The ecophysiology of the New Zealand mangrove,

Avicennia marina (Forssk.) Vierh. subsp*. australasica* (Walp.) J. Everett

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Thesis Abstract

The availability of water is an important environmental factor determining plant growth, survival, and distribution globally. From a physiological viewpoint, non-structural carbohydrates (NSCs) are necessary for plants to cope with abiotic factors such as drought and may allow us to understand how plants like mangroves are able to survive in what is seemingly an inhospitable habitat. Furthermore, the metabolic profiles of these plants may also provide relevant information as to how routine metabolic processes contribute to their success in estuarine conditions. In this thesis, I studied the role of NSCs in species distribution and survival of the New Zealand mangrove, *Avicennia marina* subsp. *australasica*.

In Chapter 2, I discuss the methods of NSC quantification used in this study. I also investigate latitudinal NSC dynamics in New Zealand mangroves. NSC quantification using enzymatic hydrolysis did not yield interpretable results for the species studied. So I show that near infrared spectroscopy is a useful method to estimate NSC content in the species. My results show that total carbon content was significantly affected by season (summer vs. winter) across the latitudinal gradient. Total carbon content was increased in the southernmost sites in summer. In summer, only leaf total NSC content was increased in the southernmost sites in comparison to the northern sites. Whereas in stem cores, total NSC content was neither affected by season nor latitude.

In Chapter 3, I elucidate the role of different NSC levels in the physiological responses of the New Zealand mangroves to drought and salinity. Plants with different NSC levels were obtained through a light swapping treatment. Low NSC (L-NSC) plants were grown in low light conditions during the second half of the light swapping treatment, whereas high NSC (H-NSC) plants were those that were grown

in high light conditions in the second half of the manipulation. I show that during drought and saline conditions, higher NSC levels help in maintenance of physiological functioning, however, growth parameters remained unaffected in both L-NSC and H-NSC plants. My results show that most variables such as stem water potential and hydraulic conductivity were affected by three-way or four-way interactions along with main effects of either initial NSC level, salinity, drought, and time. Plants with high initial NSC had higher water potential and conductivity under high drought and salinity. Plants with high NSC also had higher survival rates under drought and elevated salinity levels. In Chapter 4, when describing metabolomic responses to drought and salinity, I show that high NSC plants have lower abundance of α-ketoglutarate than the low NSC plants under high drought and salinity conditions. H-NSC plants also had higher abundance of soluble sugars under high drought and salinity aiding osmotic adjustment.

In conclusion, this thesis contributes to our knowledge of NSCs in mangrove ecophysiology and improves our general understanding of NSC dynamics in plants. My work extends the established paradigms of plant physiological responses of terrestrial tropical and temperate tree species under abiotic stress, especially drought and salinity, to mangroves. The results from my work can be further explored and incorporated into vegetation modelling, useful in prediction of future mangrove or other tree species distribution.

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Attestation of authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published by another person (except where explicitly defined), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

(Sridevi Ravi)

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Co-Authored works

Chapter	Authors	Contribution	Total	Signature
Chapter 3	Sridevi Ravi	Study design, Experiment, Lab analyses, Data analyses, Writing	80%	
	Martin Bader	Data analyses	4%	
	Tim Young	Lab analyses, Review/edit	3.5%	
	Mark Duxbury	Lab analyses	2%	
	Mike Clearwater	Hydraulic conductivity analysis	2%	
	Cate Macinnis-Ng	Review/edit	2.5%	
	Sebastian Leuzinger	Study design, Review/edit	6%	
Chapter 4	Sridevi Ravi	Study design, Experiment, Lab analyses, Data analyses, Writing	80%	
	Tim Young	Data analyses, Review/edit	8%	
	Van Thao Nguyen	Lab analyses	1.5%	
	Mark Duxbury	Review/edit	1%	
	Cate Macinnis-Ng	Review/edit	2.5%	
	Andrea C. Alfaro	Review/edit	5%	
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To my parents, Amma, Appa and to my plants

Chapter 1 – General introduction

“The true delight is in the finding out rather than in the knowing”.

- Isaac Asimov

1.1 Abiotic stress in plants and modelling of plant drought effects

Plants are an important component of the global carbon cycle (Bonan, 2008a). As sessile organisms, plant species have evolved a variety of physiological, biochemical, molecular and morphological adaptations in response to the environment in which they are growing (Ahmad & Prasad, 2011). Understanding the physiological and biochemical responses of plants to different abiotic factors potentially leading to plant mortality is important for vegetation modelling (Hartmann et al., 2018a). Abiotic stress is defined as the negative impact of physical factors on plants in a specific environment (Mantri et al., 2012). Drought, salinity, heat and, radiation are major abiotic factors influencing plant growth and survival (Figure 1.1). Each one of these abiotic factors alone or in combination affect plant physiological responses and can influence mortality mainly through dehydration. To enhance our knowledge on plant responses to abiotic stress under natural conditions, tools such as stable isotopes, radiocarbon dating are suitable (Chaves, Maroco, & Pereira, 2003). Additionally, combining the metabolomics, genomics, proteomics and transcriptomics approaches will also aid us in the understanding of plant stress tolerance (Mantri et al., 2012).

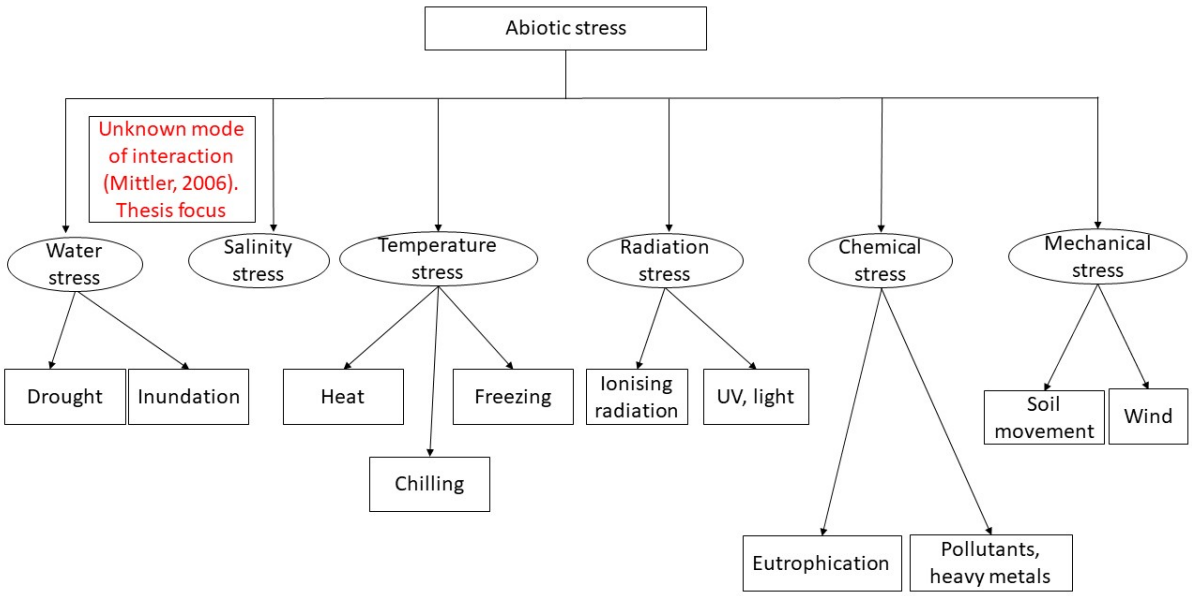


Figure 1.1 Stress in plants. Adapted from Akula & Ravishankar, (2011); Mittler (2006).

Drought is the most important abiotic stress affecting plant life and it is well studied (Anderegg et al., 2015; Hartmann et al., 2018a). Although the consequences of plant mortality events under drought and other abiotic stress factors are apparent, there is limited understanding of the linkages between physiological mechanisms causing plant mortality (McDowell et al, 2008; Sala, Piper & Hoch, 2010; Mitchell et al, 2013; Powell et al., 2013). The existing mechanistic explanations from most studies also lack evidence for long term effects of drought (Rosas et al., 2013). In order to forecast drought-induced tree mortality global scale monitoring of trends in forest mortality is necessary (Hartmann et al., 2018a). Moreover, we need to underpin causes of tree mortality based on observation and manipulation experiments by investigating the underlying physiological factors/mechanisms leading to tree mortality (Hartmann et al., 2018a).

Tree growth models involving the soil-atmosphere –plant continuum often take into account the photosynthate production, carbon fluxes at the input stage (leaf carbon intake) and, the allocation to different plant organs. Individual tree growth

models take into account increase in height, diameter at breast height (DBH) and, biomass yield. Although, such models are developed for single species at one time, they can be incorporated to a large number of species and on a broadscale (Le Roux, Lacointe, Escobar-Gutierrez, & Le Dizes, 2001). Such models mechanistically explain tree growth by deriving useful mathematical relationships for the different carbon fluxes (Le Roux et al., 2001). These tree growth models, are applicable only at a coarse scale, when finer scale interpretations involving root architecture, soil moisture variations, incorporation of different interacting environmental variables and stress factors (drought, ozone, heavy metals, heat, temperature, nutrient availability) occur, the models fail to predict tree growth responses accurately (Allen et al., 2010; Le Roux et al., 2001; McDowell, 2008).

One of the most important drawbacks associated with the tree growth models is the lack of understanding carbon reserve/storage dynamics within the plant when plants are exposed to abiotic stress. Unless these fluxes and pools are well explained, we are far from explaining tree growth and mortality to changing environments (Richardson et al., 2013). Very few studies have included/ modelled non-structural carbohydrate pools in their growth models. For example, Sampson et al. (2001) modelled the labile carbon pool in relation to net canopy photosynthesis in loblolly pine. The differences between fertilizer treatments was assessed by empirical measurements of tissue carbon. It was shown that carbon availability from storage was increased by 4% in fertilized pine (Sampson et al., 2001). Including the carbon storage/reserve mobilization dynamics under different environmental factors is very important. Radiocarbon dating techniques and significant improvements achieved in NSC measurements have paved the way to better understand carbon dynamics. Richardson et al. (2015) used ^{14}C to measure non-structural carbohydrates (NSCs) age in wood and followed a ‘two pool’ model, consisting of a young and dynamic ‘fast’

pool and a relatively older ‘slow’ pool. Growth is supported as NSC is drawn from the fast pool first and only once the fast pool is depleted, the slow pool is accessed. Therefore, new growth will be aided by older NSC from the slow pool. Thus, with the inclusion of the reserve component in plant growth models and Dynamic Global Vegetation models, better predictions and modelling of forest responses to disturbance (abiotic stress) and carbon fluxes can be expected. Halophytes are less represented in these models since most research focuses on physiological responses of terrestrial species (Sippo et al., 2018). Since salinization increase is predicted as a result of climate change (Munns &Tester, 2008), understanding salt tolerance in combination with other abiotic stressors is of utmost importance (Flowers & Muscolo, 2015).

Climate change and land use changes have resulted in global scale vegetation mortality affecting plant productivity and threatening food security (Mantri et al., 2012). Several recent research efforts have focused on unravelling the cascading events that lead to plant mortality eventually when exposed to abiotic and biotic stress factors. Drought, salinity, heat, UV radiation, ozone, chilling, heavy metal exposure, reactive oxygen species (ROS) exposure, nutrient non-availability are the major abiotic stress factors that are identified and that potentially affect a plant’s functioning in an ecosystem. Plants, especially halophytes have simultaneously evolved a variety of physiological, morphologic, genetic and metabolic adaptations to overcome these stresses (Flowers, 1977; Munns & Tester, 2008). Some of these adaptations are common to both abiotic and biotic stress factors.

Numerous studies have elucidated the physiological and genetic effects on the plants from stress factors in isolation. From a physiological perspective, very few studies have quantified the interactive effects of stress factors. Though it is known that the plant responses to interactive effects are different to plant responses to stress

factors in isolation, research has mainly focused on factors such as drought, ozone stress or salinity alone. For example, in tobacco, gene expression was different when exposed to drought and heat stress in combination than when exposed to the individual stressors (Rizhsky, Liang & Mittler, 2002). The combinatory effect increased respiration rates and lowered photosynthesis rates accompanied by higher leaf temperature. Also, some trancripts such as alternative oxidase, glutathione peroxidase, phenylalanine ammonia lyase etc. were induced only under drought and heat in combination (Rizhsky et al., 2002). In contrast, transcripts that are normally induced only under drought (e.g., catalase, glycolate oxidase) and only under heat shock (e.g., thioredoxin peroxidase and ascorbate peroxidsase) were inhibited in this study (Rizhsky et al., 2002). So, identifying and relating the plant physiological responses and environmental factor interactions is required and is also one of the challenges in plant physiology. This thesis focuses on the interaction of drought and salinity that affect a plant's physiological functioning (Figure 1.1).

1.2 Relationship between drought and salinity:

Drought and salinity affect plant growth and distribution. Salinity affects 10 – 20% of arable land globally reducing crop yield and climate change is likely to increase soil salinisation (Munns & Tester, 2008). Salinity and drought cause osmotic stress and trigger similar physiological, biochemical and molecular responses in plants (Munns, 2002). Salinity and drought both cause low soil water potential. The difference between the two lies in the total amount of water available in the soil. Under drought, plant water availability decreases over time and under salinity, physiological drought stress is induced, when abundant water is available but salt presence limits plant water uptake (Leksungnoen, 2012).

Salinity causes three major problems in higher plants (Leksungnoen, 2012).

1) The osmotic pressure in the external solution can exceed the osmotic pressure in the plant cells causing it to adjust osmotically in order to avoid dessication.
2) The higher ionic content of Na in the salt solution hinders the uptake of other ions such as potassium, chlorine etc.
3) High levels of salt content induce toxicity in plant cells affecting metabolism.

1.2 Drought adaptations and salinity tolerance of halophytes

Drought generally reduces plant growth, impairs water relations and water use efficiency of plants, mineral uptake, and assimilation rates. Broadly, drought affects carbon fixation through stomatal or non-stomatal limitations. Stomatal closure occurs under mild to moderate drought. In other cases, non-stomatal limitations such as decreased enzyme and impaired ATP synthesis, and heavy oxidative loads are present (Aroca, 2012).

Plants possess morphological, anatomical, physiological and molecular adaptations to cope with drought or salinity stress. Morphological adaptations include, reduced leaf size or area, yellowing or wilting of leaves. A reduction in biomass and, increase in root length also occurs (Aroca, 2012). Halophytes and some glycophytes also possess salt glands involved in salt secretion.

When plants are exposed to drought, plants exhibit mechanisms of drought escape, avoidance or tolerance (Hara et al., 2012). Drought escape is when a plant completes its life cycle before drought onset, e.g., plants with extremely short life cycles may die off during drought, recovering from the soil seedbank once rain arrives. Drought avoidance is when plants remain hydrated even under drought. Drought tolerance is when plants survive and maintain cellular hydration under extreme water deficit conditions for extended time periods (Hara et al., 2012).

Salinity exerts an osmotic and ionic effect on plants. A salt sensitive plant (glycophytes) cannot control Na^+ transport which can cause ion toxicity. Also, plants need to maintain low levels of Na^+ for metabolic reactions to take place (Mantri et al., 2012). Similar to drought, plants employ the strategies of avoidance and tolerance when exposed to salinity. Avoidance involves the mechanism of keeping away sensitive plant tissues from salt areas of high salt concentration (reviewed in Ahmad & Prasad, 2011). Tolerance involves the exclusion of ions from either roots or cytoplasm. It also represents the degree to which plants adjust osmotically without growth reduction. (reviewed in Ahmad & Prasad, 2011; Munns and Tester, 2008). For example, halophytes store Na^+ ions in vacuoles rather than the cytoplasm. Transport membrane proteins aid in ion exclusion and H^+ is taken up in exchange for Na^+. This process is energy driven, by the proton motive force of plasma membrane H^+-ATPase (Munns & Tester, 2008; Slama et al., 2015).

The vacuole is also involved in cell turgor regulation and expansion and has 20 times greater fluid volume than the cytoplasm. The ionic concentration found here can be estimated for the plant cell as a whole. Even at high ion concentrations, metabolic functions occur in halophytes (Strogonov, 1964; cited in Flowers, 1977). Halophytes are able to maintain growth under higher salt concentrations by storing the bulk of ions in vacuoles and osmotic adjustment within the cellular cytoplasm is brought about by compatible osmotica (Ball, 1988; Flowers, 1977; Munns & Tester, 2008; Reef et al., 2012).

During drought or salinity or both abiotic stresses, plants adapt physiologically and biochemically through osmotic adjustment, an antioxidant defence system and, interactions or crosstalk of different metabolites (Yang & Guo, 2018). Similar to drought, salinity also causes cellular dehydration. To overcome dehydration, plants

accumulate organic compounds (osmolytes) such as sugars, proline, quarternary and other amines, and organic acids (Ahmad & Prasad, 2011; Gil et al., 2013). The accumulation of these osmolytes reduces the water potential of the cell, facilitating osmotic adjustment (Ahmad & Prasad, 2011; Parida & Das, 2005). Under growing conditions of tolerable levels of salt content, nutritional and osmotic effects are more pronounced than salt toxicity. When tolerable levels are exceeded, the salt solution enters the transpiration stream causing damage to all plant organs (Flowers, 1977; Parida & Das, 2005). Accumulation of osmolytes also aids in cold acclimation. Cold acclimation is the phenomenon by which plants increase freezing tolerance to low non-freezing temperatures (<10°C; Ahmad & Prasad, 2011). Under salt stress, for example, osmotic adjustment allows homeostasis, while freeze-induced osmotic stress relies on avoidance of ice nuclei formation (reviewed by Ahmad & Prasad, 2011). Accumulation of osmolytes such as sugars plays an important role in freezing tolerance by contributing to osmoregulation, cryoprotection or signalling molecules (Ahmad & Prasad, 2011; Parida & Das, 2005).

In addition to cellular dehydration, elevated levels of ROS (Reactive Oxygen Species) are found in plants exposed to different types of abiotic stress, such as drought and salinity. They are produced in mitrochondria, peroxisomes and chloroplasts. However, overproduction causes oxidative damage to cell membranes and eventually cell death. Plants produce both enzymatic and non-enzymatic metabolites that play a role in ROS signalling (reviewed by Ahmad & Prasad, 2011; Yang &Guo, 2018).

The plant physiological and biochemical responses to drought, salinity, cold and other abiotic factors can be either species - specific or general (Mantri et al. 2010; cited in Ahmad & Prasad, 2011). Some osmolytes may be accumulated in halophytes

and not in other plant species. For example, sorbitol is a well-known osmolyte found in *Plantago spp.* whereas glycine betaine is known to accumulate in accumulate in *Avicennia marina* (Gil et al., 2013; Parida & Jha, 2010).

1.3 Mechanisms of plant mortality during drought

Different mechanisms are linked to plant mortality during drought and species-specific effects are suggested (McDowell et al., 2008., Palacio et al, 2014; Sala et al, 2012). McDowell et al. (2008) formulated two main hypotheses on the basis of drought stress response. The 'hydraulic failure hypothesis' predicts that during drought reduced soil water availability together with high evaporative demand causes xylem conduits to become air-filled, creating embolism and eventually, cell death (McDowell et al., 2008, 2011). The 'carbon starvation hypothesis' states that there is less carbon uptake (photosynthesis) due to closure of stomata under water stress or drought, and therefore less carbon is available for the plants' metabolic needs (Adams et al., 2013; McDowell et al., 2008; Wiley & Helliker, 2012). Depending on whether the species is isohydric or anisohydric the relative contributions of each of these processes to mortality will differ. Isohydric plant species are capable of stomatal regulation to limit transpiration. So, they have relatively constant leaf water potential both during drought and conditions of abundant water availability. Whereas, anisohydric species have more variable leaf water potential, with less stomatal down regulation. Biotic stress (pathogen attack) will also intensify hydraulic failure or cause carbon starvation either by obstructing xylem vessels affecting water transport or by increasing resin production respectively (McDowell et al., 2008). Plants are more susceptible to biotic stress when either hydraulic stress or the onset of carbon starvation or both results in production of less defensive compounds, which are used

by the plant to cope with different environmental stressors (McDowell et al., 2008; Powell et al., 2013; Gessler, Schaub & McDowell, 2017).

The carbon assimilated by the plants through photosynthesis is used for various functions (Figure 1.2). During plant growth the assimilated carbon is primarily converted to six carbon sugars (glucose) and associated isomers which constitute the NSC pool (Chapin et al., 1990; Hoch et al., 2002). Simultaneous growth respiration (for synthesizing new materials from assimilates) and maintenance respiration take place. Meanwhile, the NSC pool is used for various functions, which is described separately (section 1.4.1). Whether the flow of carbon to storage is an active or passive is poorly explained and is also an important focus of this thesis (Sala et al., 2012).

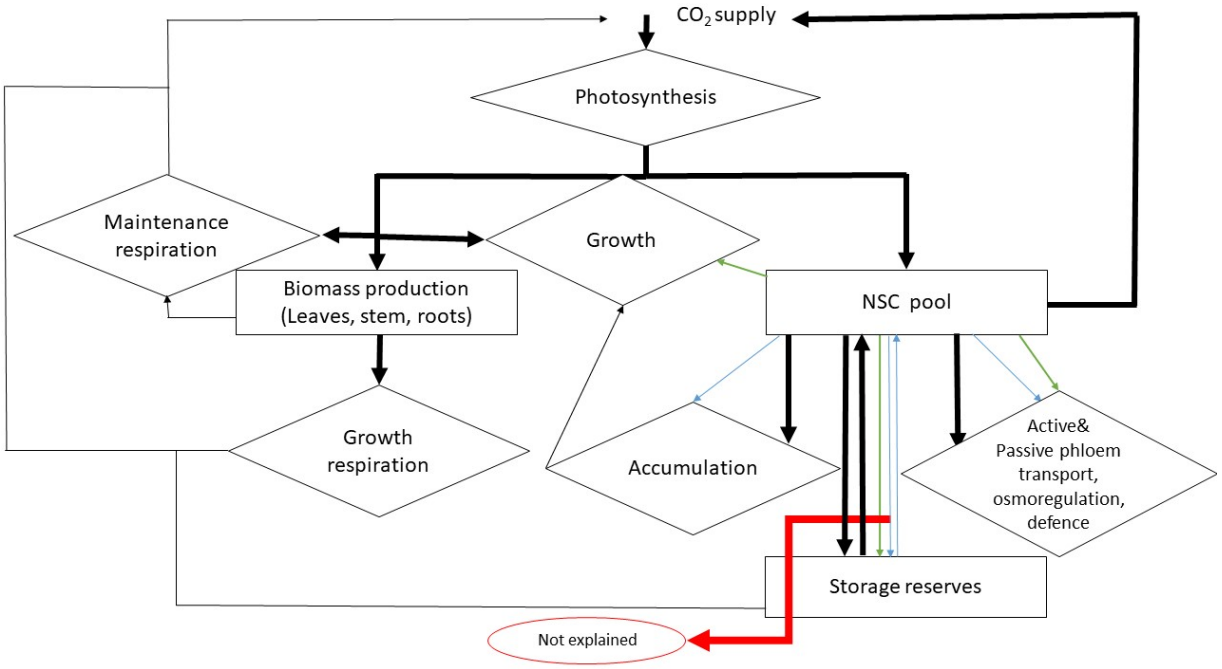


Figure 1.2 Carbon flow in plants, showing NSC formation and functions (adapted from, Chapin, et al, 1990; Le Roux et al., 2001; Sala et al., 2012; Richardson et al., 2013; Fischer et al, 2015; Hartmann & Trumbore, 2016). The thick black arrows and lighter arrows indicate carbon flow and plant information flows (plant functions). The diamonds represent carbon fluxes and the rectangles represent carbon pools. The green arrows indicate active NSC functions and blue arrows indicate passive functions. The active NSC storage function indicated in red arrow is the area of much needed research.

Sevanto et al. (2014) investigated the two hypotheses outlined above when pine trees are exposed to drought and shade. The occurrence of carbon starvation alone was not found. Instead, carbohydrate transport and consumption were significantly affected by water availability. The importance of the role of phloem in sugar transport, and also the xylem-phloem water transport was recognised. Under drought, the faster dying trees succumbed to hydraulic failure, but death ensued in those that had no access to carbohydrate reserves. The slowly dying trees showed symptoms of both hydraulic failure and carbon starvation. The shaded, well-watered trees trees died of carbon starvation. The study found that hydraulic failure is linked to a loss of carbon content affecting osmoregulation. It is important to study the effect of other linked solutes like potassium, sodium, amino acids in addition to carbohydrates (Gil et al., 2013; Sevanto et al., 2014). Cellular level control of plant metabolism is also important in drought processes (Niinements, 2016; Sevanto et al., 2014). The interaction of the two mechanisms (hydraulic failure/carbon starvation) is likely, and the relative contributions of either will depend on the environment to which the plant is exposed to.

The carbon starvation hypothesis was questioned by Sala et al. (2010). Only when the carbon source is highly affected (shade or defoliation), will the carbon storage processes be affected and this is unlikely in field conditions. In contrast, when the carbon sink activity is affected, such as by drought, the plants survive depending on the sink demand (i.e. duration of drought; Sala et al., 2010, 2012). For example, when drought duration is less (2-6 weeks), carbon balance is maintained, under a stable stored carbon pool, plants are able to survive. In other cases, when drought duration is extended (for example, 6-20 weeks), though plants are less able to

maintain the carbon balance, the pool size is enough for the plant to draw upon for production of compounds that enables it to survive (Sala et al., 2010).

When carbon balance becomes less within the plant (which is called depletion process; Sala et al., 2010), and sink demand increases (for growth and metabolism) the reserves are all exhausted and plants die. Also, mobilisation of carbon is affected under drought and may lead to plant death (Andregg et al., 2012; Ryan & Asao, 2014). However, even after tree mortality carbon reserves are present, so trees may actively allocate carbon at the expense of growth (Sala et al., 2012). It still remains to be deciphered how active carbon storage affects plant physiological responses to abiotic stress, in particular, drought (Korner, 2003; McDowell, 2013; Sala et al., 2012; Tomasella et al., 2017). Plants store carbon mainly as non-structural carbohydrates for use in growth and metabolic functions (NSCs, mainly glucose, fructose, sucrose and starch; Chapin et al., 1990; Hoch et al., 2002). NSCs play a role in mortality mechanisms and understanding their allocation/usage in plant physiological processes is crucial. The main focus of this thesis is to better understand the role of non-structural carbohydrates (NSCs) in plant physiology, their storage dynamics and also the role of other metabolites in addition and complementary to the NSCs in New Zealand mangroves.

1.4 Non-structural carbohydrates (NSCs) – Introduction, functions and importance in plant drought effects: An overview

The role of NSCs or carbohydrates in halophytic salt tolerance needs more investigation even if they occur in low concentrations (Gil et al., 2013). This thesis particularly focuses on the role of NSCs in New Zealand mangroves to combined abiotic stresses, drought and salinity.

Studies on carbohydrate reserves date back to as early as the start of the 19th century. Plant reserves were first defined as “those carbohydrates and nitrogen compounds elaborated, stored and utilized by the plant itself as food for maintenance and for the development of future top and root growth” (Graber et al., 1927; reviewed in White, 1973). Later, Weinmann (1947) [cited in White, 1973] redefined plant reserves as ”total available carbohydrates” which is convertible to plants’ energy requirements. The term total non-structural carbohydrates (TNSCs) which is also the main focus of this thesis was suggested by Smith, (1969). By observing the reserve changes (%TNSC) we can understand the role of these NSCs in plant vigour maintenance (National Research Council, 1962, White 1973). But in order to know the role of NSCs in plant survival, we must also account for other parameters such as leaf, root area, light conditions, nutrients and, morphological factors which are inevitable (White, 1973).

Carbohydrates contain carbon, oxygen and, hydrogen atoms. They are hydrates of carbon with the empirical formula $C_m(H2O)_n$. Examples are sugars, starch and cellulose. Also called as saccharides carbohydrates are divided into mono, di, oligo and poly saccharides. Monosaccharides are the basic sugar units as they cannot be further hydrolysed as can glucose, fructose, galactose and, mannose. These combine glycosidically to yield disaccharides. Dissacharides of this type include sucrose, lactose (Tiwari & Rana, 2015). Some disaccharides like maltose, cellobiose are hydrolysis products of polysaccharides such as starch and cellulose. Oligosaccharides contain approximately two to ten monosaccharides joined together by chemical bonds, for example, raffinose. Polysaccharides are hetero-genous macromolecules containing long chains of monosaccharide units. Their hydrolysis results in monosaccharides and oligosaccharides. Unlike mono and di-saccharides they are often insoluble in water. They provide energy and are useful in storage

(starch) of maintenance of structure (cellulose). Photosynthesis by plants yield glucose which are mainly stored as the polysaccharide, starch. Starch is composed of amylose and amylopectin. Amylose and amylopectin are composed of alpha type glucose molecules unlike cellulose, which have beta type glucose molecules. Unlike mono and di-saccharides starch is often insoluble in water. Polysaccharides provide energy and are useful in storage (starch) of maintenance of structure (cellulose) in plants (Tiwari & Rana, 2015; Slama et al., 2015).

Even after 100 years of research since 1920s we are still unable to fully understand the plant carbon storage let alone the predicaments in quantifying it (Germino, 2015). Chapin et al. (1990) attempted to classify carbon storage by analogous comparison to economy (Figure 1.3). Here storage was defined as "resources that build up in the plant and can be mobilised in the future to support biosynthesis for growth or other plant functions". Chapin et al. (1990) proposed three different storage classes. This was also dependant on whether the plant carbon demand exceeds the supply. When the supply exceeds the demand NSC 'accumulates' (Figure 1.3). This is a passive process and these compounds do not directly promote growth. The 'reserve' formation of the NSC pools are compounds that directly interfere with growth (the active storage activity as pointed out by Sala et al. (2010). The other part of the NSC pool are compounds that not only contribute to growth immediately but are also stored as compounds that can support future growth and re-mobilised for use as C-source for other sinks. These 'recyclable' compounds could potentially be lost as litter (Chapin et al., 1990). From an ecologists' perspective, the recyclable and the reserve part of NSC are often included, but not accumulation.

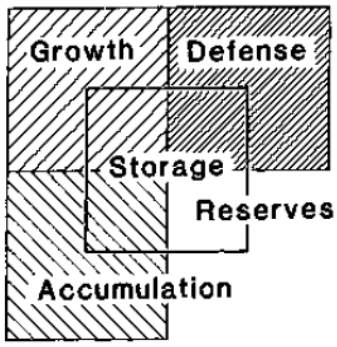


Figure 1.3 Chapin et al. (1990). Description of NSC storage pools formation.

When under drought, plants have shown to accumulate NSC stores as a result of decreased sink activity (particularly growth; Galvez et al., 2011; Maguire & Kobe, 2015; Mitchell et al., 2013; O'Brien et al., 2014). NSC has been shown to increase drought survival (Adams et al., 2013; Anderegg et al., 2012; O'Brien et al., 2014). It is important to measure the plant carbon and water status directly (carbon reserves and hydraulic conductivity respectively) rather than measuring fluxes indirectly (gas exchange, stomatal conductance; Anderegg et al., 2012). NSCs play an important role in osmoregulation, storage, defence and, transport. They can be affected by environmental variables such as drought, temperature, salinity, pathogen exposure and also the age of the plant (seedlings vs mature trees). Most studies are carried out in seedlings owing to the long growth periods of plants and also for logistical reasons. Field studies are often limited by factors such as site accessibility and presence of many confounding variables. Rosas et al. (2013) studied NSC dynamics in Mediterranean woody species after a 14 year long drought. Low concentrations followed the extremely long drought period and also led to the inability of the trees to survive following recurrent periods of drought. This is in contrast to the short-term studies that almost always show increased NSC concentrations following stress exposure such as drought (O'Brien et al., 2014).

After almost four decades we are still debating as to determining the critical level of NSC at which plant mortality occurs. NSC concentrations differs between species, seasons, plant parts. It is also affected by environmental variables and also the age of the plant (seedlings vs mature trees) making it more complex to determine the lethal NSC level (Dietze et al., 2014; Germino, 2015; Hoch et al., 2002; White, 1973). NSCs are thought to reflect the balance between carbon supply and demand (Germino, 2015; Hoch et al., 2002). Also, plants tend to actively store NSCs which aid long term survival (Wiley & Helliker, 2012; Sala et al., 2012). Global scale tree mortality under drought has triggered intense research in this area. NSC dynamics in both observational studies and under controlled conditions of NSC responses to different abiotic stress have been the focus of recent research (Klein, 2015; Hartmann et al., 2018a). Understanding NSC dynamics along with other plant physiological changes may potentially reveal the mechanisms leading to mortality under drought per se and other environmental factors as a result of projected climate change (Hoch et al., 2002).

When conifer species were subjected to complete darkness of varying periods (one, three, six, nine and twelve species), resprouting occurred after 9 weeks, but not after 12 weeks. After twelve weeks, no plant survival occurred. Plants stored NSC compromising growth (Weber et al., 2018). Defoliation in Mediterranean Scots pine increase their carbon uptake under drought stress. Although carbon uptake is increased, they generally cannot maintain a favourable C balance, since cell turgor maintenance and osmotic balance require energy which is drawn upon from carbon reserves. Higher water loss decreases their hydraulic safety margins putting them at risk of hydraulic failure. A feedback loop between carbon starvation and hydraulic failure occurs (Anderegg et al., 2012; Salmon et al., 2015; Tomasella et al., 2017).

Non-defoliated trees were similar to isohydric species. Although the trees will face carbon deficits in the longer term, non-defoliated trees are less prone to mortality from hydraulic failure (Salmon et al., 2015).

1.4.1 Functions of non-structural carbohydrates (NSCs)

Different functions of NSCs, particularly during drought as reviewed by Hartmann & Trumbore (2016) are highlighted here:

1) *Metabolism*

Photosynthesis enables the assimilation of carbon with the loss of water through the leaf stomatal pores (transpiration). To control for water loss, vascular plants employ the physiological mechanism of stomatal regulation. This also occurs when there is soil water deficit or when vapour pressure deficit is high. Different environmental factors (eg. temperature, precipitation) will influence carbon uptake. During the initial drought phase plants employ physiological adaptations or mechanisms (stomatal regulation, hormone signalling) that allow photosynthesis. As a result, the non-structural carbohydrate (NSC) pools increase in the initial phases of drought since growth is compromised earlier than the photosynthetic process (Korner, 2003; Muller, 2011). Many studies have shown that plants accumulate NSC stores as a result of decreased sink activity (particularly growth) during drought (Galvez et al., 2011; Maguire & Kobe, 2015; Mitchell et al., 2013; O'Brien et al., 2014), whereas, under longer drought periods, an increase in catabolic processes like respiration, reduces NSC pools leading to plant death (McDowell et al., 2008, 2011).

NSCs provide the basis for production of compounds and energy required for biosynthesis and cellular maintenance (30-40%; Poorter et al. 1990; reviewed in Hartmann & Trumbore, 2016). Plant metabolism leads to the production of compounds (anabolism) or breakdown of larger molecules for energy (catabolism).

Glycolysis and tricarboxylic acid are the different pathways of respiration that generate energy in the form adenosine tri-phosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Carbohydrates are the precursor molecules for all the two pathways. Drought affects plant metabolism by stomatal closure, potentially leading to carbon starvation and also by affecting carbon transport via phloem causing impaired NSC mobilisation because of less water availability. Additionally, higher temperatures that accompany drought conditions further affect metabolic activities within the plant, eventually leading to plant mortality (Adams et al. 2009; Ryan, 1991). Severe drought alone reduces respiration, especially root respiration with simultaneous reduction in root carbon (Hartmann et al., 2013). Although plants can use alternative substrates like lipids, drought affects metabolic processes like photosynthesis and respiration and not only NSC mobilization.

2) *Transport*

Non-structural carbohydrates are measured in different plant experiments as an indicator of the source to sink demand of carbon in the plant. They represent the mobile carbon pool composed of free sugars and starch. Phloem is responsible for sugar transport in plants. Studies have explored the nature of this phloem transport under different abiotic and biotic stress (Peuke et al. 2014; cited in Hartmann & Trumbore, 2016). By creating root and shoot anoxia in *Ricinus communis* it was found that sugar transport was affected in both root and shoot by the anoxic conditions. However, increased sugar concentrations were found in root (Peuke et al., 2014; in Hartmann & Trumbore, 2016).

Sugars are loaded into the phloem by one of the three mechanisms: active and passive symplastic loading or apoplastic loading. Apoplastic loading occurs via

sucrose transporters and requires energy. Symplastic loading through polymer trapping is where sucrose is converted to oligosaccharides in the companion cells elevating the sugar concentration gradient in the phloem. Symplastic loading through diffusion occurs when there is a high concentration gradient of sucrose or sugar alcohols without polymer trapping. Essentially, phloem transport is thought to occur by pressure build up in the source tissues such as leaves and taken to sink organs, such as stem and roots (Munch, 1930; cited in Hartmann & Trumbore, 2016). In the source tissues, water moves from the cell into the sap (phloem solution) through osmosis. Then the sap moves down to the sink tissues and the sugars move back into the sink cells releasing the pressure. The hydrostatic pressure required for this movement is driven by water movement from the adjacent xylem into the phloem. Less availability of water during drought, therefore impairs phloem transport. Phloem viscosity increases causing a reduction in turgor affecting sap transport (Sevanto et al., 2014). NSC aids the maintenance of this transport by removing embolism by the influx of low molecular weight sugars from adjacent non-embolised conduits (Sevanto, 2014; Hoch et al., 2002). Some studies suggest that embolism refilling is overestimated due to methodological artefacts (Cochard & Delzon, 2013; cited in Hartmann & Trumbore, 2016). Trees also maintain living xylem ray and axial parenchyma cells that help in embolism repair and for osmoregulation and protection (Plavcová & Jansen, 2015).

3) *Storage*

Chapin et al. (1990) defined NSC storage as “resources that build up in the plant and can be mobilised in the future to support biosynthesis for growth or other plant functions”. Depending on whether plant carbon demand exceeds the supply, Chapin et al. (1990) identified three different storage classes. i) NSC accumulation occurs

when supply exceeds demand (a passive process). The accumulated compounds do not promote growth. ii) Part of the NSC pool is set aside as reserves (reserve formation) and these compounds directly interfere with plant growth (the active storage process, as pointed out by Sala et al. 2010). Chapin et al. (1990), also identified (iii) third NSC pool, apart from accumulation and reserve formation. These compounds not only contribute to current plant growth but are also utilised as compounds that can support future growth (recycling). Whole-plant physiologists and ecologists include only reserve formation and recycling whilst defining storage, often excluding accumulation.

The role of NSC storage when trees are exposed to drought is unclear and therefore the mechanisms of tree mortality are inconclusive (Klein, 2015). Short term studies on seedlings have shown increased NSC concentrations in the plant as a result of sink limitation (Korner, 1998 & 2003; Mitchell et al., 2013). Contrastingly, Wiley & Helliker (2012) suggest that plants have evolved to upregulate storage/reserve formation in order to avoid carbon starvation and not only as a result of sink limitation (Wiley & Helliker, 2012). Upregulation of storage in trees is often seen as a mechanism to overcome unfavourable conditions (Sala et al., 2012). This is evidenced by studies reporting increased NSC concentrations whilst source activities are reduced, for example by defoliation (Wiley & Helliker, 2012). Thus different theories exist on the carbon storage/reserve formation and long term studies on mature trees and NSC storage mechanisms are necessary.

4) *Defence*

Plants produce compounds directly involved in growth (primary metabolites) and those that play a role in a variety of functions in defence and stress tolerance. All these metabolites are ultimately derived from photosynthetic products. Therefore,

NSC will also affect the production of these metabolites also and particularly under drought, there will be a reduction in the allocation to the production of secondary metabolites. The production of volatile organic compounds, like jasmonic acid, through the jasmonate pathway for example, is upregulated for the protection against reactive oxygen species. The interaction of NSC availability under drought stress with such pathways is not known.

5) *Osmoregulation*

Plants synthesise osmotically active solutes to lower the solute potential of the cell to ensure plant survival under abiotic stress. Examples are sugars (glucose, sucrose), proline, glycine betaine, mannitol. Non-structural carbohydrates are involved in the synthesis of these solutes and declines in water availability will affect osmoregulation through decreased production of these solutes (Chaves et al., 2003; Slama et al., 2015). Whether plants actively store NSC, for using sugars as osmolytes which aid in survial is still to be unravelled (Sala et al., 2012; Adams et al., 2013; Germino, 2015; Quentin et al., 2015).

6) *Export*

NSC storage, accumulation and transport differs between above and below-ground tissues, especially under drought (Hartmann et al., 2013). Root carbon is important and many important ecological processes occur in the roots. Rhizosphere carbon may account for up to 10% of the net primary productivity (Philips et al., 2011; reviewed in Hartmann & Trumbore, 2016). Although its importance is known, root exudates are rarely studied because of impedances from sample collection to method of analysis (Kannenberg & Philips, 2017). Plants allocate NSC to mychorrizhae and

bacteria at the root level also. Fungi provide the plants with minerals and nutrients and plants return carbohydrates. The exchange occurs through cell membranes, whether plants or fungi control this exchange is not yet fully understood (Fitter et al., 2011; cited in Kannenberg & Philips, 2017). Roots exude a diverse class of compounds, both primary and secondary metabolites and there is C cost to the plant. *Populus tremoides* seedlings showed more root exudation under drought, shade and cold temperatures. Although assimilation rates were low with reduced growth rate, exudation increased. The root sugars and exudation exhibited a significant linear relationship showing root exudates are derived from root NSC (Karst et al., 2017). The relationship between NSC and root exudation is to be understood for better C flux modelling (Kannenberg & Philips, 2017; Kannenberg et al., 2018; Hartmann & Trumbore, 2016).

1.5 Additional plant metabolites

NSCs are the primary substrate for the synthesis of other plant metabolites. Plants have both primary and secondary metabolism and the terms were first introduced by Kossel in 1891 (Mothes, 1980; reviewed in Hartmann, 2007). Metabolomics is the study of the intermediary and end products of metabolism present in cells, tissues and biofluids. These metabolites can be profiled to investigate plant processes such as growth, reproduction and development. Primary metabolite compounds are used for plant growth directly, for example, carbohydrates, amino acids and lipids. Secondary metabolites are those compounds that are not involved in the process of plant growth directly, however they are inevitable for plant physiological functioning and associated with plant defence and adaptation (Akula & Ravishankar, 2011; Hartmann, 2007; McKiernan et al, 2014; Ninemets, 2016; Tiwari & Rana, 2015). The study of plant secondary compounds initiated with the isolation

of morphine from the ‘opium poppy’ plant in 1806 (Hartmann, 2007). The first synthetic secondary compound was indigo prepared by Von Baeyer in 1886. Although the compounds were reported, Julius Sachs (a founding father of plant physiology) identified later that the role of secondary metabolites in the economy of the plant is largely unknown. Sachs’ defined this as: “One can designate as by-products of metabolism such compounds which are formed during metabolism, but which are no longer used in the formation of new cells. Any importance of these compounds for the inner economy of the plant is so far unknown”.

Today more than 200,000 plant secondary products are structurally elucidated. The functional, physiological, structural and evolutionary aspects of the secondary products are also investigated. However, the role of secondary metabolites in response to environmental stress is rarely explored and is an important aspect for understanding plant physiological mechanisms (Niinemets, 2016).

1.5.1 Plant Secondary metabolites

Biosynthetic derivation of primary metabolites leads to the production of secondary metabolites. Phenolics, alkaloids, sterols, steroids, essential oils are some of the secondary metabolites (Tiwari & Rana, 2015).

Based on the biosynthetic origin, secondary metabolites can be classified as,

1) Flavanoids and allied phenols and polyphenols
2) Terpenoids
3) Nitrogen containing alkaloids and sulphur-containing compounds

In the early 20th century plant biochemistry research was just confined to identifying the different classes of compounds (such as terpenes, alkaloids, phenolic compounds etc.) and assigning them to the various plant kingdoms and also the differences in allocation to the different tissues. Recent developments in

instrumentation has improved the isolation of more of these compounds and with the development of radio-active labelled precursors since 1950, the biosynthetic mechanisms of the metabolites could better be addressed. Commercial interest in such compounds has resulted in the application of plant tissue culture techniques to produce metabolites that are bioactive (Tiwari & Rana, 2015). Additionally, it is quite difficult to identify plant metabolites and assign them to specific plant functions. This is due to the fact that, those compounds that are involved in primary functions such as growth, are also synthesised by pathways other than the carbohydrate, lipid and protein metabolism (Firn & Jones, 2009). For example, gibberellins are hormones that function as endogenous regulators but are synthesised via the isoprenoid pathway. The importance and necessity of identifying these metabolites for plant ecological and physiological functioning has gained prominence lately (McKiernan et al.2014; Niinemets, 2016). These other metabolites (terpenoids, phenolics), may play a role in defence, signalling in metabolic pathways, and also the regulation of cellular metabolic activity through plant hormones.

Abiotic and biotic factors potentially affect plant physiology by interfering with metabolic pathways and metabolic derivatives. Understanding these cellular levels of drought response in plants is also important (Niinemets, 2016; Sevanto et al., 2014), in addition to drought effects on photosynthesis, stomatal conductance, biomass effects, root length and other morphological and anatomical parameters (Sevanto et al., 2014). Under drought as NSCs accumulate, larger carbon compounds (secondary metabolites) such as isoprenoids and phenolics also tend to accumulate. There is evidence of enhanced phenolic concentrations in Mediterranean plants and eucalypts respectively (Llusia & Penuelas, 1998; cited in McKiernan et al. 2014). But the same study by McKiernan et al. (2014) witnessed reduced levels of terpenoids. Additionally, McKiernan et al. (2016) reported the

differential response of secondary metabolites to drought and rewatering, for example, the enhanced tannin levels and the non-enhanced levels of terpenes. Thus it is evidenced that metabolic responses are more complex with different by products associated with multiple pathways. The implications of these responses are yet to be understood (Niinemets, 2016).

The interplay of different abiotic stresses can cause different physiological responses (stomatal conductance, gas exchange) in comparison to the effect of a single factor. Similarly, combination of stress factors also creates a reprogrammed plant metabolomic network that enables plant survival, the mechanisms of which is to be explored (Ninemets, 2016). In chickpea, fungal infection triggered metabolomic responses similar to what high salinity conditions would cause (reviewed by Mantri et al., 2012). Therefore, studying effects of stress factor combinations in one species responsible for both phenotypic and genotypic modification is crucial (Granda *et al*. 2014; reviewed by Niinemets, 2016). By investigating genetic, physiological and biochemical variations under different environmental conditions, plastic components of trait variation conferring drought resistance to the species can be understood. (Niinemets, 2016; Yang & Guo, 2018).

1.6 Quantification of non-structural carbohydrates (NSCs)

During the past five years, several reviews (Germino, 2015; Hartmann and Trumbore, 2016; Pinkard, 2018), as well as original research articles (Landhausser et al., 2018; Quentin et al., 2015. 2017; Ramirez et al., 2015) have highlighted issues around NSC analysis methods and data interpretation. Among these, Quentin et al. (2015) analysed the differences in the NSC results of Eucalyptus species across 29 laboratories using different methods. They summarised the different extraction

protocols and quantification methods for sugars and starch separately. Quentin et al. (2015) also classified the quantification methods into HPLC (high-performance liquid chromatography), HPAEC-PAD (high-performance liquid chromatography-pulsed amperometric detection), H-NMR (proton nuclear magnetic resonance), enzymatic, and colorimetric. This classification is slightly different to the one adopted by Landhausser et al. (2018), who classified the quantification methods into three categories: a) ion chromatographic methods, b) selective enzymatic methods, c) acid methods. In general, the ion chromatographic methods have high consumable and instrument costs with low sample throughput and are time consuming. The enzymatic methods have low instrument costs, but high consumable costs, with high sample throughput, but are also time consuming. The colorimetric assays have low instrument and low consumable costs with high sample throughput and are less time consuming. However, with regard to data quality, data obtained using ion chromatographic methods is considered better due to sensitivity of the data. Data obtained using enzymatic analyses are better than colorimetric assays and yield sugars that have been hydrolysed by specific enzymes. Colorimetric assays are often criticised for the fact that only a total sugar content is obtained instead of the specific sugar constituents (Raessler, 2011; Quentin et al., 2015).

The key difference among these two studies is, Quentin et al. (2015) found high variability in the results obtained even if the same sample was used in all the 29 laboratories. Conversely, Landhausser et al. (2018) found that the variation was less than 11.5% when rigorous procedures and protocols were followed for NSC estimation among the six labs participating in the study. The differences observed in NSC estimates may occur even due to different sample handling procedures (Quentin et al., 2015). However, Landhausser et al. (2018) suggest that major differences occur only during quantification of soluble sugars or starch.

Near Infrared spectroscopy (NIRS) is another method to measure NSC content. NIRS is user friendly and involves less sample processing costs and handling time in comparison to the chromatographic, enzymatic and, colorimetric assays. The use of NIRS to predict NSC content is shown by Asner et al. (2015), De Bei et al. (2017), Quentin et al. (2017), and Ramirez et al. (2015). Quentin et al. (2017) used foliar samples of *Eucalyptus globulus* Labilladière and De Bei et al. (2017) used grapevine trunks as study species for NSC estimation. Asner et al. (2015) measure leaf NSC concentrations of tropical and sub-tropical forest species in North America for monitoring forest canopy physiological changes. The developed calibration models in this study allow spectroscopic measurements of leaf NSC remotely for monitoring physiological changes in forest canopies (Asner et al., 2015). Ramirez et al. (2015) used the method to apply across more than 80 temperate tree species. This shows that NIRS can be used to predict NSC content across different plant species.

1.7 Why mangroves as a study system?

Mangroves cover an area of ten million hectares globally and provide vital ecosystem services. Of the total mangrove cover, an estimated 36,000 ha. have been lost since the 60’s and recent climate extremes have caused mangrove dieback in Australia in the Gulf of Caribbean (Sippo et al., 2018). Mangrove dieback is unreported in many areas and a global synthesis of mangrove mortality causes is lacking (Sippo et al., 2018). This is because most global research efforts on the physiological mechanisms contributing to forest mortality have concentrated on terrestrial tropical and temperate species.

Halophytes serve as convenient model organisms for studying interacting factors as they are naturally salt tolerant (Gil et al., 2013). Because halophytes exist in extreme saline conditions, studying the physiological and biochemical mechanisms aids in understanding their mortality mechanisms. Additionally, this can be extended in a molecular perspective, wherein the desired traits can be used in breeding techniques to produce species that are more salt and drought resistant.

Thesis structure

The main aim of this thesis is to assess the role of non-structural carbohydrates (NSCs) in New Zealand mangrove physiology under experimental and natural conditions. In the general introduction (Chapter 1), the thesis context was laid out and the general importance of non-structural carbohydrates in plant physiology and under changing abiotic environmental factors was described. The thesis consists of three experimental Chapters. Since significant methodological predicaments were encountered in the NSC analyses, Chapter 2 describes why the existing methodologies were not suitable for the present study. I explain the process for the selection of the wet chemistry method best suited for mangroves. I also explore the possibility of near infrared spectroscopy (NIR) as a potential tool in NSC analysis. I discuss the development of a calibration model by comparing NIR and the selected wet chemistry method, used for prediction of NSC concentrations. Using the developed model, the changes in NSC content of New Zealand mangroves across latitude and season are discussed.

Chapter 3 describes an experimental setup to test the role of NSCs in the physiology of New Zealand mangroves under the interactive effects of drought and salinity. A method of alternately exposing plants to high sunlight and deep shade to achieve different NSC levels is used to manipulate NSC concentrations in plants. Effects of this initial NSC content on height, leaf area, xylem properties and differential NSC responses to drought and salinity are discussed.

In Chapter 4, the effects of drought, salinity and high vs low initial NSC content on a range of metabolites of mangroves were tested using the same experimental setup as described in Chapter 3. Using an untargeted approach, I assessed the expression

of 150 metabolites in total (known and unknown) across different biosynthetic pathways. The functional role of metabolites that vary under these abiotic environmental conditions are described.

Chapter 5 synthesises key findings and limitations of each of the experimental Chapters. Recommendations for future research are given.

Chapter 2 – Dynamics of non-structural carbohydrates in mangroves

2.1 Abstract

In recent decades, several studies have established the importance of non-structural carbohydrates (NSCs) in physiological responses to environmental conditions across various plant species. Inspite of this, there are huge methodological inconsistencies in the quantification of NSC levels in plants. Until now, studies have focused on the role of NSCs in non-halophytes. Here, I identified the knowledge gaps and suggest an appropriate method for halophytic NSC quantification. I discuss the methodological difficulties encountered whilst I carried out my analyses and possible reasons for these. I also investigate the latitudinal NSC dynamics of carbon, nitrogen, NSC, and $\delta^{13}C$ content of New Zealand mangroves. I show that the model (correlating measured and predicted values of NSC content using Near infrared Spectroscopy) has an $R^2 = 0.831$. Using the model, I predicted NSC values for the leaf and core samples collected in four sites across New Zealand (southernmost to northernmost occurrence of this species). I found that there was an increase in leaf total NSC content during summer as opposed to winter ($P = 0.0005$). For total carbon, there was both a latitudinal and a seasonal effect, with an increase in total carbon content across latitude in summer ($P < 0.0001$) and in winter ($P = 0.009$). However, for total nitrogen and $\delta^{13}C$, there was neither an effect of season nor latitude but there was an increasing trend of $\delta^{13}C$ towards the southernmost site irrespective of the seasons. Apart from NSC, site-specific factors such as sediment texture, salinity, in addition to temperature may play a role in the latitudinal distribution of mangroves.

2.2 Introduction

2.2.1 Carbon, nitrogen, non-structural carbohydrate content, stable isotopes

Carbon is central to plant metabolism and makes up approximately 50% of a tree's dry mass (Germino, 2015; Hoch, Popp & Korner, 2003). Non-structural carbohydrates (NSCs) are a good indicator of the plant carbon balance and therefore can be used to characterise the influence of abiotic environmental factors and plant physiological responses (Dietze et al., 2014; Germino, 2015). Other nutrients such as nitrogen, phosphorous, potassium, magnesium etc. also play an important role in plant physiological processes. Among these, nitrogen and phosphorous are the two most important and limiting nutrients (Reich & Oleksyn, 2004; in Liu et al., 2018a).

Mostly, the effects of resource limitation (carbon, nitrogen or phosphorous) coupled with other abiotic factors (light availability) in both tropical and temperate species are studied in controlled conditions (Li et al., 2018; Xie et al., 2018). Xie et al. (2018) showed a significant correlation between leaf NSC allocation and C:N and N:P ratio under reduced light intensity in two shade tolerant species, *Elaeocarpus sylvestris* and *Illicium henryi*. Species-specific differences occurred under light intensity variation. Leaf NSC concentration decreased in *E. sylvestris* under 100% vs. 33% full sunlight. Under the same light conditions, constrastingly, *I. henryi* showed unchanged NSC concentrations. When light was reduced from 33% to 6% of full sunlight, *E. sylvestris* maintained stable NSC concentrations whereas those in *I. henryi* decreased. In this study, the N:P ratio decreased with decreasing light intensity only in *E. sylvestris* and not in *I. henryi.* When light intensity decreased, the C:N ratio increased in *E. sylvestris*, whereas in *I. henryi* C:N ratio decreased. Abiotic factors other than drought such as light availability also influence how plants maintain nutrient balance between leaf NSCs, C, N, P and the ratios of C:N and N:P (Xie et

al., 2018). The physiological responses of plants to different abiotic factors may play a role in determining a species distribution across latitudinal gradient. However, few studies (de Lange & De Lange, 1994; Duke et al., 1998) have explored the role of these physiological factors in setting the latitudinal limit of New Zealand mangroves. A lot of research has been carried out on the front of altitudinal tree line formation rather than latitudinal gradients (Liu et al., 2018a). The two most important hypotheses used to explain tree distribution across an altitudinal gradient are the Carbon Limitation Hypothesis (CLH) and the Growth Limitation Hypothesis (GLH). The former suggests an insufficient plant carbon balance (source limitation; Stevens and Fox, 1991; in Hoch et al., 2002) and the latter suggests that formation of new cells is impossible below a certain temperature whereas carbon gain remains unaffected. This is carbon sink-limitation rather than source limitation (Korner, 1999; in Hoch et al., 2002). Both CLH and GLH can also be used to explain how nutrients (NSCs, C, N and P) vary across a latitudinal gradient (Lintunen et al., 2016; Liu et al., 2018a).

Stable isotopes are an integrated and powerful tool to understand plant responses to different environmental factors under natural conditions, from cellular to the ecosystem level (Chaves et al., 2003). The isotopic composition of a sample is measured relative to a standard (Chaves et al., 2003). The ratio between intercellular and atmospheric CO_2 partial pressures (C_i/C_a,) [$\delta^{13}C$], is related to the intrinsic water use efficiency of plants, which is the ratio of carbon fixation (g) to the amount of water transpired (g) (Bresinsky et al., 2013; Farquhar et al., 1982). Elevated CO_2 experiments using stable isotope signatures as well as ^{13}C pulse labelling have been used to assess salinity response of mangroves (Reef et al., 2015c) and also used to understand assimilation processes in trees (Hoch & Keel, 2006; Mildner et al., 2014).

$\delta^{13}C$ values are more positive in drier sites associated with stomatal limitation (Beard, 2006; Bowling et al., 2002[in Chaves et al., 2003]). Whereas, when stomatal limitations are not present $\delta^{13}C$ values are more negative. The differences in $\delta^{13}C$ values due to the presence or absence of stomatal limitations are used to assess hydraulic limitations in forests at the physiological level (Chaves et al., 2003).

Non-structural carbohydrates and the sugar-starch partitioning show seasonal variations in tropical and temperate species (Bansal & Germino, 2009; Liu et al., 2018b). In general, NSC reserves accumulate during the growing season and are used for growth and metabolism in winter. This means that deciduous species should show stronger seasonal oscillations in comparison to evergreen species (Chapin et al., 1990). However, there are inconsistencies in these general seasonal NSC patterns which show that deciduous species exhibit less strong seasonal NSC oscillations in comparison to the evergreen species (Hoch et al., 2003; Richardson et al., 2013). For example, in both red maple *Acer rubrum* (L.) and eastern hemlock *Tsuga canadensis* (L. Carriére) of north-eastern USA, the starch and sugar concentrations across seasons were similar in both deciduous and evergreen species (Richardson et al., 2013). In another study of the conifers, *Pseudotsuga meinzeisii* and *Abies lasiocarpa,* starch was highest at the start with almost complete depletion towards the end of growing-season and soluble sugars increased until winter (Bansal & Germino, 2009). Bansal & Germino (2009) also show that seasonal patterns were more dominant than diurnal variations in these species. Liu et al. (2018b) report higher starch and NSC concentrations in the dry season and higher soluble sugars in the wet season across species of the subtropical monsoon broadleaved evergreen forests in China. A global synthesis of 121 studies covering 177 species under natural conditions show that in all species across biomes,

depletion of total NSC or soluble sugars during the seasons did not occur (Martinez-Vilalta et al., 2016). The same study showed that in temperate and boreal biomes, while starch was depleted towards the end of the growing season, soluble sugars increased. Whereas, during winter, starch increased progressively. This indicates the use of starch for future plant functional needs and the use of soluble sugars for immediate plant functions such as osmoregulation. Though Mediterranean biomes are known to have strong seasonality, this study reports low seasonal variations of NSCs in these biomes (Martinez-Vilalta et al., 2016). Species-specific variations and variation in NSC allocation, emphasize the need to assess seasonal NSC dynamics more rigorously (Hoch et al., 2003; Richardson et al., 2013; Martinez-Vilalta et al., 2016).

2.2.2 Non-structural carbohydrate analysis methods

Research on NSC dynamics and the effect of abiotic stresses on NSC in plants has been carried out for more than a decade and a number of reliable methods for the quantification of NSC exist (Hoch et al., 2002; Landhausser et al., 2018; Pinkard, 2018; Quentin et al., 2015). However, very few attempts have been made to quantify NSC in halophytes (Gil et al., 2013; Jung & Burd, 2017). Presently, it is difficult to compare NSC results among laboratories because of the differences in extraction procedures, timing of sample collection, storage, duration of drying and choice of NSC extraction technique (enzymatic or acid hydrolysis; Quentin et al., 2015, Chapter 1). Interspecies differences in plants are also a reason for the difficulty to compare NSC results. For example, the presence of salts and other antioxidants in higher concentrations can hinder the estimation of NSC content in mangroves (personal observation; Parida & Das, 2005; Popp et al., 1985). Following a standard method

applicable across different plant species can possibly eliminate methodological differences and aid in better interpretation of results.

Near infrared spectroscopy

Near infrared spectroscopy (NIRS) is a powerful analytical technique which can be used to measure the chemical composition of biological tissues. NIRS is used in plant biochemistry and has proven useful for the analysis of non-structural carbohydrates (NSCs; Quentin et al., 2017; Ramirez et al., 2015). NIRS covering the electromagnetic region of the wave spectrum from 14000 cm^{-1} to 4000 cm^{-1} is now becoming an important tool in plant metabolite analysis, although its discovery dates back to the 19^{th} century (Manley, 2014). NIRS consists of short wavelength (penetrative) spectra. The spectra are overtones of C-H, O-H and N-H bonds in comparison to MIR (Mid Infrared) spectra, which are not overtones and were used extensively to understand molecular structure and composition, before NIRS techniques and chemometrics were well developed (Conrad & Bonello, 2016; Foley et al., 1998; O'Reilly-Wasptra et al., 2013). The near infrared region can be used to interrogate vibrational properties of chemical bonds. These properties provide key information on the presence/absence of different functional groups (e.g., -OH, -COOH, -COOC-, -CH3, C=C), which together point toward characteristic features of certain chemical classes (e.g., sugars, lipids, proteins, nucleic acids). Infrared spectroscopy is Fourier transform based which uses light interference rather than dispersion when spectral measurements are made (Bates, 1976) For FT-NIR measurements, interferometers such as Michelson interferometers are used to focus the emitted light, and wavelengths are analysed simultaneously (Diem, 1993; in Conrad & Bonello, 2016). Handheld spectroscopic devices or laboratory benchtop spectrophotometers are also used to collect the NIR spectra. Although ease of

sample preparation is a potential advantage, the functional group signatures can be complex in nature, with information being contained at multiple spectral regions, often with overlapping peaks, and noisy signals. However, through careful signal processing and use of chemometric or multivariate statistical analysis, these spectra can be used to construct accurate prediction models (validated against data using wet chemistry techniques) to estimate the quantity of particular compounds of interest (e.g., total sugars in plant tissues). Therefore, the development of a reliable reference method (wet chemistry) is necessary to make useful interpretations from NIR data.

Prior to statistical analysis of NIR spectra, appropriate mathematical pre-treatment is conducted to ensure the quality and integrity of the data (e.g., first/second derivative transformation, smoothing, multiplicative scatter correction (reviewed by Foley et al., 1998; Manley, 2014). Then, chemometric techniques are used on NIR data for exploratory analysis, sample outlier detection, and quantitative prediction modelling. Common methods include principal component analysis (PCA), partial least squares regression or discriminant analysis (PLS-R, PLS-DA), and artificial neural networks (ANN). PLS-R (Partial least squares regression) has most widely been adopted for prediction modelling using NIR data (Ramirez et al., 2015; Windley & Foley, 2015). A series of paired measurements using a quantitative technique (i.e., wet chemistry) as well as NIRS on different samples provides known values for the compound of interest, which can be cross-referenced with the NIR spectral signature. This is performed by dividing the samples into a training set and a validation set, with permutation-based PLS-R modelling. The efficacy of the calibration equations that are developed and used for predictions is assessed via the standard error of cross validation (SECV), standard error of prediction (SEP), and Pearson's coefficient of determination (r^2).

The use of NIRS to characterise the chemical composition in plants is gaining traction due to its low cost, speed of analysis, and ability to gain data from plant material (whole leaf, stem, fruit) with minimal or no sample preparation. The development of hand-held spectrophotometers has also eased the data acquisition process. Some applications of NIRS and PLS-R (Partial least squares regression) modelling accurately predict different plant constituents. In leaves of five New Zealand forest species, constituents such as total nitrogen, available nitrogen, and invitro dry matter digestibility content were accurately predicted (Windley & Foley, 2015). NIRS has also been used to estimate the abundance of alkaloids and other secondary metabolites in medicinally important plant species with excellent predictive capability (reviewed by Cozzolino, 2010). For example, quercetin in *Ginkgo biloba* species was measured using NIRS, with RMSECV (root-mean-square error of cross validation) value of 0.75 (Zhou et al., 2007; in Cozzolino, 2010). Phenolics in green tea was identified using NIRS combined with a Folin–Ciocalteu assay. Essential oils, like Honghua oil were also analysed for methyl salicylate, eugenol, á-pinene with accurate predictions using NIRS (Wu et al., 2008). When using NIRS there is a tradeoff between using a standard calibration equation or tailoring an equation to a specific set of experimental conditions (Lawler et al., 2006). However, NIRS is used to predict total NSC content (soluble sugars plus starch) in atleast 82 temperate plant species with r^2 = 0.91 (Ramirez et al., 2015).

2.2.3 New Zealand mangroves distribution:

Mangrove ecosystems are primarily divided into two main regions globally: the Atlantic East Pacific (AEP) and the Indo-West Pacific (IWP), covering the northern and southern hemispheres (Duke et al., 1998). The IWP region is more species rich than the AEP, with 58 taxa whilst the AEP consists of only 13 species. On a global

and regional scale, temperature, rainfall, tides are the important climatic controls that govern mangrove distribution (Duke et al., 1998). In addition to this, propagule dispersal and establishment are also important factors (Duke, 1990; Duke et al., 1998). Temperature restricts mangrove distribution mostly to the tropics and sub-tropics, where seasonal temperature ranges do not extend beyond 10°C (Chapman, 1975,1977; reviewed by Duke et al., 1998). *Avicennia marina* is the exception that extends beyond the seasonal range of 10°C extending into the temperate zone.

The southernmost mangroves of the world *Avicennia marina* subsp. *australasica* are found in temperate northern New Zealand (Burns and Ogden, 1985). They occur along estuarine margins in the North Island from Cape Reinga (34°S) in the north to Ohiwa Harbour (38°03'S) on the east coast and Kawhia Harbour (38°05'S) on the west coast. Planted mangroves also occur further south along the Uawa estuary, Tolaga bay (38°23'S). Temperature and salinity play a role in the distribution of mangroves (Mittler, 2006; Munns, 2006; Quisthoudt et al., 2012), they influence propagule development, dispersal and establishment (Duke et al., 1998) and thus determine mangrove distribution across latitudes (Duke et al., 1998). Most studies have concentrated on the role of these climatic factors on the current distribution of New Zealand mangroves. Susceptibility or sensitivity to frost (temperatures below 2°C) is thought to be the main reason for the latitudinal limits of New Zealand distribution (Chapman and Ronaldson, 1958 [in Duke et al., 1998]). Conversely, Sakai and Wardle, (1978) found that New Zealand mangrove shoots can withstand 4 hours of freezing conditions at -3°C. Therefore, temperature extremes alone cannot be the sole driver of mangrove distribution (Quisthoudt et al., 2012). In addition, when Duke et al. (1998) investigated the global distributional limits of *Avicennia marina* as a function of phenological events, they found that the shorter growing periods in the

higher latitudes were responsible for less reproductive success of mangroves leading to a lower number of established mangrove populations. Thus, in addition to understanding the role of climatic factors in mangrove distribution it is important to investigate the simultaneous influence of physiological and site-specific factors (de Lange & de Lange, 1994).

The role of non-structural carbohydrates (NSCs) in mangrove physiology is important to the understanding of the study species tolerance to extreme conditions. Despite this, our understanding of mangroves NSC dynamics, both on a temporal and spatial scale, derived from previous studies is limited (de Lange & De Lange, 1994; Gil et al, 2013). Studying NSCs in natural conditions under different seasons aids in the understanding of their role in the plant carbon source-sink dynamics (Gil et al, 2013). In this study, I aim to suggest an appropriate method for NSC analysis in mangroves. My overall hypothesis was that temperate mangroves are not carbon limited (i.e. the total carbon (including structural carbon), NSC content is similar in the northern and southern sites). Only seasonality will influence total carbon, nitrogen and, NSC content across the latitude gradient. However, other environmental conditions such as increasing salinity and, edaphic factors may also influence the species' carbon sink activities, such as growth, and play a role in species' distribution. I further hypothesise that $\delta^{13}C$ values will increase towards the southernmost sites since they are more stressed with less stomatal conductance (Beard, 2006). I addressed the following research questions, 1) What is the most suitable wet chemistry method to determine NSC content in mangroves? 2) Is NSC content affected by a latitudinal gradient at the southernmost distribution range of the New Zealand mangrove? 3) Does seasonality affect NSC content of this species?

2.3 Materials and methods

2.3.1 Latitudinal NSC gradient - Study area

Three main study sites were chosen across a latitudinal gradient in the North Island, New Zealand (Figure 2.1). The northernmost site was at Parengarenga Harbour (34°31'0" S). A mid-latitudinal site was located at Manghawhai (36°7'35" S), which had two subsites, Jack Boyd and Molesworth. The southernmost site was chosen at Ohiwa Harbour (38°03'9" S), at the southern limit of this species' distribution. Another site, Tolaga Bay was 200 km south-east of Ohiwa Harbour, roughly at the same latitude (38° S). This site consisted of naturalised mangrove populations, planted in the 1970s. Trees were sampled at each site during both summer and winter seasons in 2017. All three main sites consisted of mixed age mangroves (Beard, 2006).

Figure 2.1 Study sites from north to south (North island, New Zealand) for determining latitudinal NSC content

The northernmost study site at Parengarenga Harbour has both tall (> 5m) and short (< 4m) trees. Trees in this site were taller than those at the southernmost sites ranging from 5 to 7 metres in height, with diameters of upto 30 cm (Beard, 2006). This study site was in the upper intertidal zone with coarse muddy substrate.

The Manghawhai estuary site has about 87 ha of mangroves. The individuals are of 3-4 m in height. Two subsites were included in this study. First, Jack Boyd which is situated in the upper estuary and second, Molesworth which is located in the middle of the estuary. Jack Boyd consists of sandy substrate whereas Molesworth had a muddy substrate.

(A)

(B)

Plate 2.1: (A) Photo of the study site at Jack Boyd, Manghawhai. (B) Molesworth, Manghawhai.

Ohiwa Harbour represents the natural limit of mangroves in New Zealand, surrounded by salt marshes and mudflats (Morrisey et al., 2007). It consists of shorter mangroves with most individuals of about 50cm height with spreading crowns of 2 or 3 metres width. The tallest individual was of 1.5m in height (Beard, 2006) and seedlings were abundant in open patches between trees. Tolaga Bay consists of naturalised populations, wherein propagules from Ohiwa Harbour were planted in the 1970s (Morrisey et al., 2007). Tolaga bay is surrounded by salt marshes, characterised by muddy substrate. This site also has both tall and short trees according to the above definition.

(A)

(B)

Plate 2.2: (A) Southernmost mangrove in New Zealand at Tolaga Bay with the researcher. (B) Mangroves at Tolaga Bay site (with fellow researchers during field work).

2.3.2 Sample collections and measurements

Environmental variables, such as temperature, rainfall and local weather conditions were taken from meteorological data providers (metservice.com; Table, 2.1) of Te Hapua, Whangharei, Gisborne recordings were used for Parengarenga Harbour, Manghawhai, and Ohiwa Harbour respectively. Te Hapua is about 72 km away from Parengarenga Harbour, Whangharei is about 30 km away from Manghawhai and Gisborne is about 150 km away from Ohiwa Harbour. Sampling was conducted during rain-free days during both summer and winter periods. At each site, leaf and wood samples were collected from ten trees along a line transect from north to south. Trees approximately 10-20 metres apart were selected for sample collection.

From each tree, sun-exposed leaves (*n* = 5-10) were collected at midday (12-4 pm approximately). Simultaneously, microcores (*n* = 2 per tree) were collected using a 'trephor' microcorer (Rossi, Anfodillo, & Menardi, 2006). The cores were 2cm in depth and 2mm in diameter. Leaf and core samples were stored on ice immediately, then microwaved for 60 seconds on the same day to stop enzymatic activity. Samples were stored on ice again until they could be analysed at the laboratory.

Soil samples (*n* = 10) were also collected at each site at a 10 cm depth for salinity measurements. Pore water salinity at each site was recorded using a standard refractometer.

2.3.3 Stable isotopes, carbon, and nitrogen content

Leaf samples from Parengarenga, Jack Boyd, Ohiwa, and Tolaga Bay sites were used for stable isotope analyses. Samples from each site for both summer (*n* = 5)

and winter (*n* = 5) were sent to the University of Waikato stable isotope unit for total carbon, nitrogen and $\delta^{13}C$ analyes.

Differences in carbon, nitrogen and stable isotope content were analysed as a function of latitude and season using flexible generalized additive models (GAMs; Wood et al., 2017). Diagnostic plots were used to assess homogeneity of variance and normality. All statistical analyses were performed using R (R 3.4.2, R Development Core Team, 2017), using the R-packages: *mgcv* (Wood et al., 2017); lmtest (Zeileis and Hothorn, 2002); *emmeans*, [Lenth, (2018).

2.3.4 NSC analysis

Once the leaf and core samples were transported to the laboratory they were placed in the drying oven at 65˚C for atleast 72 hours. The dried samples were ground using a ball mill (Retsch planetary ball mill, PM 100; Retsch GmbH, Germany) to a pore size of less than 350 microns. Ground samples were stored in vials in desiccators at room temperature until further analysis.

2.3.5 NSC method identification

Sample preparation

A sequential extraction procedure was followed to detect analytical problems in enzymatic hydrolysis and molecular absorbance was recorded using a UV-visible (UV-Vis) spectrophotometer. Normal absorption values at 340 nm for plant tissues are below 0.8 following hot water extraction during the enzymatic hydrolyis procedure (Hoch et al., 2002; Quentin et al., 2015, Landhausser et al., 2018). Firstly, I tested the effect of salt on absorption (340 nm) of terrestrial (*Laurus nobilis* L.) and mangrove leaves (*n* = 3 each). I also tested if sample dilution reduced the UV-Vis absorption values to <1in mangrove leaves. Samples were ground using a ball mill

(Retsch planetary ball mill, PM 100; Retsch GmbH, Germany) to a particle size of less than 350 microns. Deionized water (1 mL) was added to 10 mg of leaf samples. The sample rack was then placed in boiling water, extracted in steam for 30 minutes, with vortexing for 30s every 10 minutes. Then samples were centrifuged (at 16240 *g* for 3 minutes). Sample extracts (200 µL) were added to a microplate and UV-Vis aborption at 340 nm of these samples were recorded.

Salt effect on absorption at 340 nm

To test the effect of salt, I added 800 µL of deionized water and 200 µL of saltwater (35 ppt, seawater concentration), to the leaf samples. This was followed by vortexing, centrifugation and the absorption was recorded as above to see if it was more than 1.

Secondly, the hot water samples of both mangrove and terrestrial (*Laurus nobilis* L.) leaves were diluted. A quantity of 20 µL of the sample was added to 200 µL of deionized water. This solution of 200 µL was then added to the microplate and the spectrum was recorded.

Dilution effect on absorption at 340 nm

Since dilution did not reduce the 340 nm absorption values of the mangrove leaves to <1, I carried out an acetone washing experiment on the mangrove leaves. Acetone was used to remove pigments and both nonpolar and polar compounds. Only one leaf sample was used for washing and this sample was further divided into four parts of 10 mg each. One group was washed with acetone once, the second group twice, the third group thrice and the fourth group four times. Each group consisted of two replicates of 10 mg each.

Acetone wash: To each sample, 1mL of acetone was added and vortexed. The samples were then centrifuged (at 16240*g* for 3 minutes). The acetone was evaporated in a drying oven at 70°C. Deionized water (1 mL) was added to the samples and then the samples were placed in boing water and extracted in steam for 30 minutes. Sample extract of 200 µL was also added to the microplate and the UV-Vis spectrum was recorded.

Since acetone washing did not reduce the absorption values either, NSCs in the study species were later analysed colorimetrically using the anthrone reagent method (anthrone in 0.2% conc. H_2SO_4), according to Yemms & Willis (1954). Soluble sugars were extracted using 80% ethanol. Ethanol (1 mL) was added to the leaf sample, vortexed and centrifuged (16240 *g*; 3 minutes). The supernatant (500 µL) was used for spectrum recording. The remaining ethanol was dried off using a drying oven and then the pellet was used for starch analysis.

For starch analysis, I used the common enzyme based extraction using amyloglucosidase consisting of 260 mg amyloglucosidase dissolved in 52 mL of sodium acetate buffer. The pellet was first gelatinised using deionized water at 70°C for 2 hours. Amyloglucosidase was added to the leaf sample, in 1:1 ratio. These samples were incubated for 15 hours (overnight) at 50°C. The supernatants were later collected after vortexing and 200 µL of the supernatant was added to the microplate for UV-Vis spectrum recording.

2.3.6 Spectroscopy

All ground samples were analysed using a Fourier transformed infrared (FTIR) spectrophotometer (Prestige 21, Shimadzu Corporation). Absorbance spectra were collected from 3770 cm^{-1} to 12000 cm^{-1} at a resolution of 16 cm^{-1} averaged over 64

scans per sample (Figure 2.2). I recorded the entire near-infrared spectral range. Each spectrum in the study was characterised by 511 variables (wavenumbers).

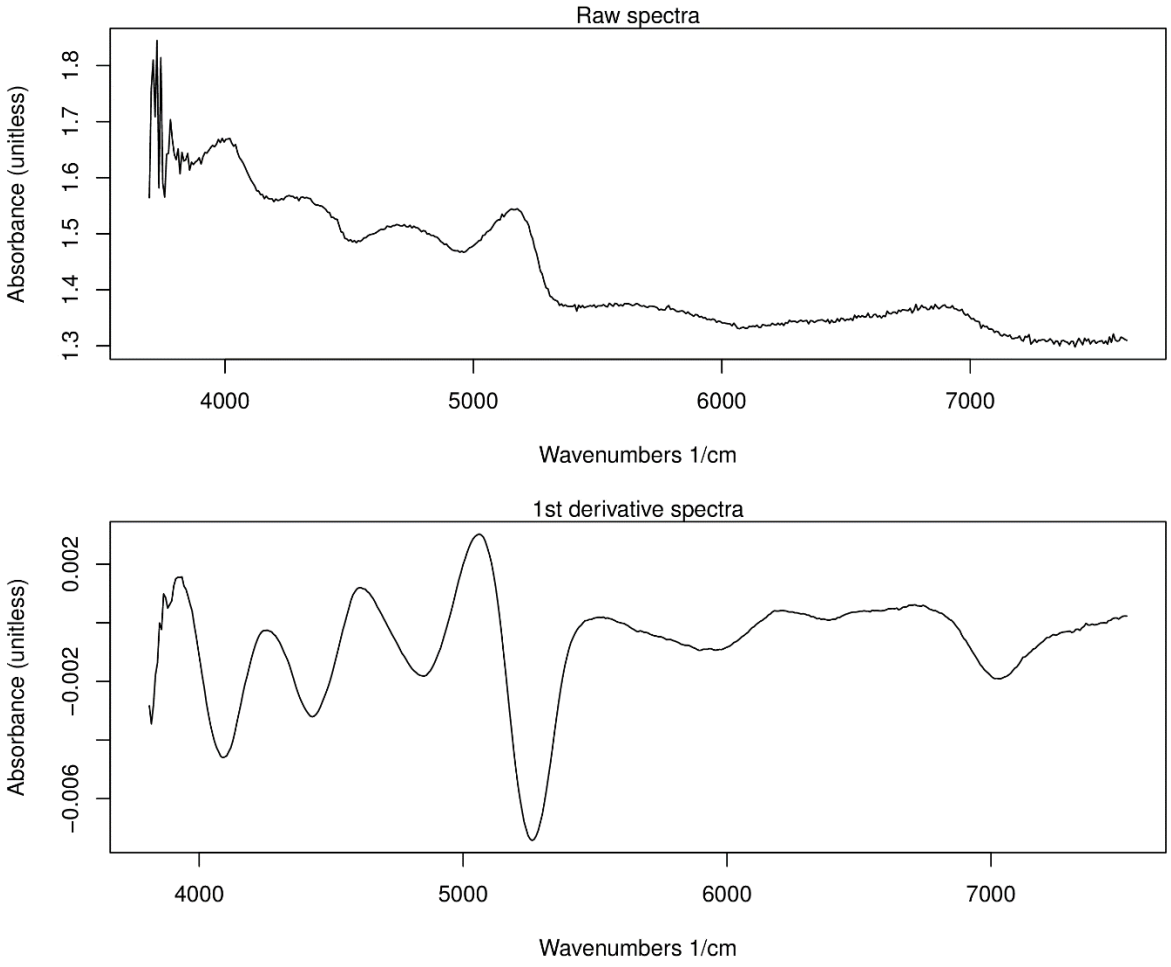


Figure 2.2 Example NIR spectrum (4000 to 7000 cm^{-1}). Top panel: Raw spectra from the instrument. Bottom panel: Spectra after application of Savitzky-Golay filter.

2.3.7 Calibration model

I used the calibration model developed from mangrove seedlings (*n* = 200) grown for atleast 16-20 weeks. These seedlings were also used for drought and salinity experiments (Chapter 3). Leaf, stem and root samples from the seedlings were collected after treatment application at week 4, 8 and 12. The collected samples were also analysed using a FTIR spectrophotometer for recording the Near-Infrared (NIR) spectra.

After recording the NIR spectra, 16% of the total samples (*n* = 82) were selected out of the total of approximately 500 (following Ramirez et al., 2015). I used the Kennard Stone algorithm for selection of the 82 samples for chemical analysis using the R package *prospectr* (Kennard & Stone, 1969; Ramirez et al., 2015; Stevens & Ramirez-Lopez, 2013). NSCs here were then analysed using the anthrone method (Yemm & Willis, 1954), as described earlier.

The NSC values obtained after the analysis were higher than 1 (more than 100% d.w.) in some cases. Therefore, for consistency and standardisation, I report NSC content values as relative values. This was obtained after dividing all the NSC (% dryweight) values by the highest NSC value.

2.3.8 Partial least squares regression

Partial least square regression (PLS-R) analysis was used to predict the NSC concentrations in the samples. Before statistical analysis, the Savitzky-Golay filtering process was used to obtain the first derivative spectra from the raw spectra. The first-derivatized spectra were used for analysis. Model selection was based on Pearson's r^2 and root-mean-squared-error of prediction (RMSEP). The calibration model was developed using the spectra of leaf, stem and root samples of mangrove seedlings as described above. PLS-R calibration equations were derived using cross validation with *n* = 3 components. When performing cross validation, the number of components is chosen. Number of components is the value at which the RMSEP value does not increase further. Using higher components (e.g. *n* = 5) results in overfitting of the model.

2.3.9 Latitudinal NSC data analysis

The PLS-R derived calibration equations were used for NSC content predictions from the NIR data across the latitudinal gradient. Total NSC (I present the total of soluble sugars plus starch) concentrations were modelled in leaves and cores separately as a function of latitude, with season as smoother terms, using flexible generalized additive models (GAMs; Wood et al., 2017). Only Parengarenga Harbour, Manghawhai, and Ohiwa Harbour were included in the additive modelling. The site Tolaga Bay was not included since this is a naturalised population (planted in 1970s) whereas Ohiwa Harbour is the natural mangrove limit in New Zealand (Morrisey et al., 2007). Diagnostic plots were used to assess homogeneity of variance and normality. All statistical analyses were performed using R (R 3.4.2, R Development Core Team, 2017).

2.4 Results

2.4.1 Carbon, nitrogen content, stable isotopes

Only leaf carbon content (including structural carbon) was significantly different across latitude in summer ($P < 0.0001$) and in winter ($P = 0.009$; Figure 2.3). Neither season nor latitude caused differences in nitrogen content and $\delta^{13}C$ values. However, the $\delta^{13}C$ values were higher at Ohiwa Harbour (-24 ‰), in comparison to Parengarenga Harbour (-26.5 ‰; Figure 2.3).

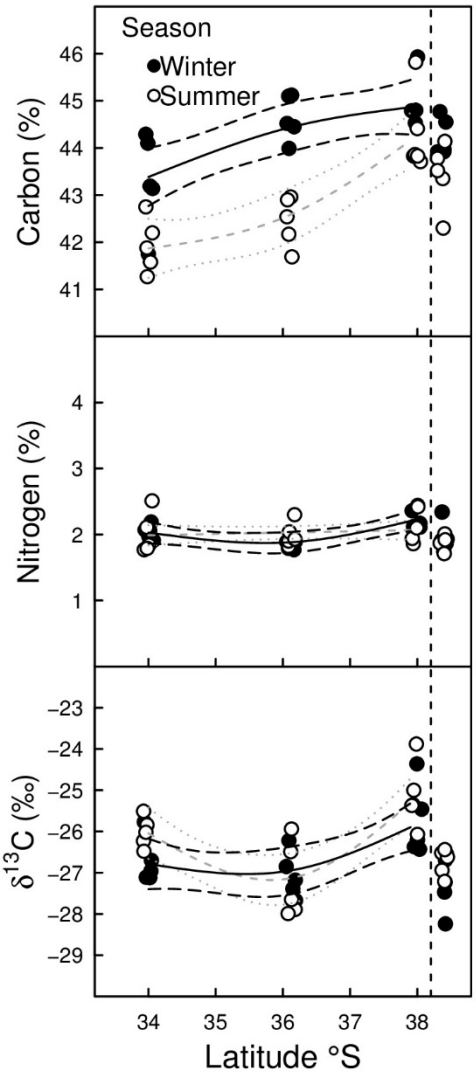


Figure 2.3 Total carbon, nitrogen and stable C isotope content of *Avicennia marina* subsp. *australasica* across a latitudinal gradient in New Zealand. The dashed line after 38°S is to differentiate between the naturalised mangrove population at Tolaga Bay and the populations at the other study sites.

2.4.2 Non-structural carbohydrate determination in mangroves

Estimation of NSC content in mangroves using NIRS as a tool is possible with an appropriate reference chemical method. I chose the anthrone method which is useful for analysing NSC content in mangroves instead of the widely followed enzymatic hydrolysis in other plant species, because of the absorption interference at 340 nm. In this study, salt did not contribute to the higher absorbance values. Diluting the samples ten times also did not reduce the absorption values. Mangrove leaves mostly had higher absorption values (>1; Figure 2.4).

I used acetone washing as I expected the absorption to decrease as the number of washes increased. However, washing the samples with acetone either once, twice, three or four times did not reduce the absorption at 340 nm to <1 (Figure 2.5).

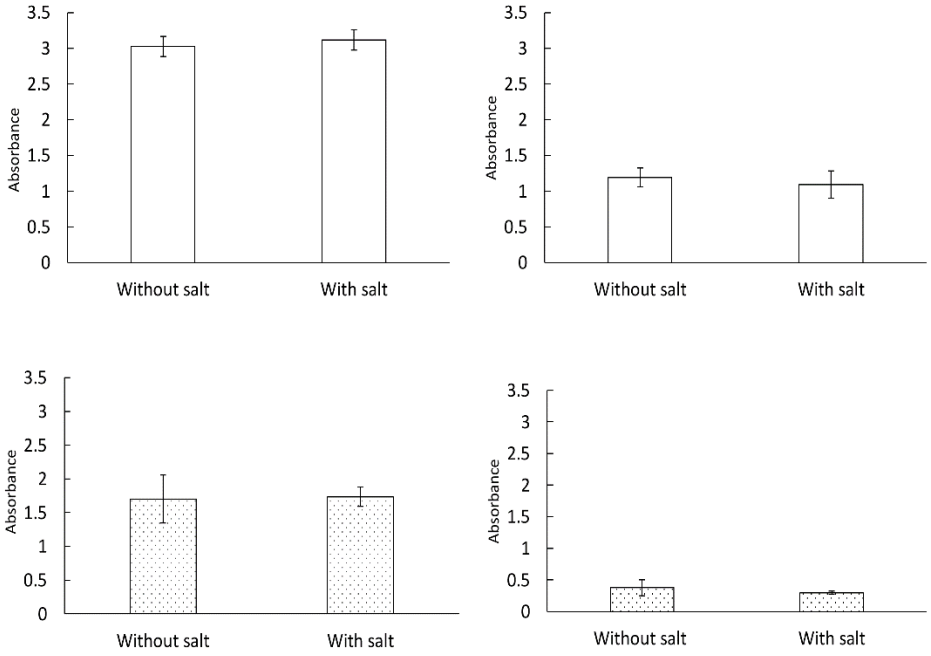


Figure 2.4 Salt and dilution effect on mangrove and terrestrial leaves. UV-Vis absorbance values at 340 nm. Plain bars represent mangrove leaves absorption. Dotted bars represent terrestrial leaves absorption. Left panel represents leaf absorption without dilution. Right panel represents leaf absorption after dilution

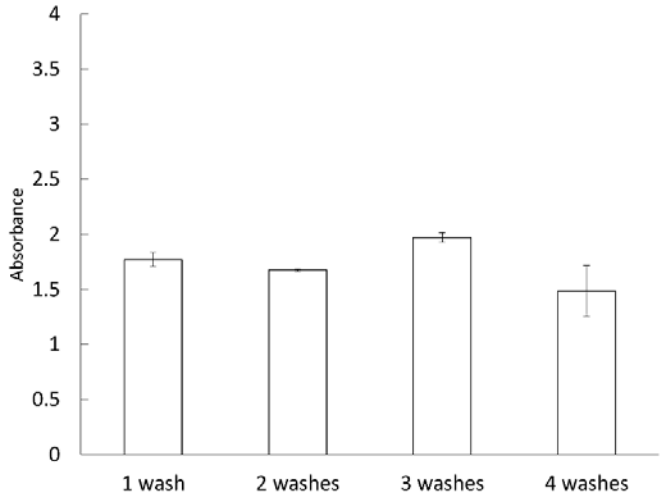


Figure 2.5 Acetone washing of the mangrove leaves. UV-Vis absorbance at 340 nm. Even after washing the samples four times with acetone, mangrove leaf absorption did not reduce to <1.

For the latitude study, I used partial least square regression to derive calibration equations and the chosen model had an $r^2 = 0.8$ and RMSEP = 0.135 (Figure 2.6). The training set in the PLS model had an $r^2 = 0.45$, with a RMSEP = 0.09. The low r^2 values in my study is associated with instrumental glitches such as replacement of tungsten lamp, IR software, firewire cable etc. during the analysis period with more than *n* = 100 spectra taken out prior to PLS-R analysis.

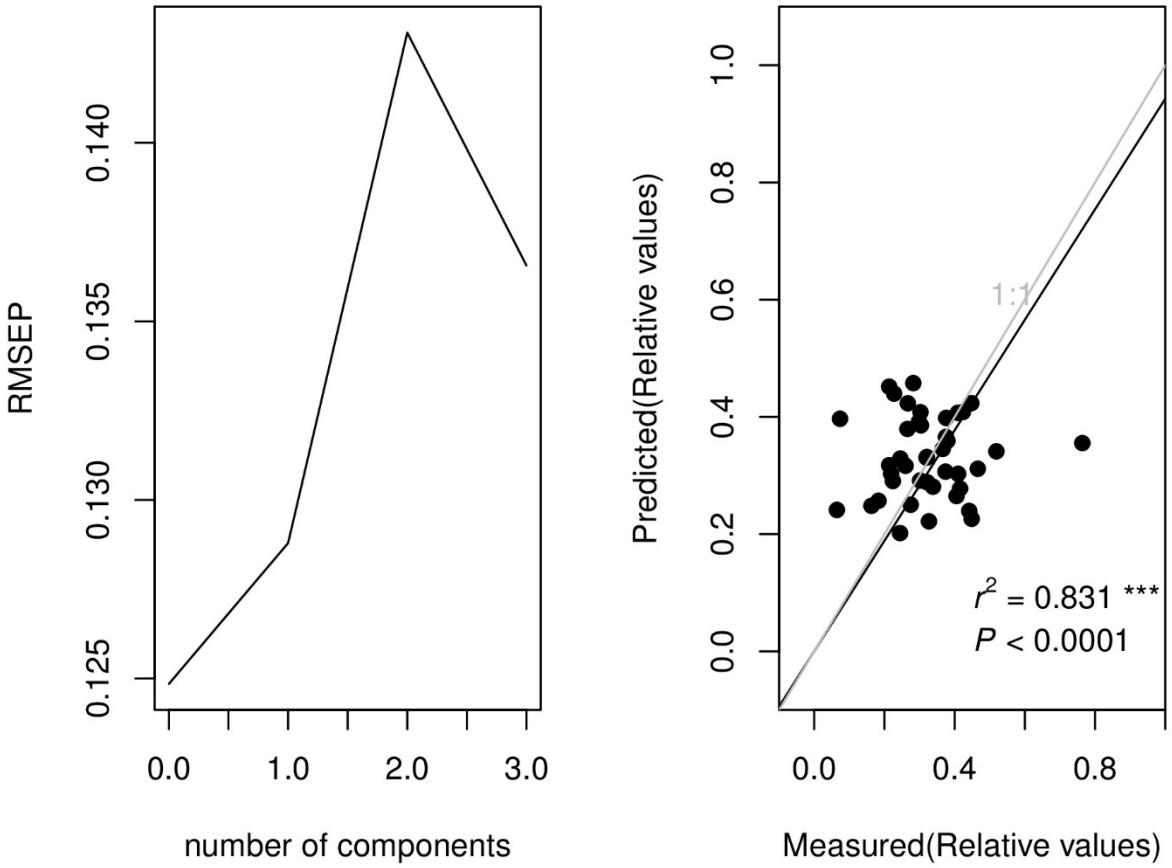


Figure 2.6 PLSR-derived calibration model for NSC content. The left hand side plot shows the chosen number of components to obtain the model. The right side plot shows the NSC content (n = 40) as predicted from NIRS calibration model after removal of the intercept.

2.4.3 Total non-structural carbohydrate concentrations

Total non-structural carbohydrate concentrations (TNSC) in both leaves and cores were modelled as a function of season with latitude as a smoother term stratified by season. In leaves, I detected a significant latitudinal TNSC difference in summer (*P* = 0.0005). The deviance explained for the leaf model was D = 30.2%. At the southern site (Ohiwa Harbour), TNSC content increased to 0.5 in summer as compared to 0.35 in winter (Figure 2.7).

For cores, there was no significant latitudinal difference in summer (*P* = 0.294) nor in winter (*P* = 0.685). The deviance explained for the cores model was D = 16.9%. At the southern site (Ohiwa Harbour), the mean concentration was 0.35 across latitude in both seasons (Figure 2.7).

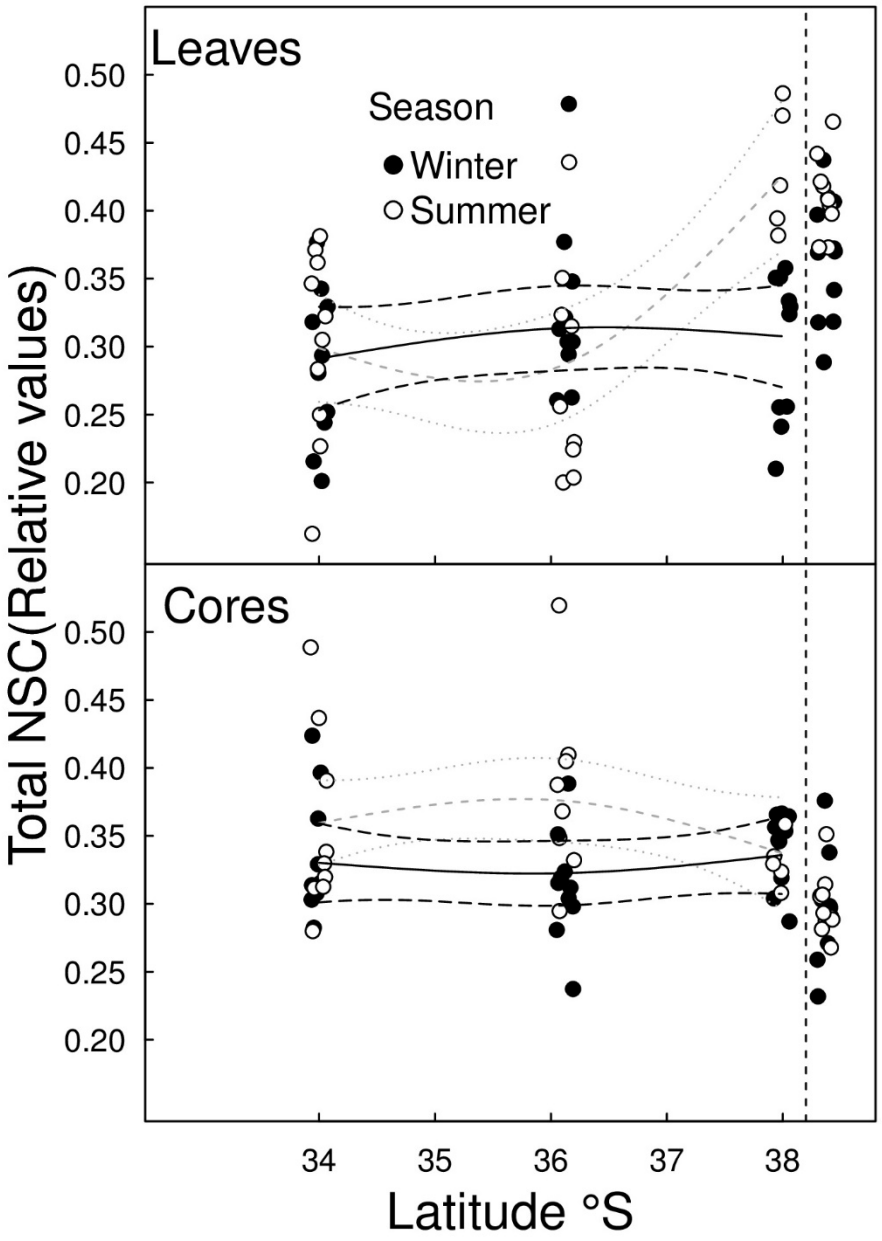


Figure 2.7. Top panel: Total leaf NSC concentration across latitude. NSC values are predicted from the PLS-R calibration model derived from mangrove seedlings (Chapter 3). Bottom panel: Total NSC concentration in the cores. Dashed lines represent GAM fits and the surrounding white dotted lines show 95% confidence intervals. Similarly, black sold lines represent GAM fits and the surrounding black dashed lines show 95% confidence intervals. The dashed line after 38°S is to differentiate between the naturalised mangrove population at Tolaga Bay and the populations at the other study sites.

Table 2.1. Climate data summary for the 1981-2010 period. Source:NIWA climate database. The months of sample collection, January, February for summer and July, August for winter are highlighted here. Data are from local weather stations, recorded by NIWA (National Institute of Water and Atmospheric Research). Te Hapua recordings were used for Parengarenga Harbour, Whangharei recordings were used for Manghawhai, and Gisborne readings were used for Ohiwa Harbour and Tolaga Bay.

		Summer			Winter		
Climate variables	Locations	January	February	March	June	July	August
Mean daily maximum temperature (°C)	Te Hapua	19.5	20	18.6	12.8	12.1	12.2
	Whangharei	19.9	20.2	18.8	12.4	11.6	11.9
	Gisborne	19.2	19.1	17.5	10.3	9.7	10.4
Mean daily minimum Temperature (°C)	Te Hapua	15.1	15.5	14.2	9.2	8.5	8.6
	Whangharei	15.5	16.1	14.7	8.7	7.8	8.2
	Gisborne	13.9	14	12.5	5.6	5.3	12.5
Mean monthly rainfall (mm)	Te Hapua	82	92.5	82	149.1	165.4	140
	Whangharei	81.2	95.2	118.1	131.5	168.6	128.4
	Gisborne	56.2	71.3	91.4	104.6	127.7	76.2
Mean daily maximum temperature	Te Hapua	19.5	20	18.6	12.8	12.1	12.2
	Whangharei	19.9	20.2	18.8	12.4	11.6	11.9
	Gisborne	19.2	19.1	17.5	10.3	9.7	10.4
Mean monthly ground frost days	Te Hapua	0	0	0	0.1	0.2	0.2
	Whangharei	0	0	0	2.9	4.4	2.4
	Gisborne	0	0	0	7.6	7.4	7.2

2.5 Discussion

First, I identified a suitable wet chemistry method for estimating non-structural carbohydrate content in mangroves. I have shown NIRS is also a valuable tool for NSC estimation alongside the anthrone method which is preferable for halophytic mangroves. In addition, I showed that New Zealand mangroves have high total carbon content both in summer and winter, in the northern sites in comparison to the southern sites. Nitrogen content did not show latitudinal trend variations in neither summer nor winter. Although, the $\delta^{13}C$ values were higher in the southern sites significant latitudinal and seasonal trends were not detected. In the following few paragraphs, I discuss the reasons for choosing the particular wet chemistry method. Then, I discuss the factors influencing temporal and spatial variation of NSC content, total carbon and nitrogen content across the latitudinal gradient of New Zealand.

Studies so far have analysed NSC only in non-halophytic species. In my study, the presence of salts in mangrove leaves did not affect absorption since the UV-Vis absorption of plain saltwater also showed no absorbance peaks at 340 nm (0.035). I thus concluded that salt was not a cause of the high absorption values we obtained when carrying out enzymatic hydrolysis. Second, I use diluted samples of both terrestrial and mangrove leaves to test if dilution caused a lower absorbance. The absorbance values were still higher (>1x) at 340 nm as shown in the results. Therefore, I used acetone washing in order to remove high molecular weight compounds and pigments. I expected that the samples washed with acetone four times would yield lower absorbance values and that this pretreatment prior to hydrolysis would lead us to follow enzymatic hydrolysis protocol, following Hoch et al. (2002). However, even after four washes, there was no reduction in the absorbance values. Hence, I did not carry out the free sugar extraction procedure for

these samples. Since I was not successful in removing the pigments and polyphenols that interfere with the enzymatic assay, I was unable to obtain reliable results using enzymatic hydrolysis. In this specific case, I hypothesise that the presence of polyphenols (characteristic absorption at 330 nm) causes a high background absorption which hinders the spectrometric detection of a further increase of absorption due to the formation of NADH $+H^+$ when glucose-6-P is converted to gluconate-6-P at 340 nm. As a result, I chose to use the standard anthrone method for extraction and quantitative determination of sugars and starch. This is a colorimetric method based on the formation of a blue green complex (combination of furfural derivatives and anthrone), measured at 630 nm. The acid acts on glucose molecules and results in 5-hydroxymethyl furfural after a dehydration reaction. I used this as the reference method for predicting total NSC content using partial least squares from the NIR spectra recorded from the same samples.

Total NSC content usually consists of starch and the soluble sugars glucose, fructose, and sucrose. The anthrone method is non-specific and acts on all sugars rather than only glucose, fructose, and sucrose. When estimating NSC content, getting the individual sugar concentrations is not mandatory (Edwards, Downie & Clingeleffer, 2010). For my study, I also followed the suggested ethanol extraction with minor modifications for soluble sugars quantification. I followed a three times sequential extraction of soluble sugars with 80% ethanol for my work (superior extraction method; Quentin et al., 2015). Ethanol dissolves most free sugars but not many of the hemicelluloses. This provides an advantage of reducing the interfering substances in NSC quantification (Hoch et al., 2002; Quentin et al., 2015; Raessler, 2011). Whereas, water extracts fructans and soluble starches, which is the extraction procedure normally followed in enzymatic hydrolysis (Edwards, Downie &

Clingeleffer, 2011; Hoch et al., 2002). Similarly, for starch estimation in my study, starch was broken down to glucose, using amyloglucosidase and then the corresponding glucose content was quantified using anthrone method. Prior to soluble sugar and starch extraction, I microwaved the samples immediately after sample collection to stop enzymatic activity. However, the samples had a pore size of <0.35 mm instead of the suggested <0.15mm (Quentin et al., 2015). Regardless of the quantification method followed, studies are still struggling to obtain reliable starch estimates during NSC estimation (Hoch et al., 2002; Quentin et al., 2017). Quentin et al. (2017) applied NIRS to estimate leaf NSC of *Eucalyptus globulus* Labill. Starch models had a low R^2 value of 0.5 only in comparison to total sugars which had a R^2 of 0.7. It was suggested that the protocols/methods available for starch analysis must be amended with improved quantification methods (Hoch et al., 2002; Quentin et al., 2017; Ramirez et al., 2015). In general, HPLC (High performance liquid chromatography; Quentin et al., 2015, 2017) is recommended for quantification of NSCs. However, HPLC is quite time consuming and expensive in comparison to the methods followed in my study. Colorimetric methods have some drawbacks, for example, the gross estimation of soluble sugars rather than characterization of individual sugars. These drawbacks were reduced by comparing to different concentrations of a known standard, such as glucose (Quentin et al., 2015). Additionally, the use of enzyme extraction for starch rather than acid hydrolysis in this study was an advantage: cellulose, which is not part of NSCs, was not broken down confounding the NSC estimation further. Also, the usage of toxic chemicals was reduced.

To my knowledge, NSC content as such has been estimated in the saltmarsh *Spartina alterniflora* both above-ground and below-ground (Jung & Burd, 2017), also

in rhizomes of the seagrass *Zostera muelleri* (Sorensen et al., 2018) and in other glycophytes. Similarly, only few studies have examined the factors controlling the distribution of New Zealand mangroves, most have identified a climatic control, particularly temperature (Beard, 2006; de Lange & De Lange, 1994). However, it is suggested that coastal processes affect propagule dispersal and thereby have more control over their distribution in comparison with climatic factors (de Lange & De Lange, 1994). Also, mangroves grow when given suitable conditions. For example, they grow when planted in more southern locations, e.g., Tolaga Bay in New Zealand. The present limits of distribution are also likely due to the lack of suitable habitats for propagule establishment within a timeframe of 3-5 days. The focus of the present study was not on the effect of temperature in defining the southern limits. Also, from additional data collected (Table, 2.1 [see Results section]), on the differences between mean daily maximum and minimum temperatures in summer and winter in all the sites, it was not possible to draw strong conclusions on temperature effects. However, the mean monthly ground frost days may also play an important role in New Zealand mangrove distribution. During my study, the mean monthly ground frost days were more than 7 in the southernmost site (Ohiwa Harbour) whereas in the northern site (Parengarenga Harbour) it was less than 1 day.

Species' latitude and altitude gradients are mainly driven by temperature (Liu et al., 2018a). The altitude tree-line hypotheses Carbon limitation hypothesis (CLH) and Growth limitation hypothesis (GLH) can also be used to explain a species' latitude gradient (Liu et al., 2018a). The increase in leaf NSC content towards southernmost sites in New Zealand mangroves is consistent with study of Liu et al. (2018 a). Liu et al. (2018a) showed that NSCs did not decrease across latitude in both mature trees and juveniles of *Quercus variabilis* Blume. However, NSCs

increased in the juveniles towards higher latitudes (Liu et al., 2018a). Therefore, New Zealand mangrove distribution is not supported by CLH consistent with Liu et al. (2018a). However, NSCs in juvenile mangroves were not measured in my study.

My results show that there is an increase in NSC concentration in summer, especially in the leaves. The southern latitudes show a much higher concentration compared to the northern ones. However, in my study, it was uncertain, whether the reported seasonal differences in NSC concentration in leaves were due to increases in soluble sugars or starch. This was mainly due to methodological predicaments during NSC estimation in this study and similar previous studies (Quentin et al., 2015, 2017; Pinkard, 2018). It is also important to measure the soluble sugar and starch concentrations/ratios separately as osmoregulation is influenced by solute content variation. For example, the ratio of raffinose to all sugars increased toward the north and south extremes showing the role of raffinose in cold and drought tolerance in *Betual pendula* and *Populus tremula* in Europe (Lintunen et al., 2016). Whereas in the same study, the ratio of pinitol to all soluble sugars did not show increases towards north and south as in raffinose. Glucose and fructose ratios were high only at mid-latitudes and the ratio of starch to all non-structural carbohydrates was high towards the north. The increase in starch may be due to the accumulation of C so that conversion to soluble sugars during winter (Lintunen et al., 2016). Similarly in examining the altitudinal dynamics shaping the climatic tree limits of Mexico, Swiss Alps and northern Sweden, it was shown that an increase in NSC and lipids occurred towards the upper limit of the treeline. The increase in NSC was due to increase in starch and lipids rather than soluble sugars (Hoch & Korner, 2003). However, teasing apart the NSCs into soluble sugars and starch was not possible in my study due to methodological issues (see results).

In a previous latitudinal study of New Zealand mangroves, it was shown that δ^{13}C depletion rate in leaves was lower (higher δ^{13}C values) in the southern latitudes compared to high depletion rates (lower δ^{13}C values) in the northern latitudes (Beard, 2006). As a consequence, closure of stomata occurs, followed by photosynthetic downregulation in southern mangroves than northern mangroves (Beard, 2006). My study is consistent with the study of Beard (2006) who also showed higher δ^{13}C values in the southern sites in comparison to northern ones in winter but not in summer. In my study, I assessed δ^{13}C values for summer also. Beard (2006) associated the higher values with decreased stomatal conductance. Their study also showed that the stomatal conductance rates were not different in summer and winter in the southernmost site, Ohiwa Harbour. Similarly, I did not detect significant seasonal differences in δ^{13}C values at all three study sites, though the values were higher in the southern sites in comparison to the northern ones in both seasons.

In terms of nutrients and other resources, New Zealand mangroves had higher total carbon content at the southern limit in this study with no variations in nitrogen content across the latitude. However ontogenetic variations may be present for nutrient assimilation. For example, higher P occurred in mature trees of *Quercus variabilis* Blume compared to juveniles (McKee et al., 2002; Liu et al., 2018a). The larger root system present in mature trees may help in increased P uptake (in Liu et al., 2018a). Nitrogen available in the soil mostly depends on atmospheric deposition and therefore nitrogen content in my study is less interpretable as the source is unknown (McKee et al., 2002).

The southern mangrove populations of New Zealand show stunted growth due to reduced sink activity and this may possibly be a reason for NSC accumulation in my study. Carbon source and sink activities are generally not uncoupled, i.e. if sugars

accumulate in the leaves then photosynthesis is down-regulated (Hoch et al., 2002; Wiley & Helliker, 2012). However, altering source strength such as defoliation causes either an increase or a decrease of carbon compounds (Li et al., 2002; Maguire & Kobe, 2015). The genus *Avicennia* is generally more salt tolerant compared to other mangrove species (Clough, 1984; Lovelock et al., 2006b; Reef et al., 2012). Reduced growth also occurs under increased salinity (Clough, 1984; Duke et al., 1998). In addition, bud mortality due to low temperatures may also explain why the southern populations at Ohiwa are rather stunted or dwarfed in nature. However, Nickerson (1980) showed that the size decrease was only apparent with no systematic trends with latitudinal increase. Similarly, de Lange & De Lange (1994) found no significant differences between latitude and mangrove height, wherein the New Zealand mangroves were divided into three classes according to their height i) stunted forms – which are < 3 m in height ii) tall forms – which are more than average height (5 m) with incomplete canopy cover and iii) normal forms – which cannot be classified ias either stunted nor tall. In addition to the role of carbon and other nutrients, other physiological factors may play an important role in shaping New Zealand mangrove distribution similar to the study by Liu et al. (2018a).

Numerous factors both climatic and site specific may interact to limit the current distribution of New Zealand mangroves. Most studies conducted so far have focused on the effect of climatic factors such as temperature and frost (Chapman and Ronaldson, 1958, cited in de Lange & De Lange, 1994; Sakai and Wardle, 1978; cited in de Lange & De Lange, 1994). Temperature is especially important at the southern limits like the present study species because of shorter main growing periods. When mean daily summer temperatures fall below 18°C, there is zero reproductive success (de Lange & De Lange, 1994). Frost is also important in

shaping the southern mangrove limit and when coastal frosts fall below -2.2°C mangroves do not occur. Although, this threshold was identified, its reliability was questioned since the data were obtained only from one site in Henderson Creek in Auckland. Additionally, Sakai and Wardle, (1978) have shown that *Avicennia* leaf tissues could not withstand -3°C for four hours. Frosts usually lower mangrove crown growth since terminal buds are affected. However, Sakai and Wardle, (1978), concluded that there is not enough evidence to say that only sensitivity of frost shapes New Zealand mangrove distribution since only one mangrove population was assessed in their study and additional site-specific effects on populations may also be present which play a role in determining the distribution.

As described earlier, the present study did not focus on the effects of temperature in defining New Zealand mangrove limits. However, during my study the southernmost site had more days of ground frost as opposed to the northern sites. Beard (2006) studied the effect of frosts on New Zealand mangrove leaves in the sites ranging from 35°S to 38°S. Frost tolerance of leaves of mature trees was determined by the conductivity method and calculation of Lt_{50} through visual assessment. Lt_{50} is the temperature at which 50% maximal damage to the leaf occurs. It was found that temperatures below-2°C increased leaf injury assessed visually. Lt_{50} ranged from -3.5°C at Tapotupotu Bay to -4.99°C and -4.68°C on the east and west coasts respectively in spring of the year 2000. However, in autumn much lower Lt_{50} values were seen. Temperature of -2.74°C was seen in the north compared to -4.76°C at Ohiwa Harbour in the south. Upper leaves in the canopy were exposed to lethal temperatures upto three times more than the lower and inner leaves. The latter part had ameliorating effects on exposure the surrounding warm water (Kuchler, 1972, cited in de Lange & De Lange, 1994; Beard, 2006,

unpublished). Temperature tolerance to almost -4°C shows that even southernmost populations have limited tolerance to frost. Perhaps, the duration and the depth of frosts may play a critical role in setting New Zealand mangrove treeline (Duke et al., 1998). This may occur if frosts are severe such that the warm water fails to buffer its effects (Duke et al., 1998). So, temperature along with other factors such as salinity, aridity may also play an important role in New Zealand mangrove distribution (Quisthoudt et al, 2012).

Physiological and site-specific factors apart from climatic ones such as availability of suitable habitats for establishment, physiological tolerance limits, and competition with other species on an individual and stand scale are important (Duke et al., 1998). Salinity is an important site-specific factor which likely influences a species' latitudinal distribution range. In the present study, the substrate salinity did not differ between the seasons across all sites. For example, the northern and southern sites showed pore water salinity values of $8^0/_{00}$ – $12^0/_{00}$ during winter. Whereas in summer, the northern and southern sites showed average pore water salinity values of $25^0/_{00}$ – $32^0/_{00}$. Similarly, a previous study by Beard (2006) also showed that substrate salinity did not affect the measured physiological responses, such as gas exchange and stomatal conductance in New Zealand mangroves across a latitudinal gradient. Probably, other site-specific factors such as sediment texture may play a role on New Zealand mangrove distribution. For example, de Lange & De Lange (1994), showed that mangroves growing on <50% muddy substrate display a stunted growth form compared to when mud concentrations were higher, no distinction between normal and tall forms was present.

2.6 Conclusion

In this study, I have shown that it is possible to determine NSC content in temperate mangroves using near infrared spectroscopy which is also an important tool for rapid and reliable estimation of NSC content. In New Zealand mangroves, there is an increasing trend of leaf NSC towards the southernmost sites in summer. Probably, a four-degree latitude variation was not sufficient in order to detect NSC variations or patterns in stem cores in addition to the leaves. Further studies should explore the effect of site-specific factors like sediment texture, salinity, and nutrient availability, in addition to temperature effects, on non-structural carbohydrate dynamics in New Zealand mangroves.

Chapter 3 – Effects of drought and salinity on New Zealand mangroves under different non-structural carbohydrate levels

3.1 Abstract

Despite a large number of studies examining the effects of abiotic stress factors on plants, the mechanistic explanations of drought-induced tree mortality remain inconclusive and even less is known about how multiple stressors interact. The role of non-structural carbohydrates (NSCs) in preventing or postponing drought mortality is gaining attention. Here I tested the role of NSCs in mitigating the effects of drought and salinity in New Zealand mangroves, *Avicennia marina* subsp. *australasica*. I experimentally manipulated NSC levels of the study species, later subjecting them to combinations of drought and salinity. Plant growth and survival rates were higher in the high-NSC group (H-NSC) under high salinity and drought conditions in comparison to the low-NSC (L-NSC) group. In the high salinity high drought conditions, at week 12, H-NSC plants were able to maintain higher stem hydraulic conductivity (281 ± 50 mmol cm^{-1} s^{-1} MPa^{-1}), compared to the L-NSC group (134 ± 40 mmol cm^{-1} s^{-1} MPa^{-1}). Although starch remained relatively constant, I found a 20% increase in soluble sugars in the H-NSC group under high drought and high salinity. My results suggest (1) an important role of NSCs in osmoregulation via mitigation of low soil water potential stress caused by drought and salinity, and (2) sink-limited growth under conditions of combined salinity and drought.

3.2 Introduction

Forests store about 45% of terrestrial carbon yet they are threatened globally (Allen et al., 2010; Bonan, 2008b). Apart from deforestation and land-use change, causes of forest decline are frequently attributed to drought (Allen et al., 2010; Anderegg et al., 2012; Galvez et al., 2011). A growing body of literature is available on the physiological processes associated with drought-related mortality, shedding light on the ramifications for forest ecosystem services such as carbon sequestration (Adams et al., 2013; Allen et al., 2010; McDowell, 2011). However, little is known about how environmental stressors interact (e.g., Leuzinger et al., 2011). In a changing climate, it is particularly important to understand which plant functional traits are affected by the interaction of environmental conditions, such as salinity, temperature, or water and nutrient availability. Such data are urgently needed, to parameterise global dynamic vegetation models and earth system models (Fischer et al., 2015; Le Roux et al., 2011; Powell et al., 2013).

McDowell et al. (2008) proposed two hypotheses to explain the effect of drought on tree mortality. First, the 'hydraulic failure hypothesis' states that a reduction in soil water availability coupled with high evaporative demand results in embolism formation (air-filled xylem conduits), and ultimately complete xylem failure. Second, the 'carbon starvation hypothesis' argues that the closure of stomata during prolonged water stress periods results in reduced carbon uptake, which eventually compromises the plant's ability to meet metabolic requirements, leading to tissue or whole plant mortality. These two processes do not act in isolation and it is important to also take phloem processes into account (Dickman et al., 2015 ; Hartmann et al., 2013; Ryan & Asao, 2014; Sala et al., 2010, 2012; Savage et al., 2015; Turgeon, 2010). For example, Sevanto et al. (2014) found that a decrease in phloem flow is

caused by changes in cell sap composition (osmotic changes). Subsequent phloem turgor loss resulted in compromised carbon transport within the plant, leading to phloem failure. This illustrates the tight interaction between plant carbon and water relations, and the fact that they need to be considered together.

Salinity is another major abiotic stress that affects plant growth in isolation or in combination with drought (Mittler, 2006). This is especially important in arid and semi-arid regions, where increasing drought can expose plants to saline conditions (Munns & Tester, 2008) or where rising water tables have mobilised soil salts, causing dryland salinity (Macinnis-Ng et al., 2016). In coastal areas, such as estuaries and salt marshes, plants are routinely exposed to high salinity levels and therefore low soil water potential. Mangroves can be exposed to high salinity, high temperatures and desiccation during low tide. The interactive effects of drought and salinity can be used to understand the ability of mangroves to survive in hostile environments. In order to cope with these extreme conditions, they have evolved a variety of morphological, anatomical, biochemical, physiological, and genetic adaptations (Ball, 1988; Parida & Jha, 2010). For example, mangroves are characterised by low transpiration rates and water uptake, with consequences for plant growth and productivity (Lovelock et al., 2006b). In addition, cell osmotic balance is modulated by a variety of osmolytes, such as glycine-betaine, proline and abscisic acid (Parida & Jha, 2010). Sugars are likely to play an important role in osmoregulation (Gil et al., 2013) in these halophytes. Non-structural carbohydrates (NSCs) are central in plant growth modelling and represent the balance between carbon uptake (source) and carbon demand (C-sink activities like growth) within the plant (Germino, 2015; Hoch et al., 2002; Korner, 2003). However, few studies have characterised sugar contents of mangroves in response to environmental stress (Parida et al., 2002, 2004b).

Non-structural carbohydrates (NSCs) can be used to help explain associations between environmental conditions and plant physiology. Large quantities of NSCs are stored by plants as a reservoir of carbon and represent the surplus of carbohydrates which remain once metabolic activity, growth, reproduction, respiration, and osmoregulation costs have been accounted for (Chapin et al., 1990; Gruber et al., 2012; Maguire & Kobe, 2015). Active storage of carbon also occurs at the cost of growth (Chapin et al., 1990; Wiley & Helliker, 2012). Accumulation of NSC occurs during periods of unsuitable growth conditions when C-sink activity (growth) is limited. For example, NSC levels increased under drought in 17 semi-deciduous Panaman forest species, indicating that environmental constraints act on sinks, rather than photosynthesis directly affecting growth (Wurth et al., 2005). In some plant species, the NSC pool may also promote plant survival under drought through the conversion of starch to sugars. For example, NSC depletion occurred when carbon became limiting under combined shade and drought in five temperate species, but again, sugars mostly increased (Maguire & Kobe, 2015). Eventually, source and sink activities will necessarily correlate (through adjustment of photosynthesis by sink activity) if carbon pools remain more or less constant. This can make it difficult to identify the underlying causality of the carbon source-sink balance. Measurements of NSCs and their main constituents (sugars and starch) across plant compartments over time are therefore important to understand what limits plant growth, and how and when plants die under unfavourable conditions. Ultimately, a good understanding of the source-sink dynamics of the NSC pool is needed to model carbon allocation and survival of plants under different environmental scenarios (Martínez-Vilalta et al., 2016).

Interactions between environmental stress and NSC content have yielded mixed findings and interpretations. Maguire & Kobe (2015), reported that drought,

defoliation and shade induced decreases in NSC concentrations in roots and stem of five temperate tree species. Substantial declines in foliar NSC content, driven by decreases in soluble sugar content occurred in *Pinus edulis* under drought (Adams et al., 2013). On the other hand, total NSC values remained constant or even increased during drought conditions due to inactive C-sinks, which suggests NSC involvement in osmoprotection (O'Brien et al., 2014). In some studies, a correlation between NSC concentrations and shade tolerance and also herbivory were identified (Myers & Kitajima, 2007; Piper, 2011). For instance, Myers & Kitajima (2007), showed that in seven shade-tolerant neotropical species, higher total NSC increased survival following defoliation, especially in the juvenile stage. They discovered that NSC concentration was related to the light requirements of the species and increased NSC concentrations were found in the roots of more shade-tolerant species. In another study involving two Nothofagus species (one drought-tolerant and one less drought–tolerant), higher total NSC content was reported in the tolerant species (Piper, 2011). This was attributed to carbon sink limitation rather than a carbon source limitation in the drought–resistant species (Sala et al., 2010; Piper, 2011). The different responses of NSC concentrations under experimental drought and other abiotic factors (e.g., light) are often owed to the complex interactions among different stress factors which are further convoluted by species-specific differences in plant traits, such as stomatal regulation (isohydric versus anishohydric), ontogeny and life history strategies (McDowell et al., 2008; Wurth et al., 2005). Additionally, the lack of standardised procedures and use of different methods to quantify or estimate NSC, makes absolute comparisons among studies difficult (Germino, 2015; Quentin et al., 2015).

To elucidate how NSCs are involved in stress tolerance, their levels can be effectively manipulated by changing ambient CO_2 levels or by changing light

conditions. For example, Alberda (1966) obtained different NSC content in rye grass by placing them in either dark or continuous light conditions. O'Brien et al. (2014), effectively manipulated NSC in tropical plant species by exposing plants from low to high light conditions and vice versa. Similarly, studies have also used low CO_2 treatments for altering NSC content of plants (Hartmann et al., 2013). By altering NSC levels, their role in the mechanisms leading to plant mortality under different abiotic factors can be understood.

The aim of this study was to investigate the physiological responses of New Zealand mangroves (*Avicennia marina* [Forssk.] Vierh. subsp. *Australasica* (Walp.) J. Everett) with different NSC levels to various drought and salinity conditions within a controlled mesocosm experiment. I hypothesized that mangroves with higher NSC levels will be more tolerant to drought and salinity stress by having higher stem hydraulic conductivity via osmoregulation. I also assume that carbon starvation in isolation is not a mechanism of plant mortality, rather, I expect NSC concentrations to increase under drought and salinity. Further, when C-sinks are blocked, I hypothesize that plants with higher NSC levels will show increased survival. I addressed the following questions 1) Is a light swapping treatment useful to manipulate NSC levels in the present species? 2) Do salinity and drought affect physiological responses similarly and are effects of drought and salinity additive or interactive? 3) Do salinity and drought influence NSC levels? 4) Do enhanced NSC levels lead to improved physiological conditions under drought and salinity conditions?

3.3 Materials and methods

3.3.1 Seedling collection and environmental conditions

Propagules of the New Zealand mangrove, *Avicennia marina* (Forssk.) Vierh. subsp. *Australasica* (Walp) J. Everett., were collected from Manghawhai estuary (36° 7′ 35″ S, 174° 34′ 29″ E), about 100 km north of Auckland, in December 2014. The study was conducted under semi-controlled conditions in a shade house research facility (University of Auckland, Tamaki campus, Auckland), and consisted of three phases (Figure 3.1a): Phase 1, germination and acclimation; Phase 2, manipulation of non-structural carbohydrates (light swapping); Phase 3, salinity × drought treatment.

Environmental conditions were monitored in the shade house throughout the entire study. Light, temperature, and relative humidity were monitored continuously using dataloggers (Hobo Pendant Temp-light Data Logger; Tidbit Mx Temperature Data logger; Hobo Temperature-RH Data Logger [Onset Computer Corporation, USA]). For phases 1 and 2 (Jan-Apr 2015; warmer months), the daily average maximum temperature was 22°C and the average minimum was 18°C. For phase 3 (May-Aug 2015; cooler months), the daily average maximum temperature was 16°C and the minimum was 9°C. For phases 1 and 2, the daily average maximum relative humidity was 84% and the average minimum was 61%. For phase 3, the daily average maximum relative humidity was 64% and the minimum was 55%. For phases 1 and 2, the details of the light conditions are explained along with manipulation of NSCs. However, during phase 3, the ambient light conditions were lower than during phases 1 and 2, as treatment application was carried out during winter (cooler months) with an approximate monthly average PPFD of 100.2 ± 4.9 (mean ± s.e.), which approximately corresponds to the light requirement of seedling growth.

Phase 1: Germination and acclimation

Propagules were placed in moist sand beds until plumules and radicles developed. Following the appearance of the first two leaves one month later, 162 seedlings were transferred to 3.7 litre pots, with each pot receiving two to three seedlings for subsequent destructive harvesting. Fine sand was used as the substrate to ensure a homogenous media for treatment application. Pots were fertilised every 15 days during phase 1 using a commercial garden fertiliser (Thrive all-purpose soluble fertilizer [Yates, New Zealand]; NPK ratio of 25:5:8.8 plus trace elements; 45 gL^{-1} in fresh water; 250 mL provided per pot per application).

Phase 2: Manipulation of NSCs

Cotyledons were removed from seedlings immediately prior to initiation of phase 2 in order to reduce potential confounding effects from maternal reserves. I set out to manipulate NSC levels by applying a light swapping treatment for 84 days following O'Brien et al. (2014), before introducing the drought and salinity treatments (Figure 3.1a and 3.1b). The acclimated 50-day-old seedlings were randomly divided into two groups of 81 plants each (162 plants in total). For the first 42 days, one group of plants was grown under ambient light conditions with a monthly average PPFD of 307.6 ± 22.8 (µmol m^{-2} s^{-1}), whereas the other group was kept under low light, corresponding to 9–10% of ambient light with a monthly average of 24.2 ± 4.9 (µmol m^{-2} s^{-1}) (Figure 3.1a). The two groups of plants were then swapped to receive the reciprocal light conditions for a further 42 days (Figure 3.1a and 3.1b). The NSC contents in stem and roots were then assessed in subsamples (n = 3 per group, per tissue) to confirm that the prescribed light treatment did indeed modify NSC levels.

Phase 3: Drought x salinity treatments

After manipulation of NSCs, I used a randomized factorial design where three levels of salinity (25% [low], 50% [medium], 75% [high] seawater) were crossed with three levels of drought (low, medium, high) and two levels of NSCs (low and high). Seedlings within each NSC group were randomly allocated to each of the drought × salinity treatments, with nine replicate pots per treatment combination. Artificial sea salt (S9883, Sigma-Aldrich, St. Louis, MO, USA) was used to prepare saline solutions at three different concentrations: low salinity (150 mM NaCl; equivalent to 25% seawater), medium salinity (300 mM NaCl; equivalent to 50% seawater), and high salinity (450 mM NaCl; equivalent to 75% seawater). To prevent potential physiological shock due to sudden salt additions, salinity treatments (low, medium and high) were ramped up gradually over one week after the end of the light swapping (Reef et al., 2012). Once target levels were reached (Week 0), pots were watered with 400 mL of the respective concentrations at weeks 4 and 8. All salinity treatments were within the salinity tolerance range of *A. marina* (Morrisey et al., 2007). During the interval between each salinity treatment, seedlings exposed to low drought conditions received tap water sprays every 3–4 days (ca. 200 mL per pot), whereas medium drought received tap water sprays every 6–7 days, and those exposed to high drought conditions did not receive any tap water between salinity treatments. Soil moisture content was measured in the extreme treatments using a soil moisture sensor (EC - 10, Decagon Devices, USA). Low salinity–low drought had higher level of soil moisture content (36.5% ± 3.2% s.e.) after 8 weeks of treatment, n = 3), whereas, high salinity–high drought had a lower level of soil moisture content (15.9% ± 2.4% s.e. after 8 weeks of treatment, n = 3). Soil water potential was measured only in the extreme treatments using soil water potential sensors (MPS-6, Decagon

Devices, USA). As expected, low salinity–low drought had a higher soil water potential (-4 MPa ± 1.5 s.e. after 8 weeks of treatment, *n* = 2), whereas, high salinity–high drought pots showed lower monthly water potential (-6.5 MPa ± 0.6 s.e. after 8 weeks, *n* = 2).

In this third phase (Figure 3.1a), destructive harvests were carried out at approximately four, eight and twelve weeks after the start of the drought and salinity treatments. At each sampling, we measured stem water potential, maximum stem hydraulic conductivity, and NSC concentrations in stems (*n* = 3 per treatment at weeks 4, 8 and 12). Non-destructive measures of stomatal conductance and growth were carried out two days before the planned harvest dates.

3.3.2 Growth and physiological measurements

Growth parameters (i.e., plant height, relative growth rate, leaf area and leaf number) were recorded before each destructive harvest. Plant height was defined as the height of the stem from above the soil surface up to the first node below the stem apex. At the same time, plants were visually assessed for wilting and mortality, following Tyree et al. (2003). Plants were classified into five classes: normal, wilted, severely wilted, nearly dead, and dead. Plants with more than four healthy green leaves and an upright stem were classified normal. Wilted plants were defined as plants with two or more healthy leaves and few yellow, spotted, or diseased leaves, and an upright stem. Severely wilted plants had only one healthy leaf and the stem was not upright in most plants. Nearly dead plants consisted of at least three black leaves and black stem. Dead plants were those that had no functional leaves and had black coloured stems.

Stomatal conductance was measured using a leaf porometer (SC-1, Decagon Devices, USA). Measurements were taken around midday (12:00–3:30 pm) from 2–

3 leaves per pot and from three pots per treatment, with data representing the mean of the means.

Gas exchange (photosynthesis measurements were made on days 28, 35, 54, 71, 82 using CIRAS 2 portable photosynthesis system (PP Systems, MA, USA). However, data are shown for week 4, week 8 and week 12 (Figure S1). Similar to stomatal conductance, averages of 2-3 leaves per pot were measured around midday (12:00–3:30 pm) from three pots per treatment. All the gas exchange and stomatal conductance measurements were made prior to each destructive harvest to measure stem water potential and hydraulic conductivity.

Stem water potential (MPa) was measured using a custom-built Scholander pressure chamber (Scholander et al., 1965). The stem of the seedling (with leaves) was excised above the soil surface and immediately placed in the pressure chamber. All measurements were taken around midday. Predawn stem water potential measurements were also carried out at weeks 4, 8, 12 (n = 24-54). Due to less interpretability, the plot is included in supplementary material along with gas exchange data (Supplementary Figure 3.1).

Xylem embolism was measured using an embolism meter (XYL'EM, INRA, Bronkhorst, France). Whole seedlings were collected at dusk and stored in plastic bags for measurements the following morning. The hypocotyl region of the stem was chosen for measurements and cut underwater. Degassed KCl (10 mM) was used as the flushing solution. The flow rate, pressure applied, water temperature, stem length and diameter were recorded. After measuring the initial flow rate, the samples were flushed at 0.1 MPa for about three minutes and the process was repeated until the flow rate no longer increased. The maximum flow rate per sample was recorded. Maximum hydraulic conductivity (K_{max}; mmol cm^{-1} s^{-1} MPa^{-1}) was calculated for each stem segment.

3.3.3 Dry weight and Non-structural carbohydrates concentrations

The collected plant samples (*n* = 3 per treatment) at each harvest were separated into leaves, stems, roots, and immediately placed on ice. Tissues were dried at 70°C to constant weight. To prepare tissues for NSC analysis, dried samples were ground to a fine powder using a ball mill (Retsch planetary ball mill, PM 100; Retsch GmbH, Germany), sieved (350 µm mesh), and stored in a desiccator until analysis.

3.3.4 Soluble sugar extraction

NSCs (total soluble sugars plus starch) were analysed colorimetrically using the anthrone reagent method, according to Yemms & Willis (1954), see Chapter 2 for details. Approximately 10mg of dry plant material were accurately weighed into 1.5 mL vials and sequentially extracted thrice with 1 mL of 80% ethanol (50°C for 45 minutes) with vortexing and centrifugation (16240 *g*; 3 minutes) in between. Supernatants from the three extractions were combined for soluble sugars analysis: 250 µL of anthrone reagent was added to 50µL of sample, boiled for 10 minutes, and the absorbance of the resulting blue green furfuraldehyde derivates (from dehydration of sugar monomers) were measured at 630 nm. Remaining ethanol was evaporated, and the dried pellet was stored for starch analysis.

3.3.5 Starch extraction

Following soluble sugar extraction, 1mL deionised water was added to the pellet and extracted at 70°C for 2 hours (gelatinization), with vortexing every 30 minutes. Samples were centrifuged (16240 *g*; 3 minutes) and 500 µL of extract was combined with 500 µL of amyloglucosidase solution (260 mg amyloglucosidase [from *Aspergillus niger*; Sigma-Aldrich A7420] dissolved in 52 mL of 0.1M Na-acetate buffer). Extracts with the enzyme were incubated in a water bath for 15 hours at 50°C, then analysed using anthrone reagent as previously outlined.

Final starch and soluble sugar concentrations were calculated via the standard curve method and normalised to sample weight. Starch is reported as percent dry weight. I report soluble sugars as relative values (relative to the highest value obtained in the study) rather than absolute values. This was necessary because of the methodological issues stated in Chapter 2, where sometimes the absolute NSC concentrations were more than 100% d.m. The relative values were calculated after normalization of the soluble sugar concentrations to the highest soluble sugar concentration.

3.4 Data analysis

NSC content data in the two plant groups obtained after light–swapping (Phase 2) was analysed by one–way ANOVA. For multifactorial experimental data (Phase 3), maximum stem hydraulic conductivity, midday stem water potential, stomatal conductance and growth parameters were modelled using a generalised least squares approach with restricted maximum likelihood (gls, R package *nlme*, [Pinheiro *et al.*, 2018]) as a function of salinity, drought, pretreatment NSC levels and time. Post treatment starch and soluble sugar content were also modelled using the same approach as a function of drought and salinity. Underlying model assumptions such as variance heterogeneity and normality were assessed using residual vs fitted plot and quantile-quantile plot respectively. Cumulative link mixed models (clmm, R package *ordinal*, [Christensen, 2018]) with flexible thresholds and a 'pot' random term were used to compare plant health among treatments. The same four explanatory variables as outlined above were used. Significant interactions were sliced following a multiple comparison procedure (R package *emmeans*, [Lenth, (2018)]). The p-values were then corrected for multiple testing using the Benjamini & Hochberg

method (1995). All statistical analyses were performed using R (R 3.4.2, R Development Core Team, 2017).

3.5 Results

3.5.1 Effect of manipulation of NSCs

The light-swapping treatment produced two groups of plants with different NSC contents due to substantial changes in the soluble sugar fraction. Seedlings that received low light conditions during the latter half of the 84 day light manipulation period had lower mean levels of starch (15% and 20% dry weight in roots and stem respectively) than those exposed to higher light conditions (22% and 33% dry weight in roots and stem respectively) (Figure 3.1c). However, these differences were non-significant between light treatment groups for each tissue type. Relative soluble sugar levels were also lower in seedlings that received low light conditions during the latter half of the 84 day light manipulation period (0.4 in roots and 0.2 in stem respectively), compared to their counterparts (0.7 in roots and 0.8 in stem respectively), with significant differences between groups for each tissue type (roots: $F_{(1, 4)} = 8$, $P = 0.047$; stems: $F_{(1, 4)} = 37.56$, $P = 0.003$; Figure 3.1c). The light swapping treatment did not influence growth (i.e., plant height, leaf area, leaf number, Supplementary Figure 3.2) or physiological properties (i.e., stomatal conductance, maximum stem hydraulic conductivity, and midday stem water potential, Supplementary Figure 3.3). This manipulation in Phase 2 resulted in two experimental groups with similar growth and physiological characteristics, but with different NSC contents.

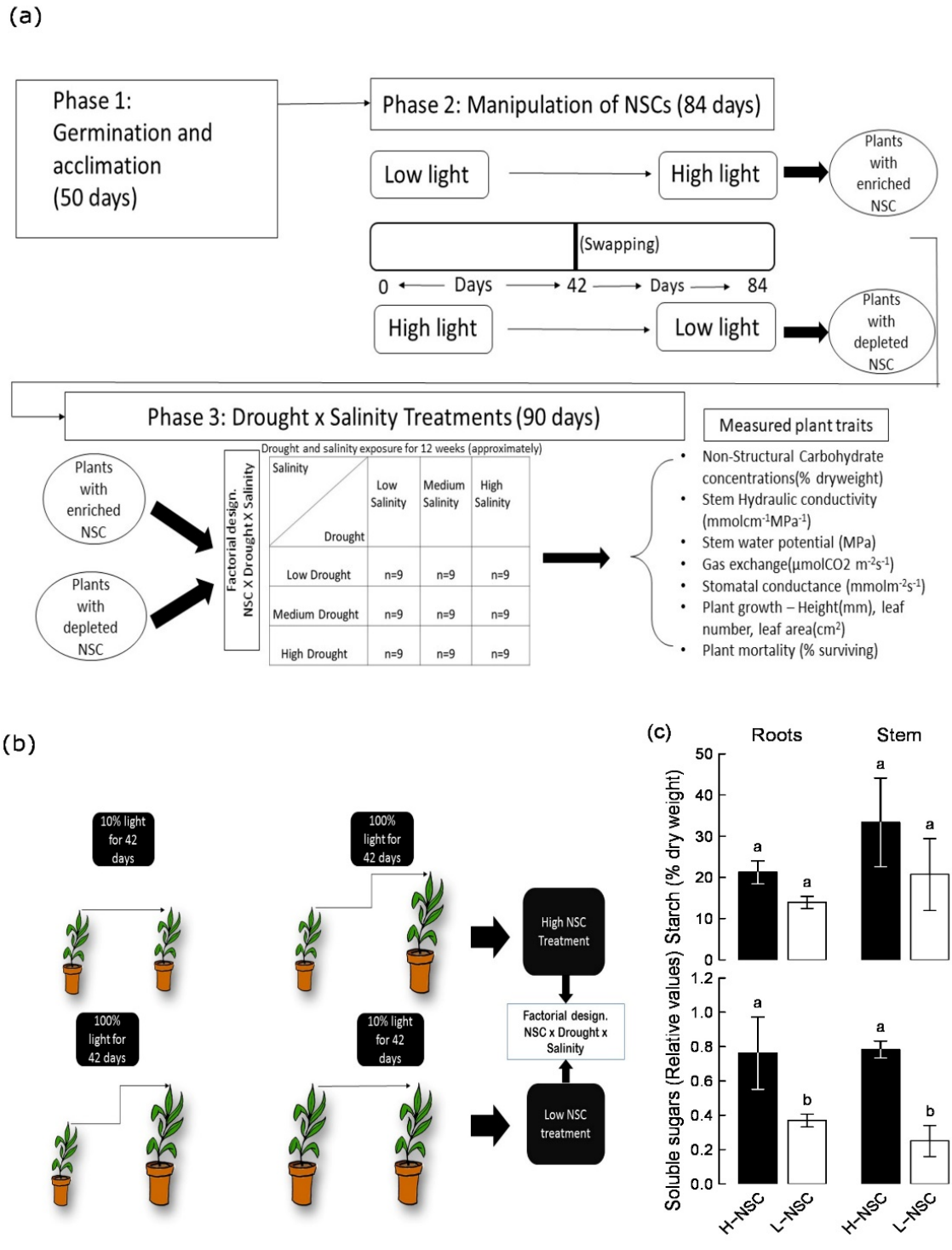


Figure 3.1 Study design. a) Schematic of the three phases of the entire study b) Schematic of NSC manipulation (Light swapping treatment). One group of plants grown in low light (PPFD = 24.2 ± 4.9 μmol m^{-2} s^{-1}) to high light (PPFD = 307.6 ± 22.8 μmol m^{-2} s^{-1}) conditions. Another group of plants grown from high light to low light conditions. Plant growth occurs only in high light and not in low light. c) Non-structural carbohydrate concentrations(separated into soluble sugars and starch here) following the shade treatment, prior to salt and drought treatments. Top row: Starch in roots and stem. Bottom row: Relative soluble sugars values in roots and stem. Data represent means ± s.e. (*n* = 3) for roots and (*n* = 3) for stem. (H-NSC is the group that received high light at the end of the NSC manipulation experiment and L-NSC is the group that received low light at the end of the manipulation experiment).

3.5.2 Treatment effects on plant vigour

Plant health was affected by a significant four-way interaction of NSC, salinity, drought and time (L = 72.12*,* df = *29, P* <0.0001). At week 4, high NSC (H-NSC) plants had a higher percentage of surviving individuals in the normal class, at high salinity. However, at week 4, the low NSC (L-NSC) group began to show increased mortality in comparison to the H-NSC group (Figure 3.2, top, middle, bottom rows: second column from the left). At week 8, mortality occurred in the H-NSC group at high salinity, whereas the L-NSC group showed mortality in low, medium and high salinity (Figure 3.2, top, middle, bottom rows: fourth column from the left). However, after prolonged drought at week 12, mortality was observed in both high and low NSC groups (Figure 3.2, top, middle, bottom rows: fifth and sixth columns from the left). Although mortality occurred in the high and low NSC groups at high salinity (22% and 33%, respectively), the H-NSC group had more plants in the top health categories and fewer plants in the severely wilted category (31%), compared to the L-NSC group where most plant were in the nearly dead (42%) and dead (33%) category (Figure 3.2, bottom row: fifth and sixth columns from the left).

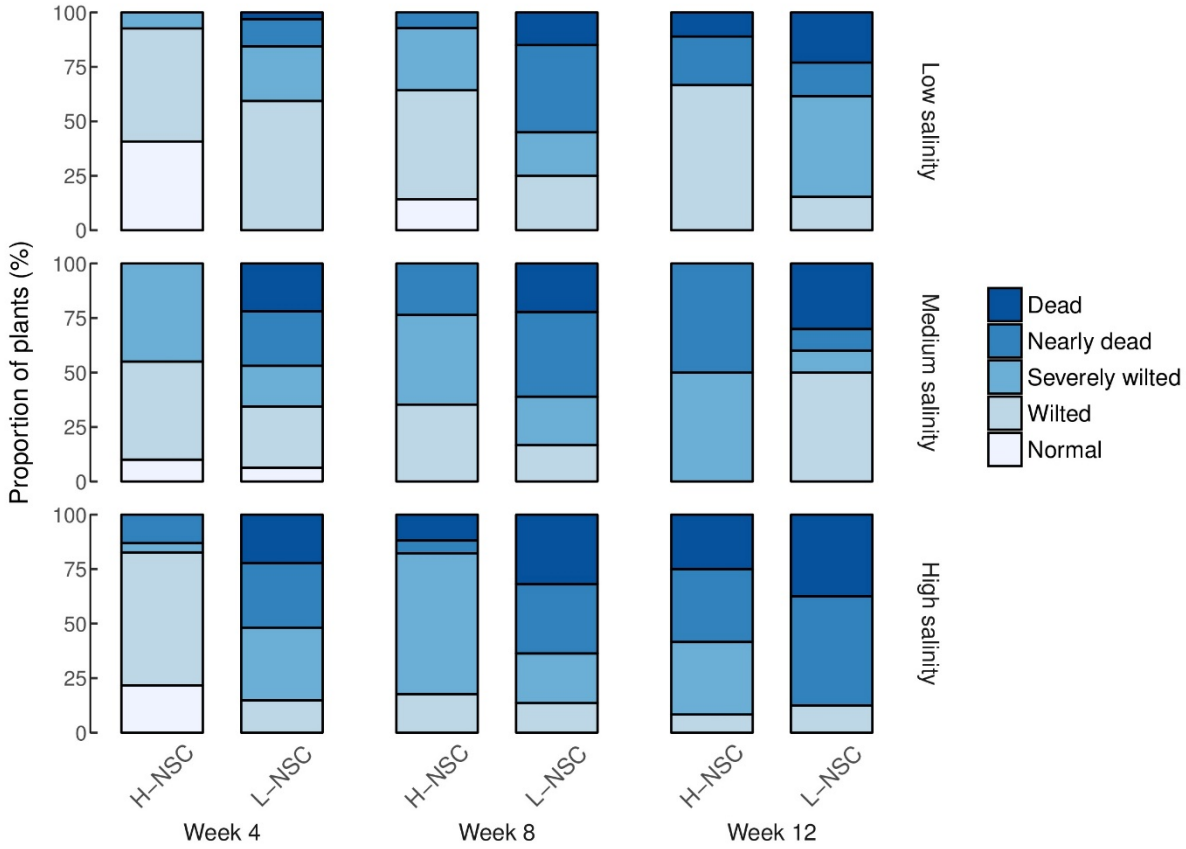


Figure 3.2 Visual assessment of seedling mortality in low salinity, medium salinity and high salinity (with the three drought treatments pooled in each salinity level). Percentage of plants surviving in five categories were assessed (following Tyree et al, 2003). i) 'normal', ii) 'wilted', iii) 'severely wilted', iv) 'nearly dead' and v) 'dead'. Top row: high (H-NSC) and low NSC (L-NSC) group in low salinity at week four, eight and twelve. Middle row: high and low NSC group in medium salinity at week four, eight and twelve. Bottom row: high and low NSC group in high salinity at week 4, 8 and 12.

3.5.3 Treatment effects on NSC concentrations

Following the drought and salinity treatment application, we detected a significant NSC × salinity interaction for starch concentrations. (L = 7.38, df = 2, *P* = 0.002). This interaction was sliced to allow comparisons between NSC and salinity levels. This was mainly driven by low NSC (L-NSC) and low salinity (*P* = 0.029). At week 4, the L-NSC plants had lower starch levels. Additionally, we detected a significant main effect of drought on starch concentrations (L = 9.88, df =2, *P* = 0.007, Table 3.1). Starch content generally remained constant or decreased in the H-NSC group during low drought (Figure 3.3b). For soluble sugars, no significant NSC × salinity interaction nor NSC × drought interaction was detected. Rather, the main effects NSC (L = 3.89, df = 1, *P* = 0.048) and drought (L = 6.97, df = 2, *P* = 0.030) were significant. In week 8, the H-NSC group maintained relatively higher sugar concentrations under low, medium and high salinity levels (Figure 3.4a). This is likely due to the conversion of starch to soluble sugars. However, high saline conditions decreased sugar concentrations in the H-NSC group especially in week 12 (Figure 3.4a). When compared to low drought treatment, soluble sugar contents decreased relatively in medium and high drought treatments, in the L-NSC group (Figure 3.4b). The H-NSC group had relatively higher soluble sugar content in all treatments (Figure 3.4b).

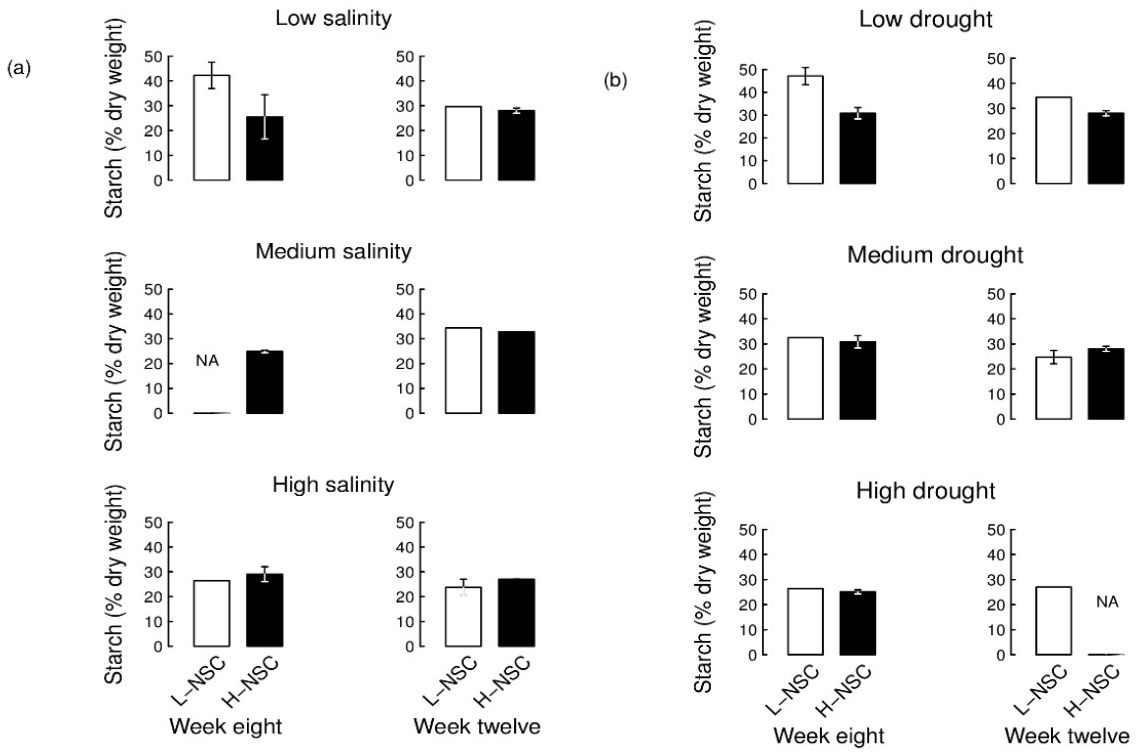


Figure 3.3 Starch concentrations in both the High NSC and Low NSC groups after treatment application. a) Starch content as a function of salinity (with the three drought treatments pooled). Low NSC-Medium salinity is missing in week 8. b) Starch content as a function of drought (with the three salinity treatments pooled). Data represent means ± s.e. (*n* = 2 to 5) of stem samples. High NSC - High drought is missing in week 12.

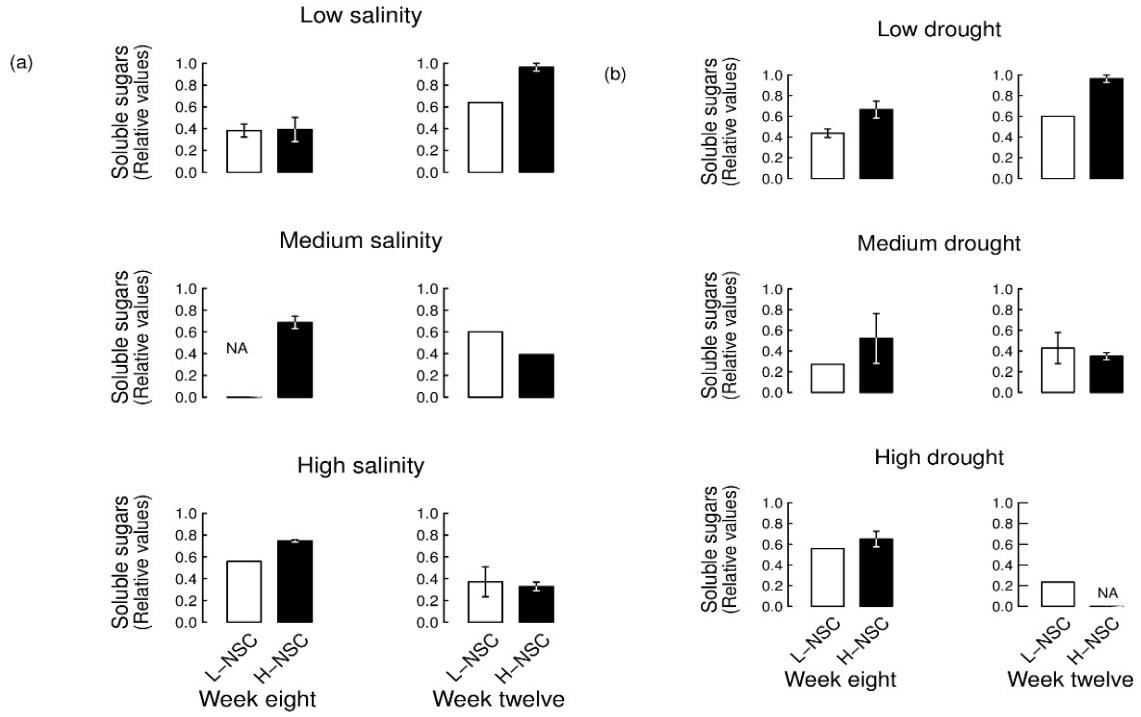


Figure 3.4 Relative soluble sugar concentrations in both the High NSC and Low NSC groups after treatment application. Soluble sugar concentrations here are plotted as relative values to the median of highest concentrations rather than absolute values. a) Soluble sugars as a function of salinity (with the three drought treatments pooled). Low NSC-Medium salinity is missing in week 8. b) Soluble sugar as a function of drought (with the three salinity treatments pooled). Data represent means ± s.e. (*n* = 2 to 5) of stem samples. High NSC - High drought is missing in week 12.

3.5.4 Treatment effects on plant growth parameters

For plant height, two significant three-way interactions were detected (NSC × drought × time term; L = 7.36, df = 4, *P* = 0.003) and a salinity × drought × time interaction (L = 30.57, df = 8, *P* = 0.001; Table 3.5). The latter interaction was sliced to allow comparisons between the timepoints within the salinity and drought levels. This interaction was driven by the low drought - high salinity treatment (*P* = 0.0109), at week 8. High NSC plants were able to grow taller (> 6 cm) than L-NSC plants in weeks 4, 8 and 12. Low NSC plants had only two surviving individuals under medium and high saline conditions at weeks 8 and 12. Mean plant height of 4 cm was

observed in the L-NSC plants under low salinity – all drought levels (Figure 3.5a, Row 1, Panel 1, 2, 3). However, at the low salinity – low drought level, the H-NSC group showed a higher plant height of more than 7 cm compared to L-NSC group which had a height of 4 cm (Figure 3.5a, Row 1, Panel, 2). Under medium and high salinity-high drought conditions, the L-NSC group showed reductions in plant height to less than 4 cm in week eight and week twelve. On the other hand, the H-NSC group showed a mean height of more than 6 cm (Figure 3.5a, Row 1, Panel, 6 and 9).

For leaf area, I identified a significant main effect of NSC (L = 23.30, df = 1, *P* = <0.001) and a significant two-way (drought × time) interaction (L = 9.43, df = 4, *P* = 0.046; Table 3.6). The interaction was mainly driven by low drought at time point 8, where leaf area decreased in L-NSC plants in low, medium and high salinities (Figure 3.5a, Row 2, Panels 1, 4, 7). In both weeks 4 and 8, the H-NSC group maintained a higher leaf area (25 cm^2) compared to the L-NSC group (15 cm^2) even at a high drought level under low and medium salinities (Figure 3.5a, Row 2, panels 3, 6, 9). Under high salinity-high drought, the H-NSC group maintained a leaf area of more than 20 cm^2, compared to the L-NSC group at all time-points (Figure 3.5a, Row 2, Panels 8 and 9).

For leaf number, a significant two-way (drought × time) interaction was detected (L = 11.0, df = 4, *P* = 0.026; Table, 3.7), driven by the highest drought level at week 12. Both the H-NSC and the L-NSC plants had six leaves on an average. However, during weeks 4 and 8, H-NSC plants had 6-8 leaves whereas leaf shedding and wilting reduced the leaf number to as low as 2 in the low NSC group, when salinity and drought increased (Figure 3.5a, Row 3, Panels 6 and 8).

3.5.5 Treatment effects on physiological responses

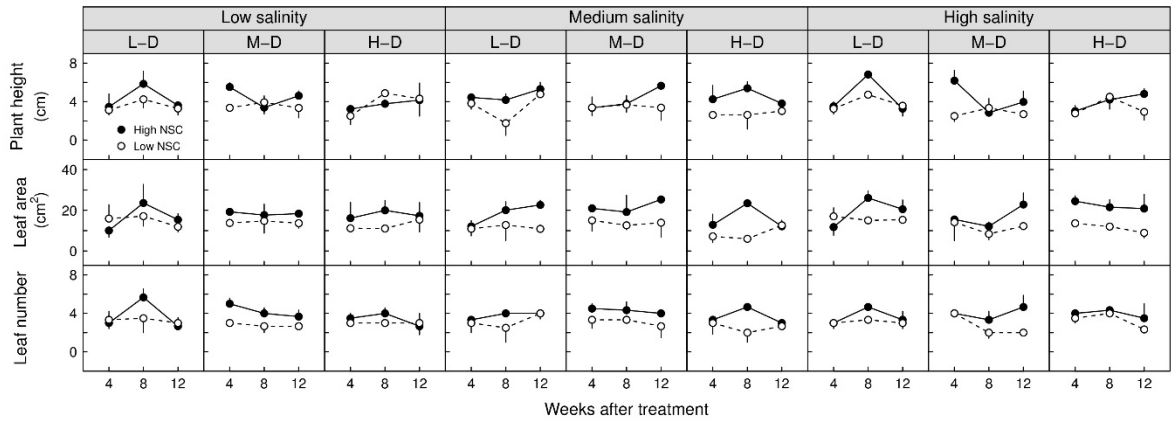
For midday stem water potential, no significant interaction terms were determined only a significant main effect of time was detected (L = 11.72, df = 2, P = 0.002; Table, 3.8). The low salinity – high drought treatment combination decreased the water potential of L-NSC plants. In week 12, under low salinity – medium drought, the H-NSC group showed higher water potentials (–0.5 MPa) compared to the L-NSC group (<–1 MPa) (Figure 3.5b, Row 1, Panel 1 and 2). Overall, medium salinity did not affect water potential. However, in week 12, under low drought the H-NSC group maintained (-0.5 MPa) as compared to the L-NSC group of (-0.8 MPa; Figure 3.5b, Row 1, Panel 4). At high salinity – high drought conditions, in week 12, only two surviving individuals were present in the L-NSC plants, while all the three individuals were present for the H-NSC plants. In week 12, however, the H-NSC plant water potentials dropped to -1.1 MPa similar to the L-NSC plants (Figure 3.5b, Row 1, Panel 7, 9).

For maximum stem hydraulic conductivity, a significant four-way interaction was detected (L = 26.61, df = 8, P = <0.001; Table 3.9). This was mainly driven by the high salinity - low drought conditions at week 8, medium drought – high salinity treatment at week 12. At time 4, low salinity did not affect the maximum stem hydraulic conductivity in both H and L-NSC groups. However, significant reductions in maximum stem hydraulic conductivity as low as 100 (mmol cm^{-1} s^{-1} MPa^{-1}) in both the H and L- NSC plants were observed under medium and high drought (week 12; Figure 3.5b, Row 2, Panels 2 and 3). Under high salinity and all drought levels, the H-NSC plants maintained higher conductivity (>400 mmol cm^{-1} s^{-1} MPa^{-1}) compared to the L-NSC plants (<200 mmol cm^{-1} s^{-1} MPa^{-1}) (Figure 3.5b, Row 2, Panel 7, 8 and 9). The L-NSC plants had only one surviving individual at week 12, under medium

salinity - high drought (Figure 3.5b, Row 2, Panel 6). Also, only one individual was also present at week 8, under low salinity - high drought (Figure 3.5b, Row 2, Panel 3).

A three-way interaction (salinity × drought × time) was detected for stomatal conductance (L = 30.57, df = 8, *P* = <0.001; Table 3.10). The interaction was sliced to allow comparison between time points within salinity and drought levels. This was driven by low and high salinity treatments, under all drought levels, at weeks 8 and 12. Both H and L-NSC plants maintained similar stomatal conductance values throughout the experiment. During week 8 and week 12, under high salinity treatments and all drought levels, low stomatal conductance of <50 (mmol $m^{-2}s^{-1}$) was observed in both the H- and L-NSC plants (Figure 3.5b, Row 3, Panels 7, 8 and 9).

(a)



(b)

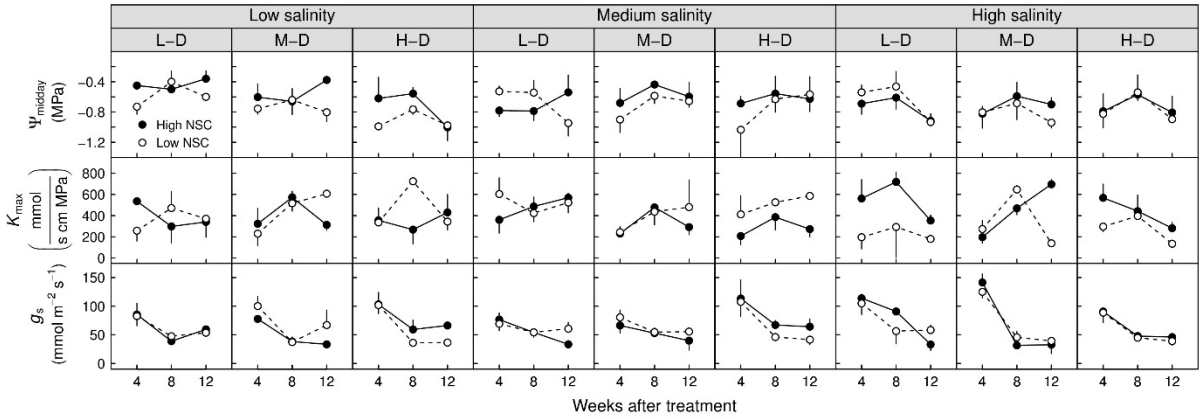


Figure 3.5 Growth and physiological measurements under different salinity and drought treatments at the 4th, 8th, 12th weeks. L–D is Low drought, M–D is Medium drought and H–D is High drought. The closed circles represent High NSC levels before treatment application and open circles represent Low NSC levels. Symbols represent means ± s.e. (*n* = 2 to 3) where surviving individuals were present. a) Growth responses at the 4th, 8th, 12th week. The first row represents plant height in cm. The second row represents leaf area per square centimetre. The third row represents leaf number in counts. b) Physiological responses at weeks 4, 8 and 12. The first row shows midday water potential (MPa). The second row represents maximum hydraulic conductivity (mmol s^{-1} cm^{-1} MPa^{-1}). The third row represents stomatal conductance (mmol $m^{-2}s^{-1}$).

3.6 Discussion

I aimed to understand the role of NSC in mitigating the adverse effects of drought and salinity on plant health, growth and water relations. Accordingly, I effectively manipulated NSC levels with a light swapping treatment for the study species, the New Zealand mangrove, *Avicennia marina* subsp. *australasica*. My hypothesis that higher NSC levels alleviate plant responses to drought and salinity conditions was confirmed. NSC concentrations also increased in both the low and high NSC groups, ruling out carbon starvation as a cause of plant mortality. Plants with higher NSC levels prior to experimentally induced drought and salinity resulted in increased plant height, leaf area and survival after the start of the treatments. High NSC levels also resulted in increased stem hydraulic conductivity and plant vigour. This is consistent with my second hypothesis that higher NSC levels prolong plant survival and aid in the maintenance of plant physiological functions.

Studies on halophytes have mostly concentrated on the effects of salinity alone (Bompy et al., 2014; Reef et al., 2012, 2015b). Here I tested the combined effect of drought and salinity since halophytic plants such as mangroves face coastal dynamics such as tidal events which influence salinity levels. Moreover, changes in rainfall patterns can also affect salinity and thus cause physiological drought (Ball, 1988, Mendez-Alonzo et al., 2016; Parida & Jha, 2010). In my experiment, maximum stem hydraulic conductivity, plant height, leaf area and stomatal conductance, all responded to interactions between some or all of the following: initial NSC level, salinity, drought and time. Two of the responses, midday stem water potential and leaf area were driven by the single factors NSC and time, respectively. Despite the importance of understanding responses to a combination of environmental factors (Mittler, 2006), particularly those that are changing rapidly under global change

(Leuzinger et al., 2011), I am unable to conclusively interpret the four-way and three-way interactions (Tables 2, 3 and 4) observed in this experiment.

Bompy et al. (2014) examined the effect of constant and fluctuating salinity on physiological responses of *Avicennia germinans*, *Rhizophora mangle* and *Laguncularia racemosa* with plant growth (relative length growth) decreasing with increasing salinity from 95% to almost 40% in *A. germinans*. In the same study, in A. germinans, total leaf area also showed reductions from (161 ± 24 cm^2 to 87 ± 18 cm^2 approximately when salinity increased from 0 mM to 685 mM. Also, when salinity was equivalent to 50% seawater, *Avicennia marina* (Forsk.) Vierh. and *Rhizophora stylosa* showed decreased growth rates compared to plants grown in 25% seawater (Clough, 1984). These growth reductions were attributed to ion toxicity rather than low water availability. A decrease in plant growth in higher salinities has also been attributed to changes in net assimilation rate and leaf area ratio (Ball, 2002; Lopez-Hoffman et al., 2006). In the present study, at the end of week 12, under high salinity-high drought conditions, the H-NSC plants had grown more (4.8 ± 0.5 cm) and had higher average leaf size (20.8 ± 7 cm^2) in comparison to the L-NSC plants (2.9 ± 0.9 cm; 8.9 ± 3 cm^2) respectively, suggesting a mitigating role of osmolytes in plant responses to abiotic stresses. Accumulation of these organic solutes may have alleviated ion toxicity, since there is an inverse relationship between the accumulation of organic solutes and ions (Briens & Larher, 1982; Gil et al., 2013). The overall reduced total leaf area per plant recorded in the present study in all the treatments is consistent with the findings of Nyugen et al. (2015) when *Avicennia marina* was grown in fresh water since the present study also involved periods of freshwater application. *Avicennia marina* is generally considered a salt tolerant species in comparison to other mangrove species (Clough 1984; Dangremond et al., 2015; Parida & Jha, 2010; Popp 1985). Some studies suggest that salinity is required at a

point in the lifecycle of mangrove for enhanced growth and physiological functions. However, long-term responses of mangrove growth to freshwater application is not well documented (Wang et al., 2011). The frequent usage of freshwater in addition to saline water in order to differentiate drought treatments in this study and the corresponding low growth rates suggest that mangroves require saline conditions for improved growth (Nyugen et al., 2015; Wang et al., 2011).

Reductions in stem hydraulic conductivity were reported in *A. germinans*, *Rhizophora mangle* and *Laguinaria racemosa* under increasing salinity which was ascribed to a lack of phosphorous (Lovelock et al., 2006a,b). It was concluded that apart from type of species and salinity, nutrient availability also influences hydraulic conductivity (Lovelock et al., 2006b). Seasonality also affects physiological processes resulting in higher hydraulic conductivity during summer in Australian *A. marina* (McClenahan et al., 2004). In my study species, maximum stem hydraulic conductivity was affected by the combination of different factors such as NSC, salinity, drought and time with high salinity levels causing the significant interaction. H-NSC plants were able to maintain higher conductivity than L-NSC plants, especially at high salinity levels. Combination of different factors such as drought, salinity, nutrient availability, often affect stem hydraulic conductivity (Dangremond et al., 2015; Lovelock et al., 2006b). Additionally, stem water potential seemed to be only affected by time rather than increasing salinity. The complex experimental design and the low replications is a drawback which limited my ability in teasing apart the main and interactive effects.

Mangroves are predisposed to have low stem water potential in comparison to other plant species since they grow in highly saline conditions naturally (Macinnis-Ng et al., 2004; McClenahan et al., 2004 ; Flowers et al., 1977; Morrisey et al., 2007; Parida & Jha, 2010). The physiological responses of halophytic species to salinity

are not instantaneous, but, delayed, involving reductions in water potential and conductivity (Donnellan Barraclough et al., 2018; Mendez-Alonzo et al., 2016). In my study, I found that the absolute midday stem water potential values were higher during the drought and salinity period compared to pre-treatment period. This was surprising and could not be explained by changes in relative humidity. At the end of the experiment (week 12), under high salinity-medium drought, stem water potential in the L-NSC plants dropped to (-0.9 ± 0.07 MPa), whereas stem water potential in the H-NSC plants was -0.7 ± 0.09 MPa. This suggests NSCs may help to maintain water potentials as drought progresses. This is consistent with studies in other temperate species (Dickman et al., 2015; Maguire & Kobe, 2015). Species-specific differences also contribute to the ability of withstanding abiotic stress such as drought and salinity. *Laguncularia racemosa* was unable to maintain water potential at higher salinity. A reduction of water potential (<-4 MPa) occurred at more than 50% seawater whereas *A.germinans* was able to maintain its water potential (>-2 MPa) at high salinities (Mendez-Alonzo et al., 2016). In general, the genus *Avicennia* is capable of maintaining higher stem hydraulic conductivity and water potential, photosynthesis and growth rates at higher salinities than other mangrove species (Clough, 1984; Reef et al., 2012). However, when salinity interacts with drought, NSC plays an important role in sustaining stem hydraulic conductivity, as shown here. Under high salinity-high drought conditions, the H-NSC plants showed higher conductivity across all weeksin comparison to the L-NSC group. The ‘well-fed’ plants (with high initial NSC concentrations in their tissues) were indeed able to maintain more favourable stem hydraulic conductivity, plant height, leaf area and overall health or vigour compared to plants depleted in NSC.

Non-structural carbohydrates are important substrates for both primary and secondary metabolism of plants and play an important role in drought-induced tree

mortality (Hartmann & Trumbore, 2016). The assimilated carbon is used for different plant functions and the synthesis of other organic compounds. Plants can also shift their substrate use under stress – from NSC to lipids (Hartmann & Trumbore, 2016; Hoch et al., 2002). Here I show increased NSC concentrations in stem in both the high and low NSC groups under the combination of drought and salinity. Starch contents decreased or remained constant during the treatment period. The degradation of starch to sugars perhaps aided plant survival through osmotic adjustment. Therefore, as shown in the present study, quantifying starch and soluble sugar content separately is essential (Quentin et al., 2015). After drought and salinity application, NSC concentrations could also have increased due to reduced C-sink activities under low soil water availability. This can happen as a consequence of carbon sink limitation and reduction in carbon transport within the plant (phloem failure; Sevanto et al., 2014). With reductions in stem hydraulic conductivity, the plant's ability to transport carbon (NSC) may also be compromised. Perhaps hydraulic and phloem impairment together acted as mechanisms leading to plant mortality under abiotic factors such as drought.

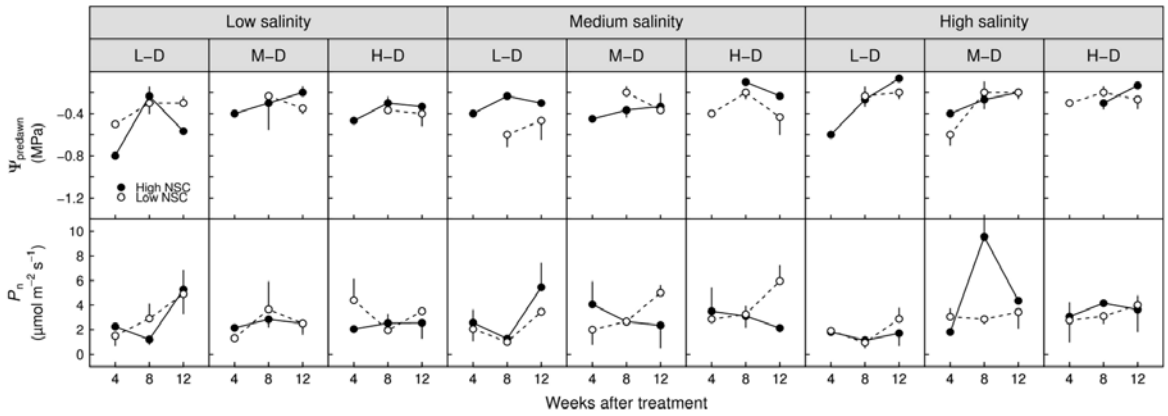
Salinity induced increases in NSC content in halophytes other than mangroves have been observed under controlled conditions (Gil et al., 2013). For example, pinitol contents increased 4.7 fold when salinity increased to 500 mM (Alla et al., 2012; Vernon & Bohnert, 1992), and sucrose contents increased fivefold in 40-day- old *Atriplex halimus* plants following a ten day drought (Alla et al., 2012). However, except for a few studies, NSCs in mangroves have never been quantified (Parida et al., 2002; Parida et al., 2004b). In *Brugiera parviflora*, similar to the findings in the present study, soluble sugar content increased, and starch decreased at 400 mM salinity (Parida et al., 2002). The present study is one of the first attempts to determine how osmolytes such as soluble sugars help mangroves cope with the

effects of combined abiotic stress. The ability of H-NSC plants to maintain higher stem hydraulic conductivity, plant height and leaf area suggests the involvement of solutes such as soluble sugars in osmotic adjustment facilitating turgor maintenance, improving plant survival and possibly preventing ion toxicity in New Zealand mangroves.

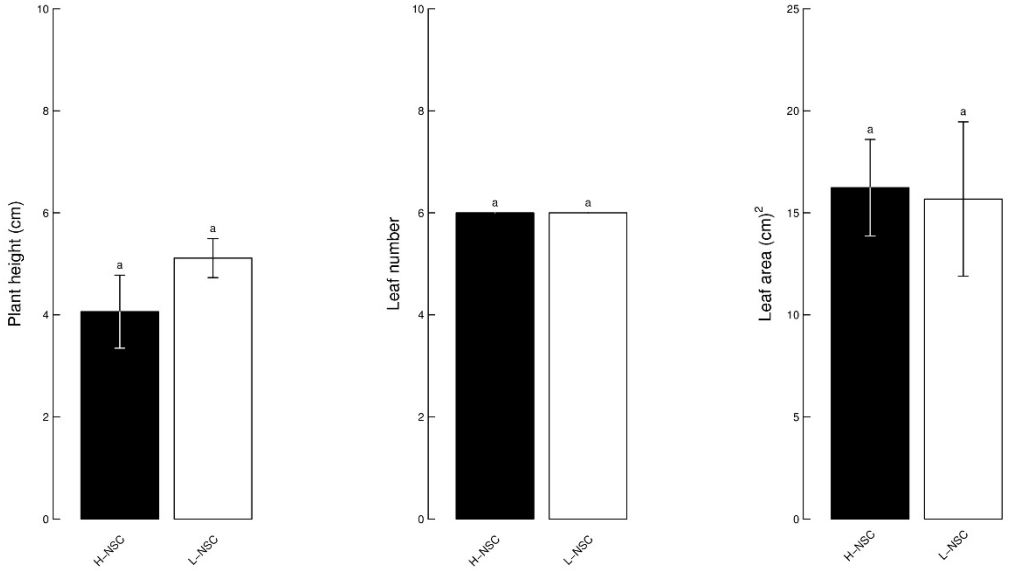
3.7 Conclusion

In conclusion, I showed that higher initial levels of NSCs play a role in mitigating adverse drought and salinity responses. Plants with higher NSC concentrations before the initiation of the salinity and drought treatment were more resilient to low soil water potential caused by these abiotic factors, likely via the conversion from starch to sugars. Further, the salinity and drought-induced increase in NSC concentrations provide strong evidence for sink-limited growth of the studied species, i.e. reduced sink activity due to decreased growth leading to NSC accumulation. I also suggest that mangroves can be used as a potential model species to study the effect of low soil water potential on tree physiology, as they grow in periodically inundated, highly homogenous stands suitable for controlled outdoor studies. Further investigations should focus on determining osmotic potential and metabolic profiles in stems and leaves, which will shed light on how higher concentrations of NSC may help to withstand low water potentials caused by drought and salinity.

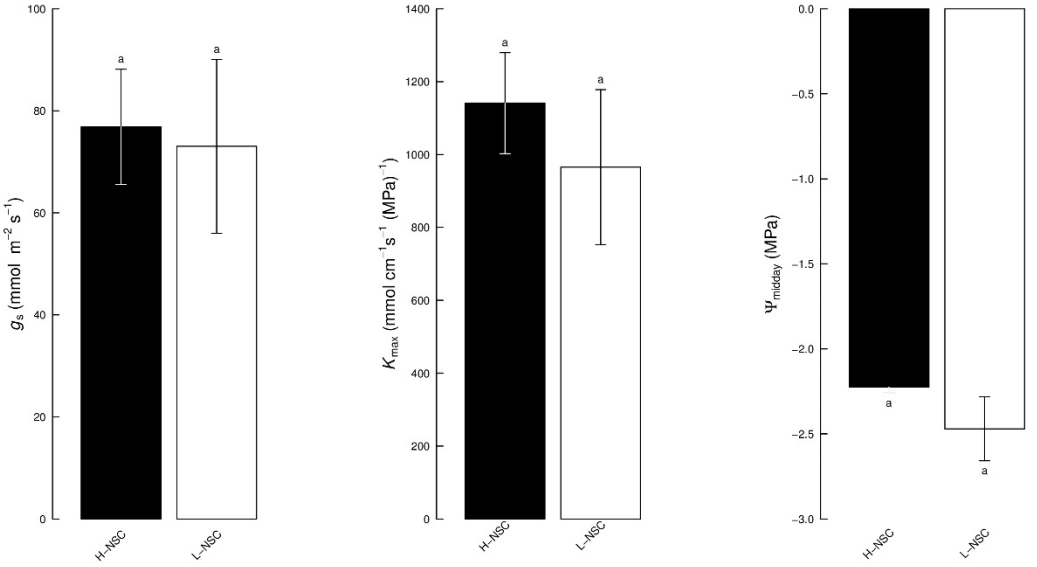
Supplementary data



Supplementary Figure 3.1 Predawn and net photosynthetic rate measurements after drought and salinity treatments. Predawn water potential measurements did not have enough replicates since a total of *n* = 20 only was measured instead of *n* = 54.



Supplementary Figure 3.2 Plant height, leaf number and leaf area prior to the salt and drought treatments. Data represent means ± s.e. (*n* = 6). H-NSC: High-NSC treatment, LNSC: Low-NSC treatment.



Supplementary Figure 3.3 Stomatal conductance (g_s), maximum hydraulic conductivity (K_{max}) midday stem water potential (Ψ) in both the high and the low NSC groups prior to the salt and drought treatments. Data represent means ± s.e. (*n* = 6 for g_s and Kmax, *n* = 3 for Ψ). H-NSC: High-NSC treatment, L-NSC: Low-NSC treatment.

Chapter 3 Results (continued)

Table 3.1 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of salinity on starch content. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity	7.38	2	0.002**

Table 3.2 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of drought on starch content. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × drought	4.16	2	0.124
Second stage			
NSC	0.89	1	0.345
Drought	9.88	2	0.007**

Table 3.3 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of salinity on relative soluble sugar content. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity	0.33	2	0.844
Second stage			
NSC	3.04	1	0.081
Salinity	0.42	2	0.807

Table 3.4 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of drought on relative soluble sugar content. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × drought	1.49	2	0.473
Second stage			
NSC	3.89	1	0.048*
Drought	6.97	2	0.030*

Table 3.5 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of NSC, salinity, drought, time and their interactions on plant height. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity × drought × time	10.12	8	0.256
Second stage			
NSC × salinity × drought	5.59	4	0.974
NSC × salinity × time	2.81	4	0.846
NSC × drought × time	7.36	4	0.003**
Salinity × drought × time	30.57	8	0.001***

Table 3.6 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of NSC, salinity, drought, time and their interactions on leaf area. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity × drought × time	5.09	8	0.747
Second stage			
NSC × salinity × drought	1.75	4	0.780
NSC × salinity × time	0.91	4	0.922
NSC × drought × time	9.10	4	0.058
Salinity × drought × time	6.03	8	0.643

Third stage			
NSC × salinity	2.24	2	0.325
NSC × drought	2.12	2	0.346
NSC × time	4.48	2	0.106
Salinity × drought	6.68	4	0.153
Salinity × time	2.62	4	0.622
Drought × time	9.80	4	0.043*
Fourth stage			
NSC	24.33	1	<0.001***
Salinity	0.33	2	0.846
Drought × time	9.43	4	0.046**
Fifth stage			
NSC	23.30	1	<0.001***
Salinity	0.30	2	0.859
Drought	1.08	2	0.580
Time	3.02	2	0.220

Table 3.7 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of NSC, salinity, drought, time and their interactions on leaf number. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity × drought × time	5.95	8	0.652
Second stage			
NSC × salinity × drought	1.09	4	0.894
NSC × salinity × time	5.47	4	0.242

NSC × drought × time	6.47	4	0.151
Salinity × drought × time	11.37	8	0.181
Third stage			
NSC × salinity	0.31	2	0.854
NSC × drought	4.15	2	0.125
NSC × time	4.52	2	0.104
Salinity × drought	2.59	4	0.627
Salinity × time	2.49	4	0.644
Drought × time	11.44	4	0.022*
Fourth stage			
NSC	25.86	1	<0.001***
Salinity	0.02	2	0.989
Drought × time	11.00	4	0.026*
Fifth stage			
NSC	24.54	1	<0.001***
Salinity	0.02	2	0.989
Drought	0.99	2	0.609
Time	6.09	2	0.047*
Sixth stage			
NSC	3.35	1	0.067
Time	11.72	2	0.0447*

Table 3.8 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of NSC, salinity, drought, time and their interactions on midday stem water potential. *L* = likelihood ratio statistic, *df* = degrees of freedom of the statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity × drought × time	8.82	8	0.358
Second stage			
NSC × salinity × drought	0.49	4	0.974
NSC × salinity × time	1.38	4	0.846
NSC × drought × time	8.11	4	0.087
Salinity × drought × time	9.97	8	0.266
Third stage			
NSC × salinity	3.00	2	0.222
NSC × drought	3.46	2	0.177
NSC × time	5.50	2	0.063
Salinity × drought	9.91	4	0.041 *
Salinity × time	7.56	4	0.108
Drought × time	4.81	4	0.306

Fourth stage			
NSC	4.03	1	0.044*
Time	12.04	2	0.002**
Salinity × drought	9.43	4	0.051
Fifth stage			
NSC	3.85	1	0.049*
Salinity	2.53	2	0.281
Drought	4.79	2	0.091
Time	12.24	2	0.002**
Sixth stage			
NSC	3.35	1	0.067
Time	11.72	2	0.002**

Table 3.9 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of NSC, salinity, drought, time and their interactions on maximum hydraulic conductivity. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity × drought × time	26.61	8	0.0008***

Table 3.10 Likelihood ratio test results from a backwards selection procedure applied to a generalised least-squares model testing the effects of NSC, salinity, drought, time and their interactions on stomatal conductance. *L* = likelihood ratio statistic, *df* = degrees of freedom of the *L* statistic. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

Dropped term	*L*	*df*	*P*
First stage			
NSC × salinity × drought × time	14.41	8	0.077
Second stage			
NSC × salinity × drought	5.59	4	0.974
NSC × salinity × time	2.81	4	0.846
NSC × drought × time	7.36	4	0.087
Salinity × drought × time	30.57	8	0.0001***

Chapter 4 – The role of different metabolites in New Zealand mangroves using untargeted metabolomics

4.1 Abstract

Mangroves employ a range of biochemical mechanisms to counteract the extreme environments where they are present. Plant metabolomics allows us to investigate the role of different metabolites in plant physiological responses to abiotic stress factors. Previous studies have revealed that levels of non-structural carbohydrates in plants are associated with stress tolerance. Here, I manipulated levels of non-structural carbohydrates (NSCs) in the New Zealand mangrove, *Avicennia marina* subsp. *australasica*, through a light swapping regime. In a subsequent drought x salinity experiment, I then monitored the leaf metabolite profiles of two the NSC groups (high-NSC and low-NSC). My results show that fourteen metabolites, belonging to multiple biochemical pathways, were significantly affected by one or more of these factors. The manipulation of non-structural carbohydrates led to increased abundance of amino acids in the low-NSC (L-NSC) but not in the high-NSC (H-NSC) plants. After treatment application, under high drought conditions, the L-NSC plants had higher abundances of leucine and valine in comparison to those with the H-NSC phenotype. The L-NSC plants also had higher abundances of putrescine and aminoadipic acid when exposed to high salinity. Under the combination of drought and salinity, α-ketoglutarate was reduced in plants with the H-NSC phenotype, and soluble sugars accumulated compared to those with the L-NSC phenotype. The increased soluble sugar content in the H-NSC plants can facilitate osmotic adjustment, thereby aiding their survival during low soil water potential conditions. I also detected the presence of stress-protective phenolic compounds (syringic and sinapic acids) not previously reported in mangroves. My findings show that the metabolites detected in this study and their respective

metabolic pathways, play a significant role in salinity and drought stress tolerance of *A. marina* subsp. *australasica*, providing new information to better understand mangrove mortality.

4.2 Introduction

Mangroves are intertidal halophytes that experience periodic inundation and high salinity conditions. In light of increasing threats due to climate change and rising sea levels, mangroves are likely to experience increased mortality (Lovelock et al., 2017). Despite their importance in plant physiology, the functional roles of different primary and secondary metabolites in abiotic stress responses for many plant species is poorly understood (McKiernan et al., 2014; Niinemets, 2016). Characterising the myriad of cellular and biochemical fluctuations in response to different stressors will help to interpret changes in physiological parameters such as leaf water potential, stomatal conductance, and gas exchange (Deshmukh et al., 2014).

Recent research efforts have focused on the role of plant metabolites in ecological and physiological functioning (McKiernan et al., 2014; Niinemets, 2016). Plant metabolomics research has provided advancements in the discovery of novel plant metabolites, metabolic pathways, and their associated functions such as coping with temperature and oxidative stress (Alseekh & Fernie, 2018). Primary metabolites are required for growth and maintenance of cellular functioning. For example, increases in concentrations of simple sugars (glucose, sucrose, fructose) and starch are associated with increased leaf area (Galvez et al., 2013). Secondary metabolites are those involved in plant defence, hormonal regulation, and protein synthesis. For example, abscisic acid is an important plant hormone, which regulates stomatal closure during drought (Chaves, Maroco & Pereira, 2003; Grant, 2012).

Non-structural carbohydrates (NSCs) such as glucose, sucrose, fructose, starch are primary metabolites, and their concentrations can reveal important insights regarding their use as osmolytes for drought survival. For example, variations in NSC levels have proved useful for investigating plant/tree mortality under abiotic stress such as drought, low/high temperature, increased precipitation and biotic factors such as pathogen attacks (Dietze et al., 2014; Germino, 2015; Hoch et al., 2002; Wurth et al., 2005). NSCs have been observed to either accumulate (O'Brien et al., 2014; Wurth et al., 2005) or deplete in response to drought in terrestrial plant species (Adams et al., 2013; Ivanov et al., 2019; Tomasella et al., 2017). They also influence the accumulation/depletion of other primary and secondary metabolites, such as amino acids, isoprenoids, and phenolics (Dietze et al., 2014; Niinemets, 2016; Wurth et al., 2005). In order to further advance knowledge regarding the physiological stress responses in plants, broader analyses of other metabolites and their associated biochemical pathways is needed to gain deeper understanding at the metabolic level.

Mangroves exist within extreme environments and employ similar biochemical mechanisms as terrestrial plant species to tolerate water-limited conditions and maintain physiological homeostasis (Aroca, 2012; Ahmad, Azooz & Prasad, 2013). For example, mangroves accumulate osmolytes such as glycine betaine, which enables them to thrive in saline conditions (reviewed by Parida & Jha, 2010). Similarly, investigations on the effect of abscisic acid on salinity tolerance of *Avicennia marina* (Forssk.) Vierh. revealed that exogenous application of abscisic acid reduced sap flow and transpiration rates that are typical of plant drought responses (Reef et al., 2012). In addition, in a study on 23 Australian mangrove species, metabolites such as proline and methylated quartenary ammonium compounds (MQAC) were found to be present only in a few species (*Avicennia marina*, *A. eucalyptifolia*, *Acanthus ilicifolius*, *Heritiera littoralis*, and *Hibiscus*

tiliaceius), whereas the remaining species accumulated low molecular weight carbohydrates in high quantities, with mannitol and pinitol being the most predominant (Popp, Larher & Weigel, 1985).

Previous studies have examined the effect of salinity and drought in isolation on the plant metabolome (Alla et al., 2012; Gagneul et al., 2007; Johnson et al., 2003, Parida et al., 2002; Parida et al., 2004b). However, when salinity co-occurs with drought, different metabolic adjustments may transpire, with either synergistic (conserved) or antagonistic (divergent) responses occuring (Sanchez et al., 2008b). Synergistic metabolic responses occur when two or more species show accumulation or depletion of similar metabolites. Antagonistic metabolic responses are those where metabolite profiles of different plant taxa show opposing trends (Munns 2002; Sanchez et al., 2008b & 2012). In mangroves, high salinity conditions usually cause accumulation of pinitol, mannitol, glycine betaine, and proline (reviewed in Parida & Jha, 2010; Kumari & Parida, 2018). The production and roles of different metabolites in plant abiotic stress responses are complex, but recent advances in analytical instrumentation make it possible to identify a wide array of plant metabolites and their associated functions (Alseekh & Fernie, 2018).

In this contribution, stress response patterns in primary and secondary metabolites of the New Zealand mangrove, *Avicennia marina* subsp. *australasica* were investigated under varying levels of drought, salinity, and NSC concentration. I first manipulated NSC levels in mangrove seedlings by exposing them to different light conditions, then subjected them to a combination of salinity and drought treatments to investigate their effects on a comprehensive range of metabolites using an untargeted GC/MS-based metabolomics approach. My overall hypothesis was NSC content plays a fundamental role in stress tolerance via a restructuring of central metabolic pathways, identifiable by changes within the mangrove metabolome. I

hypothesized that i) An altered light regimen (light swapping) will cause differences in metabolite accumulation via restructuring of energy and carbohydrate metabolic pathways. ii) After drought and salinity treatment application, seedlings with higher initial NSC content will have higher levels of primary and secondary metabolites. For example, in the extreme treatment combination (high salinity–high drought) increased metabolite accumulation, such as proline may occur.

4.3 Materials and methods

4.3.1 Plant material

New Zealand mangrove propagules were collected from Manghawhai Estuary, northern New Zealand, in December 2014. After initial acclimation and growth, 162 seedlings were placed in 3.7 litre pots with fine sand as the substrate in a shade house (University of Auckland, Tamaki campus). All 162 pots were fertilised every 15 days using commercial garden fertiliser (Thrive all-purpose soluble fertilizer [Yates, New Zealand]; NPK ratio of 25:5:8.8 plus trace elements; 45 gL^{-1} in fresh water; 250 mL provided per pot per application). Environmental conditions (light, temperature and relative humidity) in the shade house were monitored throughout the study period (Hobo Pendant Temp-light Data Logger; Tidbit Mx Temperature Data logger; Hobo Temperature-RH Data Logger [Onset Computer Corporation, USA]). For Jan-Apr 2015, the daily average maximum temperature was 27°C and the average minimum was 18°C. The daily average maximum relative humidity was 84% and the average minimum was 61%. For May-Aug 2015, the daily average maximum temperature was 15°C and the minimum was 9°C. The daily average maximum relative humidity was 64% and the minimum was 55%. The duration of the study from propagule collection, to the final harvest lasted from December 2014 – August 2015.

4.3.2 Manipulation of NSCs

To reduce potential confounding effects from maternal reserves (i.e., to prevent plants from using cotyledonary reserves), cotyledons were removed from all the seedlings immediately prior to NSC manipulation. Before the drought × salinity treatment application, plants with different levels of NSC were obtained by applying a light swapping treatment for 84 days (O'Brien et al., 2014). After 50 days of initial acclimation and growth, seedlings were randomly divided into two groups of 81 plants each (162 plants in total). For the first 42 days, one group of plants was grown under ambient light conditions with a monthly average PPFD of 307.6 ± 22.8 (μmol m^{-2} s^{-1}), whereas the other group was kept under low light, corresponding to 9–10% of ambient light with a monthly average PPFD of 24.2 ± 4.9 (μmol m^{-2} s^{-1}). The two groups of plants were then swapped to receive the reciprocal light conditions for a further 42 days (Figure 3.1b, see Chapter 3). This resulted in two groups of plants with different NSC levels (Figure 3.1c, see Chapter 3).

4.3.3 Treatment application

After manipulation of NSCs, seedlings were exposed to drought × salinity treatments for 12 weeks. A randomized factorial design was used where three levels of salinity (i.e., Low: 25% seawater, medium: 50% seawater, and high: 75% seawater) were crossed with two levels of drought (low and high), and with two levels of initial endogenous NSC content (low and high). Seedlings within each NSC group were randomly allocated to each of the drought × salinity treatments, with three replicate pots per treatment combination. Saline solutions at three concentrations were prepared using artificial sea salt (S9883, Sigma-Aldrich, St. Louis, MO, USA): low salinity (150mM NaCl), medium salinity (300 mM NaCl), and high salinity (450mM NaCl). Pots were watered every with 400 mL (water holding capacity of pots) of the respective salinity solutions at the start of the experiment, then at weeks 4 and 8. In between salinity treatments, seedlings exposed to low drought conditions received tap water every 3–4 days (200 mL via spray bottle), whereas those exposed to high drought conditions did not. The salinity concentrations used in this experiment were within the salinity tolerance range of *A. marina* (Morrisey et al., 2007).

4.3.4 Sample collection

A first set of leaf samples was collected from the seedlings for metabolomic analysis at the end of the light swapping treatment. Two to three leaves from each plant were collected from both the high NSC (H–NSC; *n* = 3 plants each) and low NSC (L–NSC; *n* = 3 plants each) treatments for analysis.

Further sampling of leaves took place after drought and salinity treatments had been in place for 12 weeks. Two to three leaves from each plant were collected from each treatment combination (*n* = at least 3 each). The leaf samples were snap frozen in liquid nitrogen and stored at -80°C until extraction and further analysis.

4.3.5 Metabolite Analysis

Leaf metabolite profiling was conducted according to protocols described in detail by Young et al. (2017) and Zarate et al. (2017). Briefly, freeze-dried leaf samples (~10 mg) were co-extracted with an internal standard (d4-alanine or D-Ribitol) using an established methanol-water method. To increase volatility of the organic acid and carbohydrate constituents, extracted metabolites were derivatized via methyl chloroformate (MCF) alkylation and N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) trimethylsilylation (TMS), respectively (Villas-Bôas et al., 2011). Compounds were analysed via gas chromatography mass spectrometry using a Thermo Trace GC Ultra system for the MCF derivatives, and an Agilent GC7890 + MSD5975 for the TMS derivatives. Instrument setups are outlined in Young et al. (2017) and Zarate et al. (2017). Spectral preprocessing was performed using the Automated Mass Spectral Deconvolution and Identification System (AMDISV2.66) software. Chemstation software (Agilent Technologies) and customised R xcms-based scripts (Aggio et al., 2011) were used to identify metabolites by interrogating

in-house libraries of MCF- and TMS-derivatised compounds constructed using pure standards. Records were quality assessed and manually checked for presence of contaminants, with aberrant records being removed. Metabolite peak intensities within each dataset were normalised against the relevant internal standard to compensate for potential technical variations (e.g. variable metabolite recoveries), and to sample-specific biomass.

4.4 Data analysis

To provide visualisation of the metabolome coverage across plant cellular pathways, pathway mapping was performed on metabolites able to be matched to entries within the KEGG database (reference plant model: *Arabidopsis thaliana*) using Interactive Pathway Explorer v3 (iPATH3; Darzi et al., 2018). Statistical analysis of metabolite data after manipulation of NSCs was conducted using Metaboanalyst 4.0 (Chong et al., 2018), and the R package *nlme* (Pinheiro et al., 2018). For multivariate analyses, data were log-transformed and mean-centred to reduce heteroscedasticity and provide a Gaussian distribution of metabolite abundances.

After manipulation of NSCs (prior to the drought × salinity treatments), combined heatmap and hierarchical cluster analysis (Euclidian distance; Wards criterion) of the top 50 metabolites ranked by their t-test statistics was conducted to provide an intuitive visualisation of expression patterns and between-sample variability. Quantitative Enrichment Analysis (QEA [Xia & Wishart, 2010]) and Network Topology Analysis (NTA [Nikiforova & Willmitzer, 2007]) were used as pathway analysis methods to investigate functional relationships among the annotated metabolites and highlight differential regulation within these collections at the pathway level. Biochemical pathways in the KEGG database involving two or more annotated metabolites with simultaneous QEA p-values < 0.05, QEA false

discovery rates [FDRs] < 0.1, and with NTA Pathway Impact (PI) scores > 0.1 were considered as potential primary target pathways of interest.

After drought × salinity treatments, metabolites were modelled using a generalised least squares approach with restricted maximum likelihood (gls, R package *nlme*; Pinheiro et al., 2018) as a function of pre-treatment NSC level, salinity and drought. Variance heterogeneity and normality were assessed using residual vs. fitted plots and quantile-quantile plots respectively for testing violation of model assumptions. For cases where significant interactions were present, these were sliced using a multiple comparison procedure (R package *emmeans*; Lenth, 2018). The resulting p-values from the multiple comparison procedure were corrected for multiple testing using the Benjamini & Hochberg method (1995). Additionally, because of multiple hypothesis testing involving 104 metabolites, Bonferroni correction was used to reduce type I errors, with a marginal significance level (10%, $P \leq 0.0009$). These analyses were performed using R (R 3.4.2, R Development Core Team, 2017).

4.5 Results

A total of 149 metabolites including unknowns were detected in *Avicennia marina* subsp. *australasica* leaves. Of these, 69 metabolites, broadly covering a number of modules (collection of manually defined functional units) in the KEGG global metabolic network could be mapped onto the KEGG plant reference pathway (Figure 4.1). These metabolites belong to different metabolic pathways with ~35% involved in energy metabolism, ~38% in amino acid metabolism, ~15% in carbohydrate metabolism, and ~10% involved in biosynthesis of secondary plant metabolites such as phenolic acids.

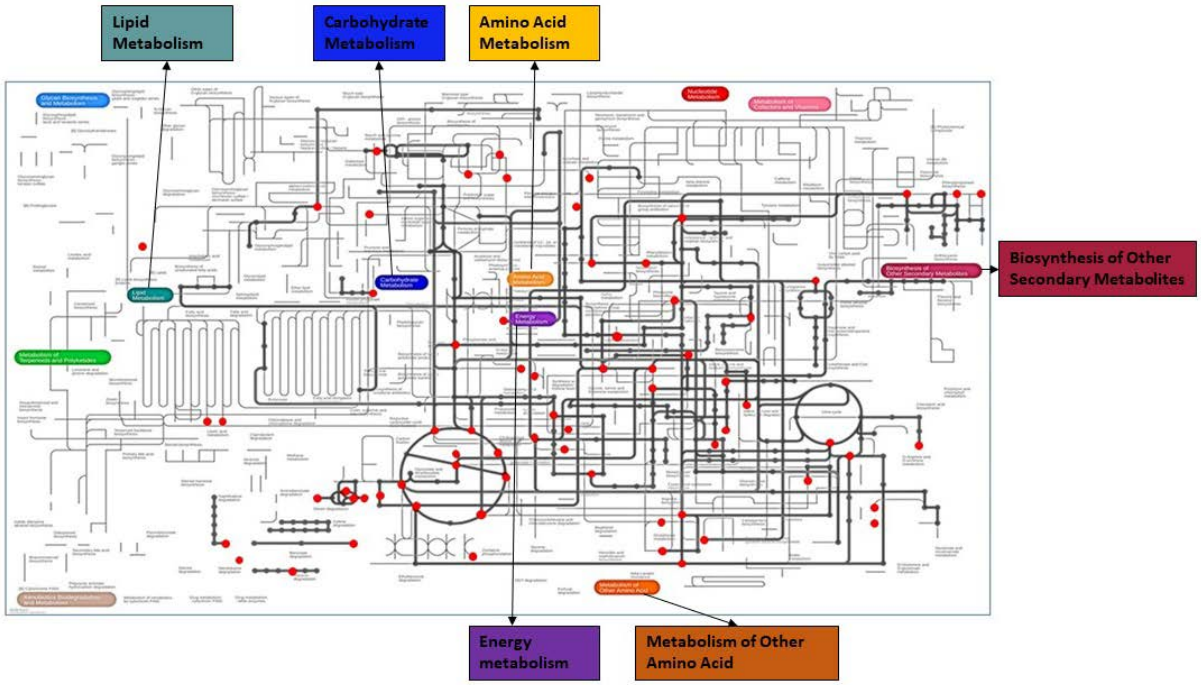


Figure 4.1. Projection of annotated metabolites in mangrove (*Avicennia marina* subsp*. australasica*) leaves overlaid on the KEGG global pathway map of *Arabidopsis thaliana*. Red circles = metabolite matches (*n* = 69): black edges = obtained coverage of KEGG pathway modules with one or more matching compounds. Metabolic pathways that are discussed are enlarged.

After the light swapping treatment to manipulate NSC levels, 30 metabolites including unknowns were identified as being significantly different (t-test; $P < 0.05$) between the high- and low-NSC groups (Figure 4.2). A combined heat map and HCA (Hierarchical cluster analysis) using the top 50 metabolites ranked by their t-test p-values show distinct grouping based on their sample class membership (Figure 4.2). In the high NSC group, the sugars rhamnose, ribose, xylose, myo-inositol, and glyceric acid were substantially more abundant than in the low NSC group; being primarily responsible for the higher total NSC content. Levels of arabinose, fructose, mannose, galactose, and glucose did not differ between NSC-manipulated groups, but levels of sucrose were higher in the low NSC group.

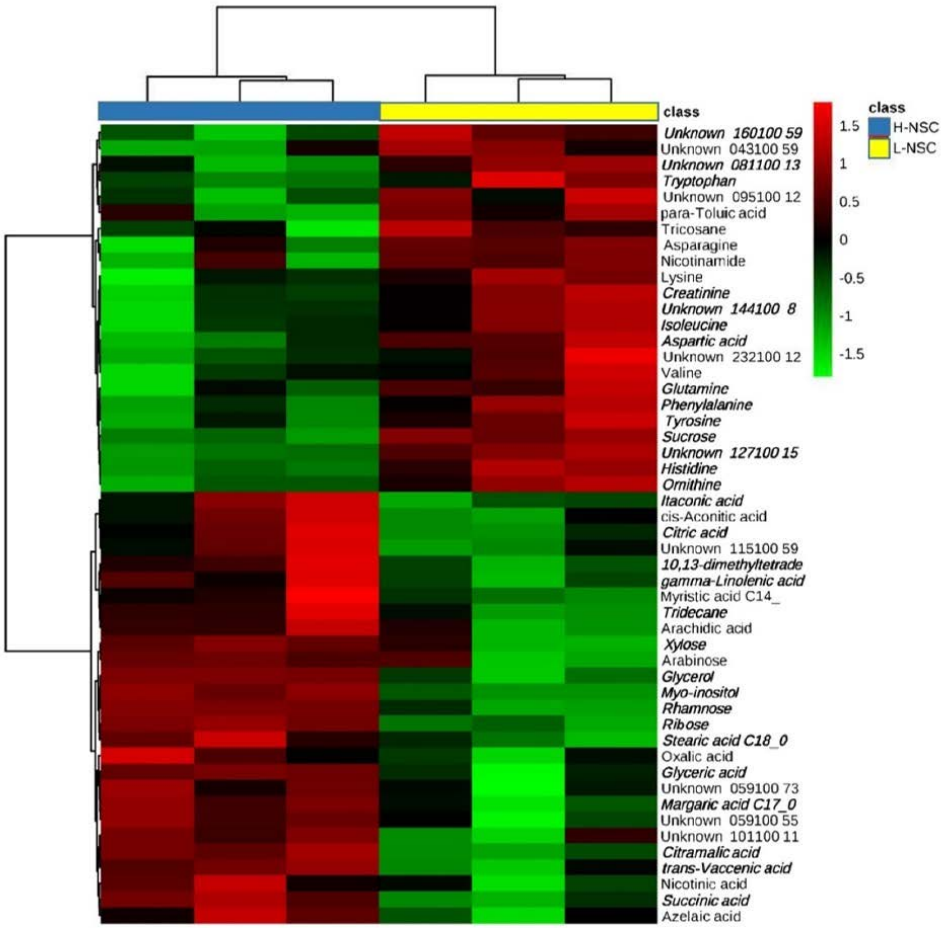


Figure 4.2. Effect of light-swapping regime on mangrove leaf metabolome via heatmap and hierarchical cluster analysis (Euclidean distance; Ward's criterion) of the top 50 metabolites ranked by their t-test p-values. Thirty metabolites (in bold) were identified as being significantly altered ($P < 0.05$) by the light treatment. Columns represent samples (blue = High-NSC group; yellow = Low-NSC group), and rows represent metabolites. The green/black/red colour scale represents standardised (log-transformed and mean-centred) abundance data, where red = higher values, and green = lower values.

Topology-based pathway analysis was conducted to reveal the most relevant pathways associated with the light swapping treatment regime (Figure 4.3; Supplementary table, 4.1). From within the KEGG database, a total of 53 biochemical pathways were recognised which contained one or more of the annotated metabolites detected. The pathways including two or more detected metabolites and with simultaneous QEA p-values < 0.05, QEA FDR values < 0.1 and NTA Pathway Impact (PI) values > 0.1 were screened as potential primary target pathways of interest relating to the treatment effect (drought and salinity). According to this selection, 10 biochemical pathways were identified with evidence for differential regulation between NSC treatments, comprising: inositol phosphate metabolism, tyrosine metabolism, TCA cycle, glycine, serine and threonine metabolism, phenylalanine metabolism, glyoxylate and dicarboxylate metabolism, isoquinoline alkaloid biosynthesis, phenylpropanoid biosynthesis, tyrptophan metabolism, carbon fixation. Twelve further pathways that were identified statistically via QEA (P < 0.05), but did not meet one or more criteria in the ideal impact assessment were screened as potential secondary target pathways of interest, comprising: starch and sucrose metabolism, nicotinate and nicotinamide metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, glycerolipid metabolism, nitrogen metabolism, tropane, piperidine and pyridine alkaloid biosynthesis, pyruvate metabolism, ubiquinone and other terpenoi-quinone biosynthesis, aminoacyl-tRNA biosynthesis, glucosinolate biosynthesis, biosynthesis of unsaturated fatty acids, lysine biosynthesis.

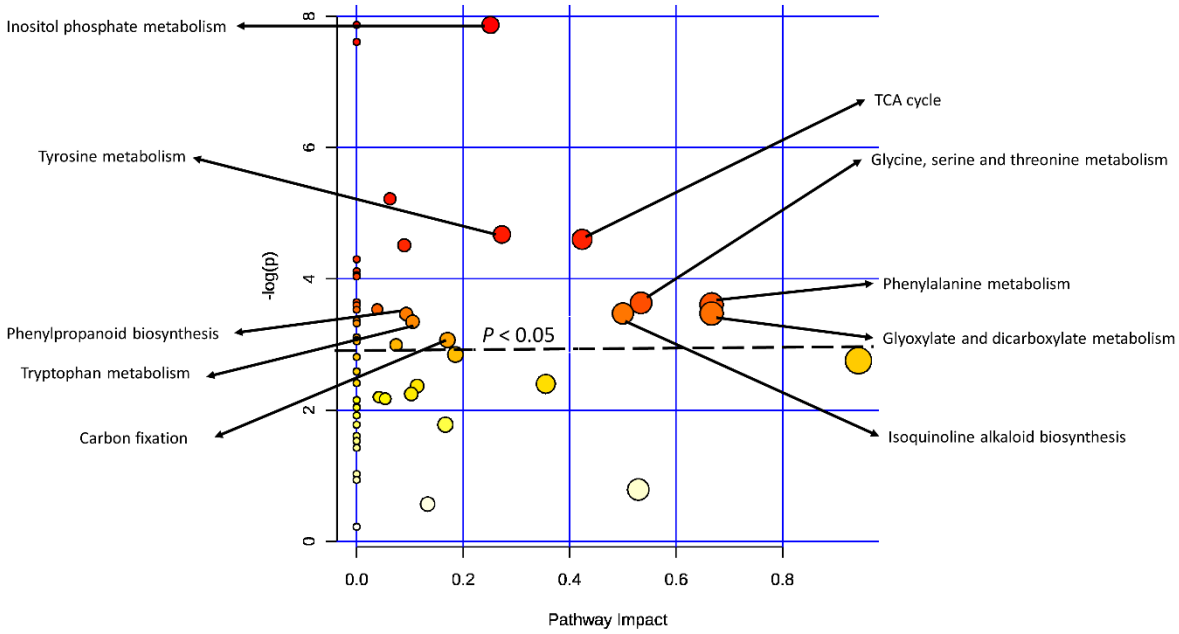


Figure 4.3. Topology-based pathway analysis showing metabolic networks in mangrove seedlings significantly altered by the light-swapping technique to manipulate levels of non-structural carbohydrates. The most impacted metabolic pathways are designated by the volume and the colour of the spheres (yellow = least relevant; red = most relevant; small circles = low pathway impact scores, large circles = high pathway impact scores) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA), respectively. Dotted line represents the significance cutoff at $P < 0.05$.

After the drought and salinity treatments, each metabolite was affected by either an interaction effect of NSC, drought and salinity or just the main effect of each factor. I also report metabolites that are prominent in the plant metabolomics literature. The details of this generalised least squares analysis are presented in Supplementary Table, 4.2 (Appendix).

A total of fourteen compounds were either significantly affected by three-way (NSC × salinity × drought) interactions and main effects (Table 4.1). Three of them belonged to the energy metabolism, the TCA (Tricarboxylic) cycle. 2-ketoglutamarate abundance was affected by NSC × drought interaction. The interaction was driven by low and high drought conditions in the L-NSC group which had lower abundance. The abundance of α-ketoglutarate was affected by low salinity–high drought conditions. The L-NSC group had higher abundance of α-ketoglutarate than the H-NSC group. Malonic acid, which is also an intermediate in the malonic acid pathway leading to the formation of phenolics, was significantly affected by drought only and not salinity.

Other carbohydrates and amino acids that were significantly affected by interactions or main effects are: Galactose (galactose metabolism) abundance was affected by the NSC × salinity interaction. This interaction was driven by high salinity in the H-NSC group which had higher abundance. Glycerol abundance was significantly affected by drought only. Four amino acids differed significantly between treatments: Leucine and valine which are synthesized via Valine, leucine, isoleucine biosynthetic pathway. Also, putrescine which is synthesized via arginine and proline metabolism and also glutathione metabolism. Leucine and valine were affected by the NSC × drought interaction. For both compounds, the interaction was driven by high drought conditions. The L-NSC plants had higher abundance of both the compounds. Putrescine and aminoadipic acid (lysine biosynthetic pathway) were

affected by NSC × salinity interaction. For putrescine, the interaction was driven by low and medium salinity conditions, whereas for aminoadipic acid the interaction was driven by high salinity conditions. The H-NSC plants had lower abundance of putrescine and, aminoadipic acid in comparison to the L-NSC plants.

Other compound classes, such as phenolic acids, were affected by three-way (NSC × salinity × drought) interactions. Two of them included para-toluic acid and syringic acid. Under medium salinity – high drought conditions, the abundance of para-toluic acid was high in the H-NSC plants and low in the L-NSC plants. Whereas, the abundance of syringic acid was similar in the H-NSC and L-NSC plants. Additionally, nicotinic acid (nicotinamide metabolism and the tropane, piperidine and pyridine alkaloid biosynthesis) and tridecane (hydrocarbon, intermediate in essential oil synthesis) were identified. The abundance of nicotinic acid was increased in the L-NSC plants under high saline conditions. The abundance of tridecane was affected by medium and high salinity associated with low drought conditions. The L-NSC plants had higher abundance of tridecane in comparison to the H-NSC plants.

Table 4.1. Metabolites that were significantly affected (Bonferroni corrected; *P*<0.0009) after drought and salinity treatments (tested interactions = NSC × salinity × drought, NSC × salinity, NSC × drought, salinity × drought; tested main effects = NSC, salinity, drought).

Metabolite	Biochemical pathways	Interactions and main effects
Malonic acid	1. Tricarboxylic acid cycle (TCA) 2. Malonic acid pathway 3. Acetate-malonate pathway	Drought
α-ketoglutarate	1. TCA cycle 2. Pentose & glucoronate interconversions 3. Ascorbate & aldarate metabolism 4. Alanine, aspartate & glutamate metabolism 5. Lysine biosynthesis 6. Lysine degradation 7. Histidine metabolism 8. Glyoxylate & dicarboxylate metabolism	NSC × salinity × drought
α-ketoglutamarate	9. Unknown	NSC × drought
Glycerol	10. Galactose metabolism	Drought
Galactose	Galactose metabolism	NSC × salinity
Leucine	Valine, leucine & isoleucine biosynthesis	NSC × drought
Putrescine	1. Arginine & proline metabolism 2. Glutathione metabolism	NSC × salinity
p-Toluic acid	3. Biosynthesis of secondary metabolites	NSC × salinity × drought
Syringic acid	4. Aminobenzoate degradation	NSC × salinity × drought
Nicotinic acid	5. Nicotinate & nicotinamide metabolism 6. Tropane, piperidine & pyridine alkaloid 7. biosynthesis	NSC × salinity
Tridecane	8. Intermediate in essential oil synthesis	NSC × salinity × drought

4.6 Discussion

My hypothesis that light swapping causes accumulation of metabolites other than NSCs was confirmed. There were significant differences between the NSC-manipulation treatments in arabinose and xylose (which are not detectable when using enzymatic hydrolysis), along with an array of other metabolites. Following the drought and salinity treatments, the abundance of fourteen metabolites were significantly affected by interactions of NSC, salinity, and drought. This confirms my other hypothesis that salinity and drought affect metabolite accumulation by altering associated biochemical pathways.

To the best of my knowledge, this is the first metabolomics-based study to show metabolite differences following experimental NSC manipulation in plants. My light swapping treatment led to differential NSC contents, as previously shown in Alberda (1966) and O'Brien et al. (2014) who used the light swapping method in perennial ryegrass and ten shade-tolerant tropical species, respectively, for NSC manipulations. In addition, a range of other sugars and metabolites were also differently modulated between the two plant groups. For example, xylose, arabinose, rhamnose, ribose glycerol, and myo-inositol were more abundant in the high NSC group compared to the low NSC group. Conversely, amino acids such as lysine, creatinine, ornithine, isoleucine, valine, glutamine, phenylalanine were more abundant in the low NSC group. In other studies, increased accumulation of sugars and polyols occurred in mangroves and other halophytes such as *Atriplex halimus* following either drought or salinity stress (Alla et al., 2012; Kumari & Parida, 2018; Parida et al., 2002; Sanchez et al., 2008b).

Several studies have examined the effect of either drought or salinity in isolation. However, when both treatments are applied together the interpretation of

plant physiological responses becomes increasingly difficult to elucidate, involving the synthesis, use and redistribution of many metabolites which belong to multiple biochemical pathways (Flowers & Colmer, 2008; Munns & Tester, 2008; Slama et al., 2015). The biphasic model of plant salt tolerance also suggests that although the initial plant responses to drought and salinity stresses are similar, long-term responses are not (Munns, 1986; Munns & Tester, 2008). In the present study, with the combination of drought and salinity stressors, a more generic response was found with metabolites belonging to different pathways, not salt or drought specific. I have shown how primary metabolic pathways respond to interacting stress factors and overlap with other metabolic pathways leading to the production of different metabolites (Aroca, 2012; Kumari & Parida, 2018; Sanchez et al., 2008a,b). I observed changes in amino acid, sugar and organic acid metabolic pathways consistent with other studies (Alla et al., 2012; Munns & Tester, 2008; Sanchez et al., 2008b).

The amino acid proline is an important osmolyte and antioxidant in plants (Slama et al., 2015), and is typically accumulated by many plant species in response to environmental stresses (Kishor et al., 2005; Parida & Jha, 2010). In the present study, the L-NSC plants had higher abundance of proline in the medium salinity–high drought compared to the H-NSC plants. As salinity increased from low to medium saline conditions, proline likely acted as an osmoprotectant. In *Bruguiera parviflora*, it was shown that proline content increased gradually as salinity increased from low (100 mM) to medium (200 mM) but decreased at higher salinity (400 mM; Parida et al., 2002). In other halophytes (*Atriplex halimus* and *Lepidium crassifolium*), proline accumulation has been observed under high salt conditions and long term drought (Alla et al., 2012; Murakeozy et al., 2003). In *Limonium latifolium*, proline and other metabolites contributed less than 25% to the osmoregulatory activity compared to

sugars and hexoses, revealing that soluble carbohydrates are also very important (Gagneul et al., 2007).

In most plants, proline accumulation is accompanied by increased levels of carbohydrates during stress. For example, in *Bruguiera parviflora*, proline and total sugar contents increased concomitantly after 45 days of 400 mM salt exposure (Parida et al., 2002). Similarly, in *Atriplex halimus*, exposure to 550 mM salt and 10 days drought simultaneously increased levels increased of proline and sucrose (Alla et al., 2012). Although saline conditions induce a nitrogen deficient environment, halophytes tend to accumulate nitrogenous compounds such as amino acids (Mansour, 2000; Murakeozy et al., 2003). The species in the present study belongs to the physiotype that accumulates both carbohydrates and nitrogenous compounds in higher quantities, as is also seen in the seagrass *Triglochin maritima* (Briens & Larher, 1982). Proline may also provide NAD^+ and $NADP^+$ for photosynthetic and respiratory process (Slama et al., 2015), and plays an important role in stabilising membranes and proteins (Ashraf & Foolad, 2007; Parida & Jha, 2010). In my study of New Zealand mangroves, the accumulation of proline and sugars in H-NSC plants may have contributed to protein stabilisation of sub-cellular structures and an enhanced capacity for osmotic adjustment.

In the present study, the organic acids α-ketoglutarate and α-ketoglutamarate, the carbohydrate galactose, and the sugar alcohol glycerol were significantly affected by one or more of the factors (NSCs, drought and salinity). This is consistent with a previous study on *Populus euphratica* leaves, where exposure to heat and high salinity caused decreases in levels of α-ketoglutarate the soluble sugar fructose, and the sugar alcohol mannitol, along with concomitant increases in the sugar alcohol glycerol and its oxidation product glyceric acid (Brosche et al., 2005; cited in Sanchez et al., 2008b). These changes were attributed to high levels of sodium in the soil

(Brosche et al., 2005; reviewed by Sanchez et al., 2008b). Additionally, in *Lotus japonicas*, physiological responses to high salinity exposure include organic acid depletion and accumulation of amino acids and sugars (Sanchez et al., 2008a & 2012). In the present study, α-ketoglutarate (a key regulator of carbon and nitrogen metabolic interactions) accumulated in mangroves with the L-NSC phenotype compared their H-NSC counterparts under low salinity – high drought conditions.

Levels of α-ketoglutarate decrease under nitrogen starvation, which is usually associated with hypersaline conditions (Stitt & Fernie, 2003). In the present study, the abundance of α-ketoglutarate was affected as drought increased from medium to high, under low saline conditions. I also detected significantly higher levels of free amino acids under increased salinity and drought, which is consistent with limited soil nitrogen availability (Tschoep et al., 2009). In plants grown under mild but continuous nitrogen limitation, total FAA levels increased in tissues due to remobilisation of stores, and decreased utilisation of AAs for protein synthesis and growth (Krapp et al., 2011). Amino acid accumulation is a widespread osmoregulatory mechanism of *Avicennia marina* (Parida & Jha, 2010).

I also detected increased synthesis of the phenolic compounds sinapic and syringic acids in both the L-NSC and H-NSC plants. Phenolics function as antioxidants, absorb UV light, and tend to be highly abundant in certain mangroves species (Asha et al., 2012; Suh et al., 2014). These compounds assist plants in coping with environmental stress through scavenging harmful levels of reactive oxygen species (ROS) and providing UV protection (Goleniowski et al., 2013). Salt and drought stress are well-known to induce overproduction of ROS in plants (reviewed by De Carvalho, 2008; Miller et al., 2010). ROS are critical secondary messengers and the level of steady-state cellular ROS is used by plants to monitor their intracellular level of stress (Miller, 2002). When ROS rises beyond manageable

limits, oxidative damage to DNA, membrane lipids, and proteins occur. Siani et al. (2018) recently demonstrated that salt-tolerant rice cultivars have higher levels of basal ROS than their salt sensitive counterparts, but also express higher activities of ROS regulatory enzymes to keep the system in check. It is thought that hydrogen peroxide in particular can activate diverse signal transduction pathways involved in salt stress amelioration through enhancing several defense-related mechanisms (Sadak, 2016). Sinapic and syringic acids in *Avicennia marina* subsp. *australasica* offers antioxidant potential to manage excess ROS production during abiotic stress events and protects against harmful UV levels which can be intense within the mangroves' distribution range during summer months (Liley & McKenzie, 2006).

4.7 Conclusion

In conclusion, this study is a first attempt to comprehensively profile metabolites in response to interacting abiotic stress factors in mangroves. My findings indicate that light swapping differentially modulates synthesis and use of numerous sugars and other metabolites in addition to the usually characterised sugars in plant ecophysiological studies. The abundance of primary metabolites in mangroves influences secondary metabolite accumulation, helping to grant enhanced salinity tolerance and enabling them to thrive in highly inundated saline conditions. Future studies should investigate seasonal effects in addition to elucidating responses to abiotic factors. Metabolite profiling of mature trees under natural conditions is a way forward to better understand the biochemical mechanisms that halophytes employ to cope with extreme environmental conditions.

Supplementary information

Supplementary Table. 4.1 List of identified metabolites, t-test results, and Pathway analysis results.

Compound name	HMDB identifier	KEGG identifier	Log 2 FC	p.value	SAM	
					p.value	q.value
10,13-dimethyltetradeca-noic acid	-	-	0.85493	0.038339		
N-(Carboxymethyl)-L-alanine	HMDB0033550					
2,4-Di-tert-butylphenol	HMDB0013816	-				
2-Aminoadipic acid	HMDB0000510	C00956				
2-Hydroxybutyric acid	HMDB0000008	C05984				
2-Hydroxyglutaramic acid	-	-				
2-Isopropylmalic acid	HMDB0000402	C02504				
2-ketoglutamarate	HMDB0000208	C00026				
2-Oxoglutaric acid	HMDB0000208	C00026				
2-Phosphoenolpyruvic acid	HMDB0000263	C00074				
3-Methyl-2-oxopenta-noic acid	HMDB0000491	C03465				
4-Aminobutyric acid	HMDB0000112	C00334				
4-Hydroxycinnamic acid	HMDB0002035	C00811				
Alanine	HMDB0000161	C00041				
Arachidic acid	HMDB0002212	C06425				
Asparagine	HMDB0000168	C00152			0.018	0.0801
Aspartic acid	HMDB0000191	C00049	-1.4175	0.0136	0.016	0.0801
Azelaic acid	HMDB0000784	C08261				
Benzoic acid	HMDB0001870	C00180				
Butylated hydroxytoluene	HMDB0033826	C14693				
cis-Aconitic acid	HMDB0000072	C00417				
Citraconic acid	HMDB0000634	C02226				
Citramalic acid	HMDB0000426	C00815	0.79339	0.0019		
Citric acid	HMDB0000094	C00158	0.90799	0.0458		
Conjugated linoleic acid	HMDB0005047	-				
Creatinine	HMDB0000562	C00791	-1.8323	0.0446	0.0293	0.0801
Cysteine	HMDB0000574	C00097				
Dibutyl phthalate	HMDB0033244	C14214				
Dehydroascorbic acid	HMDB0001264	C00425				
Dodecane	HMDB0031444	C08374				
Dodecanoic acid	HMDB0000638	C02679				

Compound name	HMDB identifier	KEGG identifier	Log 2 FC	p.value	SAM	
					p.value	q.value
Ferulic acid	HMDB0000954	C01494				
Fumaric acid	HMDB0000134	C00122				
gamma-Linolenic acid	HMDB0003073	C06426	0.69544	0.0445		
Glutamic acid	HMDB0000148	C00025				
Glutamine	HMDB0000641	C00064	-1.6685	0.0447	0.0313	0.0801
Glutaric acid	HMDB0000661	C00489				
Glutathione	HMDB0000125	C00051				
Glyceric acid	HMDB0000139	C00258	1.5479	0.0366		
Glycine	HMDB0000123	C00037				
Glyoxylic acid	HMDB0000119	C00048				
Heptadecane	-	-				
Histidine	HMDB0000177	C00135	-3.801	0.0054	0.0013	0.0392
Hydroxybenzoic acid	HMDB0000500	C00156				
Isocitric acid	HMDB0000193	C00311				
Isoleucine	HMDB0000172	C00407	-1.8394	0.0498	0.0326	0.0801
Itaconic acid	HMDB0002092	C00490	1.1254	0.0447		
Lactic acid	HMDB0000190	C00186				
Leucine	HMDB0000687	C00123				
Lysine	HMDB0000182	C00047			0.0213	0.0801
Malic acid	HMDB0000744	C00711				
Malonic acid	HMDB0000691	C00383				
Margaric acid	HMDB0002259	-	0.6946	0.0323		
Myristic acid	HMDB0000806	C06424				
NADP_NADPH	-	-				
Nicotinamide	HMDB0001406	C00153			0.0306	0.0801
Nicotinic acid	HMDB0001488	C00253				
Nonacosane	HMDB0034288	C08384				
Octanoic acid	HMDB0001954					
Ornithine	HMDB0000214	C00077	-2.9393	0.0123	0.006	0.0735
Oxalic acid	HMDB0002329	C00209				
Oxaloacetic acid	HMDB0000223	C00036				
Palmitelaidic acid	HMDB0012328	-				
para-Toluic acid	HMDB0029635	C01454				
Pentadecane	-	-				
Phenethyl acetate	HMDB0033945	C12303				
Phenylalanine	HMDB0000159	C00079	-3.6192	0.0255	0.01	0.0735

Compound name	HMDB identifier	KEGG identifier	Log 2 FC	p.value	SAM	
					p.value	q.value
Proline	HMDB0000162	C00148				
Putrescine	HMDB0001414	C00134				
Pyroglutamic acid	HMDB0000267	C01879				
Salicylic acid	HMDB0001895	C00805				
Serine	HMDB0000187	C00065				
Sinapic acid	HMDB0032616	C00482				
Stearic acid	HMDB0000827	C01530	0.6565	0.0252		
Succinic acid	HMDB0000254	C00042	0.9679	0.0071		
Syringic acid	HMDB0002085	C10833				
Threonine	HMDB0000167	C00188				
trans-Cinnamic acid	HMDB0000930	C00423				
trans-Vaccenic acid	HMDB0003231	C08367	0.7395	0.0192		
Tricosane	-	-				
Tridecane	HMDB0034284	C13834	0.6611	0.0486		
Tryptophan	HMDB0000929	C00078	-2.4223	0.0465	0.026	0.0801
Tyrosine	HMDB0000158	C00082	-1.9599	0.0310	0.0193	0.0801
Valine	HMDB0000883	C00183				
Vanillic acid	HMDB0000484	C06672				
Glycerol	HMDB0000131	C00116	1.4435	0.0041	0.008	0.0735
Phosphate	HMDB0001429	C00009				
Xylose	HMDB0000098	C00181	0.9420	0.0475		
Arabinose	HMDB0000646	C00259				
Ribose	HMDB0000283	C00121	1.0558	0.0004	0.0066	0.0735
Tartaric acid	HMDB0029878	C02107				
Ribitol	HMDB0000508	C00474				
Rhamnose	HMDB0000849	C00507	2.8143	0.0035	0.0006	0.0392
Fucitol	-	-				
Fructose	HMDB0000660	C02336				
Mannose	HMDB0000169	C00159				
Galactose	HMDB0000143	C00984				
Glucose	HMDB0000122	C00031				
Mannitol	HMDB0000765	C00392				
Myoinositol	HMDB0000211	C00137		0.00038279		
Sucrose	HMDB0000258	C00089	-1.3242	0.00029247	0.0046667	0.073564

Pathway analysis results

Primary interest pathways	Total compounds	Hits	Raw p	FDR	Impact
Inositol phosphate metabolism	24	1	0.0003	0.0087	0.2513
Tyrosine metabolism	18	3	0.0093	0.0829	0.2727
Citrate cycle (TCA cycle)	20	8	0.0100	0.0829	0.4235
Glycine, serine and threonine metabolism	30	6	0.0264	0.0829	0.5341
Phenylalanine metabolism	8	3	0.0271	0.0829	0.6666
Glyoxylate and dicarboxylate metabolism	17	6	0.0310	0.0829	0.6666
Isoquinoline alkaloid biosynthesis	6	1	0.0310	0.0829	0.5
Phenylpropanoid biosynthesis	45	5	0.0352	0.0832	0.1050
Tryptophan metabolism	27	1	0.0465	0.0900	0.1705
Carbon fixation in photosynthetic organisms	21	4	0.0580	0.1025	0.1855

FDR = False Discovery Rate

Secondary interest pathways	Total compounds	Hits	Raw p	FDR	Impact
Starch and sucrose metabolism	30	3	0.01099	0.082957	0.08938
Nicotinate and nicotinamide metabolism	12	2	0.016295	0.082957	0
Phenylalanine, tyrosine and tryptophan biosynthesis	21	4	0.017265	0.082957	0
Glycerolipid metabolism	13	2	0.017675	0.082957	0
Nitrogen metabolism	15	4	0.026201	0.082957	0
Tropane, piperidine and pyridine alkaloid biosynthesis	8	2	0.02738	0.082957	0
Pyruvate metabolism	21	4	0.029161	0.082957	0.03867
Ubiquinone and other terpenoid-quinone biosynthesis	23	2	0.029335	0.082957	0
Aminoacyl-tRNA biosynthesis	67	18	0.031305	0.082957	0.09302
Glucosinolate biosynthesis	54	5	0.034755	0.083234	0
Biosynthesis of unsaturated fatty acids	42	3	0.03612	0.083234	0
Lysine biosynthesis	10	2	0.050204	0.091753	0.07407

Chapter 5 – General discussion

Your assumptions are your windows to the world. Scrub them off every once in a while, or the light won't come in.

- *Isaac Asimov*

This thesis describes NSC dynamics of New Zealand mangroves and is the first study to investigate NSC dynamics across season, latitude (Chapter 2) and also NSC responses to both drought and salinity in seedlings and mature trees (Chapters 2 and 3). Further, NSC responses to abiotic stress and the role of NSC in exchanges of metabolites between biochemical pathways due to additive or interactive effects of salinity and drought is also shown (Chapter 4). Both these abiotic stress factors may act on one or more biochemical pathways leading to either increase or decrease in metabolite abundance. In Chapter 2, I showed that leaf NSC content increases with increasing latitude in the summer in New Zealand mangroves. However, wood NSC content did not vary across latitude in my study. In Chapter 3, I manipulated NSC using light swapping treatment. The light swapping treatment resulted in plants with different NSC levels. The main finding from Chapter 3 is that plants with high NSC levels were able to maintain higher hydraulic conductivity and stem water potential in treatments with low soil water potential (high salinity – high drought). However, growth parameters remained the same between plants with high and low NSC levels in all of the drought (low, medium, high) and salinity treatments (low, medium, high). In Chapter 4, I showed differential regulation of metabolites other than NSCs in both the high and low NSC plants. A decrease in organic acid accumulation is a common mechanism of plant salinity tolerance (Sanchez et al., 2008b). The key finding of this Chapter is that, plants with high NSC had low levels of α-ketoglutarate and low NSC plants had high levels of α-ketoglutarate. Plants with high NSC also had high levels of soluble sugars which may have enhanced osmotic adjustment leading to their longer survival than plants with low NSC.

First, my work shows the role of NSCs (soluble sugars) when mangroves are exposed to different environmental conditions (seasons, drought, salinity). Accumulation of soluble sugars accumulation is important in halophytes even if they accumulate in low concentrations in comparison to nitrogenous compounds (proline, glycine betaine) which are the most common compatible solutes in *Avicennia* species (Hibino et al., 2001; Gil et al., 2013). Also, inorganic ion accumulation (maintenance of a high K^+:Na^+ ratio) is one of the mechanisms of salt tolerance and halophytes such as *Limonium latifolium* accumulate higher concentrations of these ions in comparison to organic solutes, such as soluble carbohydrates (Gagneul et al., 2007). Synthesis of organic solutes demands energy and accumulation of inorganic ions are a better option (reviewed in Gil et al., 2013). However, in the same species, when salinity increased, compartmentalisation of organic solute accumulation was altered (Gagneul et al., 2007). Therefore the role of soluble sugar (carbohydrate) accumulation in halophytic ecophysiology cannot be neglected (Gil et al., 2013) and my work contributes to this understanding.

Although research on the roles of NSC in abiotic stress has been carried out for more than 30 years, the issues of quantification of NSC still remain. Unless we resolve the methodological predicaments meaningful estimations or predictions on NSC data cannot be performed (Germino, 2015; Pinkard, 2018). Initially, when the scope of this thesis was laid out, the methodological issues were not formulated as research questions. I was aiming to follow the enzymatic hydrolysis protocol, which is conducted in more than 50% of studies related to NSCs. As the work progressed, the issues became so prominent that almost a year of work went into identifying issues and ways to solve them allowing me to dedicate a separate chapter (Chapter 2) to this work. Personally, this was the most challenging part of the work. The developed methods for NSC estimation were also used for Chapters 3 and 4.

Traditional NSC protocols (enzymatic hydrolysis) as used in most (atleast 50%) earlier ecophysiological studies did not yield satisfactory results in my specific case. All these studies have used non-halophytes, both tropical and temperate species, in understanding NSC responses to drought, defoliation and other abiotic stresses or factors. Few studies have explored NSC variations across altitude and latitude (Hoch et al., 2002, 2003; Lintunen et al., 2016). When I applied the same protocol to my study species, a halophyte, methodological issues arose (Chapter 2). In order to obtain consistent NSC data across various studies we need to reorganise the existing methodological framework (Pinkard, 2018). Few studies have explored the accumulation of soluble sugars (part of NSCs) as osmolytes in halophytes. Some studies have shown increased soluble sugar accumulation in response to increasing salinity. As discussed before, among this only some studies such as those conducted by Alla et al. (2012), Gagneul et al. (2007), Parida et al. (2002, 2004b) used halophytic species. Extrapolating the data from glycophyte responses to plant salt tolerance can be difficult. My study contributes to this knowledge gap as the study species is the halophytic New Zealand mangrove. Regarding methodology, I also attempted to use near infrared Spectroscopy (NIRS) to estimate NSC content. As discussed earlier, NIRS is preferred for its ease of use and the ability to process large numbers of samples in a small amount of time. NIRS has been the subject of many reviews, however technical papers have also established the importance of NIRS specially over the last decade (O'Reilly-Wapstra et al., 2013; Windley & Foley, 2015). Because of its versatility and ease of use, I tried to apply this method for my study species, the New Zealand mangrove. But inorder to develop a calibration model to predict NSC content using NIRS, initially part of the samples need to be analysed via an appropriate wet chemistry method. In my case, because of issues in determining an appropriate wet chemistry method, a lot of the samples could not be used for the

analyses. My data set was not as large as the one of Ramirez et al. (2015). As described in Chapter 2, instrumental glitches during the measurement period led me to remove more than 50 spectra. Chemometrics was carried out only for the remaining dataset. In order for NIRS models to become universal larger datasets collected over many years (> 20 years) are required (Cozzolino, 2010; Landhausser et al., 2018). In addition, models generated for estimating NSC content come from specific experimental conditions (Quentin et al, 2017). Also, calibration model performance differs when the reference method is changed. For example, correlating NIR data with anthrone method may yield different results in comparison to correlating GC-MS data with NIR data (Quentin et al., 2017). In my case, I tried correlating the GC-MS data with NIR data. However, because I conducted GC analyses on the samples from the end of the experiment (week 12) and only on leaves, correlating this smaller dataset with the NIR data was difficult to obtain predictive models. This dataset was even smaller than the one I had used for predicting variation of NSCs in Chapter 2. Therefore, for my work I used the model described in Chapter 2. Possibly, the correlations with my GC-MS and NIR data would have yielded different results with respect to NSC concentrations.

In my study, as described in Chapter 2, I have applied the sample handling procedures spelt out by Quentin et al. (2015) to the best possible extent, by microwaving the samples immediately after collection to stop enzymatic activity, followed by oven drying. The different drying procedures such as oven or freeze drying may also cause differences in the NSCs quantified. Quentin et al. (2015) suggest the use of the freeze drying method. However, I used oven drying due to the availability and ease of use. This is not crucial given the findings of Landhausser et al. (2018), that quantification step is more important than sample handling. NSC

quantification is further confounded by the fact that responses (sugars accumulated) may differ across plant species. Although the study of Quentin et al. (2015) covered only one species, Landhausser et al.'s (2018) study encompassed three species comprising two angiosperms, and one gymnosperm. Therefore, their finding that only the quantification step is vital is of paramount importance. For my study, as described in Chapters 2 and 3, I was interested in looking at NSC trends either across latitude, or when exposed to drought and salinity. I did not look into quantifying specific sugars, due to the methodological issues as discussed in Chapter 2. However, these protocols are time consuming and involve using expensive reagents. More so, the quantification steps can be justified based on a particular study's research questions. When estimating NSC content (soluble sugars and starch), the greatest variation in quantification occurs in soluble sugars. This is because different methods allow the quantification of different pools of sugars (Dietze et al. 2014; Gil et al., 2013; Landhausser et al., 2018). For example, sugar alcohols which are also part of the mobile carbon pool do not get quantified in spectrophotometric methods (Raessler, 2010). Studies exploring trends in sugar accumulation can do with estimating total sugars whereas studies assessing specific metabolite roles have to adhere to methods that allow quantification of specific sugars, for example, the chromatographic methods (Raessler, 2010; Raessler et al., 2011). Using HPLC technique for quantification would have yielded better soluble sugar estimates. However constraints such as non-availability of instruments and high sample processing costs made it non-feasible to adopt this method for my study (Quentin et al., 2015).

Chapters 3 and 4 of this thesis are consistent with the comprehensive study by Parida et al. (2002) on the mangrove *Bruguiera parviflora*, where changes in the

total sugar, total phenol and total amino acids contents were studied under salinity conditions. Changes in total soluble sugar content occurred only at day 45 in 400 mM salinity when a 2.5 fold increase in sugar content occurred. Additionally, they found that chlorophyll content decreased at 400 mM salinity, whereas carotenoid content did not increase. This study did not investigate the effect of sugar alcohols. Mangroves are known to accumulate sugar alcohols in addition to glycine betaine and proline for osmotic adjustment (Hibino et al., 2001; Parida & Jha, 2010). The response of NSC to experimental drought, defoliation and shade has been studied extensively (Adams et al., 2017; Galvez et al., 2013; Hartmann et al., 2013; Piper, 2011). But, only few studies have explored plant responses to interactions of drought and salinity. Most studies resort to studying plant responses to a single factor for easy interpretation of data. Though both drought and salinity cause growth reduction via water or osmotic stress, the presence of higher ratios of Na^+:Ca^{2+} for example, in the soils also causes ion toxicity and affects metabolic components of plant growth (Hu & Schmidhalter, 2005). Studying interactive effects helps us with interpreting mechanisms of mortality since plants are exposed to numerous factors in nature. In chapter 3, I showed that high NSC aids the plant in the maintenance of water potential and maximum hydraulic conductivity during high salinity coupled with high drought. However, growth parameters such as leaf area and plant height were similar in the H-NSC and L-NSC plants. One of the reasons could be less nutrients available as this was not monitored during the experimental phase. Addition of nitrogen, in my case could have increased growth rates in my experimental study (Chapter 3). Under saline conditions, plants lower growth rates before the effect of minimal nutrient supply comes in. In Chapter 4, I show the increased abundance of amino acids in L-NSC plants consistent with the study of Krapp et al. (2011). Plants may have lowered

growth rates in order to cope with the combined effects of drought and salinity, especially in the extreme treatment: high salinity – high drought.

Proline, glycine betaine, mannitol are frequently reported osmolytes in *Avicennia* species (Hibino et al., 2001; Parida & Jha, 2010). Soluble sugars (part of NSCs) are also important osmolytes in halophytes (Gil et al., 2013). My work not only shows the importance of NSC in mangroves in the maintenance of stem water potential, hydraulic conductivity and survival (Chapter 3), but also its role in regulation of other metabolites in mangroves (Chapter 4). Plants with high NSC (H-NSC) survived longer under drought and salinity treatment consistent with the study of O'Brien et al. (2014). H-NSC plants had high abundance of soluble sugars and low abundance of α-ketoglutarate (Tricarboxylic acid cycle). L-NSC plants had vice versa abundance of these two compounds. The role of soluble sugars in osmotic adjustment is well known. I have shown that the accumulation of NSC also causes increased or decreased production of other metabolites which potentially affects long term survival (Niinemets, 2016). The detection of phenolic acids may also have implications for survival of the species (for example through ROS scavenging, see Chapter 4, page 136) under extreme climate conditions. However, data collection in different seasons is required to fully understand the metabolic functions of the different compounds. In *Eucalyptus globulus* when water availability was low, both decreased phenolic concentrations and C:N ratios was shown by McKiernan et al. (2014).The same study also showed that water limitations did not reduce major terpenes and overall secondary metabolite concentrations remained unaffected whilst growth decreased. In my study also, the survival was enhanced through metabolic readjustment in plants, however, growth rates remained similar in both low and high NSC plants. Perhaps, in my study the plants accumulated soluble sugars and phenolic acids as a consequence of reduced C – sink activity, or even that the

plants actively accumulated these solutes to counteract salinity and drought effects (Flowers & Colmer, 2008; Parida & Jha, 2010; Gil et al., 2013). Additionally, mangroves have slower growth rates (Clough, 1984). Though the saline conditions were not as high as those experienced by these plants in natural conditions, metabolic readjustment may have played a significant role and the plants have decreased growth rates. A linkage of the mortality mechanisms (impaired phloem transport and hydraulic failure may have played an important role in the early death of low NSC plants (Sevanto et al., 2014; Ivanov et al., 2019; Weber et al., 2019). In my study, there is no evidence for carbon starvation (Sala et al., 2012). Hydraulic failure and decreased phloem transport may be a reason for early mortality of the L-NSC plants (Savage et al., 2015).

NSC responses have been studied in other species in response to drought under controlled conditions mainly (Hartmann et al., 2013; Maguire & Kobe, 2015; Sala et al., 2012). Some studies show leaf NSC responses, although stem/root NSC data are considered more stable. Plants may also show differences in allocation, for example, some species may accumulate more NSC in roots rather stem or leaf (Cruz & Moreno, 2001). The allocation of NSC may also differ according to ontogeny. Seedlings may exhibit different allocation patterns in comparison to mature trees. The concentrations of sugars and starch may differ in the heart and sap wood (Piispanen & Saranpaa, 2001). In silver birch, the total amount of glucose and sucrose were highest in the cambium while starch was higher in the pith (Piispanen & Saranpaa, 2001). Therefore, when assessing NSC, whole plant NSC data yields better interpretation of results. However, in mature trees obtaining root NSC is difficult and in some cases even leaves. For my seedling experiment (Chapters 3 and 4), I collected leaf, root and stem samples for NSC assessment. However, whilst solving

methodological issues a lot of the samples were sacrificed and therefore I showed only stem NSC data with enough replicates in comparison to the leaves and roots. NSC assessment in roots would have led to better interpretation of NSC allocation under drought and salinity in my study (Chapter 3).

Mangrove mortality (both in Australia and Brazil) due to extreme climatic conditions results in the loss of valuable ecosystem services (Lovelock et al., 2017; Servino et al., 2018; Sippo et al., 2018). Normalised difference vegetation index, using near infrared spectrum is a proxy to monitor temporal mangrove canopy changes. However, remote sensing of these changes over long time spans requires the construction of robust predictive models using reliable biochemical and physiological data. For NSC estimation using remote sensing techniques, the development of models using actual plant NSC wet chemistry data is essential and this data has to be collected over the long term (Asner et al., 2015). Though *Avicennia* has a widespread distribution because of its salt tolerance capacity. It can exclude salt through salt glands. Species such as *Rhizophora* spp. are less tolerant and prone to mortality (Servino et al., 2018). Interestingly, some studies show that Rhizophora has increased antioxidant activity and higher phenolic content (Suh et al., 2014. Perhaps as salinity increases, these species accumulate more phenols reducing growth and in the long term, carbon assimilation may decrease with increased ion toxicity leading to the death of the species. Similarly, in this study species, the New Zealand mangroves the accumulation of phenolic compounds may help them to cope in stressful environments.

5.1 Suggestions for further research

For future research, an improvement in methods to estimate non-structural carbohydrates (NSCs) is suggested in order to obtain a universal method that can be

applied across different research laboratories. Near infrared spectroscopy (NIRS) is a useful approach as it can be applied in many plant species and also halophytes (Ramirez et al., 2015). However, when using NIRS it is important to obtain large datasets with a sample size of 300 to 400 at least. Such large datasets will lead to robust calibration models that can be used for future studies. In addition, the wet chemistry data can be obtained via GC-MS and correlated with NIRS which will lead to better calibration models (Quentin et al., 2017). However, GC-MS and the other widely applied methods such enzymatic hydrolysis require the use of expensive chemicals ($800 for 500 µL) for c. 100 samples. If the NIRS method becomes well developed, then sample processing becomes efficient with less costs and shorter handling time. In addition, sample collection for NSC analysis across latitude may have been carried out every two months instead of just two timepoints (summer and winter). This may have led to better interpretation of NSC dynamics in New Zealand mangroves. Studying interactive effects gives us a better insight in to plant physiological functioning. However, in Chapter 3, higher replication in treatments (*n* = atleast 5) would have lead resulted in more interpretable data. Additionally, two levels of drought and salinity each (low, high) would have sufficed. Better interpretation of three and four way interactions for the physiological and growth variables could be made if the number of drought and salinity treatments were less. Mangroves have lower growth rate and therefore the treatment (drought × salinity) period of the experiment could have been extended to atleast 30 weeks. However, logistics and time were a constraint. If the experiment had run longer, in Chapter 4, samples for metabolomics analyses may have been collected every 12 weeks to better interpret the biochemical mechanisms that this species employs to counter abiotic stress (salinity, drought) tolerance. In addition, seasonal variation of metabolites could have also been carried out. This can also be done in the field

across latitude which would give us insight into the dynamics of NSCs and other metabolites under natural conditions.

Understanding plant responses to different environmental conditions is crucial, because plants are an important part of the global carbon cycle. Halophytes are remarkable species and employ biochemical, morphological, anatomical, physiological mechanisms to counteract the environmental conditions where other species cease to exist. Non-structural carbohydrates help plants to cope with different abiotic factors such as drought and salinity. This work helps us to understand the role of NSCs in New Zealand mangrove ecophysiology which will aid us to better comprehend mangrove mortality and improve global modelling efforts to predict future mangrove distribution

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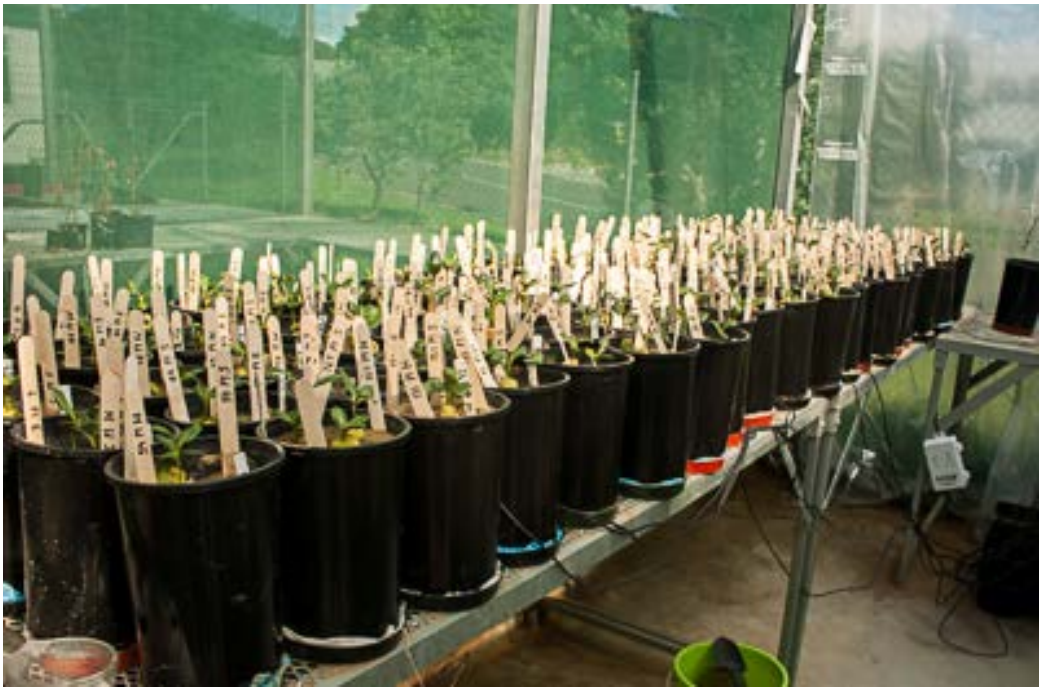
Appendix

(A)

(B)

Appendix Plate (A) and (B). During fieldwork for collection of samples for the latitudinal study.

(C)



Appendix Plate (C). High NSC plants (Plants that received ambient light conditions).

(D)

Appendix Plate (D). High NSC plants (Plants that received ambient light conditions).

(E)

Appendix Plate (E) Low NSC plants (Plants inside the shade cloth that received low light conditions).

(F)

Appendix Plate (F). Low NSC plants (Plants inside the shade cloth that received low light conditions).

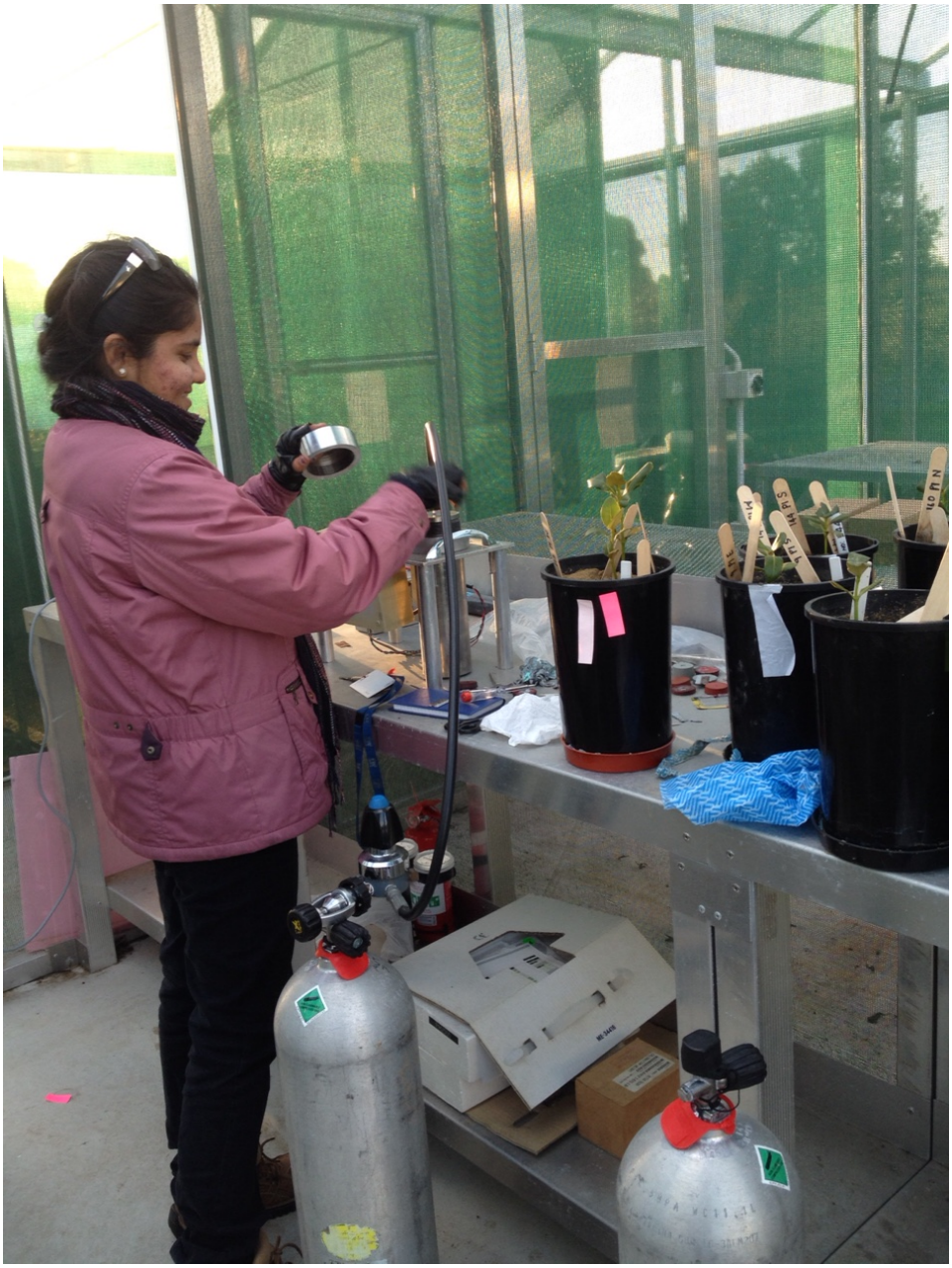
(G)

Appendix Plate (G). High NSC plants before treatment application.

(H)

Appendix Plate (H). Low NSC plants before treatment application.

(I)



Appendix Plate (I). Stem water potential measurements.

(J)

Appendix Plate (J). Seedlings during predawn stem water potential measurements.

(K)

Appendix Plate (K). During growth measurements.

(L)

Appendix Plate (L). During hydraulic conductivity measurements at the University of Waikato

Supplementary Table. 4.2 Chapter 4 - Results of 'gls' analysis Likelihood ratio test results using a backwards selection procedure applied to a generalised least squares model to test the effects of NSC, salinity, drought on each metabolite expressed. L = likelihood ratio statistic, df = degrees of freedom of the L statistic. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Metabolite	Interaction/main effect	df	L.ratio	P.value
Sucrose	nsc×salinity×drought	2	3.93	0.1395
	nsc×salinity	2	2.36	0.3071
	nsc×drought	1	2.42	0.1195
	salinity×drought	2	0.97	0.6155
	nsc	1	0.7	0.3999
	salinity	2	0.37	0.8305
	drought	1	0.01	0.9158
Arabinose	nsc×salinity×drought	2	2.43	0.2955
	nsc×salinity	2	4.1	0.1283
	nsc×drought	1	0.24	0.6204
	salinity×drought	2	1.66	0.4358
	nsc	1	3.79	0.0513
	salinity	2	0.71	0.7011
	drought	1	7.48	0.006**
Ribose	nsc×salinity×drought	2	1.39	0.4982
	nsc×salinity	2	4.67	0.0967
	nsc×drought	1	0.11	0.7398
	salinity×drought	2	0.26	0.8755
	nsc	1	5.16	0.0299*
	salinity	2	4.16	0.1249
	drought	1	6.91	0.008**
Proline	nsc×salinity×drought	2	6.6	0.0368*
	nsc×salinity			
	nsc×drought			
	salinity×drought			
	nsc			
	salinity			
	drought			
Xylose	nsc×salinity×drought	2	0.68	0.7096
	nsc×salinity	2	2	0.3654
	nsc×drought	1	0.07	0.7891
	salinity×drought	2	0.77	0.6776
	nsc	1	5.69	0.017*
	salinity	2	1	0.604
	drought	1	3.57	0.058

Metabolite	Interaction/main effect	df	L.ratio	P.value
Rhamnose	nsc×salinity×drought	2	1.95	0.3759
	nsc×salinity	2	12.93	0.001*
	nsc×drought	1	0.02	0.8702
	salinity×drought	2	1.22	0.543
	nsc	1	1.23	0.266
	salinity	2	5.59	0.0609
	drought	1	1.25	0.2627
Mannose	nsc×salinity×drought	2	1.73	0.4205
	nsc×salinity	2	3.47	0.1757
	nsc×drought	1	0.06	0.8003
	salinity×drought	2	3.45	0.1774
	nsc	1	0.008	0.9269
	salinity	2	5.56	0.0618
	drought	1	4.2	0.0403
Fructose	nsc×salinity×drought	2	0.15	0.9239
	nsc×salinity	2	9.83	0.007**
	nsc×drought	1	4.26	0.038*
	salinity×drought	2	0.26	0.8756
	nsc	1	1.49	0.222
	salinity	2	1.15	0.5616
	drought	1	1.69	0.1927
Glycerol	nsc×salinity×drought	2	4.76	0.0924
	nsc×salinity	2	5.34	0.0691
	nsc×drought	1	0.33	0.5609
	salinity×drought	2	3.77	0.1513
	nsc	1	2.04	0.1522
	salinity	2	7.08	0.0289
	drought	1	13.15	0.0002***
Galactose	nsc×salinity×drought	2	0.93	0.6259
	nsc×salinity	2	17.99	0.0001***
	nsc×drought	1	1.52	0.2168
	salinity×drought	2	0.84	0.6545
	nsc			
	salinity			
	drought			
Ribitol	nsc×salinity×drought	2	1.99	0.3681
	nsc×salinity	2	0.7	0.7027
	nsc×drought	1	0.06	0.8015
	salinity×drought	2	0.42	0.8086
	nsc	1	8.36	0.0038**
	salinity	2	5.78	0.0553

Metabolite	Interaction/main effect	df	L.ratio	P.value
Ribitol	drought	1	3.26	0.0708
Fucitol	nsc×salinity×drought	2	4.2	0.1223
	nsc×salinity	2	0.77	0.6784
	nsc×drought	1	0.77	0.3784
	salinity×drought	2	2.56	0.2778
	nsc	1	0.72	0.3953
	salinity	2	0.31	0.854
	drought	1	7.67	0.005**
Glucose	nsc×salinity×drought	2	0.55	0.7574
	nsc×salinity	2	6.86	0.0323
	nsc×drought	1	2.72	0.0988
	salinity×drought	2	1.01	0.6008
	nsc	1	2.07	0.1499
	salinity	2	1.28	0.5254
	drought	1	1.44	0.2286
Mannitol	nsc×salinity×drought	2	0.17	0.9175
	nsc×salinity	2	1.42	0.4916
	nsc×drought	1	1.02	0.3117
	salinity×drought	2	0.95	0.6212
	nsc	1	0.01	0.9193
	salinity	2	5.56	0.0617
	drought	1	4.2	0.0403*
Myoinositol	nsc×salinity×drought	2	5.32	0.0699
	nsc×salinity	2	4.27	0.1176
	nsc×drought	1	5.57	0.0182
	salinity×drought	2	1.45	0.4841
	nsc	1	1.44	0.2289
	salinity	2	5.8	0.0549
	drought	1	0.86	0.3523
Lactic acid	nsc×salinity×drought	2	2.85	0.2403
	nsc×salinity	2	0.1	0.9499
	nsc×drought	1	1.75	0.1853
	salinity×drought	2	1.16	0.5573
	nsc	1	0.09	0.7606
	salinity	2	3.06	0.2162
	drought	1	4.86	0.0274
Malic acid	nsc×salinity×drought	2	2.15	0.3407
	nsc×salinity	2	5.49	0.064

Metabolite	Interaction/main effect	df	L.ratio	P.value
Malic acid	nsc×drought	1	5.96	0.0146
	salinity×drought	2	1.41	0.4922
	nsc	1	1.93	0.164
	salinity	2	8.13	0.0171
	drought	1	4	0.0454
Tartaric acid	nsc×salinity×drought	2	2.18	0.3361
	nsc×salinity	2	5.94	0.0512
	nsc×drought	1	4.85	0.0276
	salinity×drought	2	0.47	0.7885
	nsc	1	3.23	0.0722
	salinity	2	3.51	0.1727
	drought	1	7.61	0.0057
Citric acid	nsc×salinity×drought	2	5.27	0.0721
	nsc×salinity	2	7.32	0.0256
	nsc×drought	1	5.24	0.022
	salinity×drought	2	0.47	0.7885
	nsc	1	1.55	0.212
	salinity	2	3.77	0.1516
	drought	1	2.57	0.1088
Phosphate	nsc×salinity×drought	2	3.53	0.1705
	nsc×salinity	2	8.28	0.01587*
	nsc×drought	1	4.84	0.0277
	salinity×drought	2	6.38	0.041*
	nsc	1		
	salinity	2		
	drought	1		
Asparagine	nsc×salinity×drought	2	0.15	0.9237
	nsc×salinity	2	2.99	0.2241
	nsc×drought	1	6.37	0.0116*
	salinity×drought	2	0.61	0.7368
	nsc	1	5.94	0.0147
	salinity	2	2.08	0.3518
	drought	1	8.76	0.003**
Fumaric acid	nsc×salinity×drought	2	0.92	0.6282
	nsc×salinity	2	0.77	0.6798
	nsc×drought	1	6.61	0.0101*
	salinity×drought	2	0.35	0.8389
	nsc	1		
	salinity	2		
	drought	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Glycine	nsc×salinity×drought	2	0.9	0.636
	nsc×salinity	2	0.81	0.666
	nsc×drought	1	1.54	0.2136
	salinity×drought	2	5.42	0.0663
	nsc	1	2.01	0.156
	salinity	2	5.33	0.0694
	drought	1	4.2	0.0403*
Phenylalanine	nsc×salinity×drought	2	0.75	0.6855
	nsc×salinity	2	4.7	0.0951
	nsc×drought	1	4.13	0.0421*
	salinity×drought	2	1.15	0.5617
	nsc	1		
	salinity	2		
	drought	1		
Salicylic acid	nsc×salinity×drought	2	1.66	0.436
	nsc×salinity	2	1.46	0.4807
	nsc×drought	1	0.97	0.3243
	salinity×drought	2	1.29	0.5244
	nsc	1	1.01	0.3127
	salinity	2	4.13	0.1265
	drought	1	0.04	0.83
Succinic acid	nsc×salinity×drought	2	1.55	0.4594
	nsc×salinity	2	1.58	0.4517
	nsc×drought	1	10.6	0.001**
	salinity×drought	2	4.37	0.112
	nsc	1		
	salinity	2		
	drought	1		
Citric acid (mcf derivatization)	nsc×salinity×drought	2	1.67	0.4318
	nsc×salinity	2	7.41	0.0245*
	nsc×drought	1	7.91	0.004**
	salinity×drought	2	4.41	0.1101
	nsc	1		
	salinity	2		
	drought	1		
Creatinine	nsc×salinity×drought	2	1.23	0.5386

Metabolite	Interaction/main effect	df	L.ratio	P.value
Creatinine	nsc×salinity	2	10.78	0.004**
	nsc×drought	1	3.65	0.0559
	salinity×drought	2	5.46	0.0649
	nsc	1		
	salinity	2		
	drought	1		
Cysteine	nsc×salinity×drought	2	1.41	0.4922
	nsc×salinity	2	4.34	0.1139
	nsc×drought	1	0.42	0.5168
	salinity×drought	2	0.11	0.945
	nsc	1	0.23	0.6273
	salinity	2	0.77	0.6793
	drought	1	0.78	0.3747
Dibutyl phthalate	nsc×salinity×drought	2	8.12	0.0172*
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Dehydroascorbic acid	nsc×salinity×drought	2	1.34	0.5093
	nsc×salinity	2	0.51	0.7747
	nsc×drought	1	4.38	0.0362*
	salinity×drought	2	2.85	0.2398
	nsc	1		
	salinity	2		
	drought	1		
Dodecane	nsc×salinity×drought	2	2.71	0.2576
	nsc×salinity	2	3.31	0.1907
	nsc×drought	1	0.45	0.501
	salinity×drought	2	1.42	0.4913
	nsc	1	0.04	0.8298
	salinity	2	1.28	0.5262
	drought	1	0.42	0.5148
Dodecanoic acid	nsc×salinity×drought	2	1.41	0.494
	nsc×salinity	2	1.98	0.3701
	nsc×drought	1	0.57	0.4466
	salinity×drought	2	1.06	0.5886
	nsc	1	1.25	0.2618
	salinity	2	2.55	0.2781
	drought	1	1.57	0.4099

Metabolite	Interaction/main effect	df	L.ratio	P.value
Ferulic acid	nsc×salinity×drought	2	8.16	0.0168*
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Fumaric acid	nsc×salinity×drought	2	0.92	0.6282
	nsc×salinity	2	0.77	0.6798
	nsc×drought	1	6.61	0.0101*
	salinity×drought	2	0.35	0.8389
	nsc	1		
	salinity	2		
	drought	1		
gamma-Linolenic acid	nsc×salinity×drought	2	6.97	0.0306*
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Glutamic acid	nsc×salinity×drought	2	0.51	0.7715
	nsc×salinity	2	3.67	0.1589
	nsc×drought	1	6.42	0.0112*
	salinity×drought	2	1.37	0.5016
	nsc	1		
	salinity	2		
	drought	1		
Glutamine	nsc×salinity×drought	2	1.78	0.4101
	nsc×salinity	2	3.63	0.1621
	nsc×drought	1	3.12	0.0768
	salinity×drought	2	0.75	0.6863
	nsc	1	5.79	0.0160*
	salinity	2	1.03	0.597
	drought	1	6.12	0.0133*
Glutaric acid	nsc×salinity×drought	2	4.79	0.0909
	nsc×salinity	2	0.69	0.7077
	nsc×drought	1	0.09	0.755

Metabolite	Interaction/main effect	df	L.ratio	P.value
Glutaric acid	salinity×drought	2	1.16	0.5576
	nsc	1	0.39	0.5311
	salinity	2	2.71	0.2575
	drought	1	0.38	0.5348
Glutathione	nsc×salinity×drought	2	1.33	0.5138
	nsc×salinity	2	3.42	0.1801
	nsc×drought	1	4.5	0.0337*
	salinity×drought	2	0.86	0.649
	nsc	1		
	salinity	2		
	drought	1		
Glyceric acid	nsc×salinity×drought	2	1.77	0.4119
	nsc×salinity	2	3.62	0.1633
	nsc×drought	1	1.67	0.1951
	salinity×drought	2	1.29	0.5232
	nsc	1	4.33	0.0372*
	salinity	2	12.41	0.002**
	drought	1	0.39	0.5351
Glycine (mcf derivatization)	nsc×salinity×drought	2	0.9	0.636
	nsc×salinity	2	0.81	0.6665
	nsc×drought	1	1.54	0.2136
	salinity×drought	2	5.42	0.0663
	nsc	1	2.01	0.156
	salinity	2	5.33	0.0694
	drought	1	4.2	0.0403*
Glyoxylic acid	nsc×salinity×drought	2	5.98	0.05
	nsc×salinity	2	4.34	0.1138
	nsc×drought	1	4.9	0.0267*
	salinity×drought	2	5.79	0.0552
	nsc	1		
	salinity	2		
	drought	1		
Heptadecane	nsc×salinity×drought	2	0.56	0.7526
	nsc×salinity	2	4.6	0.0998
	nsc×drought	1	5.71	0.0168*
	salinity×drought	2	3.26	0.195
	nsc	1		
	salinity	2		
	drought	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Histidine	nsc×salinity×drought	2	4.77	0.092
	nsc×salinity	2	0.29	0.8639
	nsc×drought	1	6.67	0.009**
	salinity×drought	2	0.41	0.8109
	nsc	1		
	salinity	2		
	drought	1		
Hydroxybenzoic acid	nsc×salinity×drought	2	5.34	0.0689
	nsc×salinity	2	2.15	0.3407
	nsc×drought	1	0.57	0.4481
	salinity×drought	2	2.61	0.2707
	nsc	1	2.73	0.0984
	salinity	2	2.43	0.296
	drought	1	4.85	0.0275*
Isocitric acid	nsc×salinity×drought	2	0.8	0.6703
	nsc×salinity	2	2.92	0.2315
	nsc×drought	1	2.79	0.0943
	salinity×drought	2	4.35	0.1134
	nsc	1	0.06	0.7955
	salinity	2	4.13	0.1263
	drought	1	0.87	0.3489
Isoleucine	nsc×salinity×drought	2	1.13	0.0566
	nsc×salinity	2	8.52	0.014*
	nsc×drought	1	5.23	0.0221*
	salinity×drought	2	3.33	0.1889
	nsc	1		
	salinity	2		
	drought	1		
Itaconic acid	nsc×salinity×drought	2	1.56	0.4577
	nsc×salinity	2	6.5	0.0385
	nsc×drought	1	10.15	0.001*6
	salinity×drought	2	6.09	0.0474
	nsc	1		
	salinity	2		
	drought	1		
Lactic acid (mcf derivatization)	nsc×salinity×drought	2	4.78	0.0913
	nsc×salinity	2	1.69	0.4284
	nsc×drought	1	1.82	0.1765
	salinity×drought	2	1.8	0.4048
	nsc	1	0.18	0.6711
	salinity	2	1.18	0.5528
	drought	1	0.69	0.4053

Metabolite	Interaction/main effect	df	L.ratio	P.value
Leucine	nsc×salinity×drought	2	0.43	0.805
	nsc×salinity	2	7.26	0.0264*
	nsc×drought	1	9.82	0.001**
	salinity×drought	2	4.03	0.1329
	nsc	1		
	salinity	2		
	drought	1		
Lysine	nsc×salinity×drought	2	0.74	0.6905
	nsc×salinity	2	1.25	0.5335
	nsc×drought	1	9.23	0.002**
	salinity×drought	2	0.76	0.6809
	nsc	1		
	salinity	2		
	drought	1		
Malic acid (mcf derivatization)	nsc×salinity×drought	2	0.01	0.9908
	nsc×salinity	2	0.88	0.6415
	nsc×drought	1	6.96	0.008**
	salinity×drought	2	1.17	0.5548
	nsc	1		
	salinity	2		
	drought	1		
Malonic acid	nsc×salinity×drought	2	5.76	0.0559
	nsc×salinity	2	2.32	0.3119
	nsc×drought	1	0.11	0.7361
	salinity×drought	2	2.14	0.3421
	nsc	1	5.29	0.0213*
	salinity	2	0.62	0.7331
	drought	1	12.37	0.0004***
Myristic acid	nsc×salinity×drought	2	1.48	0.4761
	nsc×salinity	2	4.06	0.1311
	nsc×drought	1	7.85	0.005**
	salinity×drought	2	1.71	0.4252
	nsc	1		
	salinity	2		
	drought	1		
Margaric acid	nsc×salinity×drought	2	0.18	0.9121
	nsc×salinity	2	4.04	0.1323

Metabolite	Interaction/main effect	df	L.ratio	P.value
Margaric acid	nsc×drought	1	7.59	0.0058**
	salinity×drought	2	1.53	0.4635
	nsc	1		
	salinity	2		
	drought	1		
NADPH	nsc×salinity×drought	2	8.63	0.0133*
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Nicotinamide	nsc×salinity×drought	2	2.91	0.2328
	nsc×salinity	2	14.35	0.0007***
	nsc×drought	1	0.005	0.9435
	salinity×drought	2	6.42	0.0401*
	nsc	1		
	salinity	2		
	drought	1		
Nonacosane	nsc×salinity×drought	2	9.86	0.007**
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Octanoic acid	nsc×salinity×drought	2	0.74	0.6879
	nsc×salinity	2	1.9	0.3855
	nsc×drought	1	0.003	0.9536
	salinity×drought	2	2.04	0.3593
	nsc	1	2.12	0.145
	salinity	2	2.13	0.3432
	drought	1	0.07	0.7778
Ornithine	nsc×salinity×drought	2	0.1	0.951
	nsc×salinity	2	0.89	0.6404
	nsc×drought	1	4.73	0.0295*
	salinity×drought	2	0.41	0.8118
	nsc	1		
	salinity	2		
	drought	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Oxalic acid	nsc×salinity×drought	2	2.62	0.2691
	nsc×salinity	2	9.45	0.008**
	nsc×drought	1	2.8	0.0938
	salinity×drought	2	1.89	0.388
	nsc	1		
	salinity	2		
	drought	1		
Oxaloacetic acid	nsc×salinity×drought	2	8.42	0.0148*
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Palmitelaidic acid	nsc×salinity×drought	2	3.71	0.1562
	nsc×salinity	2	0.54	0.7624
	nsc×drought	1	0.64	0.4208
	salinity×drought	2	5.81	0.0546
	nsc	1	0.005	0.9384
	salinity	2	0.65	0.7197
	drought	1	1.68	0.1949
Para toluic acid	nsc×salinity×drought	2	14.09	0.0008***
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Pentadecance	nsc×salinity×drought	2	4.42	0.1094
	nsc×salinity	2	0.49	0.7805
	nsc×drought	1	2.23	0.1351
	salinity×drought	2	0.51	0.7741
	nsc	1	1.4	0.2365
	salinity	2	0.2	0.9007
	drought	1	1.51	0.2189
Phenethyl acetate	nsc×salinity×drought	2	7.07	0.0290*
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Phenethyl acetate	salinity	2		
	drought	1		
Putrescine	nsc×salinity×drought	2	0.47	0.7904
	nsc×salinity	2	16.79	0.0002***
	nsc×drought	1	8.75	0.003**
	salinity×drought	2	10.68	0.004**
	nsc	1		
	salinity	2		
	drought	1		
Pyroglutamic acid	nsc×salinity×drought	2	1.23	0.5403
	nsc×salinity	2	3.45	0.1779
	nsc×drought	1	7.95	0.004**
	salinity×drought	2	1.76	0.4132
	nsc	1		
	salinity	2		
	drought	1		
Serine	nsc×salinity×drought	2	3.79	0.1503
	nsc×salinity	2	6.87	0.0321*
	nsc×drought	1	4.61	0.0316*
	salinity×drought	2	9.84	0.0072**
	nsc	1		
	salinity	2		
	drought	1		
Sinapic acid	nsc×salinity×drought	2	9.38	0.009**
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Stearic acid	nsc×salinity×drought	2	5.79	0.0552
	nsc×salinity	2	5.13	0.0767
	nsc×drought	1	9.99	0.001**
	salinity×drought	2	5.33	0.0694
	nsc	1		
	salinity	2		
	drought	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Syringic acid	nsc×salinity×drought	2	14.17	0.0008***
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Threonine	nsc×salinity×drought	2	1.34	0.5114
	nsc×salinity	2	8.56	0.0138*
	nsc×drought	1	3.46	0.0628
	salinity×drought	2	3.01	0.2217
	nsc	1		
	salinity	2		
	drought	1		
trans Cinnamic acid	nsc×salinity×drought	2	10.14	0.006**
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
trans Vaccenic acid	nsc×salinity×drought	2	5.46	0.0651
	nsc×salinity	2	0.43	0.8051
	nsc×drought	1	5.97	0.0144*
	salinity×drought	2	0.35	0.837
	nsc	1		
	salinity	2		
	drought	1		
Tricosane	nsc×salinity×drought	2	1.68	0.4302
	nsc×salinity	2	0.48	0.7849
	nsc×drought	1	0.008	0.9267
	salinity×drought	2	1.9	0.3866
	nsc	1	0.54	0.4612
	salinity	2	1.01	0.6025
	drought	1	4.29	0.0382*
Tridecane	nsc×salinity×drought	2	14.35	0.0007***
	nsc×salinity	2		
	nsc×drought	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Tridecane	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Tryptophan	nsc×salinity×drought	2	10.3	0.005**
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
Tyrosine	nsc×salinity×drought	2	1.72	0.4217
	nsc×salinity	2	3.53	0.1704
	nsc×drought	1	3.01	0.0822
	salinity×drought	2	7.12	0.0283*
	nsc	1		
	salinity	2		
	drought	1		
Valine	nsc×salinity×drought	2	2.92	0.2321
	nsc×salinity	2	12.25	0.002**
	nsc×drought	1	10.88	0.0009***
	salinity×drought	2	5.24	0.0727
	nsc	1		
	salinity	2		
	drought	1		
Vanillic acid	nsc×salinity×drought	2	5.25	0.072
	nsc×salinity	2	0.38	0.8243
	nsc×drought	1	1.13	0.2872
	salinity×drought	2	2.06	0.3554
	nsc	1	2.22	0.1359
	salinity	2	1.57	0.4556
	drought	1	1.54	0.2142
10, 13 dimethyltetradecanoic acid	nsc×salinity×drought	2	3	0.2221
	nsc×salinity	2	1.54	0.46
	nsc×drought	1	3.8	0.0509
	salinity×drought	2	1.86	0.3942
	nsc	1	0	0.9965
	salinity	2	3.59	0.1659
	drought	1	0.04	0.8276

Metabolite	Interaction/main effect	df	L.ratio	P.value
N-carboxymethyl-L--alanine	nsc×salinity×drought	2	4.63	0.0983
	nsc×salinity	2	9.96	0.006**
	nsc×drought	1	6.01	0.0141*
	salinity×drought	2	5.01	0.0816
	nsc	1		
	salinity	2		
	drought	1		
2, 4 Di-tert-butylphenol	nsc×salinity×drought	2	2.29	0.3171
	nsc×salinity	2	1.79	0.4068
	nsc×drought	1	0.16	0.6885
	salinity×drought	2	3.08	0.2139
	nsc	1	0.19	0.6622
	salinity	2	1.24	0.5379
	drought	1	0.23	0.6246
Aminoadipic acid	nsc×salinity×drought	2	3.03	0.2189
	nsc×salinity	2	16.67	0.0002***
	nsc×drought	1	13.2	0.0002***
	salinity×drought	2	5.77	0.0556
	nsc	1	0.12	0.7256
	salinity	2	2.73	0.2543
	drought	1	0.6	0.4365
Hydroxybutyric acid	nsc×salinity×drought	2	3.67	0.1592
	nsc×salinity	2	0.19	0.9073
	nsc×drought	1	0.79	0.3729
	salinity×drought	2	4.17	0.1237
	nsc	1	1.64	0.1999
	salinity	2	0.12	0.9384
	drought	1	0.53	0.4624
Hydroxyglutaramic acid	nsc×salinity×drought	2	0.03	0.9811
	nsc×salinity	2	2.44	0.294
	nsc×drought	1	0.15	0.6919
	salinity×drought	2	1.96	0.3745
	nsc	1	0.3	0.5829
	salinity	2	8.29	0.0158*
	drought	1	0.74	0.3886
Isopropylmalic acid	nsc×salinity×drought	2	0.13	0.9356
	nsc×salinity	2	1.59	0.4501
	nsc×drought	1	2.56	0.109
	salinity×drought	2	5.78	0.0554
	nsc	1	2.73	0.0981

Metabolite	Interaction/main effect	df	L.ratio	P.value
Isopropylmalic acid	salinity	2	0.6448	0.7244
	drought	1	0.71	0.3965
2-ketoglutamarate	nsc×salinity×drought	2	1.32	0.5161
	nsc×salinity	2	10.8	0.004**
	nsc×drought	1	11.19	00008***
	salinity×drought	2	5.34	0.0689
	nsc	1	0.15	0.6967
	salinity	2	0.64	0.723
	drought	1	0.96	0.3261
2-Oxoglutaric acid	nsc×salinity×drought	2	19.66	5.361e-05 ***
	nsc×salinity	2		
	nsc×drought	1		
	salinity×drought	2		
	nsc	1		
	salinity	2		
	drought	1		
2-Phosphoenol pyrUVic acid	nsc×salinity×drought	2	0.002	0.9986
	nsc×salinity	2	7.17	0.0276*
	nsc×drought	1	0.84	0.3587
	salinity×drought	2	1.61	0.4453
	nsc	1		
	salinity	2		
	drought	1		
3-methyl-2-oxo-pentanoic acid	nsc×salinity×drought	2	0.86	0.6476
	nsc×salinity	2	1.76	0.4145
	nsc×drought	1	0.93	0.3347
	salinity×drought	2	1.22	0.5425
	nsc	1	0.14	0.7075
	salinity	2	3.69	0.1573
	drought	1	0.12	0.7277
Aminobutyric acid	nsc×salinity×drought	2	1.26	0.5302
	nsc×salinity	2	1.07	0.5828
	nsc×drought	1	3.8	0.051
	salinity×drought	2	3	0.2225
	nsc	1	6.36	0.0116*
	salinity	2	1.41	0.492
	drought	1	7.61	0.0057**

Metabolite	Interaction/main effect	df	L.ratio	P.value
4-Hydroxycinnamic acid	nsc×salinity×drought	2	3.47	0.1763
	nsc×salinity	2	7.35	0.0253*
	nsc×drought	1	0.44	0.5024
	salinity×drought	2	6.53	0.0380*
	nsc	1		
	salinity	2		
	drought	1		
Alanine	nsc×salinity×drought	2	2.53	0.2822
	nsc×salinity	2	6.11	0.0470*
	nsc×drought	1	4.29	0.0382*
	salinity×drought	2	4.39	0.1109
	nsc	1		
	salinity	2		
	drought	1		
Arachidic acid	nsc×salinity×drought	2	0.34	0.8396
	nsc×salinity	2	1.16	0.5581
	nsc×drought	1	2.23	0.1346
	salinity×drought	2	1.28	0.5252
	nsc	1	0.23	0.6292
	salinity	2	9.16	0.0102*
	drought	1	0.66	0.4145
Azelaic acid	nsc×salinity×drought	2	1.37	0.5035
	nsc×salinity	2	2.31	0.3149
	nsc×drought	1	0.56	0.454
	salinity×drought	2	1.06	0.5875
	nsc	1	1.14	0.2837
	salinity	2	2.74	0.2536
	drought	1	0.74	0.3867
Benzoic acid	nsc×salinity×drought	2	1.4	0.4957
	nsc×salinity	2	4.66	0.097
	nsc×drought	1	0.09	0.7553
	salinity×drought	2	5.64	0.0594
	nsc	1	1.15	0.2818
	salinity	2	1.64	0.4404
	drought	1	2.26	0.1326
Butylated hydroxytoluene	nsc×salinity×drought	2	1.96	0.375
	nsc×salinity	2	7.55	0.0229*
	nsc×drought	1	3.61	0.0572
	salinity×drought	2	4.05	0.1319
	nsc	1		
	salinity	2		
	drought	1		

Metabolite	Interaction/main effect	df	L.ratio	P.value
Cis-aconitic acid	nsc×salinity×drought	2	1.28	0.5264
	nsc×salinity	2	9.08	0.0106*
	nsc×drought	1	8.66	0.003**
	salinity×drought	2	5.92	0.0516
	nsc	1		
	salinity	2		
	drought	1		
Citraconic acid	nsc×salinity×drought	2	1.88	0.3898
	nsc×salinity	2	2.86	0.2381
	nsc×drought	1	8.38	0.003**
	salinity×drought	2	3.93	0.1398
	nsc	1		
	salinity	2		
	drought	1		
Citric acid(mcf derivatization)	nsc×salinity×drought	2	1.67	0.4318
	nsc×salinity	2	6.61	0.0366*
	nsc×drought	1	7.1	0.007**
	salinity×drought	2	4.41	0.1101
	nsc	1		
	salinity	2		
	drought	1		
Conjugated linolenic acid	nsc×salinity×drought	2	1.7	0.426
	nsc×salinity	2	0.31	0..8554
	nsc×drought	1	5.16	0.0231*
	salinity×drought	2	1.97	0.3732
	nsc	1		
	salinity	2		
	drought	1		