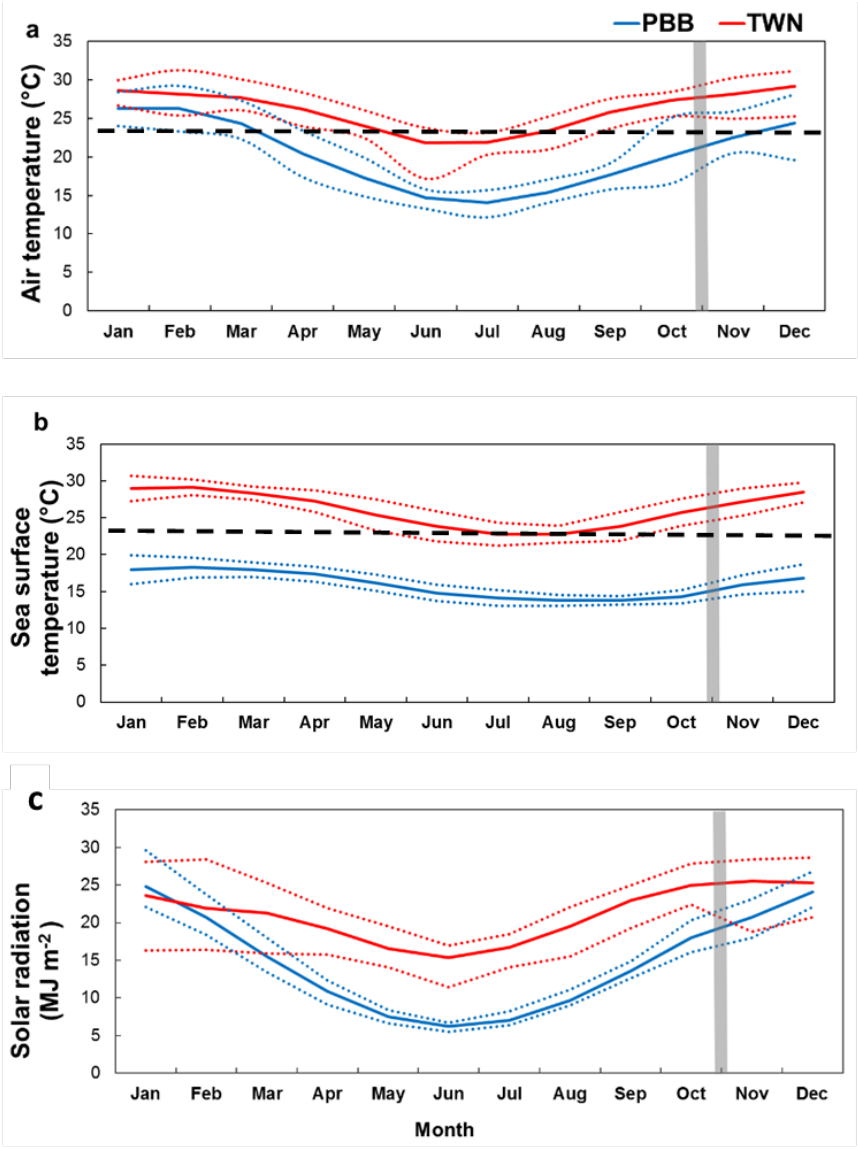

Figure 4.3 Monthly mean environmental variables in - Townsville (TWN) and - Port Phillip Bay (PPB) for **a)** air temperatures; **b)** sea surface temperatures; and **c)** solar radiation. Mean values from cover data from 1998 to 2017 (TWN station: Townsville, QLD and PPB station: Geelong North, VIC) published by the Australian Bureau of Meteorology (http://bom.gov.au). Dotted lines denote the minimum and maximum values. The grey vertical line represents the time of sampling. Black dashed line represents *Z. muelleri* thermal optima for growth (Collier *et al*. 2017).

4.3.1.2 Site conditions

Measured temperatures at sediment surface (range from 15.72°C to 30.95°C) and in sediments (range from 15.23°C to 30.95°C) was significantly different

($F_{[3,6]}$=511.05, p <0.001) between the four sites at the time of sampling (Table 4.2). Site PPB2 had significantly higher sediment mud/silt content (8.25 ± 0.83%) than all other sites, and PPB1 (5.08 ± 0.87%) had higher sediment mud/silt than TWN2 (3.18 ± 0.73%, Table 4.2). The SOM percentage content was significantly higher in PPB2 (6.25 ± 0.9%) than PPB1 (3.68 ± 0.56%) and TWN1 (3.70 ± 0.53%, Table 4.2). The content of nitrogen species including NH_3, NO_x, nitrite and nitrate were similar in the porewater of the four sites (Table 4.2). Additionally, sediment pH levels ranged from 6.42 to 6.83, however, were not significantly different between sites (Table 4.2).

Table 4.2 Results of one-way ANOVAs comparing environmental variables between sites. Bold font and * denote statistical differences (F < 0.05). Where significant differences between sites occur, similar letter (^{a,b,c,d}) indicate that mean values are not significantly different between specific sites (P > 0.05 in Tukey HSD post-Hoc tests).

		TWN1 (a)		**TWN2 (b)**		**PPB1 (c)**		**PPB2 (d)**		**One-way ANOVA**		
Variable	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	df	F-stats	P-value
Sediment surface temperature (C°)	6	27.18	± 0.44^{a}	30.95	± 0.76^{b}	15.72	± 0.20^{c}	19.03	± 0.24^{d}	3	511.05	***<0.001**
In-sediment temperature (C°)	6	27.83	± 0.30^{a}	31.28	± 0.25^{b}	15.23	± 0.37^{c}	17.52	± 0.62^{d}	3	360.33	***<0.001**
Mud/silt (%)	6	4.12	± 0.82^{ab}	3.18	± 0.73^{b}	5.08	± 0.87^{a}	8.25	± 0.83^{c}	3	7.362	***0.002**
SOM (%)	6	3.70	± 0.53^{a}	4.94	± 0.12^{ab}	3.68	± 0.56^{a}	6.25	± 0.92^{b}	3	4.151	***0.019**
pH	6	6.42	± 0.15	6.62	± 0.14	6.83	± 0.03	6.77	± 0.05	3	3.052	0.052
NH_4^+ (mg L^{-1})	6	0.863	± 0.18	0.502	± 0.15	0.462	± 0.11	0.519	± 0.20	3	1.282	0.308
NO_2^- (mg L^{-1})	6	0.004	± 0.00	0.006	± 0.00	0.006	± 0.00	0.003	± 0.00	3	1.326	0.294
NO_3^- (mg L^{-1})	6	0.048	± 0.01	0.047	± 0.01	0.050	± 0.01	0.038	± 0.00	3	0.293	0.830

4.3.2 Influence of spatial scale on non-structural carbohydrate partitioning

The overall mean tNSC was 193.53 ± 10.88 mg g^{-1}. A multilevel regression model including only the random factor "site" (Model 0) produced the best fit to tNSC, suggesting that there was significant clustering between the intercepts of the four sites (Table 4.3). However, only 5% of the variation of tNSC (pseudo r^2 = 0.05, Table 4.3) could be explained by this model and is therefore not a strong model for predicting this dependent variable and a Tukey HDS test did not identify significant differences in tNSC amongst sites (Figure 4.4).

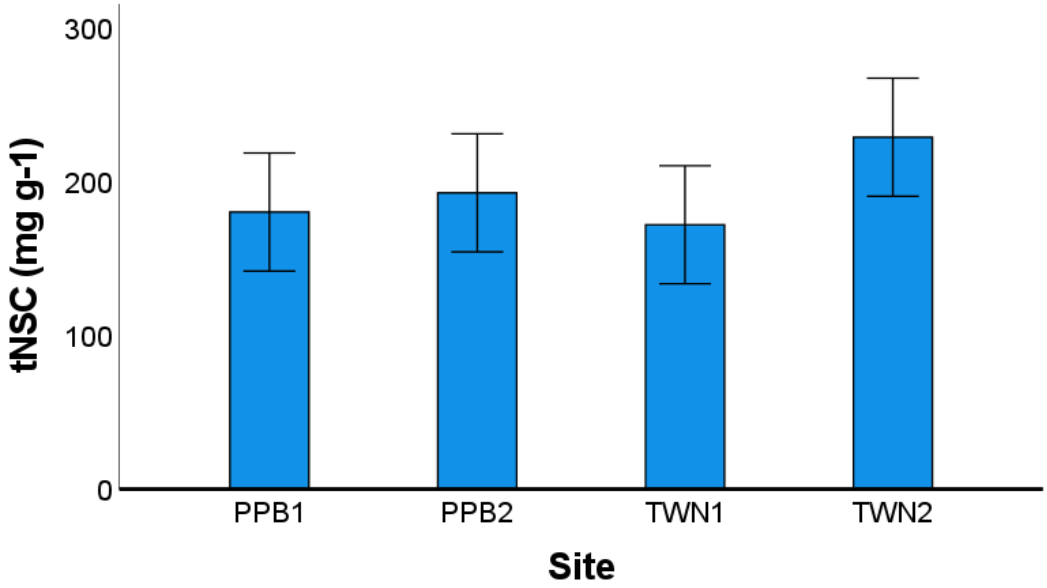

Figure 4.4 Mean total non-structural carbohydrate (tNSC mg g^{-1} DW rhizome) of *Zostera muelleri* measured in the edge and mid-meadow of four sites across two regions (Port Phillip Bay (PPB) and Townsville (TWN)) in Australia). Error bars denote standard errors of means.

Table 4.3 Multilevel regression model used to test for significant relationships between the dependent variables (sucrose, starch, tNSC and Su:St) and the fixed factors "region" (two levels = TWN/PPB) and "meadow" (two levels= mid/edge). A null model was fitted using "site" (four levels) as a nested factor to account for spatial autocorrelation (Model 0). The fixed factor 'region" was added to Model 1 and both fixed factors plus their interaction (region, meadow, region x meadow) to Model 2. The best fitting model is indicated by the lowest BIC in **bold** for each dependent variable.

		Model 0					**Model 1**					**Model 2**				
Dependent	**Independent**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**p**
tNSC	Intercept (Site)	193.530	10.880	17.800	4.000	**<0.001**	186.610	14.570	12.806	4.000	**<0.001**	45.710	12.230	3.740	24.000	**0.001**
	Region (TWN)						13.950	20.610	0.677	4.000	0.536	68.020	17.300	3.930	24.000	**0.001**
	Meadow (edge)											32.280	17.300	1.870	24.000	0.070
	Region x meadow											-57.360	24.460	-2.340	24.000	**0.028**
	Variance components															
	Intercept (Site)	104.800	10.240				56.110	7.490				138.900	11.790			
	Residual	2211.400	47.030				2211.400	47.030				1714.400	41.400			
	BIC	**263.480**					266.220					267.490				
	Deviance	253.900			21.000		253.500			20.000		0.593			18.000	
	Pseudo-R2 (fixed effects)	0.000					0.020					248.400				
	Pseudo-R2 (total)	0.050					0.050					0.210				

Table 4.3. continued

		Model 0					Model 1					Model 2				
Dependent	**Independent**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**P**	**Estimate**	**SE**	**t-value**	**d.f**	**p**
Sucrose	Intercept (Site)	96.030	13.550	7.085	4.000	**0.002**	115.040	13.660	8.419	4.000	**0.001**	109.710	14.720	7.449	5.370	**0.001**
	Region (TWN)						-38.020	19.330	-1.968	4.000	0.121	-26.320	20.830	-1.263	5.370	0.258
	Meadow (edge)											10.660	10.990	0.970	20.000	0.344
	Region x meadow											-23.420	15.540	20.000	-1.506	0.148
	Variance components															
	Intercept (Site)	667.600	25.840				306.200	17.500				313.100	17.690			
	Residual	403.900	20.100				403.900	20.100				362.400	19.040			
	BIC	**231.230**					231.700					235.890				
	Deviance	221.700			21.000		219.000			20.000		216.800			18.000	
	Pseudo-R2 (fixed effects)	0.000					0.350					0.380				
	Pseudo-R2 (total)	0.620					0.630					0.670				

Table 4.3. continued

		Model 0					Model 1					Model 2				
Dependent	**Independent**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**p**
Starch	Intercept (Site)	81.520	11.050	7.379	4.000	**0.002**	61.851	9.602	6.441	24.000	**0.000**	45.710	12.230	3.737	24.000	**0.001**
	Region (TWN)						39.336	13.580	2.897	24.000	**0.008**	68.020	17.300	3.932	24.000	**0.001**
	Meadow (edge)											32.280	17.300	1.866	24.000	0.074
	Region x meadow											-57.360	24.460	-2.345	24.000	**0.028**
	Variance components															
	Intercept (Site)	287.200	16.950				0.000					0.000	0.000			
	Residual	1206.100	34.730				1106.000	33.260				897.600	29.960			
	BIC	251.480					**249.040**					250.370				
	Deviance	241.900			21.000		236.300			20.000		231.300				
	Pseudo-R2 (fixed effects)	0.000					0.270					0.410				
	Pseudo-R2 (total)	0.190					0.270					0.410				

Table 4.3. continued

		Model 0					Model 1					Model 2				
Dependent	**Independent**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**p**	**Estimate**	**SE**	**t-value**	**d.f**	**p**
Su:St	***Fixed effects***															
	Intercept (Site)	1.519	0.410	3.702	4.000	**0.021**	2.192	0.331	6.630	4.000	**0.003**	2.503	0.366	6.848	5.920	**0.001**
	Region (TWN)						-1.348	0.468	-2.883	4.000	**0.045**	-1.705	0.517	-3.298	5.920	0.017
	Meadow (edge)											-0.623	0.312	-1.996	20.000	0.060
	Region x meadow											0.715	0.441	1.620	20.000	0.121
	Variance components															
	Intercept (Site)	0.614	0.784				0.160	0.400				0.170	0.412			
	Residual	0.351	0.593				0.351	0.593				0.292	0.540			
	BIC	62.300					**61.000**					63.600				
	Deviance	58.800			21.000		48.300			20.000		44.600			18.000	
	Pseudo-R2 (fixed effects)	0.000					0.480					0.530				
	Pseudo-R2 (total)	0.560					0.640					0.700				

The overall mean sucrose content was 96.03 ± 13.55 mg g^{-1}, however, a multilevel regression model containing only the nested factor 'site', suggested that there was significant clustering amongst sites (Model 0, see Table 4.3). The random effects of site explained 62% of the variation of sucrose estimates. A Turkey HDS post hoc test demonstrated that the mean sucrose in TWN1 (50.87 ± 7.48 mg g^{-1}) was significantly lower than in Site TWN2 (103.17 ± 5.77 mg g^{-1}, P < 0.001), PPB1 (122.98 ± 8.00 mg g^{-1}, P = 0.001) and PPB2 (107.11 ± 10.77 mg g^{-1}, P = 0.001) (see Figure 4.5).

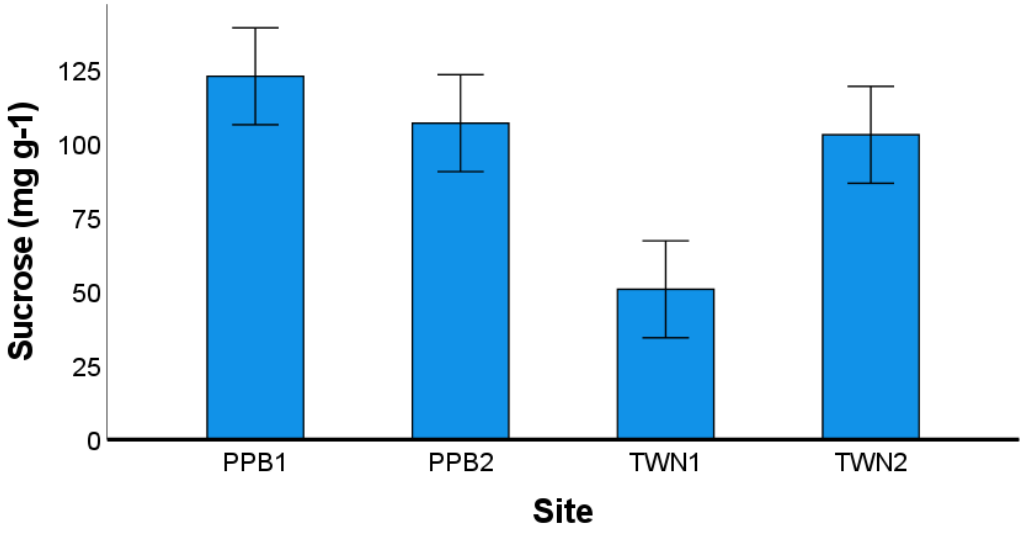

Figure 4.5 Mean sucrose content (mg g^{-1} DW rhizome) of *Zostera muelleri* in four sites across two regions (Port Phillip Bay (PPB) and Townsville (TWN)) in Australia. Error bars denote standard errors of means.

The overall mean starch content was 81.52 ± 11.05 mg g^{-1}, however, Model 1, suggested a significant effect of region, which explained 27% of the variation in starch estimates (Table 4.3). As such, the tropical region TWN had an average starch content of 101.19 ± 13.58 mg g^{-1} which was significantly higher than the average starch content of 61.86 ± 9.60 mg g^{-1} in the temperate region in PPB (p = 0.008, Figure 4.6).

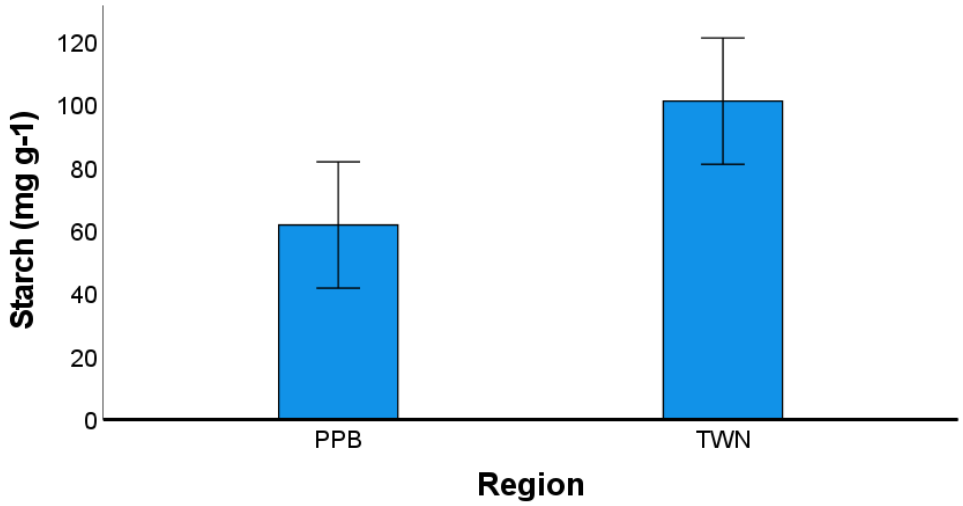

Figure 4.6 Mean starch content (mg g^{-1} DW rhizome) of *Zostera muelleri* measured in two regions (Port Phillip Bay (PPB) and Townsville (TWN)) of Australia. Error bars denote standard errors of means.

Model 1 also produced the best model to predict sucrose to starch ratio (Su:St), with 64% of the variation of Su:St explained by the fixed effects of region when the random effects of site was considered (Table 4.3). The average Su:St in TWN was 0.84 ± 0.47 which was significantly lower than 2.19 ± 0.32 in PPB (P = 0.045, Figure 4.7).

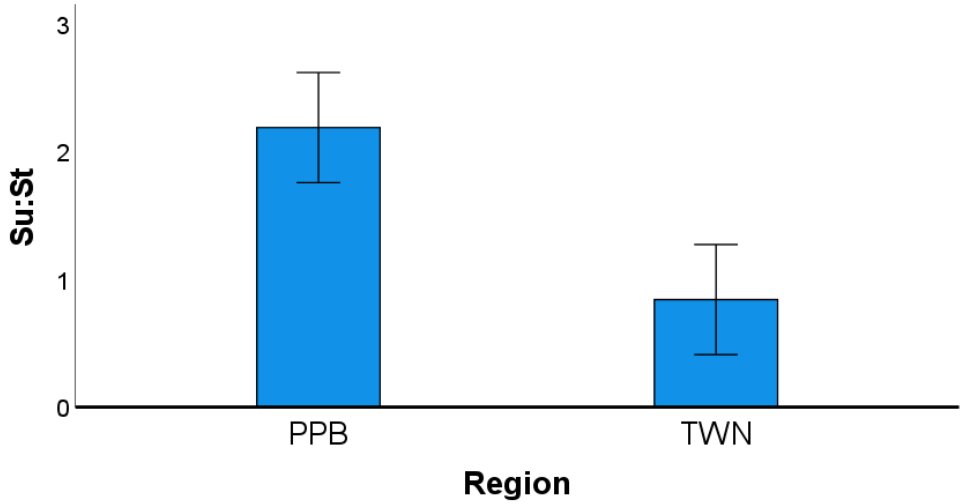

Figure 4.7 Mean sucrose to starch ratio (Su:St) of *Zostera muelleri* measured in two regions (Port Phillip Bay (PPB) and Townsville (TWN)) of Australia. Error bars denote standard errors of means.

4.3.3 Predicting seagrass non-structural carbohydrates reserves

Table 4.4 summarises the correlations between environmental conditions and NSC allocation and Table 4.5 the correlation outcomes between morphometric conditions and NSC allocation. Sucrose levels are negatively correlated to sediment

surface temperature ($r = -0.44$, $P = 0.03$), in-sediment temperature ($r = -0.48$, $p = 0.02$) and porewater NH_4^+ content ($r = -0.45$, $P = 0.03$), and positively correlated to sediment pH levels ($r= 0.51$, $P = 0.01$). Starch levels are positively correlated to sediment surface temperatures ($r = 0.57$, $P < 0.001$) and in-sediment temperatures ($r = 0.50$, $P = 0.01$) (Table 4.4). There were no statistical correlations between any environmental variables and tNSC.

Table 4.4 Correlations between non-structural carbohydrates (sucrose, starch and tNSC) and environmental variables. **Bold** font and * indicates significant Pearson's correlation coefficient (r) at P-values < 0.05.

Environmental variable	**Sucrose**		**Starch**		**tNSC**	
	R	P-value	r	P-value	r	P-value
Surface temperature	**-0.44**	***0.03**	**0.57**	***0.00**	0.28	0.19
Sediment temperature	**-0.48**	***0.02**	**0.50**	***0.01**	0.20	0.36
pH	**0.51**	***0.01**	-0.22	0.30	0.10	0.63
Mud content	0.39	0.06	-0.21	0.34	0.01	0.97
Sand content	-0.23	0.29	0.29	0.17	0.14	0.50
Silt content	-0.12	0.59	-0.25	0.24	-0.28	0.18
SOM	0.23	0.29	0.19	0.37	0.29	0.17
NH_4^+	**-0.45**	***0.03**	-0.14	0.50	-0.39	0.06
NO_2^-	0.40	0.05	0.03	0.91	0.28	0.19
NO_3^-	0.16	0.46	0.16	0.45	0.23	0.28

Table 4.5 Correlations between non-structural carbohydrates (sucrose, starch and tNSC) and biological variables. **Bold** font and * indicates significant Pearson correlation coefficient (r) at P-values < 0.05. Abbreviations include aboveground to belowground biomass ratio (ABR).

Biological variable	**Sucrose**		**Starch**		**tNSC**	
	R	P-value	r	P-value	r	P-value
Above ground biomass	0.21	0.32	0.12	0.57	0.19	0.37
Below ground biomass	0.29	0.17	0.03	0.90	0.16	0.47
total biomass	0.26	0.22	0.07	0.75	0.17	0.42
ABR	**-0.46**	***0.02**	0.39	0.06	0.09	0.66
Rhizome diameter	0.09	0.69	0.10	0.64	0.11	0.60
Internode distance	0.06	0.80	-0.40	0.05	-0.28	0.19
Blade width	-0.33	0.12	**0.50**	***0.00**	0.24	0.26
Blade length	-0.09	0.67	-0.02	0.93	-0.11	0.62
Root length	-0.03	0.13	0.26	0.22	0.05	0.82

A significant negative correlation exists between ABR and rhizome sucrose content ($r = -0.47$, $P = 0.02$), whereas starch is significantly correlated to the width of blades ($r = 0.5$, $P < 0.05$). Similar to environmental variables, tNSC was not related to any of the morphometric variables (Table 4.5).

A stepwise linear regression analysis was performed for rhizome sucrose content (Table 4.6). The resulting model showed that the combination of sediment pH ($\beta = 0.5$, $P = 0.005$) and seagrass ABR ($\beta = -0.45$, $P = 0.01$) was able to predict 47% of the variation of rhizome sucrose content ($F_{[2, 21]} = 9.18$, $r^2 = 0.47$, $P = 0.01$, Table 4.6), as shown in Equation 4.4.

Equation 4.4:

Rhizome sucrose content = -257.87 + 57.73*pH – 31.48*ABR

A stepwise linear regression analysis was also performed for rhizome starch content. The best fitting model included only one predictor variable; sediment surface temperature ($F_{[2, 21]} = 10.85$, $r^2 = 0.33$, $P = 0.003$, Table 4.6) which was able to explain 33% of the variance of rhizome starch content as demonstrated in Equation 4.5.

Equation 4.5:

Rhizome starch content = -2.34 + 3.61*sediment surface temperature (°C)

Table 4.6 Significant multiple linear regression models calculated with a forward stepwise approach, to predict the dependent variables sucrose and starch by a combination of environmental and morphometric variables. Above to belowground biomass ratio = ABR. Significant models $P < 0.05$.

	Unstandardized coefficients		**Standardised coefficients**					
Model Variables	B	SE(±)	Beta (β)	SE(±)	F	P-value	R^2	AIC
Sucrose								
Constant	-257.87	122.41						
pH	57.73	18.27	0.50	0.16	9.99	0.005	0.47	184.37
ABR	-31.48	11.10	-0.45	0.16	8.05	0.010		
Starch								
Constant	-2.34	26.34						
Sediment surface temperature	3.61	1.10	0.57	0.17	10.85	0.003	0.33	195.79

4.4 Discussion

4.4.1 Spatial variation in the partitioning of non-structural carbohydrates

Measurements of non-structural carbohydrate (NSC) reserves can be useful tools to estimate seagrass resilience; however, a considerable amount of uncertainty exists about the expected NSC reserves and in particular the partitioning of specific carbohydrate groups (e.g., see Chapter 2). Total NSC contents were similar when measured at different spatial scales (Figure 4.4) and averaged 193.53 ± 10.88 mg g^{-1} overall. However, sucrose reserves were significantly influenced by site effects, indicating that processes occurring at this scale are likely to influence sucrose accumulation. The significant effect of site was due to the tropical site TWN1 having significantly lower sucrose levels than the remaining three sites (Figure 4.5). Starch, on the other hand, appeared to be affected by large-spatial scale processes as seagrass in the tropical region TWN had significantly higher starch contents, and thus, significantly lower Su:St compared to seagrass in the temperate region PPB (Figure 4.6 and 4.7).

Zostera muelleri is considered a temperate/subtropical species and as such exists on the edge of its distribution range in tropical regions, where average temperatures (Figure 4.3) often exceed its thermal optima for growth (Campbell *et al.*, 2006; Collier *et al.*, 2011; York *et al.*, 2013; Collier *et al.*, 2017). This study was conducted in late spring when the standing stock of tropical *Z. muelleri* is known to peak (McKenzie, 1994). However, it was undertaken before the expected summer peak in aboveground biomass in temperate regions (Larkum *et al.*, 1984; Kerr & Strother, 1990). This lag in peak aboveground biomass is likely reflected in NSC reserves. In the tropical meadows, *Z. muelleri* is facing a long period of both air temperatures and SST above its thermal optimum for growth, whereas meadows in Port Phillips Bay, are facing increasing solar radiation but air temperatures and SST are below thermal optimum (Figure 4.3). Summer conditions may, therefore, be considered more favourable in higher latitudes, whereas winter conditions favour seagrass in lower latitudes. Accumulation of NSC reserves may therefore continue to rise throughout summer in PPB seagrasses (as observed in Tauranga, New Zealand, in Chapter 3) and exceed that of the potentially declining NSC reserves of tropical TWN seagrasses by the end of the growing season in autumn.

When measured in autumn, rhizome starch content of the congener *Z. noltii* displays a positive relationship with latitude; however, this relationship does not exist in spring (Soissons *et al.*, 2018b). The latitudinal study by Soissons *et al.* (2018b) was published after the fieldwork component for this study was conducted, and this information was, therefore not considered when designing the current study. In this study, higher latitude seagrass had significant lower starch levels which may be due to seasonal mobilisation of starch reserves to ensure winter survival and regrowth in spring. Sampling in autumn (end of the growing season) may likely reveal an opposite trend in starch reserves (i.e. significantly higher in temperate seagrass) as observed for *Z. noltii* (Soissons *et al.*, 2018b). Unfortunately, limited resources did not allow for seasonal sampling events to occur in this current study. To confirm this hypothesis, it is recommended that sampling should be expanded to include seasonal sampling.

4.4.3 Predicting non-structural carbohydrate reserves of seagrass

Sucrose content was correlated to several environmental variables, including sediment surface temperature, in-sediment temperature, porewater NH_4^+ content, and sediment pH levels (Table 4.6). Increasing temperatures (both surface and in-sediment) appeared to decrease levels of sucrose in seagrass rhizomes. This pattern is not surprising given that respiration rates exceed photosynthetic rates once a thermal optimum is exceeded (Collier *et al.*, 2011; York *et al.*, 2013; Collier *et al.*, 2017). In contrast, starch content appeared to increase in higher temperatures (sediment surface temperatures $r = 0.57$, $P < 0.001$ and in-sediment temperatures $r = 0.50$, $P = 0.01$) and may, therefore, prove valuable as long-term energy storage in tropical seagrass, enabling survival during high-temperature events, such as heatwaves. As described previously, starch has been linked to the seasonal survival of temperate *Z. noltii,* enabling survival through unfavourable growing conditions in winter and regrowth in spring (Govers *et al.*, 2015; Soissons *et al.*, 2018b). As such, starch accumulation may be critical for tropical populations in the cooler winter and spring months to survive the hot summers, whereas, temperate population accumulate in summer and autumn to survive the cold winters. Again, seasonal sampling events across large spatial scales are necessary to confirm this hypothesis.

A negative relationship existed between sucrose and porewater NH_4^+. Adverse effects of NH_4^+ have been linked to the mortality of the congener *Z. marina* at concentrations as low as 25 µM, with effects being more severe in sandy substrates and higher temperatures (van Katwijk *et al.*, 1997). Alternatively, the Mediterranean seagrass *P. oceanica* does not experience significant mortality in response to experimental addition of NH_4^+(Invers *et al.*, 2004). In this study, the negative relationship between sucrose reserves and porewater NH_4^+ levels infers that mobilisation of energy stores may meet the increased metabolic demands associated with ammonium assimilation and further highlight the association between sucrose reserves and *Z. muelleri* resilience. However, it is noted that NSC reserves of *Z. muelleri* in Tauranga Harbour, New Zealand, appear to increase with porewater nutrients (Chapter 3).

Higher levels of sediment pH were correlated to increasing sucrose contents ($r = 0.51$, $P = 0.01$). Reduction of nitrogen to ammonium occurs during the bacterial breakdown of organic carbon in the sediments which usually coincides with the bacterial reduction of sulphur to phototoxic hydrogen sulphide (Phillips *et al.*, 1997). Because of this increased microbial respiration, elevated CO_2 levels may lead to the acidification of sediments that is generally associated with eutrophication. Water-column acidification, reflecting increased CO_2 availability to the aboveground photosynthetic tissues, has previously been associated with elevated NSC contents of otherwise CO_2-limited seagrass (Campbell & Fourqurean, 2013; Zimmerman *et al.*, 2017). Due to diurnal variations in seagrass productivity and community respiration, water column pH may fluctuate by as much as 0.5 to >1 pH units daily, with higher variations in seagrass meadows existing on the range of tolerance levels (Olsen *et al.*, 2018). The results in this study indicate that sediment acidification may have an opposite effect to water-column acidification (e.g., Campbell & Fourqurean, 2013; Zimmerman *et al.*, 2017), however, the adverse effects of low sediment pH, is probably associated with elevated exposure to phototoxins and increased metabolic rates due to higher O_2 exchange between above and belowground biomasses during times of anoxia.

Sediment pH and seagrass ABR explained 47% of sucrose reserve variations (Table 4.7). Sediment pH explained the majority of the variation. Thus, environments in which sediment pH levels are constant, the ABR may explain some of the remaining

variations in sucrose responses (Equation 4.4). High ABR has previously been highlighted as an adaptation of *Z. muelleri* to low light environments (Abal *et al.*, 1994; Kohlmeier *et al.*, 2014; Maxwell *et al.*, 2014) and the negative relationship between sucrose reserves and ABR, as expressed in the SLR model in this study, may reflect the impacts of environmental disturbances on *Z. muelleri's* carbon balance in low pH environments.

The best-fitting model for predicting rhizome starch content included only one independent variable; sediment surface temperatures, which explained 33% of the starch variation (Equation 4.5). Temperatures are often related to large-scale processes associated with climatic forcing, and thus, indicates that starch contents of *Z. muelleri* are likely to vary along a latitudinal gradient.

4.5 Conclusion

In this chapter, two main objectives were investigated pertaining to the partitioning of NSC in *Z. muelleri*. Despite environmental processes varying across spatial scales, tNSC contents were similar within and between meadows and across large spatial scales (Figure 4.4). However, sucrose contents appeared to be influenced by small spatial scale processes as significant differences were seen between sites and were best explained by the two variables; sediment pH and seagrass ABR. In contrast, starch contents were influenced by large-scale processes linked to temperatures, and as such, tropical seagrass meadows contained significant higher starch levels than temperate counterparts and as such had a lower Su:St when measured in spring. The ability to moderate NSC partitioning, therefore, appears to be crucial to the opportunistic *Z. muelleri* that inhabits a range of environments.

The measurement of a few easily attained environmental and morphometric variables (pH, sediment surface temperature and ABR) can be used to predict NSC content and, thus, monitor *Z. muelleri* health. Yet, to increase the confidence in the NSC prediction models, and thus, increase the use of this as a management tool, it is recommended that seagrass meadows across the entire environmental envelope should be included in future model development. Furthermore, a seasonal extension of this current study with a focus on seasonal sampling is recommended to gain a full understanding of how the temporal patterns of NSC partitioning in different climatic regions.

Chapter 5
General conclusions

Intertidal seagrass (*Zostera muelleri*) meadow in Tauranga Harbour, New Zealand. Photo: Sørensen, S. T.

5.1 Overview of research contributions

This thesis comprises three inter-linked studies describing observational, manipulative and analytical experiments, aimed collectively at exploring the spatial variation of *Zostera muelleri* non-structural carbohydrate (NSC) reserves and resilience to acute sedimentation. The focus on sedimentation is driven by common predictions of the ongoing increases of this disturbance due to extreme weather events linked to climate change and growing anthropogenic pressure on coastal systems.

Within the literature, a number of approaches have been used to quantify NSC reserves (Chapter 2). Following experimental comparisons of individual analytical steps of the most commonly used methods, I demonstrated that estimates of NSC by different methodologies are not comparable (Figure 5.1). The inconsistencies between methods extended to all stages of the analytical process, including the solvents used for soluble NSC extraction, starch gelatinisation and hydrolysis, as well as the specific quantitation assays used. For example, the most commonly used assay; the phenol-sulfuric assay, deviated by more than 15% of the expected carbohydrate levels, whereas the far less used assay by high-performance liquid chromatography (HPLC) estimated NSC contents within 1.1% of the expected levels. Based on the literature review and analytical experiments, a five-step standard protocol was developed (Chapter 2). This protocol will enable researchers to adopt a single method to ensure comparable estimates across and between laboratories, thereby alleviating the variability linked to analytical inconsistencies. Consequently, increasing the application of NSC reserves as accurate measures of health and resilience. The newly developed method was applied in chapters 3 and 4 to measure *Z. muelleri* NSC reserves in different environments (Figure 5.1).

The results in Chapter 3 demonstrated that phenotypic plasticity increase resilience of seagrass to burial events. As such, single and repeated burial treatments caused a significant reduction in relative shoot cover in two out of the three studied sites. However, repeated burial events (two burial events 33-days apart) only caused a significantly greater reduction than a single burial treatment in one out of three

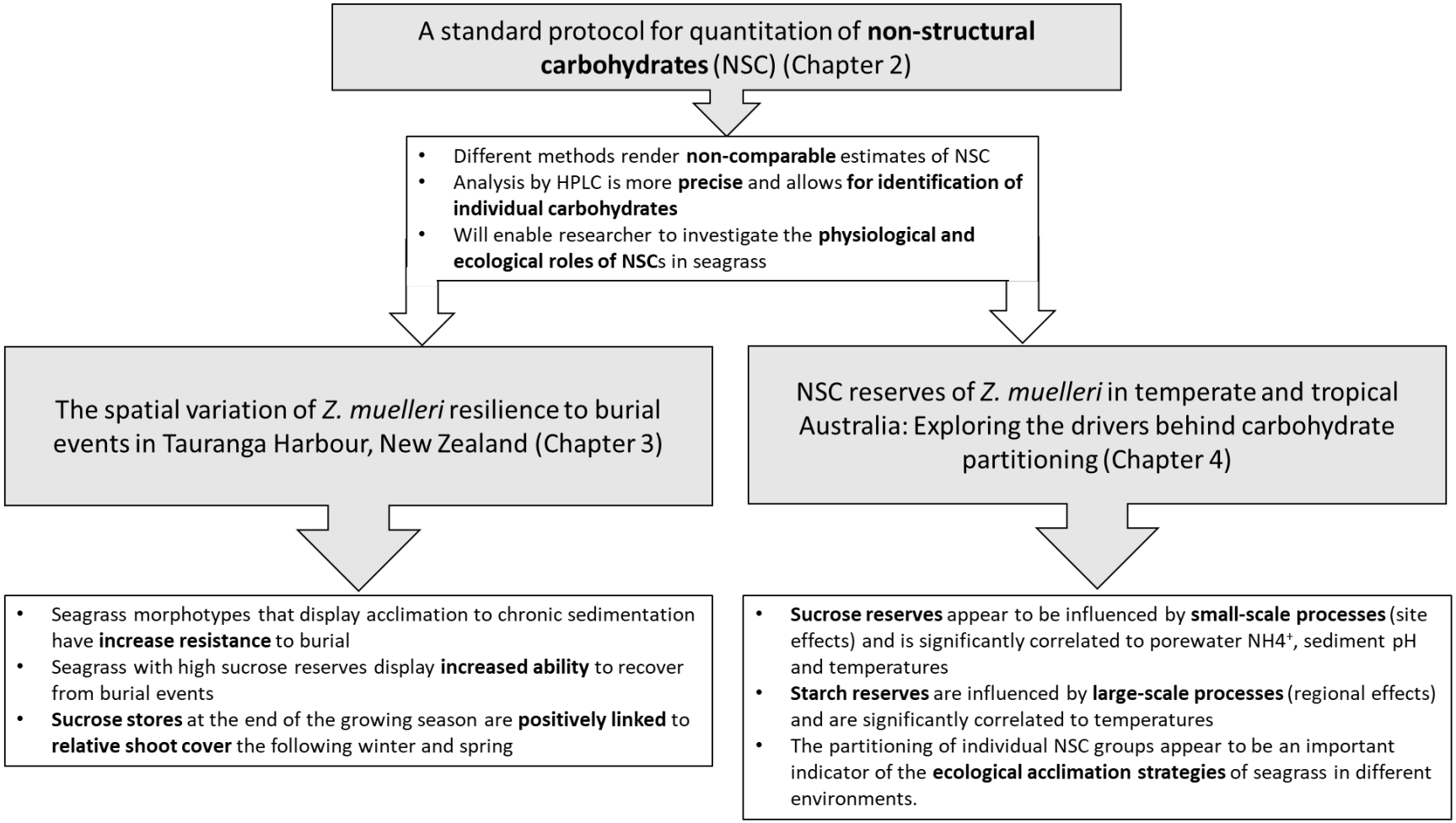

Figure 5.1. Diagrammatic model of thesis outcomes and contributions.

sites (Figure 3.10). Large morphotypes (indicating acclimation to a chronically degraded environment), displayed a high level of resistance to burial (no treatment effects), whereas smaller morphotypes (indicating little or no chronic exposure to environmental disturbances) had a significantly lower resistance as well as the slowest rates of recovery (up to 251 days). Seagrass that displayed some acclimation to chronic environmental impacts (intermediate morphotype) also had low resistance to burial, however, experienced faster recovery rates following burial events (single burial recovered 63-days, repeated burials recovered by 168-days, Figure 3.10). A significant positive relationship was established between sucrose levels at the end of the growing season (February) and relative shoot cover in winter and spring, however, mobilisation of NSC reserves was not detected in response to burial in any of the three sites. The apparent lack of treatment effect in NSC reserves is hypothesised to be due to the sampling occurring too long after the burial event (30 days after burial). Mesocosm experiments demonstrated that sucrose reserves were mobilised within 10-days of severe stress (Appendix C, Figure C.9). As the experimental units were not subject to prolonged burial (natural remobilisation of sediments allowed) and clonal integration kept intact, the 30-day period following the burial event in summer may have been sufficient to restock NSC reserves.

The two mechanisms of resilience; resistance and recovery (Levin & Lubchenco, 2008; Côté & Darling, 2010; McClanahan *et al.*, 2012), both varied spatially in response to burial and appeared to increase in seagrass that existed in chronically impacted environments. The results of this study appear to agree with another recent study of *Z. muelleri* in Australia that demonstrated that seagrass in chronically disturbed environments had significantly lower genotypic diversity but displayed a higher level of resilience to extreme flooding events (Connolly *et al.*, 2018b). And as such, it appears that *Z. muelleri* responds in a more counter-intuitive manner to increased chronic disturbances, where less tolerant individuals are gradually removed from a system, thereby leaving a less diverse but more disturbance resilient population (Côté & Darling, 2010). It is, however, import to note that this study did not identify a tipping point at which a system change occurred, as described by Holling 1973, and thus, did not fully explore the resistance of *Z. muelleri* to burial events. When a threshold is met, further addition of sediment would potentially have long-term detrimental effects, as it would disrupt important

feedback systems due to shifts in microbial and benthic-fauna communities, fragmentation and thus isolation from seagrass recruits (Kilminster *et al.*, 2015; Angeler & Allen, 2016; Connolly *et al.*, 2018a; O'Brien *et al.*, 2018) and may result in less productive unvegetated, or algae-dominated mudflats (Kendrick *et al.*, 2002; Unsworth *et al.*, 2015).

Despite a lack of treatment effect on NSC reserves, summer sucrose levels were significantly related to the aboveground survival rates the following winter (r^2 = 0.54) and spring (r^2 = 0.66) (Figure 3.13). This link between sucrose levels and survival rates throughout the growing year, highlights the importance of the timing of disturbances (particularly anthropogenic disturbance that can be scheduled to occur at a particular time in the season), as resilience may follow similar seasonal trends of NSC reserves. Recovery times have previously been linked to the timing of a disturbance of temperate *Z. marina* beds (Soissons *et al.*, 2016). Soissons *et al.* (2016) found that recovery rates were fastest when *Z. marina* was disturbed in the early growing season and slowest when disturbed in the mid-growing season when the Leaf Area Index peaked. The results of Chapter 3 indicate that prediction of *Z. muelleri* recovery is likely to be linked to seasonal trends of sucrose reserves. Longer-term studies (a minimum of three years of fieldwork) are required to determine the seasonal implications of burial and were not the focus of this study.

Chapter 4 was a tentative investigation of the natural variation of NSC reserves of *Z. muelleri* across different spatial scales, encompassing both temperate and tropical meadows in Australia. Australia was selected as New Zealand does not provide tropical ecosystems for comparison against the temperate sites. The literature suggests that higher latitude seagrass may accumulate more starch (Soissons *et al.*, 2018b) and sucrose stores (Drew, 1978a). As the two studies by Soissons *et al.* (2018b) and Drew (1978a) only measured either sucrose or starch, it was until now unknown how the tNSC pool would differ across large-spatial scales including high and low latitude meadows. The results from this thesis demonstrated that tNSC contents did not vary across different spatial scales when measured in spring, but the contents of the individual carbohydrate groups differed significantly across different spatial scales (Table 4.3). Sucrose reserves varied significantly between sites and appeared to decrease with increasing porewater NH_4^+ contents, seagrass ABR, temperatures and decreasing sediment pH. As such, environmental

variables appeared to the main drivers of sucrose accumulation. Starch contents varied at the regional scale and were significantly higher in the tropical populations in Townsville compared to temperate populations in Port Phillip Bay and were positively related to temperatures. It was hypothesised that temperate and tropical *Z. muelleri* accumulate and mobilise starch reserves in opposite seasons (tropical populations in winter/spring and temperate in summer/autumn) however, a seasonal study is needed to confirm this.

The ability of seagrass to modulate NSC allocation appear to be of particular importance to seagrass' ability to adapt to a diverse range of environments. As such, this research demonstrates that the opportunistic seagrass species *Z. muelleri* acclimates to its environment by altering its morphometry as well as the partitioning of its energy reserves. It describes for the first time how prior acclimation to chronic environmental disturbances increases the resilience of *Z. muelleri* to both single and repeated burial events (Chapter 3). Furthermore, it highlights that rhizome sucrose reserves are linked to *Z. muelleri* resilience, which emphasises the potential of NSC reserves as a robust tool to measure and predict *Z. muelleri* resilience (Chapter 3). The spatial (environmental conditioning) and temporal (annual variation in NSC reserves following seasonal trends) illustrates that *Z. muelleri* resilience to burial events (and other disturbances) is likely to follow similar trends (Chapter 3).

Existing seagrass NSC estimates from the literature are highly variable (Appendix A), but, Chapter 2 demonstrates that this inconsistency may be linked to the variable use of analytical methods. Using the newly developed standard protocol presented in Chapter 2, it is demonstrated that tNSC contents of *Z. muelleri* across large-spatial scales in Australia are statistically similar in spring (overall mean = 193.59 ± 10.04 mg g^{-1} DW, see Chapter 4). In fact, these tNSC levels were statistically similar to temperate *Z. muelleri* from Tauranga Harbour in New Zealand (Chapter 3) when measured in spring (198.98 ± 19.92 mg g^{-1} DW, $F_{[2, 47]} = 0.13$, $P = 0.88$). However, tNSC levels ranged from 474.18 to 36.08 mg g^{-1} DW over a full growing season in Tauranga Harbour and varied significantly between sites. These results indicate that NSC accumulation and partitioning is influenced by environmental

factors and that these processes are likely to influence *Z. muelleri* populations similarly over large spatial scales.

5.2 Recommendations for future research

The experimental burial treatments in Chapter 3, occurred at a time of high annual shoot cover and carbon reserves, which presumably coincide with a time of elevated resilience. It is expected that the effects of burial may be more severe if treatments were to occur during a crucial time of NSC accumulation (earlier in the growing season), especially at a site that experiences high seasonal fluctuation (e.g., Soissons *et al.*, 2016; Wu *et al.*, 2017). Thus, it would be valuable to repeat the burial treatments at various seasons to assess how the timing of burial affects *Z. muelleri* responses. Additionally, natural sedimentation events may occur on a more extensive scale than that which was simulated in this study (1 m x 1 m). In large-scale events, the availability of viable propagules and unaffected rhizomes would be limited, thereby, reducing the likelihood of recruitment into affected areas as well as subsidies of carbon reserves via clonal integration. Consequently, large-scale events may result in meadow fragmentation that further reduces the resilience of ecosystems (Young *et al.*, 1996; Thrush *et al.*, 2008; Gera *et al.*, 2013).

The scaling up of small-scale experiments to large, more common scale disturbance levels is one that environmental managers struggle with as this may have long-term detrimental impacts on the studied environment as well as being more costly. For example, an experiment designed to mimic larger-scale catastrophic depositions of terrestrial sediments onto intertidal mudflats in New Zealand required the use of a concrete mixing truck and a helicopter (Thrush *et al.*, 2003). However, that study successfully demonstrated that an increase in the magnitude of a disturbance (sediment deposition) significantly increase the time of recovery of the macrobenthos (Thrush *et al.*, 2003).

Natural burial events are generally triggered by the runoff and influx of terrestrial sediments that are high in mud and silt particles, organic matter and nutrients (e.g., Short, 1987; Terrados *et al.*, 1998). In this study, washed silica sand purchased from a landscaping retailer was used to eliminate any unknown variables associated with using site-specific sediments. A recent mesocosm study found that fine sediments increased the negative effect of burial on both *Z. muelleri* and *H. ovalis* when

compared to burials by coarser sediments (Benham *et al.*, 2019). In a different study with different species, burial by two different sediment types (sand and silt-clay) caused similar effects on *Z. marina* (Mills & Fonseca, 2003). Experimental nutrient additions have been found to increase the negative effects of burial on *P. oceanica* (Ceccherelli *et al.*, 2018) and *C. nodosa* (Balestri & Lardicci, 2014). Thus, the addition of nutrient-rich sediment in a burial event within a seagrass meadow in already elevated nutrient conditions (high sediment regime) may exaggerate the effect of burial. Future studies should aim to include nutrient-rich, fine-particle sediments in experimental burial treatments *in situ* to test for the interactive effect of burial and sediment type on *Z. muelleri* along an environmental gradient.

The repeated burial treatments in Chapter 3 were limited to two burial events one-month apart. The period between burial events (for the repeated burial treatment) was based on the average time (calculated over ten years) between heavy rainfall events (which typically trigger sedimentation influx to the estuary that was being studied) in the region. However, it is noted that burial events may occur more frequently due to the current weather patterns experienced in New Zealand. For example, in 2017 cyclone Debbie (9th April 2017), cyclone Cook (14th April 2017), and cyclone Donna (11th May 2017) all landed on New Zealand shores within a close temporal sequence. These tropical-strength storms resulted in three seagrass burial events five days and then 27 days apart in estuaries in the Coromandel and Bay of Plenty, New Zealand (Martin, pers. comm; Campbell, pers. obsv.). Thus, the repeated burial treatments simulated in Chapter 3, potentially underestimate the stochasticity of burial events that occur in current New Zealand seagrass meadows but it does represent the average over the last 10-years.

The increased longevity of effects of repeated burial events as observed in one out of three sites in Chapter 3, may result in decreased genotypic diversity, habitat loss, fragmentation and impacts on flowering (Thrush *et al.*, 2008; Munkes *et al.*, 2015; Côté-Laurin *et al.*, 2017; Connolly *et al.*, 2018a). Therefore, a higher number of successive burial events are necessary for the repeated burial treatments to simulate the recent weather patterns and assess the cumulative impacts of increasing burial frequencies associated with predicted climate changes.

Seasonal repetition of burial treatments will allow for the assessment of the seasonal variation in resilience, which presumably follows the seasonal trends sucrose reserves. A great proportion of this thesis was aimed at developing a method for measuring NSC in seagrass and it was not designed to be a seasonal assessment. However, to gain a clearer understanding of the seagrass resilience to burial it would be important to repeat this study at all seasons (for three consecutive years) and in addition, include sites from other harbours and estuaries with aims to encompass the full environmental envelope of *Z. muelleri* is needed to test if the disturbance-response trends observed in Tauranga Harbour are spatially transferable.

Another important finding of this research was the identification of the environmental and biological variables that influence sucrose and starch reserves (Chapter 4). As such it was determined that sucrose accumulation differs at site level, and that sediment pH and above to belowground biomass ratio explained 47% of the variation of sucrose ($r^2 = 0.47$). Chapter 3 demonstrated that a significant relationship exists between sucrose and seagrass cover, thus understanding the feedback systems that control *Z. muelleri* energy reserves is crucial to the understanding of seagrass resilience and will allow the development of predictive models. Models offer cost-effective ways to assess scenarios at larger scales, and models are now more frequently developed to predict the variability of numerous seagrass indicators under various circumstances (e.g., Eldridge *et al.*, 2004; Newell & Koch, 2004; Short *et al.*, 2007; Canal-Vergés *et al.*, 2014; Macreadie *et al.*, 2014b; Wu *et al.*, 2017; Jayathilake & Costello, 2018; Stankovic *et al.*, 2018; Vieira *et al.*, 2019). However, the ability of numerical models to simulate real-life scenarios depends on the quality of the data that a model is based upon.

This thesis provides novel insights for the future development of models for prediction of seagrass resilience. First, it developed a standard protocol for the estimation of NSC reserves (Chapter 2), which will ensure that the reported variability of seagrass NSC reserves are not due to analytical inconsistencies and, therefore, remove some of the uncertainty relating to the ecological and physiological roles of sucrose and starch specifically. Furthermore, the thesis found a significant linear relationship between seagrass NSC reserves (sucrose) and seasonal change in seagrass cover (Chapter 3) and produced models for the prediction of sucrose and starch reserves (Chapter 4). The linear regression models

produced in Chapters 3 and 4 can be linked to predict seagrass resilience. As an example, Stankovic *et al.* (2018) linked three linear regression models that predicted: 1) plant biomass; 2) plant carbon content; and 3) organic carbon in sediments, to model carbon content in sediments at a landscape scale. However, before linking the models developed in Chapters 3 and 4, it is necessary to increase the confidence in the individual predictive model units (i.e., seagrass sucrose prediction, and seagrass cover prediction).

Chapter 3 illustrated that NSC reserves vary significantly seasonally. Investigations of the seasonal variations in the predictive relationships of sucrose and starch by environmental and biological variables are, therefore, necessary. Future studies should occur over consecutive growing seasons along a more stratified latitudinal gradient, as results in Chapter 4 indicate that temperate and tropical seagrass have opposite seasonal trends of NSC accumulation. Furthermore, it is important to ensure that the full spectrum of *Z. muelleri's* environmental envelope is included in order to increase the confidence of the effects of each independent variable measured. Additional environmental variables are recommended for inclusion in future investigations of predictive models for sucrose and starch including; salinity, sediment sulphide content, phosphorus content, and water column turbidity.

Salinity is known to affect sucrose due to the additional function of sucrose as an organic osmolyte (Gil *et al.*, 2011; Olsen *et al.*, 2016). High levels of hydrogen sulphide in sediments may assimilate in seagrass tissue and act as a phototoxin (Kilminster *et al.*, 2008; García *et al.*, 2013; Brodersen *et al.*, 2015; Martin *et al.*, 2019) and phosphorus may limit seagrass productivity, especially in tropical carbonate sediments (Short, 1987; Lee *et al.*, 2007). Furthermore, turbidity levels directly influence seagrasses' ability to photosynthesise during submersion (Vermaat *et al.*, 1997; Longstaff & Dennison, 1999; Newell & Koch, 2004). The inclusion of these variables in future regression analysis may increase the confidence of the models for prediction of sucrose and starch reserves of *Z. muelleri*.

The relationships between NSC reserves and *Z. muelleri* resilience should be further explored as Chapter 3 found that summer sucrose accumulation was a significant predictor of seagrass cover in winter and spring. Starch has previously been suggested to be the determining carbohydrate reserve for seasonal survival in the

congener *Z. noltii* sampled in higher latitudes (Govers *et al.*, 2015; Soissons *et al.*, 2018b). The significant relationship between seagrass cover and sucrose should, therefore, be explored across a broader latitudinal gradient encompassing *Z. muelleri* meadows in higher latitudinal extremes to determine whether the role of sucrose is replaced by starch when nearer to its distributional limits. With ongoing exploration and understanding of the mechanics of seagrass energy reserves, models will become more precise in their predictions, which is necessary for these to be useful management tools, (e.g., Fong *et al.*, 1997). As such, the results from this study will aid the development of future models to predict seagrass resilience on a large-spatial landscape-scale and may enable identification of conservation hotspots (seagrass meadows in need of protection) and, thus, improve the management of these critical marine angiosperms.

Appendices

Appendix A

Summary of methods used for non-structural carbohydrate estimation from the existing literature (*Chapter 2*).

A literature search was conducted to identify the methods used to quantitate non-structural carbohydrates using Web of Science literature database (May 2018). Two sets of keywords were used. The first set consisted of “seagrass” and “eelgrass”, whilst the second set consisted of “non-structural carbohydrates”, “carbohydrates” and “sugars”. Studies that undertook NSC quantitation and contained an adequate amount of detail pertaining to these methods (79 of 227 studies) were compiled and examined to determine the common analytical procedures in use. Tables A.1 presents a summary of the methods used to estimate soluble sugars or sucrose, whereas Table A.2 lists methods for starch estimation. Finally, Table A.3 summarises methods used to estimate total NSC (tNSC) contents, without differentiating between soluble sugars and starches. These tables were submitted as supplementary material with the publication by (Sørensen *et al.*, 2018).

Table A.1. List of assays (anthrone, phenol-sulfuric, resorcinol and 3-methyl-2-benzothiazolinonehydrazone (MBTH), and chromatographic assays: gas-liquid chromatography (GC) and high-performance liquid chromatography (HPLC)) used in the existing literature for the analytical quantitation of soluble non-structural carbohydrates (all soluble sugars or sucrose) listed by seagrass species with supporting references. Min and max mean estimates are standardized to mg g^{-1} of tissue (leaf, sheath, above ground, rhizome, root, below ground or whole plant). State of tissue are either dry weight (DW), fresh weight (FW) or extracted dry weight (EDW). * Indicates approximate estimates obtained from graphs in referenced material.

					Sugars mg g^{-1}					
Species	**Assay**	**Carbohydrate**	**Tissue**	**State**	**min**		**max**		**Location**	**Study**
Amphibolis antarctica	GC	Sugars	Leaf	EDW	18				Not specified	Drew (1983)
Amphibolis griffithii	GC	Sucrose	Leaf	DW	10	-	100		Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Leaf	DW	20	-	52	*	Australia	Mackey *et al.* (2007)
	Phenol-sulfuric	Sugars	Rhizome	DW	25	-	170	*	Australia	Mackey et al. (2007)
Cymodocea nodosa	GC	Sucrose	Leaf	DW	67				Malta	Drew (1978a)
	GC	Sugars	Leaf	EDW	86				Not specified	Drew (1983)
	Resorcinol	Sugars	Leaf	DW	25	-	30	*	Spain	Malta *et al.* (2006)
	GC	Sugars	Leaf	DW	30	-	70	*	Mediterranean	Pirc (1989)
	Anthrone	Sugars	Leaf	DW	8	-	10		Portugal	Sandoval-Gil *et al.* (2012)
	Anthrone	Sugars	Leaf	DW	16	-	25	*	Spain	Sandoval-Gil *et al.* (2014)
	Phenol-sulfuric	Sugars	Leaf	DW	40	-	80	*	Portugal	Silva *et al.* (2013)
	Anthrone	Sugars	Above	DW	1	-	8		Gran Canaria, Spain	Tuya *et al.* (2013)
	Resorcinol	Sugars	Rhizome	DW	20	-	235	*	Spain	Malta et al. (2006)
	GC	Sugars	Rhizome	DW	20	-	240	*	Mediterranean	Pirc (1989)
	Anthrone	Sugars	Rhizome	DW	133	-	177		Portugal	Sandoval-Gil et al. (2012)
	Anthrone	Sugars	Rhizome	DW	30	-	120	*	Spain	Sandoval-Gil et al. (2014)
	Phenol-sulfuric	Sugars	Rhizome	DW	40	-	80	*	Portugal	Silva et al. (2013)
	Resorcinol	Sugars	Root	DW	25	-	30	*	Spain	Malta et al. (2006)
	GC	Sugars	Root	DW	20	-	80	*	Mediterranean	Pirc (1989)
Cymodocea rotundata	GC	Sucrose	Leaf	EDW		> 100			Not specified	Drew (1983)
Cymodocea serrulata	GC	Sucrose	Leaf	DW		14			Saudi Arabia	Drew (1980)
	GC	Sugars	Leaf	EDW		38			Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Above	DW		92			Philippines	Tomasko *et al.* (1993)
	GC	Sucrose	Rhizome	EDW		43			Saudi Arabia	Drew (1980)

Table A.1. continued.

					Sugars mg g^{-1}					
Species	**Assay**	**Carbohydrate**	**Tissue**	**State**	**min**		**max**		**Location**	**Study**
Enhalus acoroides	GC	Sugars	Leaf	EDW		39			Not specified	Drew (1983)
Halodule pinifolia	Phenol-sulfuric	Sugars	Aboveground	DW		163			Philippines	Tomasko et al. (1993)
	Phenol-sulfuric	Sugars	Rhizome	DW	20	-	40		Australia	Longstaff and Dennison (1999)
Halodule uninervis	GC	Sucrose	Leaf	EDW		>			Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Aboveground	DW		290			Philippines	Tomasko et al. (1993)
Halodule wrightii	GC	Sucrose	Leaf	DW		12			Seychelles	Drew (1980)
	GC	Sugars	Leaf	EDW		50			Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Rhizome	DW	400	-	550	*	Florida, US	Dawes and Lawrence (1980)
Halophila decipiens	GC	Sugars	Leaf	EDW		306			Not specified	Drew (1983)
Halophila ovalis	GC	Sucrose	Leaf	DW		19			Australia	Drew (1980)
	GC	Sucrose	Leaf	EDW		>			Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Leaf	DW	15	-	32	*	Australia	Longstaff *et al.* (1999)
	Anthrone	Sugars	Aboveground	DW	15	-	18		Australia	Kilminster *et al.* (2008)
	GC	Sucrose	Rhizome	EDW		846			Australia	Drew (1980)
	Phenol-sulfuric	Sugars	Rhizome	DW	13	-	32	*	Australia	Longstaff et al. (1999)
	Phenol-sulfuric	Sugars	Root	DW	5	-	16	*	Australia	Longstaff et al. (1999)
	Anthrone	Sugars	Belowground	DW	68	-	79		Australia	Kilminster et al. (2008)
Halophila stipulacea	GC	Sucrose	Leaf	EDW		>			Not specified	Drew (1983)
Heterozostera	GC	Sucrose	Leaf	DW		73			Australia	Drew (1980)
	GC	Sucrose	Leaf	EDW	10	-	100		Not specified	Drew (1983)
Phyllospadix torreyi	GC	Sucrose	Leaf	DW		98			California, US	Drew (1980)
	GC	Sugars	Leaf	EDW		388			Not specified	Drew (1983)
Posidonia australis	HPLC	Sucrose	Leaf	DW	3	-	20	*	Australia	Cambridge *et al.* (2017)
	GC	Sucrose	Leaf	DW		25			Australia	Drew (1980)
	GC	Sucrose	Leaf	EDW	10	-	100		Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Leaf	DW	129	-	682		Australia	Ralph *et al.* (1992)
	GC	Sucrose	Leaf	DW		65			Seychelles	Drew (1980)
	GC	Sucrose	Leaf	EDW	10	-	100		Not specified	Drew (1983)

Table A.1. continued.

					Sugars mg g^{-1}					
Species	**Assay**	**Carbohydrate**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Posidonia oceanica	Anthrone	Sugars	Leaf	DW	28	-	88		Spain	González-Correa *et al.* (2009)
	Resorcinol	Sugars	Leaf	DW	25	-	30	*	Spain	Hernan *et al.* (2017)
	Anthrone	Sugars	Leaf	DW	8	-	23	*	Spain	Marín-Guirao *et al.* (2017)
	GC	Sugars	Leaf	DW	12	-	37		Italy	Pirc (1985)
	GC	Sugars	Leaf	DW	5	-	40	*	Mediterranean	Pirc (1989)
	Anthrone	Sugars	Leaf	DW	6	-	12		Portugal	Sandoval-Gil et al. (2012)
	Anthrone	Sugars	Leaf	DW	11	-	17	*	Spain	Sandoval-Gil et al. (2014)
	Enzymatic	Sugars	Leaf	DW		150			Italy	Scartazza *et al.* (2017)
	GC	Sugars	Sheath	DW	26	-	143		Italy	Pirc (1985)
	Enzymatic	Sucrose	Rhizome	DW	120	-	180	*	Italy	Scartazza et al. (2017)
	Resorcinol	Sugars	Rhizome	DW	70	-	110		Spain	Delgado *et al.* (1999)
	Anthrone	Sugars	Rhizome	DW	40	-	130	*	Spain	Gacia *et al.* (2012)
	Anthrone	Sugars	Rhizome	DW	5	-	48	*	Spain	González-Correa *et al.* (2008)
	Anthrone	Sugars	Rhizome	DW	25	-	85	*	Spain	González-Correa *et al.* (2009)
	Resorcinol	Sugars	Rhizome	DW	20	-	25	*	Spain	Hernan et al. (2017)
	Anthrone	Sugars	Rhizome	DW	30	-	150	*	Spain	Marin-Guirao et al. (2017)
	GC	Sugars	Rhizome	DW	16	-	237		Italy	Pirc (1985)
	GC	Sugars	Rhizome	DW	10	-	90	*	Mediterranean	Pirc (1989)
	Resorcinol	Sugars	Rhizome	DW	70	-	180	*	Spain	Ruiz and Romero (2001)
	Resorcinol	Sugars	Rhizome	DW	40	-	220		Spain	Ruiz *et al.* (2009)
	Anthrone	Sugars	Rhizome	DW	38	-	58		Portugal	Sandoval-Gil et al. (2012)
	Anthrone	Sugars	Rhizome	DW	95	-	135	*	Spain	Sandoval-Gil et al. (2014)
	Resorcinol	Sugars	Root	DW	80	-	100	*	Spain	Hernan et al. (2017)
	GC	Sugars	Root	DW	5	-	70	*	Mediterranean	Pirc (1989)
Posidonia sinuosa	Phenol-sulfuric	Sugars	Leaf	FW	4	-	12		Australia	Collier *et al.* (2008)
	Phenol-sulfuric	Sugars	Leaf	DW	8	-	40	*	Australia	Collier *et al.* (2009)
	Phenol-sulfuric	Sugars	Rhizome	FW	18	-	93		Australia	Collier et al. (2008)
	Phenol-sulfuric	Sugars	Rhizome	DW	86	-	270		Australia	Collier et al. (2009)

Table A.1. continued.

					Sugars mg g^{-1}					
Species	**Assay**	**Carbohydrate**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Syringodium filiforme	GC	Sucrose	Leaf	DW	10	-	100		Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Rhizome	DW	360	-	490	*	Florida, US	Dawes & Lawrence (1980)
Syringodium isoetifolium	GC	Sucrose	Leaf	DW		0			Seychelles	Drew (1980)
	GC	Sucrose	Leaf	EDW		> 100			Not specified	Drew (1983)
	Phenol-sulfuric	Sugars	Above	DW		169			Philippines	Tomasko et al. (1993)
Thalassia hemprichii	GC	Sucrose	Leaf	DW		19			Saudi Arabia	Drew (1980)
	GC	Sucrose	Leaf	DW		12			Seychelles	Drew (1980)
	Anthrone	Sugars	Leaf	DW	85	-	135	*	Zanzibar,	Deyanova *et al.* (2017)
	Phenol-sulfuric	Sugars	Above	DW	92				Philippines	Tomasko et al. (1993)
	Anthrone	Sugars	Rhizome	DW	80	-	210	*	Zanzibar,	Deyanova et al. (2017)
	Anthrone	Sugars	Rhizome	DW	70	-	120	*	Zanzibar,	Eklöf *et al.* (2008)
	Anthrone	Sugars	Root	DW	30	-	55	*	Zanzibar,	Deyanova et al. (2017)
Thalassia testudinum	Phenol-sulfuric	Sugars	Leaf	DW	25	-	120		Florida, US	Dawes *et al.* (1979)
	GC	Sugars	Leaf	EDW	100				Not specified	Drew (1983)
	Anthrone	Sugars	Above	DW	15	-	23	*	China	Jiang *et al.* (2013a)
	MBTH	Sugars	Rhizome	DW	175	-	225	*	Florida, US	Campbell and Fourqurean (2013)
	Phenol-sulfuric	Sugars	Rhizome	DW	110	-	350	*	Florida, US	Dawes & Lawrence (1980)
	MBTH	Sugars	Root	DW	55	-	60	*	Florida, US	Campbell & Fourqurean (2013)
	Anthrone	Sugars	Below	DW	58	-	88	*	China	Jiang *et al.* (2013a)
Thalassodendron ciliatum	GC	Sucrose	Leaf	DW		3			Seychelles	Drew (1980)
	GC	Sugars	Leaf	EDW		39			Not specified	Drew (1983)
Thallassia hemprichii	GC	Sucrose	Leaf	EDW		> 100			Not specified	Drew (1983)
Zostera augustifolia	GC	Sucrose	Leaf	DW		127			Scotland	Drew (1980)
Zostera marina	GC	Sucrose	Leaf	DW	184	-			England	Drew (1980)
	Resorcinol	Sugars	Leaf	FW	7	-	30	*	Monterey, US	Alcoverro *et al.* (1999)
	Phenol-sulfuric	Sugars	Leaf	DW	32	-	144		Chesapeake	Burke *et al.* (1996)
	Anthrone	Sugars	Leaf	FW	17	-	164	*	Mexico	Cabello-Pasini *et al.* (2002)
	Anthrone	Sugars	Leaf	DW	42	-	130	*	Mexico	Cabello-Pasini *et al.* (2004)

Table A.1. continued.

					Sugars mg g^{-1}					
Species	**Assay**	**Carbohydrate**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Zostera marina	Phenol-sulfuric	Sugars	Leaf	DW	84	-	361		Ireland	Dawes and Guiry (1992)
	GC	Sugars	Leaf	EDW		27			Not specified	Drew (1983)
	HPLC	Sugars	Leaf	DW	107	-	161		Oregon, US	Kaldy *et al.* (2013)
	Resorcinol	Sugars	Leaf	FW	14	-	82		California, US	Palacios and Zimmerman (2007)
	Resorcinol	Sugars	Leaf	DW	30	-	341	*	Finland	Salo *et al.* (2015)
	Phenol-sulfuric	Sugars	Leaf	DW	30	-	70	*	Portugal	Silva et al. (2013)
	Anthrone	Sugars	Leaf	FW	3	-	29		North Carolina, US	Touchette and Burkholder (2002b)
	HPLC	Sugars	Leaf	DW		1			Denmark	Vichkovitten and Holmer (2004)
	Anthrone	Sugars	Leaf	FW	74	-	86		Monterey, US	Zimmerman *et al.* (1989)
	Resorcinol	Sugars	Leaf	FW	33	-	46	*	Monterey, US	Zimmerman *et al.* (1995)
	GC	Sucrose	Rhizome	EDW	159	-			England	Drew (1980)
	HPLC	Sucrose	Rhizome	DW	140	-	333		Oregon, US	Kaldy et al. (2013)
	Resorcinol	Sugars	Rhizome	FW	43	-	103	*	Monterey, US	Alcoverro et al. (1999)
	Phenol-sulfuric	Sugars	Rhizome	DW	116	-	229		Chesapeake Bay, US	Burke et al. (1996)
	Resorcinol	Sugars	Rhizome	DW	200	-	500	*	Sweden	Eriander (2017)
	HPLC	Sugars	Rhizome	DW	163	-	392		Oregon, US	Kaldy et al. (2013)
	Resorcinol	Sugars	Rhizome	DW	140	-	400	*	Baltic Sea	Munkes *et al.* (2015)
	Resorcinol	Sugars	Rhizome	DW	50	-	240	*	Finland	Salo et al. (2015)
	Phenol-sulfuric	Sugars	Rhizome	DW	10	-	100	*	Portugal	Silva et al. (2013)
	Anthrone	Sugars	Rhizome	DW	9	-	32		North Carolina, US	Touchette & Burkholder (2002)
	HPLC	Sugars	Rhizome	DW		29			Denmark	Vichkovitten & Holmer (2004)
	Anthrone	Sugars	Rhizome	DW	101	-	124		Monterey, US	Zimmerman et al. (1989)
	HPLC	Sucrose	Root	DW	31	-	57		Oregon, US	Kaldy et al. (2013)
	Resorcinol	Sugars	Root	FW	1	-	7	*	Monterey, US	Alcoverro et al. (1999)
	Phenol-sulfuric	Sugars	Root	DW	23	-	52		Chesapeake Bay, US	Burke et al. (1996)
	HPLC	Sugars	Root	DW	33	-	84		Oregon, US	Kaldy et al. (2013)
	Resorcinol	Sugars	Root	DW	4	-	35	*	Finland	Salo et al. (2015)

Table A.1. continued.

					Sugars mg g^{-1}					
Species	**Assay**	**Carbohydrate**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Zostera marina	HPLC	Sugars	Root	DW		6.82			Denmark	Vichkovitten & Holmer (2004)
	Resorcinol	Sugars	Root	DW	3	-	4	*	Monterey, US	Zimmerman et al. (1995)
	HPLC	Sugars	Whole plant	DW	203	-	268		Oregon, US	Kaldy (2014)
Zostera muelleri	GC	Sucrose	Leaf	EDW	10	-	100		Not specified	Drew (1983)
	GC	Sucrose	Rhizome	EDW		171			Australia	Drew (1980)
	Phenol-sulfuric	Sugars	Rhizome	DW	15	-	47	*	Australia	Maxwell *et al.* (2014)
	GC	Sucrose	Leaf	DW		47			Australia	Drew (1980)
Zostera noltii	GC	Sucrose	Leaf	DW		164			Scotland	Drew (1980)
	GC	Sucrose	Leaf	EDW		> 100			Not specified	Drew (1983)
	Resorcinol	Sugars	Leaf	DW	30	-	65	*	Spain	Brun *et al.* (2003)
	Phenol-sulfuric	Sugars	Leaf	DW	2.0	-	70	*	Portugal	Cabaço and Santos (2007)
	Phenol-sulfuric	Sugars	Leaf	DW	89	-	148		Ireland	Dawes & Guiry (1992)
	Resorcinol	Sugars	Leaf	FW	6 d^{-1}	-	15 d^{-1}	*	Netherlands	Egea *et al.* (2018)
	GC	Sugars	Leaf	DW	35	-	85	*	Mediterranean	Pirc (1989)
	Resorcinol	Sugars	Above	DW	83	-	220		Spain	Brun *et al.* (2002)
	Resorcinol	Sugars	Above	DW	20	-	120	*	Spain	Brun *et al.* (2008)
	Resorcinol	Sugars	Above	DW	18	-	88	*	Spain	Olivé *et al.* (2007)
	Resorcinol	Sugars	Above	DW	100	-	160		Spain	Peralta *et al.* (2002)
	HPLC	Sugars	Above	DW	2	-	60		Netherlands	Vermaat and Verhagen (1996)
	Resorcinol	Sugars	Rhizome	DW	60	-	280	*	Spain	Brun et al. (2003)
	Phenol-sulfuric	Sugars	Rhizome	DW	10	-	90	*	Portugal	Cabaço & Santos (2007)
	Resorcinol	Sugars	Rhizome	FW	9 d^{-1}	-	21 d^{-1}	*	Netherlands	Egea et al. (2018)
	Resorcinol	Sugars	Below	DW	58	-	225		Spain	Brun et al. (2002)
	Resorcinol	Sugars	Below	DW	20	-	171	*	Spain	Brun *et al.* (2008)

Table A.2. List of assays (anthrone, phenol-sulfuric, resorcinol and 3-methyl-2-benzothiazolinonehydrazone (MBTH), and chromatographic assays: gas liquid chromatography (GC) and high performance liquid chromatography (HPLC)) used in the existing literature for the analytical quantitation of starch listed by seagrass species with supporting references. Min and max mean estimates are standardized to mg g^{-1} of tissue (leaf, sheath, above ground, rhizome, root, below ground or whole plant). State of tissue are either dry weight (DW), fresh weight (FW) or extracted dry weight (EDW). * Indicates approximate estimates obtained from graphs in referenced material.

				Starch mg g^{-1}					
Species	**Assay**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Amphibolis griffithii	Phenol-sulfuric	Leaf	DW	110	-	250	*	Australia	Mackey et al. (2007)
	Phenol-sulfuric	Rhizome	DW	22	-	62	*	Australia	Mackey et al. (2007)
Cymodocea nodosa	Anthrone	Leaf	DW		4			Spain	Malta et al. (2006)
	Anthrone	Leaf	DW		63		*	Spain	Mascaro *et al.* (2009)
	Anthrone	Leaf	DW	3	-	4		Mediterranean	Pirc (1989)
	Anthrone	Leaf	DW	15	-	18		Portugal	Sandoval-Gil et al. (2012)
	Anthrone	Leaf	DW	28	-	41	*	Spain	Sandoval-Gil et al. (2014)
	Phenol-sulfuric	Leaf	DW	6	-	11	*	Portugal	Silva et al. (2013)
	Anthrone	Rhizome	DW		4			Spain	Malta et al. (2006)
	Anthrone	Rhizome	DW	3	-	7		Mediterranean	Pirc (1989)
	Anthrone	Rhizome	DW	37	-	133		Portugal	Sandoval-Gil et al. (2012)
	Anthrone	Rhizome	DW	15	-	50	*	Spain	Sandoval-Gil et al. (2014)
	Phenol-sulfuric	Rhizome	DW	28	-	38	*	Portugal	Silva et al. (2013)
	Anthrone	Root	DW		4			Spain	Malta et al. (2006)
	Anthrone	Root	DW	1	-	9		Mediterranean	Pirc (1989)
Halophila ovalis	Phenol-sulfuric	Leaf	DW	72	-	78	*	Australia	Longstaff *et al.* (1999)
	Phenol-sulfuric	Rhizome	DW	50	-	60	*	Australia	Longstaff et al. (1999)
	Phenol-sulfuric	Root	DW	83	-	100	*	Australia	Longstaff et al. (1999)
Halophila spinulosa	Enzymatic	Below	DW	13	-	25	*	Netherlands	Chartrand *et al.* (2018)
Posidonia oceanica	Anthrone	Leaf	DW	42	-	58		Spain	González-Correa *et al.* (2008)
	Anthrone	Leaf	DW	6	-	28		Spain	González-Correa *et al.* (2009)
	Anthrone	Leaf	DW	0	-		*	Spain	Hernan *et al.* (2017)
	Anthrone	Leaf	DW	2	-	20	*	Italy	Pirc (1985)
	Anthrone	Leaf	DW	6	-	9		Mediterranean	Pirc (1989)

Table A.2. continued

				Starch mg $^{g-1}$					
Species	**Assay**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Posidonia oceanica	Anthrone	Leaf	DW	8	-	12		Portugal	Sandoval-Gil et al. (2012)
	Enzymatic	Leaf	DW	20	-	25		Italy	Scartazza *et al.* (2017)
	Anthrone	Sheath	DW	5	-	20		Italy	Pirc (1985)
	Anthrone	Rhizome	DW	69	-	78		Spain	Delagado et al. (1999)
	Anthrone	Rhizome	DW	43	-	54		Spain	Gonzales-Correa et al. (2008)
	Anthrone	Rhizome	DW	6	-	30	*	Spain	Gonzáles-Correa et al. (2009)
	Anthrone	Rhizome	DW	30	-	40	*	Spain	Hernan et al., (2017)
	Anthrone	Rhizome	DW	42	-	62	*	Spain	Marín-Guirao *et al.* (2017)
	Anthrone	Rhizome	DW	3	-	90		Italy	Pirc (1985)
	Anthrone	Rhizome	DW	1	-	74		Mediterranean	Pirc (1989)
	Anthrone	Rhizome	DW	25	-	75	*	Spain	Ruiz and Romero (2001)
	Anthrone	Rhizome	DW	35	-	61		Spain	Ruiz *et al.* (2001)
	Anthrone	Rhizome	DW	20	-	320	*	Spain	Ruiz *et al.* (2009)
	Anthrone	Rhizome	DW	36	-	63		Portugal	Sandoval-Gil et al. (2012)
	Anthrone	Rhizome	DW	20	-	75	*	Spain	Sandoval-Gil et al. (2014)
	Enzymatic	Rhizome	DW	150	-	175	*	Italy	Scartazza et al. (2017)
	Anthrone	Root	DW	15	-	20	*	Spain	Hernan et al. (2017)
	Anthrone	Root	DW	1	-	6		Mediterranean	Pirc (1989)
Posidonia sinuosa	Phenol-sulfuric	Leaf	FW	17	-	32		Australia	Collier et al. (2008)
	Phenol-sulfuric	Leaf	DW	30	-	85	*	Australia	Collier et al. (2009)
	Phenol-sulfuric	Rhizome	FW	4	-	12		Australia	Collier et al. (2008)
	Phenol-sulfuric	Rhizome	DW	15	-	50	*	Australia	Collier et al. (2009)
Thalassia hemprichii	Anthrone	Leaf	DW	20	-	40	*	Zanzibar, Tanzania	Deyanova et al. (2017)
	Anthrone	Rhizome	DW	20	-	100	*	Zanzibar, Tanzania	Deyanova et al. (2017)
	Anthrone	Rhizome	DW	30	-	40	*	Zanzibar, Tanzania	Eklöf *et al.* (2008)
Thalassia testudinum	Anthrone	Above	DW	17	-	25	*	China	Jiang et al. (2013)
	Anthrone	Below	DW	58	-	70	*	China	Jiang et al. (2013)

Table A.2. continued

				Starch mg g^{-1}					
Species	**Assay**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Zostera marina	Phenol-sulfuric	Leaf	DW	18	-			Chesapeake Bay, US	Burke et al. (1996)
	Anthrone	Leaf	FW	1	-	8	*	Mexico	Cabello-Pasini *et al.* (2002)
	Phenol-sulfuric	Leaf	DW	9	-	12	*	Portugal	Silva et al. (2013)
	Anthrone	Leaf	FW	4	-	6		Monterey, US	Zimmerman et al. (1989)
	Phenol-sulfuric	Rhizome	DW	37	-	60		Chesapeake Bay, US	Burke et al. (1996)
	Anthrone	Rhizome	DW	6	-	16	*	Baltic Sea	Munkes et al. (2015)
	Phenol-sulfuric	Rhizome	DW	8	-	13	*	Portugal	Silva et al. (2013)
	Anthrone	Rhizome	DW	6	-	7		Monterey, US	Zimmerman et al. (1989)
	Phenol-sulfuric	Root	DW	122	-	134		Chesapeake Bay, US	Burke et al. (1996)
Zostera muelleri	Phenol-sulfuric	Rhizome	DW	56	-	80	*	Australia	Maxwell et al. (2014)
Zostera noltii	Anthrone	Leaf	DW	2	-	6	*	Spain	Brun et al. (2003)
	Phenol-sulfuric	Leaf	DW	80	-	180	*	Portugal	Cabaço & Santos (2007)
	Anthrone	Leaf	FW	6 d^{-1}	-	15 d^{-1}	*	Netherlands	Egea et al. (2018)
	Anthrone	Leaf	DW	5	-	9		Mediterranean	Pirc (1989)
	Anthrone	Above	DW	4	-	6		Spain	Brun et al. (2002)
	Anthrone	Above	DW	3	-	8	*	Spain	Olive et al. (2007)
	Anthrone	Above	DW	2	-	4		Spain	Peralta *et al.* (2002)
	HPLC	Above	DW	0	-	16		Netherlands	Vermaat and Verhagen (1996)
	Anthrone	Rhizome	DW	2	-	16	*	Spain	Brun et al. (2003)
	Phenol-sulfuric	Rhizome	DW	200	-	620	*	Portugal	Cabaço & Santos (2007)
	Anthrone	Rhizome	FW	5 d^{-1}	-	16 d^{-1}	*	Netherlands	Egea et al. (2018)
	Anthrone	Rhizome	DW	19	-	73	*	North Sea	Govers *et al.* (2015)
	Anthrone	Rhizome	DW	0	-	600	*	West coast of continental Europe	Soissons *et al.* (2018b)
	Anthrone	Below	DW	8	-			Spain	Brun et al. (2002)
	Anthrone	Below	DW	2	-	5	*	Spain	Olive et al. (2007)
	Anthrone	Below	DW	40	-	118		Mediterranean	Pirc (1989)
	HPLC	Below	DW	10	-	48		Netherlands	Vermaat and Verhagen (1996)

Table A.3. List of assays (anthrone, phenol-sulfuric, resorcinol and 3-methyl-2-benzothiazolinonehydrazone (MBTH), and chromatographic assays: gas liquid chromatography (GC) and high performance liquid chromatography (HPLC)) used in the existing literature for the analytical quantitation of total non-structural carbohydrates (tNSC = soluble NSC + starch) listed by seagrass species with supporting references. Min and max mean estimates are standardized to mg g^{-1} of tissue (leaf, sheath, above ground, rhizome, root, below ground or whole plant). State of tissue are either dry weight (DW), fresh weight (FW) or extracted dry weight (EDW). * Indicates approximate estimates obtained from graphs in referenced material. ** Standardization made by assuming that reported µmol carbon estimates were sucrose equivalents.

					tNSC mg $^{g-1}$					
Species	**Sugars**	**Starch**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Cymodocea nodosa	Resorcinol	Anthrone	Rhizome	DW	27	-	105		Spain	Mascaro *et al.* (2009)
	Resorcinol	Anthrone	Rhizome	DW	50	-	116		Spain	Mascaró *et al.* (2014)
	Resorcinol	Anthrone	Rhizome	DW	30	-	110	*	Spain	Sanmartí *et al.* (2014)
Halophila ovalis	Anthrone	Anthrone	Leaf	DW		68			Australia	Eklöf *et al.* (2009)
	Anthrone	Anthrone	Rhizome	DW		126			Australia	Eklöf *et al.* (2009)
Posidonia oceanica	Resorcinol	Anthrone	Leaf	DW	11	-	45	*	Spain	Alcoverro *et al.* (2001)
	Resorcinol	Anthrone	Leaf	DW	8	-	23	*	Mediterranean	Gacia *et al.* (2007)
	Resorcinol	Anthrone	Leaf	DW	20	-	80	*	Mediterranean	Invers *et al.* (2004)
	Anthrone	Anthrone	Leaf	DW	25	-	30	*	Spain	Marín-Guirao *et al.* (2013)
	Resorcinol	Anthrone	Sheath	DW	28	-	45	*	Spain	Alcoverro *et al.* (2001)
	Resorcinol	Anthrone	Sheath	DW	22	-	48	*	Spain	Manzanera *et al.* (1998a)
	Resorcinol	Anthrone	Rhizome	DW	44	-	164	*	Spain	Alcoverro *et al.* (2001)
	Resorcinol	Anthrone	Rhizome	DW	75	-	240	*	Mediterranean	Gacia *et al.* (2007)
	Anthrone	Anthrone	Rhizome	DW	132	-	173		Spain	Gera *et al.* (2013)
	Resorcinol	Anthrone	Rhizome	DW	60	-	210	*	Mediterranean	Invers et al. (2004)
	Anthrone	Anthrone	Rhizome	DW	105	-	145	*	Spain	Marin-Guirao et al. (2013)
	Resorcinol	Anthrone	Rhizome	DW	30	-	130		Mediterranean	Pérez *et al.* (2007)
	Resorcinol	Anthrone	Rhizome	DW	50	-	250	*	Mediterranean	Pérez *et al.* (2008)
	Resorcinol	Anthrone	Rhizome	DW	50	-	180	*	Spain	Roca *et al.* (2015a)
	Resorcinol	Anthrone	Rhizome	DW		12			Spain	Tomas *et al.* (2005)
	Resorcinol	Anthrone	Root	DW	15	-	60	*	Spain	Alcoverro et al. (2001)
	Resorcinol	Anthrone	Root	DW	40	-	130	*	Mediterranean	Invers et al. (2004)

Table A.3. continued

					TNSC mg g^{-1}					
Species	**Sugars assay**	**Starch assay**	**Tissue**	**State**	**Min**		**Max**		**Location**	**Study**
Posidonia oceanica	Resorcinol	Anthrone	Root	DW	18	-	110	*	Mediterranean	Pérez *et al.* (2007)
	Resorcinol	Anthrone	Root	DW	20	-	120	*	Mediterranean	Pérez *et al.* (2008)
Thalassia testudinum	MBTH	MBTH	Leaf	DW		50			Texas, US	Lee and Dunton (1997)
	Anthrone	Anthrone	Aboveground	DW	30	-	62	*	China	Jiang *et al.* (2013b)
	MBTH	MBTH	Rhizome	DW	110	-	212	*	Texas, US	Lee and Dunton (1996)
	MBTH	MBTH	Rhizome	DW		131			Texas, US	Lee and Dunton (1997)
	MBTH	MBTH	Root	DW	65	-	95	*	Texas, US	Lee and Dunton (1996)
	MBTH	MBTH	Root	DW		57			Texas, US	Lee and Dunton (1997)
	Anthrone	Anthrone	Belowground	DW	92	-	120	*	China	Jiang et al. (2013b)
Zostera marina	HPLC	Anthrone	Leaf	DW	36	-	70	**	Denmark	Vichkovitten *et al.* (2007)
	Anthrone	Anthrone	Rhizome	DW	44	-	80		China	Soissons *et al.* (2016)
	HPLC	Anthrone	Rhizome	DW	214	-	321	**	Denmark	Vichkovitten et al. (2007)
	HPLC	Anthrone	Root	DW	48	-	68	**	Denmark	Vichkovitten et al. (2007)

Appendix B

Assumption testing and long-term light and temperature averages in Tauranga Harbour, New Zealand (*Chapter 3*).

Assumption testing:

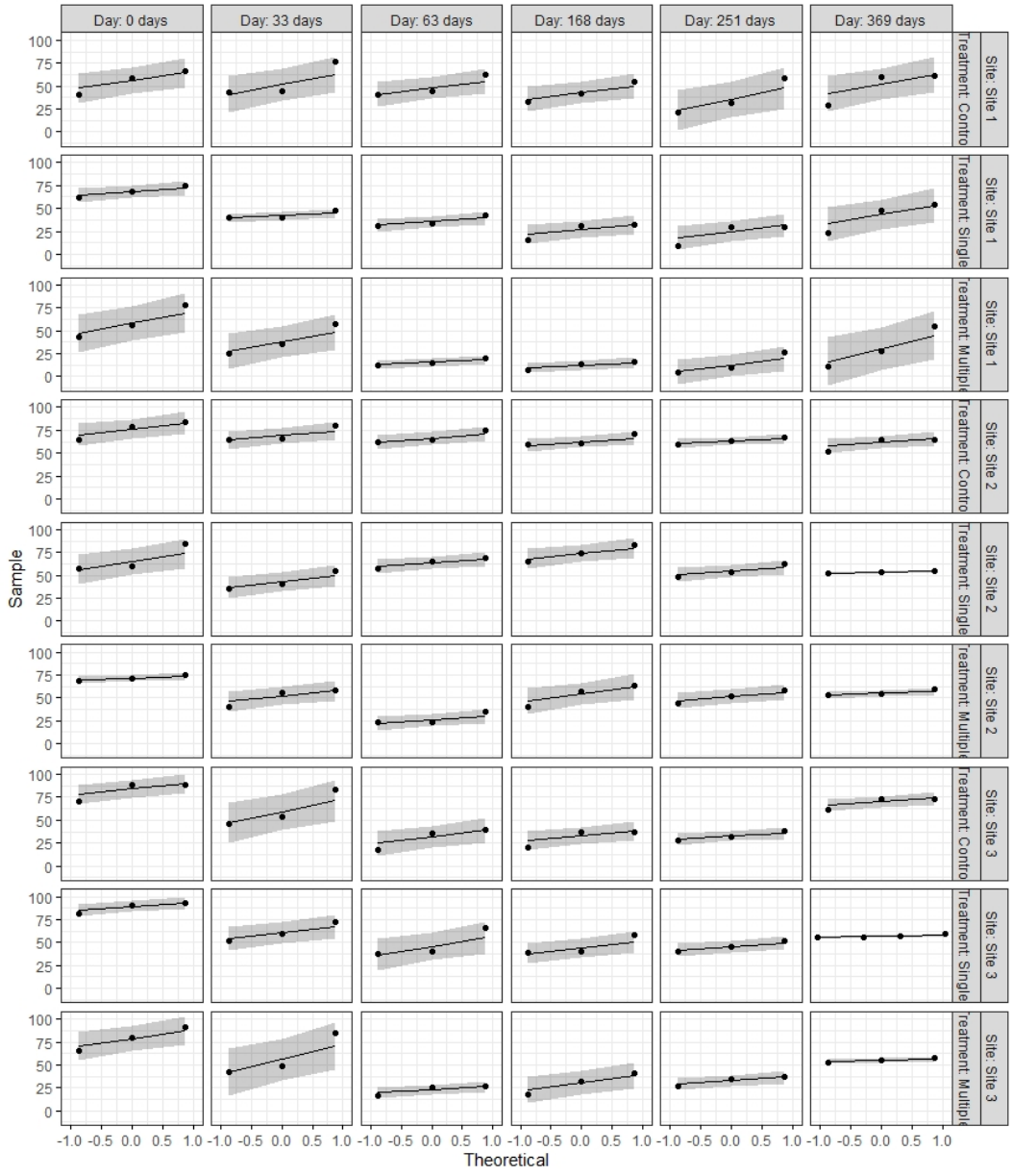

Figure B.1. Dependent variable “relative shoot cover”, group by “Site”, “Treatment” and “time” in QQ probability plot.

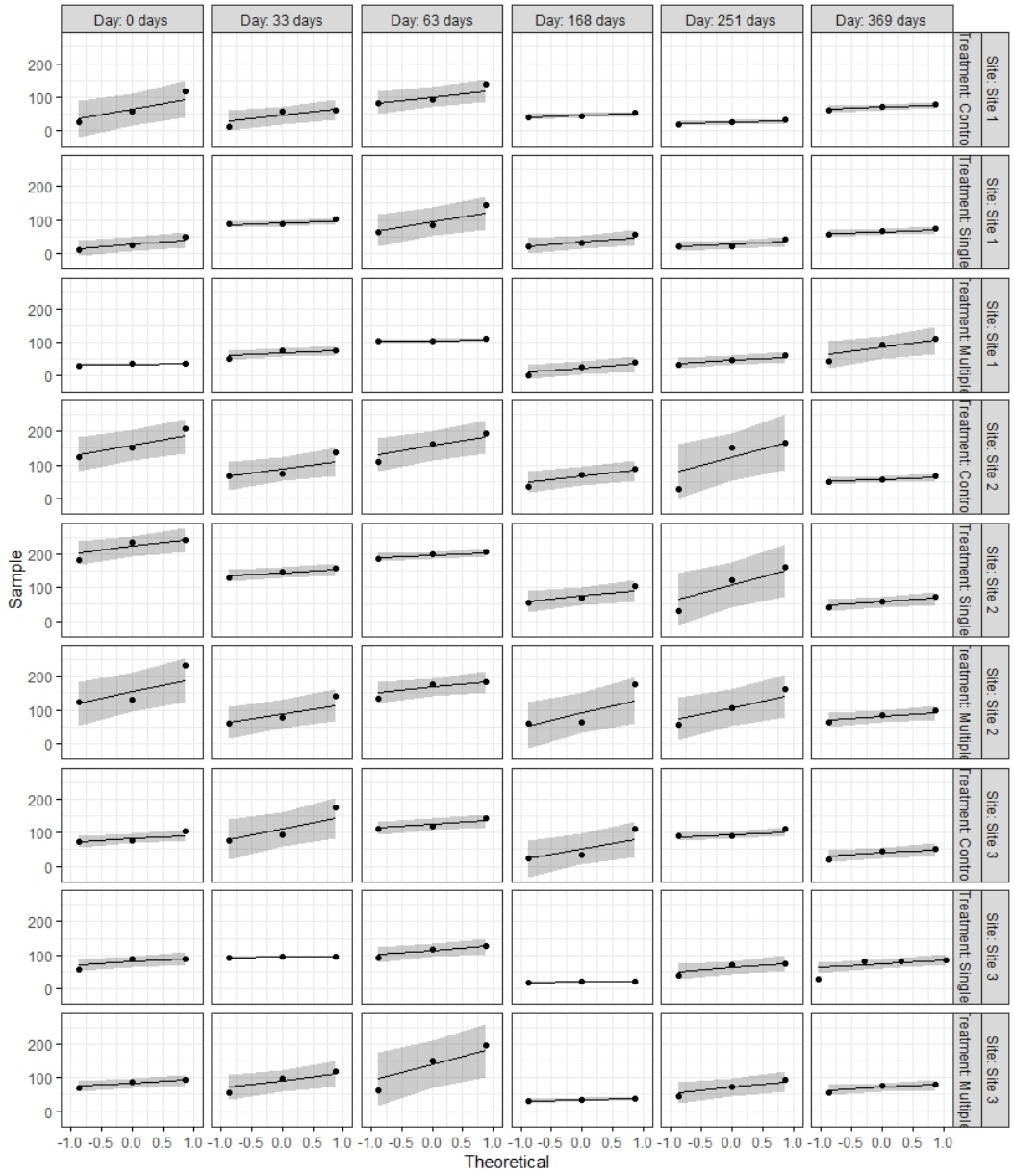

Figure B.2. Dependent variable “sucrose”, group by “Site”, “Treatment” and “time” in QQ probability plot

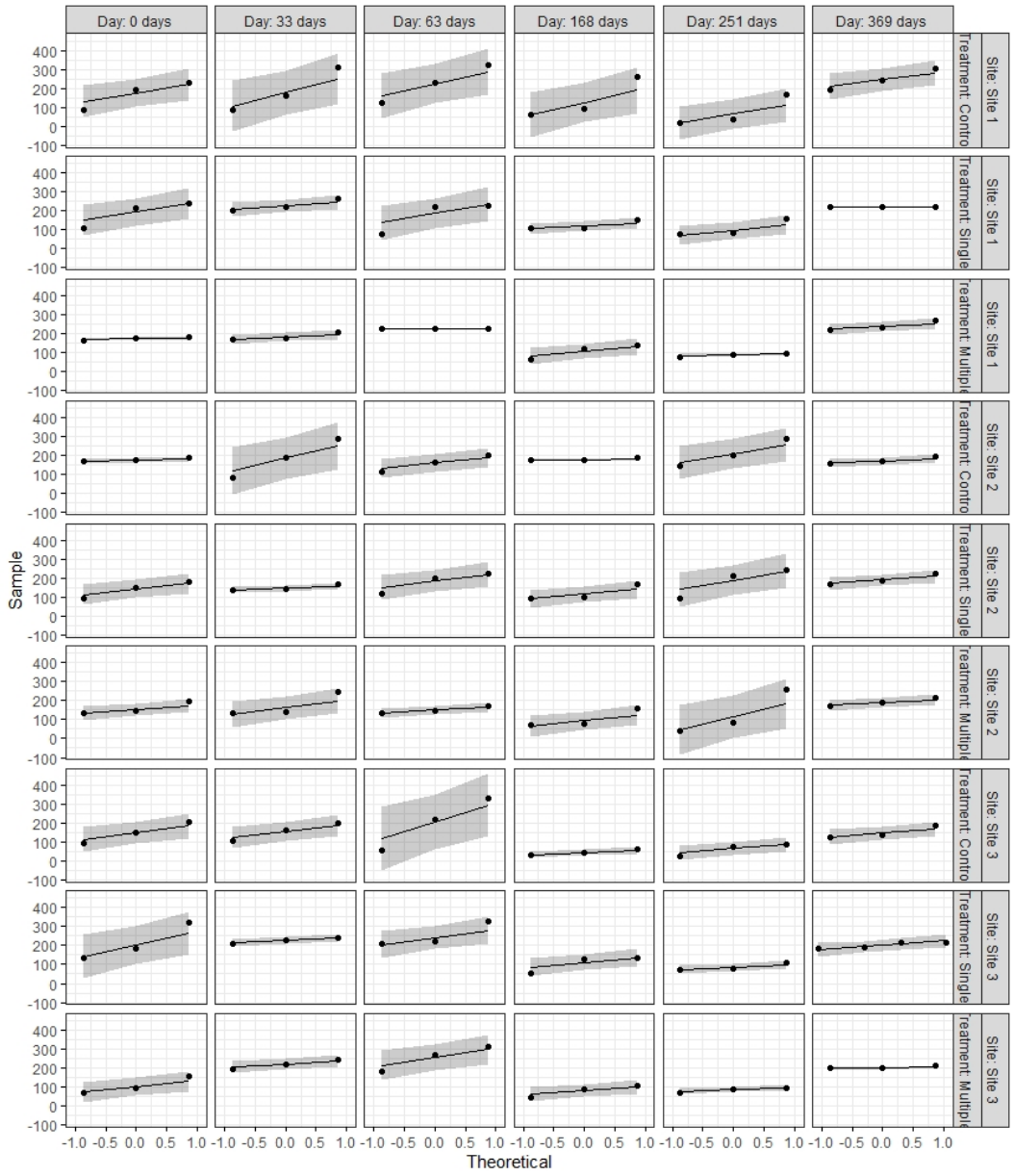

Figure B.3. Dependent variable “starch”, group by “Site”, “Treatment” and “time” in QQ probability plot.

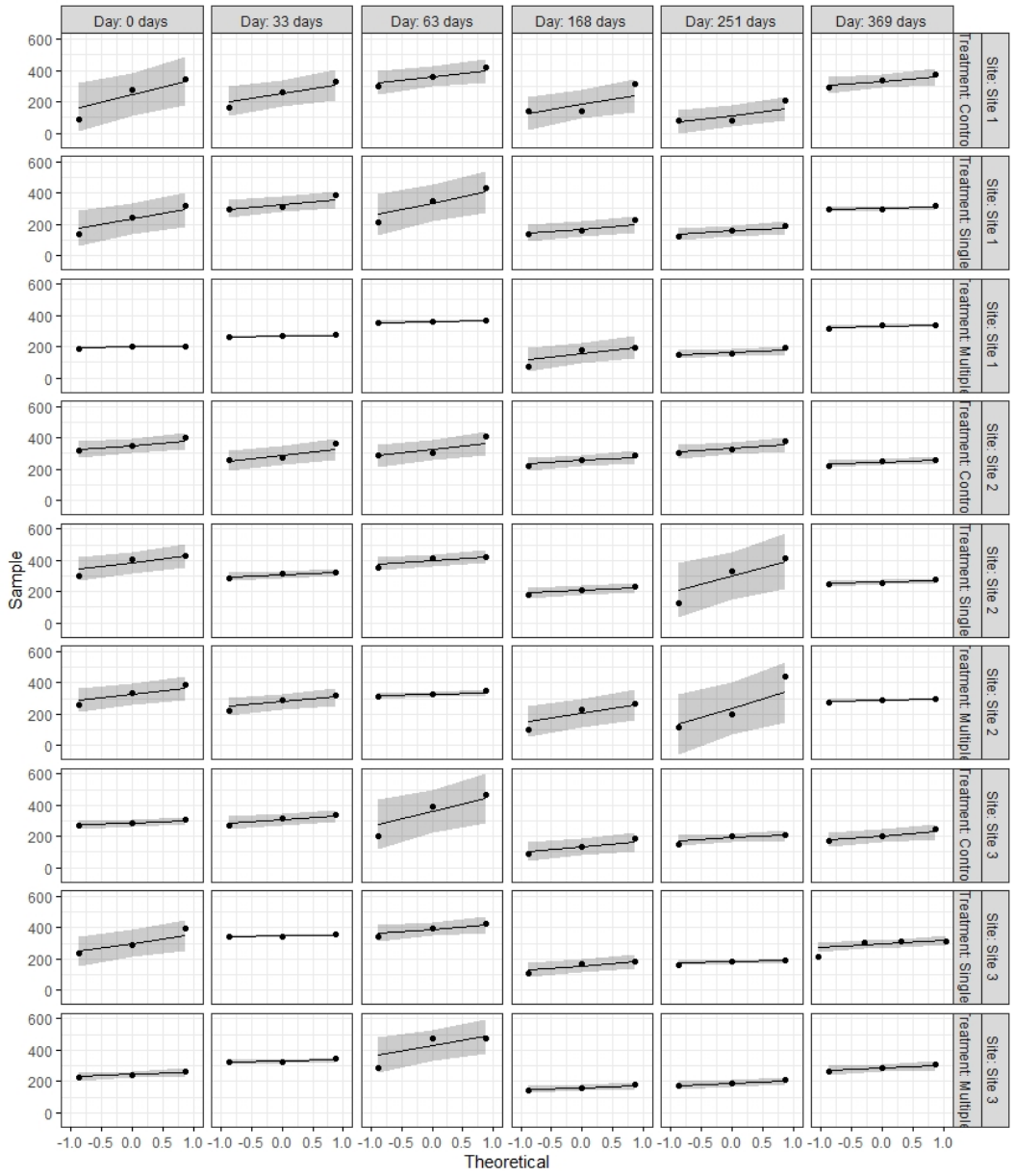

Figure B.4. Dependent variable “tNSC”, group by “Site”, “Treatment” and “time” in QQ probability plot.

Long-term averages

Long-term average air temperatures and sea-surface temperature (SST) for Tauranga Harbour are lowest in winter (July = 10°C, and 13°C respectively) and highest in summer (February = 20°C and 21°C, respectively) (Figure B.1a). The highest average monthly rainfall coincides with winter (July = 127 mm), whereas the average lowest mean rainfalls occur in spring (November = 75 mm,) (Figure B.1b). The greatest amount of daylight hours occur in the height of summer (January = 261 hrs, January) and lowest in winter (June = 135 hrs).

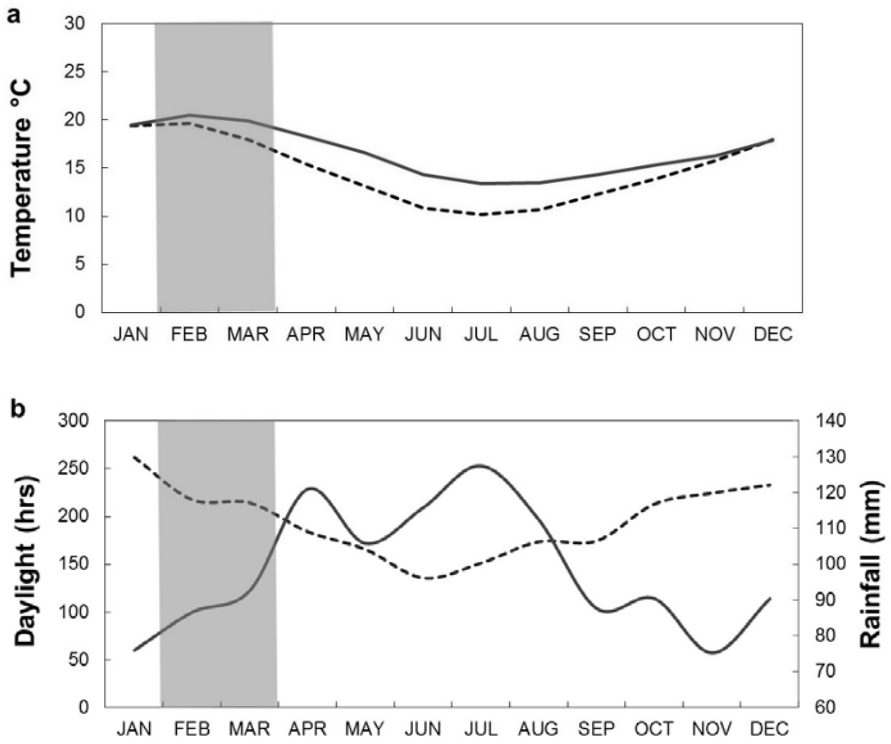

Figure B.1. Monthly averages of a) sea-surface temperature (grey line), air temperatures (dashed line) and b) rainfall (black line) and daylight hours (dashed line) in Tauranga Harbour. Grey box represents the time of experimental burial. Temperature data (2005 – 2017) sourced from Bay of Plenty Regional Council (Mount Maunganui Meteorological Station, http://monitoring.boprc.govt.nz). Mean rainfall data (1990 to 2010) sourced from the National Institute of Water and Atmospheric Research (http://niwa.co.nz).

Irradiance

Day length at the onset of the experiment was 14 hours (sunrise: 06:29 h, sunset: 20:28 h). The median photon flux in the PAR region (296.28 µmol m^{-2} s^{-1}) was similar between the three sites ($H_{[2,297]}$ = 2.43, p = 0.30), but varied significantly throughout the logged period ($H_{[32, 297]}$= 227.97, p < 0.001, Figure B.2). The daily irradiance (I_d) ranged from 5.29 to 82.41 mol m^{-2} d^{-1} from the 3rd Feb 2016 to 6th

Mar 2016. The mean I_d was 30.09 mol m^{-2} d^{-1} in Site 1, 28.28 mol m^{-2} d^{-1} in Site 2 and 24.21 mol m^{-2} d^{-1} in Site 3, which is similar to the levels previously reported from Tauranga Harbour (Kohlmeier *et al.*, 2014).

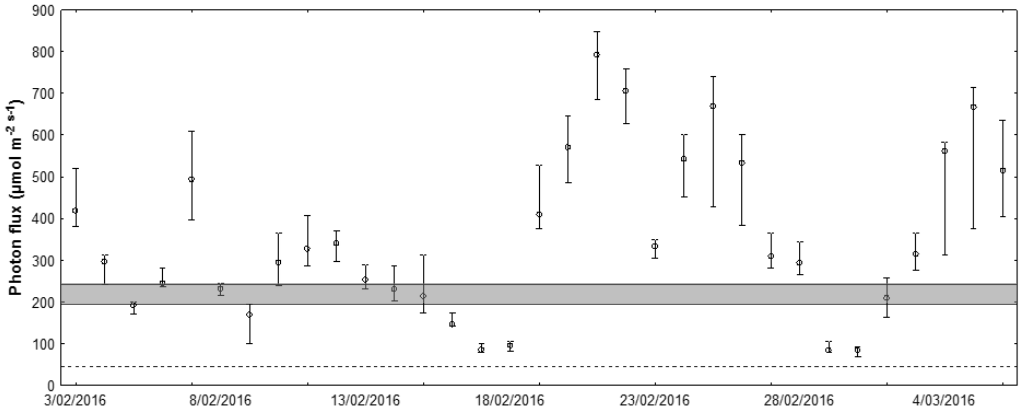

Figure B.2. Median photon flux (µmol m^{-2} s^{-1}) measured across all sites in Tauranga Harbour (3rd February 2016 to 6th of March 2016) using HOBO sensor data loggers. Error bars indicate 25% and 75% percentiles. The grey area marks the saturation irradiance range (Flanigan & Critchley, 1996) and the dashed line mark the compensation irradiance for New Zealand *Zostera muelleri* (Schwarz, 2004).

Appendix C

Effects of burial on *Zostera muelleri*; a preliminary mesocosm experiment.

Abstract

This preliminary study aimed to investigate *Z. muelleri's* response to burial events in a laboratory mesocosm experiment, to remove confounding factors that can influence results when assessed *in situ*. The study encompassed a 55-day experiment, designed to investigate the effects of burial duration and frequency. However, the effects of burial were inconclusive as all treatments (including controls) experienced significant mortalities and NSC depletion throughout the experiment.

The average soluble non-structural carbohydrate (NSC) content significantly declined from 98.18 ±11.60 to 14.40 ± 4.88 mg g^{-1} DW rhizome throughout a 10-day acclimation period. Thus, indicating that the transplantation and the mesocosm environment imposed severe stress on the transplant units. Interestingly, photosynthetic rates (Fv/Fm = 0.79) and the general appearance of plants at the onset of the experiment appeared to be consistent with that of healthy seagrasses documented *in situ.* The severe reduction in soluble NSC content of rhizomes at the onset of the experiment highlights the efficiency of this particular variable as an early indicator of stress in seagrass. Although starch was not quantified in this study, only minor increases in glucose were measured post sucrose depletion, thus starch metabolism was not detected following the depletion of sucrose stores. These results support prior results linking sucrose to *Z. muelleri* resilience, as described in Chapter 3.

Introduction

The response of seagrass to burial and the increasing frequency of these events are of significant importance to restoration ecologists (Campbell, 2016). Sedimentation regimes have changed significantly in modern time, due to the increased anthropogenic pressure on the world's coastal regions. Terrestrial sediment enters estuaries and coastal waters as a result of runoff from the land, river and stream erosions and landslides, with a large proportion of sediment influx occurring during storm events (Thrush *et al.*, 2004). Furthermore, harbour construction and dredging

cause significant increases in sediment organic matter and sediment deposition rates, especially fine sand (Roca *et al.*, 2014).

Manipulative experiments (including microcosms, mesocosms and enclosures) have become increasingly popular amongst scientists to manipulate and examine ecosystems under controlled and repeatable conditions, both in the field and in the laboratory (Petersen *et al.*, 2009). Manipulative studies in a laboratory setting enable the exclusion of external variables and isolate the physiological response to a desired disturbance or stressor. Consequently, the results act as indicators of biological/physiological behaviour as it disregards naturally occurring biotic and abiotic influences. Historically, manipulative experimental studies aiming to assess seagrasses response to burial have most commonly been conducted *in situ.* However, a handful has investigated this in laboratory/mesocosm settings (Table 1, Chapter 3). The field experiment reported in chapter 3 investigated the connection between the natural systems and the experimental systems, thus, the effects of sedimentation were applied directly to the natural environments. This preliminary study aimed to generalise the response of seagrasses beyond the systems that were directly studied. Thus the burial trials were conducted in a mesocosm laboratory setting. Specifically, this chapter aims to investigate the general response of *Z. muelleri's* to increased burial duration and frequencies, which in combination with an extensive field study (chapter 3) was intended to unravel the strength of tolerance and resilience to this specific abiotic stressor.

Methodology

Plant collection

Seagrass transplants were collected on the 5th and 6th of January 2016 from an intertidal seagrass meadow off the shores of Matapihi, Tauranga, New Zealand (37°41'24.2 S 176°10'53.4 E). This meadow was chosen as a donor site due to the dense surface cover of plants and ease of access. Collection occurred with the consent of the regional council and local hapu.

Plants were collected from the seaward growing edge of the seagrass meadow. The rhizome growing tips were located and carefully dug out by hand, ensuring that the meristem, roots and the four youngest shoots were collected per plant (sprigs). Sprigs were selected for this experiment rather than plugs (core sample transplant including rhizomes, roots and ambient sediment) as the sediments from plugs would

contain unknown variables including the content of microbes, macrofauna and contaminants. Additionally, using sprigs ensured similar age of all units, as both NSC (Chapter 2) content and photosynthetic properties are associated with plant age (Durako & Kunzelman, 2002; Enríquez *et al.*, 2002).

A total of 324 sprig units (9 per replicate) were transported in bins with ambient seawater to the University of Waikato's Coastal Marine Field Station, Tauranga, New Zealand within 2 hrs of collection. The sprig units were rinsed in several baths of particle filtered seawater (1µm mesh) before being placed in mesocosm holding tanks and left to acclimate for ten days.

Mesocosm system set-up

A total of 12 treatments (six burial treatments & six control treatments) each triplicated were established in a series of static mesocosms. The experiment was conducted in the Southern Hemisphere's summer and ran for 55 days (15/01/16 to 09/03/16). The experimental design included three burial durations (5, 10, 15 days) at two frequencies; a one-off event (one burial) or repeated events (two burials). The treatments were randomly allocated to tanks, and each treatment consisted of nine seagrass plants (sprigs), which were randomly allocated between the 36 tanks.

Each individual mesocosm was contained in a glass tank (31 x 30 x 60 cm) and installed in one of two indoor water baths (2 x 18 tanks). Each of the water baths were installed to circulate through separate refrigerators that enabled temperature control of the mesocosms (Figure C.1). The individual mesocosm units contained 40 litres particle filtered (1 µm mesh) natural seawater collected from Tauranga Harbour and 10 litres of washed fine sand (from the local landscaping retailer). All mesocosms were installed with an air stone to ensure aeration of the static system. The water bath systems were covered by black tarpaulin to ensure even distribution of artificial light (LED growing lights).

Figure C.1. Laboratory mesocosm system at the University of Waikato's Coastal Marine Field Station in Sulphur Point, Tauranga. Water baths and black cover installed to control light and temperature regime.

Average surface light was measured using a light meter (LI-250A, Li-Cor Biosciences) at 280.38 µmols per m^{-2} s^{-1} (±3.14 µmols per m^{-2} s^{-1}) and the light to dark (L:D) ratio was set to mimic the natural light regime at the seagrass collection site upon collection (14:10 hrs). Saturating irradiance for intertidal New Zealand *Z. muelleri* (former *Z. capricorni*) has been described to range from 195 to 242 µmols per m^{-2} s^{-1} (Schwarz, 2004). Salinity, dissolved oxygen (DO%), pH and temperature were measured every second day throughout the experimental period using an Aquaprobe AP-2000 (Aquaread Ltd.). Additional filtered freshwater/seawater was added to ensure steady readings of these parameters. Slow releasing nutrients were not added to the experiment as the quantification of nutrient concentration and dissolution rates in sediments required additional resources. It was assumed that the nutrients in the filtered seawater would provide the seagrasses with sufficient nutrients as a previous study found no significant difference to *Z. muelleri* morphometrics after the addition of nutrients (Rankin, 2012), however, dissolution rates were not quantified in this study.

Mesocosm treatments and sampling regime

Three of the nine plants from each treatment replicate were sampled immediately after allocation to tanks (before burial) on day 1. The remaining six plants were then randomly planted in tanks in two rows of three plants (Figure C.2). Burial treatments commenced the following day 16/01/16. A frame (2 cm height) was placed around each plant and sand filled-in to cover plants in the burial treatments, whereas control treatments were left uncovered.

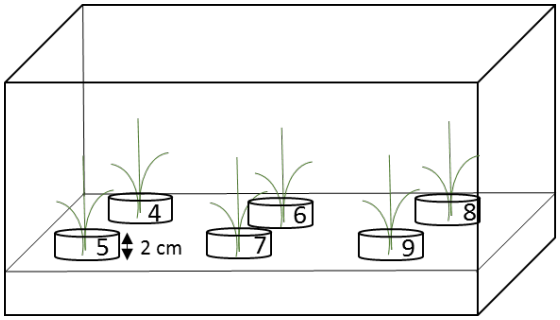

Figure C.2. Plant layout in treatment replicate. Unit 1, 2 and 3 were sampled immediately before burial commenced.

Single burials

Single burial treatments were covered by 2 cm of sand for either 5, 10 or 15 days respectively. At the end of burial, sand was gently removed ensuring meristem was not exposed in the process. After 48 hrs of settling, three plants were sampled (post-burial) from each treatment and control treatment. The remaining three plants in each replicate for both treatments and controls were sampled after 10 days of recovery (post-recovery).

Repeated burials

Repeated burials were covered by 2 cm of sand for 5, 10 or 15 days respectively. At the end of the burial period, the sand was gently removed ensuring the meristem was not exposed in the process. After 48 hrs of settling, the burial treatments were covered by 2 cm of sand for further 5, 10 or 15 days before the sand was removed again in the same manner. After 48 hrs of settling three plants were sampled (post-burial) from each treatment and control treatment. The remaining three plants in each replicate for both treatments and controls were sampled after 10 days of recovery (post-recovery)

Seagrass variables

Shoot mortality (%)

All plants had four live shoots (min. one green blade per shoot) at time of experiment start. The number of live shoots was recorded at each sampling event and shoot mortality was calculated as:

$$\textbf{Shoot mortality (\%)} = \left(\frac{Sf}{Si}\right) * 100$$

Where Sf was a final number of shoots and Si was the initial number of shoots.

Productivity

Length and diameter of rhizomes along with total weight were measured for all plants at the onset of the experiment to measure productivity. At each of the three sampling events (before burial, post-burial, post-recovery), seagrasses were scanned on a flatbed scanner and the resulting image used for later measurements of rhizome diameter, rhizome length, blade width, and blade length. Above and below ground tissue were separated and weighed using electronic scales (0.000 g). Rhizome texture and colour was also recorded.

Photosynthetic parameters

The maximum quantum yield of electron transfer (Fv/Fm) was measured in the laboratory immediately after removal from the mesocosm using a pulse-amplitude modulated (mini-PAM) fluorometer (Walz, Germany). To ensure consistency and comparability, measurements were made on the second youngest leaf on the youngest shoot of the plant, 1 cm above the leaf sheath, as fluorescence parameters are commonly associated with leaf age (Durako & Kunzelman, 2002; Enríquez *et al.*, 2002). Any external matter was removed from the leaves before being placed in a leaf clip and dark adapted for 5 minutes. The fibre-optic cable was held 5 mm from the leaf surface when conducting measurements.

Non-structural carbohydrates

Non-structural carbohydrates were extracted and analysed from seagrass rhizomes as outlined in Chapter 2. Due to severe tank effect and time constraints in this study, only select samples were analysed for NSC contents. The three subsamples from each control treatment replicate were extracted and pooled, after which three replicates were randomly selected from each day post harvesting (day 0 - 65).

Soluble sugars were extracted in a two-hour hot-water extraction post-freeze-drying for three hours. The supernatant was filtered (ash-less filter paper) before being analysed using high-performance liquid chromatography (HPLC) as described by Sørensen *et al.*, (2018). Concentrations of specific sugars were quantified using sucrose, fructose and glucose standard calibration curves.

Statistical analysis

Data was for tested for normal distribution using a Kolmogorov-Smirnov test and tested for homogeneity of variance using a Brown-Forsyth's test. As data were non-parametric, Mann-Whitney U tests were used to investigate the differences between each treatment and its specific control. Additionally, Chi-Square Median tests were used to investigate the effect of all treatments on the various seagrass variables measured.

Results

The water quality parameters measured throughout the experimental period remained close to constant (Table C.1) after some initial technical difficulties were experienced during the first three days of the experiment. The technical difficulties were due to a power outage, that disabled the refrigerators and caused the mesocosm temperature to fluctuate from 20.4°C to 30.7°C and caused disruption in the light regimes.

Table C.1. Mesocosm water quality parameters measured throughout the experimental period (n = 23), using Aquaprobe AP-2000 (Aquaread Ltd.). Values represent mean (±SE).

DO (%)	pH	Temperature (°C)	Salinity (ppt)
106.35 (±0.10)	7.69 (±0.05)	23.02 (±0.25)	±0.83)

Shoot mortality (%)

Shoot mortality was high across all treatments as 61.1% of all plants experienced ≥ 50% shoot mortality, and 44% of plants experienced 100% shoot mortality at final sampling (post-recovery). Single burial treatments caused significantly higher shoot mortalities (Figure C.3) when measured post-burial when buried for 10-days ($U_{[1]}$ = 21.0, p = 0.049) and 15-days ($U_{[1]}$ = 14.00, p =0.02). Whereas, multiple burial treatments caused significant elevated shoot mortality post-burial when

buried for 5-days ($U_{[1]}$ = 15.0, p = 0.03). Interestingly, there was a significant difference between controls and burial treatment in the 15-days multiple burial trial ($U_{[1]}$= 22.5, p = 0.03), however, the median shoot mortality was higher in control treatments (100%, 25th to 75th percentiles = 100% to 100%) than burial treatments (median = 100%, 25th to 75th percentiles = 75 to100%).

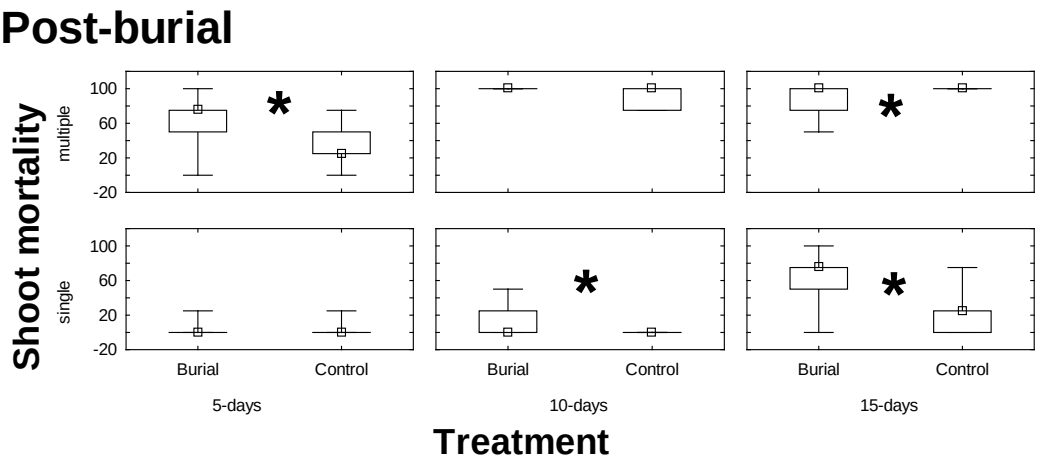

Figure C.3. Post-burial median shoot mortalities □ (box = percentiles, whiskers = min//max values) of 5, 10 and 15-days burial and control treatments in single and multiple burial events. *Indicates significant differences between burial and control treatments as results of Mann-Whitney U-tests (p < 0.05).

Following the recovery period (10-days), the 5-days multiple burial treatment was the only treatment to significantly increase shoot mortality ($U_{[1]}$ = 19.00, p = 0.04). However, this lack of treatment effect was not due to recovery but due to increased mortality of all experimental units, including controls (Figure C.4). A correlation analysis confirmed a very significant association between experimental period and mortality (R = 0.86, p = 0.0069) and a regression analysis found that time explained 74% of the shoot mortality of both controls and treatment groups (r^2 = 0.741, p < 0.0001).

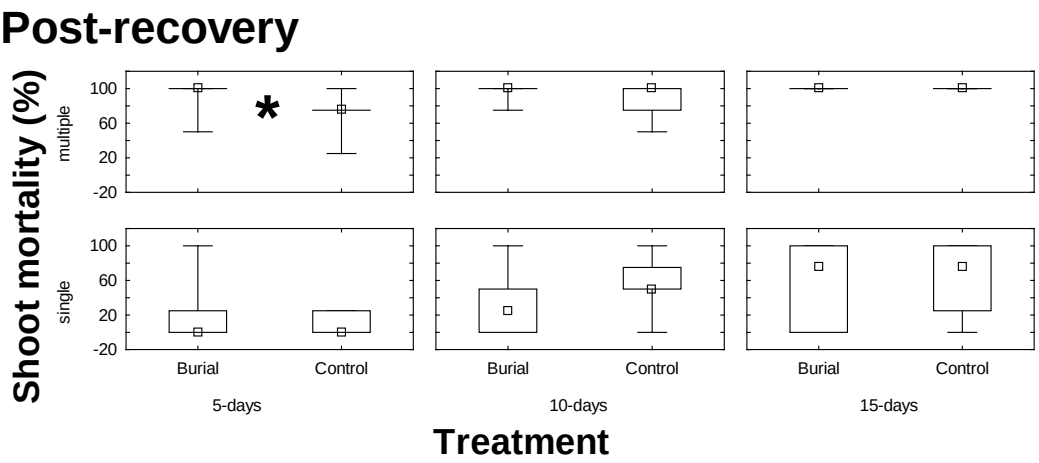

Figure C.4. Post-recovery median shoot mortalities □ (box = percentiles, whiskers = min//max values) of 5, 10 and 15-days burial and control treatments in single and multiple burial events. *Indicates significant differences between burial and control treatments as results of Mann-Whitney U-tests ($p < 0.05$).

Productivity

None of the plants in the mesocosm experiment produced new blades during the experiment and blade extension could not be measured due to the high percentage of shoot mortality. Fifty percent of all plants experienced rhizome elongation throughout the experiment, at an average 2.29 mm (±SE 0.32 mm). Despite some rhizome extension, all plants (incl. controls) experienced biomass loss throughout the experiment, averaging -0.17 g (±0.01 g). All burial treatments and controls experienced a decrease in biomass through the experimental period (Figure C.5 and C.6). However, the percentage biomass loss was significantly affected by the more severe burial treatments including the single burial of 15-days ($U_{[1]} = 8.00$, $p = 0.005$) and multiple burials of 10-days ($U_{[1]} = 6.00$, $p = 0.003$) and 15-days ($U_{[1]} = 13.00$, $p = 0.02$).

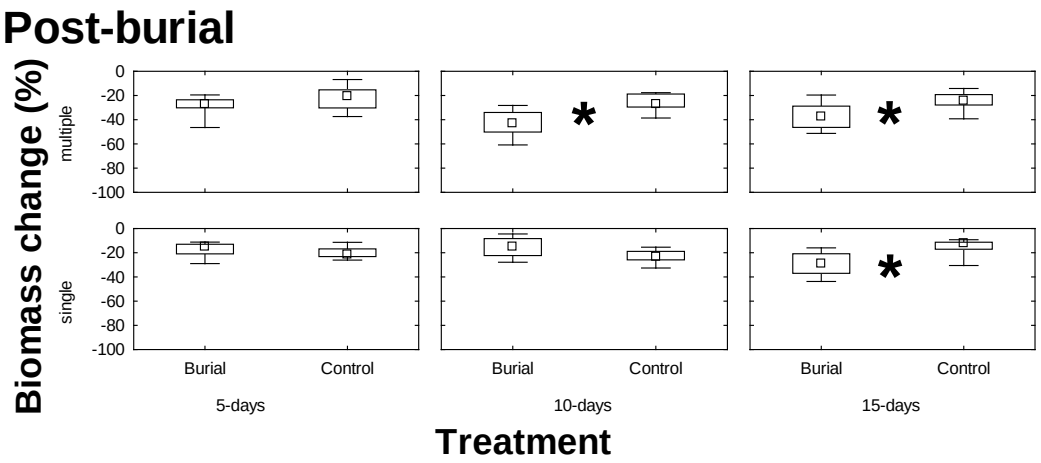

Figure C.5. Post-burial median percentage biomass change □ (box = percentiles, whiskers = min//max values) of 5, 10 and 15-days burial and control treatments in single and multiple burial events. *Indicates significant differences between burial and control treatments as results of Mann-Whitney U-tests ($p < 0.05$).

Following the 10-day recovery period, only the multiple burial treatment of 15-days was still significantly affected the percentage biomass change ($U_{[1]} = 14.00$, $p = 0.02$, Figure C.6).

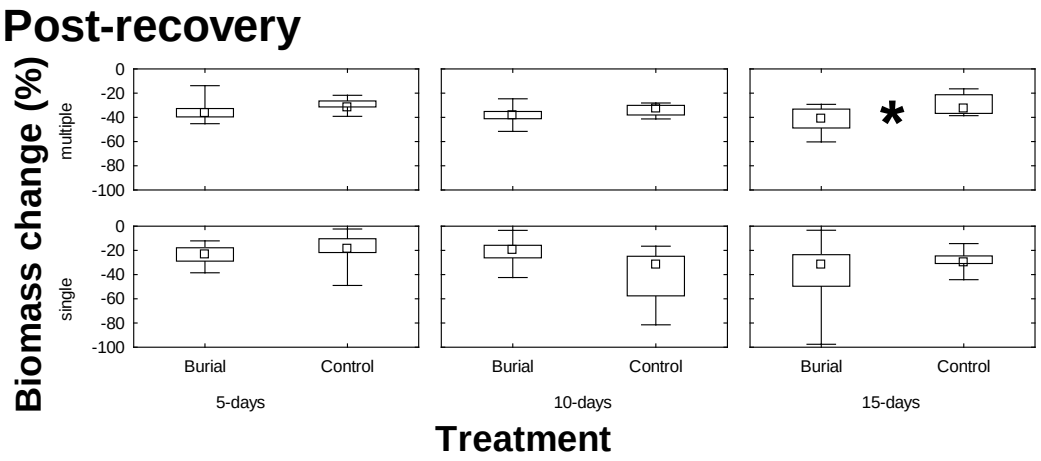

Figure C.6. Post-recovery median percentage biomass change □ (box = percentiles, whiskers = min//max values) of 5, 10 and 15-days burial and control treatments in single and multiple burial events. *Indicates significant differences between burial and control treatments as results of Mann-Whitney U-tests ($p < 0.05$).

Photosynthetic parameters

When measured at day 1 at experiment initiation, the median Fv/Fm 0.80 (ranging between 0.73 and 0.84) was similar across all treatments ($\chi^2_{[11]} = 9.97$ $p = 0.53$) indicating none- stressed plants (Turner & Schwarz 2006) . Only one treatment, single burial for 15-days, significantly decreased the photosynthetic activity of

seagrass immediate after burial treatments ($U_{[1]}$ = 9.5, p = 0.007, Figure C.7). Medians for single burial control treatments (5, 10 and 15-days controls) as well as the two shorter single burials (5 and 10-days burial) remained close to the initial average value of 0.78. However, the remainder of the longer duration treatments, including their controls, all experienced significant decreases in Fv/Fm values (Figure C.7).

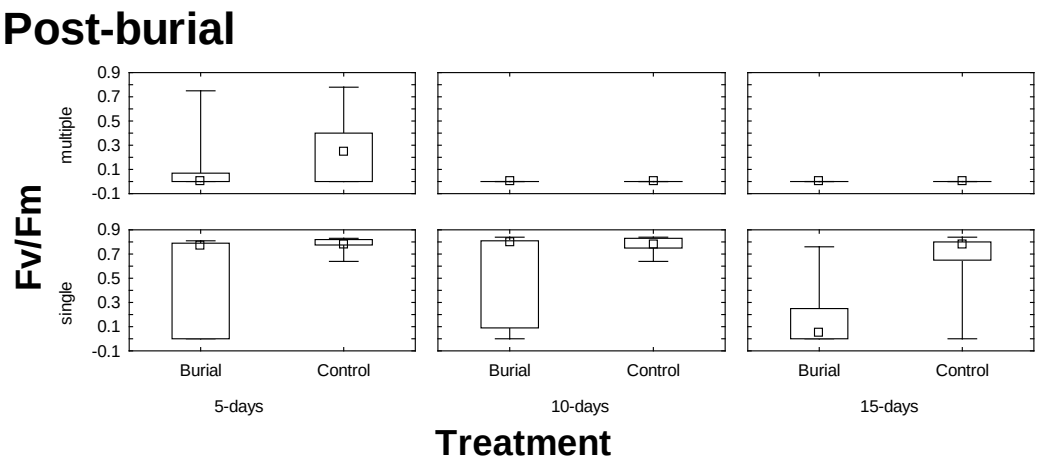

Figure C.7. Post-burial median Fv/Fm □ (box = percentiles, whiskers = min//max values) of 5, 10 and 15-days burial and control treatments in single and multiple burial events. *Indicates significant differences between burial and control treatments as results of Mann-Whitney U-tests (p < 0.05).

Throughout the 10-day recovery period, Fv/Fm values significantly declined and no significant treatment effects were detected. Fv/Fm of all single treatments and their controls were 0.28 ($25^{th}/75^{th}$ percentiles = 0.00/0.75), whilst multiple treatments and control were 0.00 ($25^{th}/75^{th}$ percentiles = 0.00/0.00). As such, Fv/Fm experienced significant declines throughout the recovery period (p < 0.001), indicating severe light stress in all seagrass units.

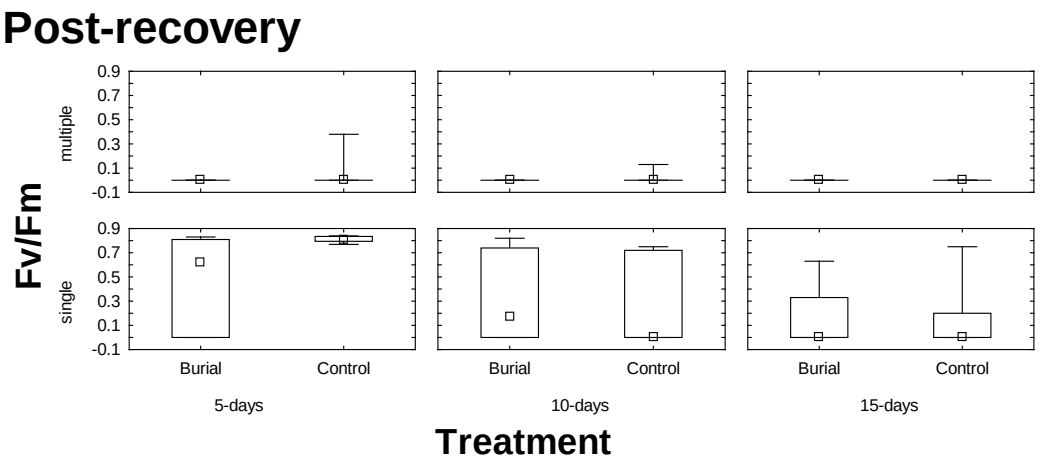

Figure C.8. Post-recovery median Fv/Fm □ (box = percentiles, whiskers = min//max values) of 5, 10 and 15-days burial and control treatments in single and multiple burial events. *Indicates significant differences between burial and control treatments as results of Mann-Whitney U-tests ($p < 0.05$).

Non-structural carbohydrates

As severe tank effects were detected in this experiment, it was decided to limit NSC reserve estimates to three samples (n=30) per experimental sampling day (df = 9) as this would allow for the documentation of soluble NSC reduction and exhaustion over time. Starch was not measured due to time constraints. Three rhizome samples were randomly selected for chemical analysis from the available control sample pool. The contents of the individual soluble sugars were plotted against days post-harvest in order to assess the effect of time in the tank (Figure C.9). Sucrose content was severely depleted between day 0 (78.11 ± 9.11 mg g^{-1}) and day 10 post-harvest (0.96 ± 0.26 mg g^{-1}). As such, sucrose contents were close to depleted by the onset of the burial trial, as the first 10 days post-harvest represent the acclimation period.

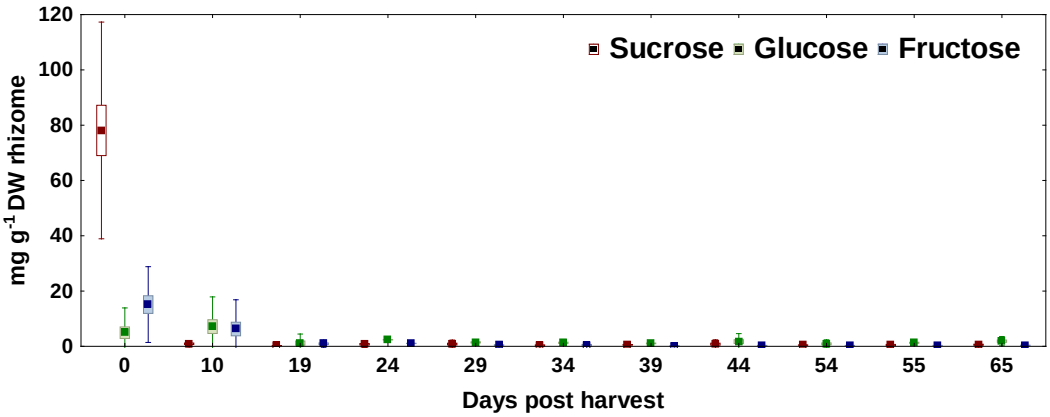

Figure C.9. Mean content of ■ sucrose, ■ glucose and ■ fructose reserves in rhizomes of *Zostera muelleri* (mg g^{-1} DW) following harvest and transplantation into mesocosm. Box represent standard error of means and whiskers 95% confidence intervals.

At the onset of the experiment, total soluble NSC had declined from 98.18 mg g^{-1} (± 11.60 mg g^{-1}) to 14.40 mg g^{-1} (SE ± 4.88 mg g^{-1}), and then consisting mainly of free glucose and fructose (7.16 ± 2.49 mg g^{-1} and 6.28 ± 2.46 mg g^{-1}, respectively Figure C.9). At day 19, soluble NSC had declined to 2.41 mg g^{-1} (± 0.92 mg g^{-1}) with the majority being glucose (1.19 ± 0.76 mg g^{-1}). Free glucose contents then increased by day 25 (2.40 ± 0.09 mg g^{-1}), potentially deriving from the breakdown of starch reserves. Between day 29 and day 65 glucose contents varied from 0.90 (± 0.34 mg g^{-1}) to 1.90 (± 0.39 mg g^{-1}) whereas sucrose and fructose contents remained < 0.8 mg g^{-1}.

Discussion

Due to the severe tank effects evident in the general decline of seagrass health indicators of the control treatments as well as the burial treatments, the effects of burial in this mesocosm experiment were deemed inconclusive. This was despite some detected significant differences ($p < 0.05$) in shoot mortality (%), biomass development and Fv/Fm parameters between treatments and controls. A range of potential explanations is suggested and discussed in this section, including general mesocosm conditions and the transplantation method.

Despite a lack of evidence to support general trends in response of seagrasses to burial, another very interesting discovery was made from this experiment; the high sensitivity of sucrose content to stress. Sucrose content in rhizomes were near depletion post 10 days of holding in mesocosms. However, none of the other parameters measured at the same time suggested declined health of transplants

following the 10-day acclimation period. The drastic decline in sucrose parallel to elevated levels in glucose and unchanged levels in fructose supports the finding from Chapter 2, suggesting that sucrose is the main NSC energy reserve in *Z. muelleri*. This discovery along with the well-defined standard methods for NSC quantification developed in Chapter 2 and the strong significant relationship between sucrose reserves and seasonal survival, now provides the basis for further studies focussing on NSC as a sensitive stress indicator

Mesocosm conditions

Technical issues experienced during the first week of the experimental period caused the temperatures to fluctuate from 20.4°C to 30.7°C and the light regimes to be interrupted from the ambient L:D ratio of 14:10 hrs. These interferences are likely to have imposed additional stress on the newly transplanted seagrass units.

The average temperature measured across three sites in Tauranga Harbour during an *in situ* study (Chapter 3), was, however, measured to fluctuate >20°C over the course of 24 hrs. A manipulative study by Kaldy (2014) found that relative daily growth rates of *Z. marina* production were positively related to temperature and no mortality was experienced due to temperature treatments. The highest temperature treatment tested in the study by Kaldy (2014) was 25°C, which was exceeded during refrigerator outage in this experiment.

York *et al.* (2013) investigated the effect of temperature on the Australian *Z. muelleri* and found that optimal growth was experienced at 27°C, whereas rapid loss of shoots occurred at 32°C. Similarly, studies of *Z. marina* found that respiration exceeds photosynthesis in temperatures above 25°C due to increased metabolism, which limits seagrass growth and cause carbon deficits (Marsh *et al.*, 1986; Zimmerman *et al.*, 1989). Additionally, reduced light levels are, as stated previously, commonly linked to seagrass decline. The light regimes (L:D ratios) were disturbed due to technical difficulties in the first three days of experimentation, however, the relative light levels were not reduced. No studies have investigated the effects of abrupt disruption of light ratios, but it is well known that photosynthetic deficits result in carbon limitations (Alcoverro *et al.*, 1999) and that leaves respond within seconds to light reductions.

Epiphytes were observed on the blades of seagrasses upon the second and third sampling effort. Despite seawater being filtered (mesh 1 μm) and plants being thoroughly rinsed prior to installation in mesocosms, epiphyte spores still appeared in the mesocosm system. Parasites and diseases are commonly avoided by installing an ultraviolet or ozone filter to the system, however, neither were available for this study. Epiphytes are known to reduce the photosynthetic rate of seagrasses by acting as a barrier to carbon uptake and by reducing light (Sand-Jensen, 1977). Increased epiphyte loads are therefore associated with decreased leaf production and are commonly linked to seagrass loss (Silberstein *et al.*, 1986). The occurrence of epiphytic organisms on the blades of seagrasses in the mesocosm, is likely to have contributed to the overall decline in seagrass health.

Altogether, the combination of temperature stress, short light regime interruptions and epiphytic load were assumed to have caused the severe stress experienced in the mesocosm plants. Additionally, the lack of supplementary nutrients most likely prevented recovery of seagrass health post onset of stress.

Transplant method

This experiment aimed to investigate the effect of burial in a controlled environment which involved transplanting experimental units in mesocosms. The reasoning for this was to enable generalisation of seagrasses response to a specific stressor. Transplanting seagrasses is, however, recognised as a complex process producing low success rates (Campbell, 2002). In this study, short sprigs were collected from the growing edge of seagrass meadows and used as the experimental units in order to minimise the unknown variables between the various units. However, *in situ* studies investigating the survival of transplanted sprigs and plugs have highlighted that there is a higher probability of survival using plugs rather than sprigs (Fonseca *et al.*, 1994; Paling *et al.*, 2007). Plugs induce less stress on the rhizomes and roots compared to sprigs and provide better anchorage in the new habitat (Walker, 1994; Paling, 1995). Furthermore, the survival rate following transplanting varies between species (Paling *et al.*, 2001) and the most appropriate transplant type appears to be species specific (Campbell, 2002).

A study using sprigs of *Posidonia oceanica* found that smaller rhizome fragments appeared to experience rapid decline in survival and growth rates within two weeks of being in aquarium settings (Marín-Guirao *et al.*, 2011). This study used rhizome

fragments bearing 40 to 60 shoots and mean growth rate and Fv/Fm of the control units ranged within that of healthy *P. oceanica* measured *in situ*. Considering the failures and successes of previous studies investigating seagrass health post transplantation, it is suggested that the use of small rhizome fragments in combination with removal of ambient sediment, caused the significant stress identified in seagrass transplant units in this study.

Sucrose metabolism

This study found a significant reduction in soluble NSC at onset of experiment when compared to that of rhizomes sampled *in situ*. The severe reduction in soluble NSC, and in particularly sucrose content, suggested that the respiration rates significantly exceeded that of photosynthesis during the 10-day acclimation period. An essential observation was that the general appearance of plants at onset of experiment (post the 10-day acclimation period) were consistent with that of seagrasses observed in the field. Furthermore, the variable Fv/Fm, which is normally considered a sensitive stress indicator (Silva *et al.*, 2009), also ranged between that of healthy plants at the onset of the experiment. Considering this, it is evident that NSC content serves as an extremely sensitive stress indicator, which further supports the importance of standardisation of quantification methods for this parameter (Chapter 2). Standardisation methods will then enable comparability of seagrass studies across space and time.

Recommendations for future research

Considering the findings discussed in the previous section, a few recommendations were made for future research. Firstly, the documentation of NSC metabolism during the critical time immediately post-transplantation for various transplantation types (sprigs, plugs, seedlings) would allow for comparison and evaluation of most appropriate transplant type as stress would be detected at much earlier stage than in traditional stress indicators. Additionally, identification of the critical level of rhizome sucrose content, from which seagrass recovery is significantly reduced, would aid future restoration efforts by enabling assessment of the immediate risk imposed on seagrass meadows health.

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