The population ecology of the seagrass, *Zostera muelleri,* in south-eastern Australia: dispersal, recruitment, growth and connectivity of a marine angiosperm

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Summary

The role of seagrass systems, including those of *Zostera muelleri,* in providing critical ecosystem services including provision of nursery habitat for economically important fish species and significant nutrient cycling services are well known (Orth et al., 2006). The current understanding of the population ecology of the species is however lacking, potentially leading to management decisions that fail to incorporate the ability of *Z. muelleri* to disperse, recruit and grow as well as the role the species plays in the development of microphytobenthic communities within seagrass systems. Important abiotic (non-living) variables that influence the growth and survival of *Z. muelleri* within the marine environment include near-shore and oceanic currents, light availability, nutrients, temperature and salinity levels, with the latter being predominantly driven by changes in freshwater inputs (Kaldy et al., 2015). Biotic factors include herbivorous predation which may assist in propagule release, competition and potential facillitatory roles of existing seagrasses that may aid in the ongoing productivity of populations (Holmgren et al., 1997).

Environmental variables can have a significant influence on the ability of *Z. muelleri* seeds to germinate and develop structures, hypocotyl hairs that may aid in the attachment of germinants to the substrate. In Chapter 2, 'Germination and early-stage development in the seagrass, *Zostera muelleri* Irmisch ex Asch. in response to multiple stressors', we investigated this influence through laboratory-based assays using altered salinity, temperature and light regimes. Our findings showed that germination declined significantly with increasing

salinity concentrations. The treatments with the highest number of seeds germinating were stored under low salinity conditions under simulated burial conditions (24-h darkness) and temperatures of either 15 or 20°C. Overall, 90% of all germination occurred in treatments of salinities of 8 ppt or less, roughly ¼ the salinity of seawater. Seeds reached germination T^{50} faster under higher temperature conditions (25°C) but had a lower number of seeds germinating overall when compared to the lower temperatures of 15 and 20°C. Germination T^{50} was a measure of the germination rate, defined as the number of days for 50% of seeds that germinated to germinate A 48-h simulated freshwater pulse (0ppt) produced a minimum three-fold increase in seed germination. *Zostera muelleri* germinants did produce hypocotyl hairs. These structures were found to reach full development when stored under simulated burial conditions (24-h darkness) at 20°C and at salinity concentrations between 2 and 16ppt. Germinants held at 20°C reached the hypocotyl hair T^{50} faster than the remaining temperature treatments. Hypocotyl hair T^{50} was determined as the number of days taken for germinants that produced hypocotyl hairs to do so. Collectively, our experiments indicate that episodic freshwater events within coastal environments strongly influence germination and early-stage seedling development within *Z. muelleri.*

In Chapter 3, 'Prolonged buoyancy and viability of *Zostera muelleri* Irmisch ex Asch. vegetative fragments indicate a strong dispersal potential' we investigated the potential for asexual vegetative fragments of *Z. muelleri* to provide a propagule source for recruitment in locations far removed from source, an important factor in the species' ability to colonise new environments. To

examine the potential of *Z. muelleri* fragments to disperse within the marine environment, fragments containing rhizomes, roots and shoots were collected as beach wrack and tested for long-term buoyancy and viability. The average proportion of potentially viable fragments collected in wrack ranged from 3.6% (SD=2.23) to 11.2% (SD=5.9). Rhizome porosity was found to be high (45.2 %) indicating lacunae (gas filled spaces within rhizomatous tissues) accounted for a large proportion of total volume within rhizomes. While there was a steady decline in the buoyancy of fragments during the ten weeks of the study, initial buoyancy was relatively high with nearly 50 % of fragments remaining positively buoyant for a period of five weeks. The viability of fragments following flotation was also high. One hundred percent of fragments (n=25 per assay) expressed formazan development within roots and rhizomes following treatment with tetrazolium violet, indicating cellular activity, for each of the studies lasting for one and three weeks respectively. A significant proportion of fragments (96%) retained their viability after a flotation period of five weeks with a significantly greater proportion of root hairs expressing formazan development when compared to the shorter flotation periods. These findings indicate that the species may be capable of prolonged periods of transport dispersal within the marine environment.

Should asexual and sexual fragments of *Z. muelleri* be able to disperse within the marine environment, understanding the extent of connectivity between populations will identify whether supplementation or recolonisation of populations by immigrating fragments is possible. In Chapter 4, ‘Population connectivity in the seagrass, *Zostera muelleri* Irmisch ex Asch. within south-

eastern Australia' we investigated the connectivity of 22 populations of *Z. muelleri* along the Victorian and Tasmanian east coast in south-eastern Australia through genetic analysis of microsatellite loci. Moderate genetic variation among most populations was observed with an overall F_{ST} value, that is comparison between pairs of populations, of 0.177 ± 0.016 (S.E.). Little differentiation was accounted for by 38% of pairwise F_{ST} and only two populations were found to have no differentiation indicating a strong degree of connectivity between some of the populations sampled. Two distinct population clusters were identified, showing a clear distinction between the central Victorian populations and those of eastern Victoria and Tasmania. One site in far west Victoria was however, placed within the eastern cluster and one central site was a site of admixture between the two clusters. Populations of *Z. muelleri* showed varying degrees of connectivity. While the Bassian Isthmus, which once connected Tasmania to mainland Australia, seems to have some influence over the historical distribution of the species, contemporary oceanic and near-shore currents are allowing for the movement of propagules between populations of *Z. muelleri* within the region. Supplementation of *Z. muelleri* populations by propagules arriving from surrounding populations can positively influence the genetic diversity and ongoing survival of the species should wide scale disturbance events occur.

Following recruitment events, the morphological and physiological characteristics and therefore phenotypic plasticity of *Z. muelleri* are a consequence of the environment in which recruitment events occur. Understanding how available nutrients can influence these characteristics can

provide an indication of the environmental condition of estuaries containing populations of *Z. muelleri*. In Chapter 5, ‘Phenotypic plasticity of the seagrass, *Zoster muelleri* Irmisch ex Asch, in south-eastern Australia’ we aimed to determine whether *Z. muelleri* displays phenotypic plasticity across six Victorian estuaries of varying condition. Data were collected across eighteen populations on the following morphological and physiological characteristics: canopy height, seagrass cover, shoot density, epiphytic algae cover, chlorophyll a concentration and seagrass biomass. There was generally a negative trend in seagrass biomass and internode lengths within estuaries of higher indicative nutrient conditions, although greater cover and a higher proportion of below- to above-ground tissue were also found under these conditions. Strong and moderate correlative relationships existed between the above-ground biomass variables of canopy height, cover abundance of seagrass, shoot density, chlorophyll a concentrations and epiphytic algal growth indicating that ongoing productivity may, in fact, be density dependent. Positive correlations were found between many variables including cover abundance and shoot density, shoot density and internode length and canopy height was positively correlated with both above-ground biomass and chlorophyll a concentrations. Negative relationships were identified between some variables including canopy height and NH4+ concentrations and internode length and the proportion of below- to above-ground biomass. We found that *Z. muelleri* displayed phenotypic plasticity and the use of the guerrilla and phalanx growth strategies of clonal plants across the studied estuaries.

Seagrasses have long been considered ecosystem engineers due to their numerous influences on the development of intertidal communities including an ability to alter flow, thereby increasing sedimentation and providing substrata for other colonising species. The development and occurrence of microphytobenthic (MPB) communities within seagrass systems also provides a range of crucial ecosystem services including the provision of considerable amounts of primary production. Seagrasses and MPB alike hold a significant place in the trophic structure of intertidal systems and their ability to transfer energy to higher tropic levels, often through complex food chains, can sustain a number of economically important fish species. In Chapter 6, 'Composition of microphytobenthic communities within seagrass systems reflects environmental condition' we used metabarcoding, a DNA based approach for profiling ecological communities of targeted taxonomic groups, to examine MPB communities within populations of *Zostera muelleri* from five Victorian estuaries of varying condition. We aimed to determine the influence of *Z. muelleri* on the development of MPB community structure. We found each estuary contained a unique MBP community, with Molecular Operational Taxonomic Units (MOTU - separately identified taxon sequences) richness, evenness and diversity also varying among the estuaries. Indicator analysis identified 229 MOTUs, which were characteristic of the five estuaries at the time of sampling. The estuaries with medium-high indicative nutrients contained the highest proportions of the Families Catenulaceae (*Amphora* spp.), Triceratiaceae (*Odontella* spp.) and Plagiogrammaceae (*Plagiogramma* spp.), while the low nutrient estuary was dominated by mesohalobes of the Chaetocerotaceae Family (*Chaetoceros*

spp.). The Naviculaceae Family (*Fistulifera* spp. and *Navicula* spp.) were ubiquitous across all estuaries. The presence of both nutrient-sensitive and nutrient-tolerant species, within the estuaries provides an indication of water quality at the time of sampling. We found that MPB community structure was not influenced the by the presence of seagrass cover. Therefore, the ability to identify and use multiple taxa in determining the condition of estuarine water quality is a novel approach that can facilitate improved management outcomes within seagrass systems.

Seagrass populations are in decline globally. An average of 110 km^2 has been lost annually over the past 30 years, with habitat modification due to nutrient enrichment and increased sedimentation being considered as major causes (Waycott et al., 2009). This research addresses some of the fundamental gaps in our understanding of critical germination, recruitment, dispersal and key aspects of population dynamics of this important species as well as the important benthic communities supported by seagrass systems. These knowledge gaps, once filled, will be fundamental to our understanding of this important species and our ability to manage and protect seagrass systems into the future.

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General declaration

I, Richard Stafford-Bell, declare that the PhD thesis entitled '**The population ecology of the seagrass, *Zostera muelleri,* in south-eastern Australia: dispersal, recruitment, growth and connectivity of a marine angiosperm**' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature: [redacted]

Date: 16/02/2016

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List of publications and conference presentations

Peer reviewed publications

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Stafford- Bell, R.E., Chariton, A.A and Robinson, R.W. (2015). Prolonged buoyancy and viability of *Zostera muelleri* Irmisch ex Asch. vegetative fragments indicate a strong dispersal potential. *Journal of Experimental Marine Biology and Ecology,* 464, 52-57.

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Stafford-Bell, R.E., Chariton, A.A, and Robinson, R.W. Longevity of *Zostera muelleri* vegetative fragments: an unrecognised potential for seagrass dispersal, in Proceedings of the Australian Marine Sciences Association (AMSA), Canberra, Australia, July 2014.

Poster presentation at conferences

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List of Awards and Funding Grants

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Chapter 1

Introduction

Though the organisms may claim our prime interest, when we are trying to think fundamentally, we cannot separate them from their special environments, with which they form one physical system' Arthur Tansley (1871-1955).

For many thousands of years, the Yunyawa people of northern Australia have had a strong link to 'underwater country' in particular *ki-maramanda* or 'the place with the seagrass' (Bradley, 2014). Understanding the relationships between seagrass loss and recruitment and management of seagrass habitats has been paramount to the ongoing survival of the Yunyawa people who have passed on a deep-seated connection with seagrass environments through countless generations (Bradley, 1997). Comparatively, the importance of seagrasses to the global environment and economy including, among other things, provision of critical nursery habitat, nutrient cycling and carbon sequestration services have only recently begun to be understood.

The marine environment provides seagrasses with a diverse range of critically important factors that determine their ability to grow, reproduce, disperse and ultimately, recruit. Critical abiotic (non-living) factors include natural disturbance events such as wave action causing seagrass removal, light availability, nutrients, temperature and salinity levels that may be altered significantly due to freshwater inputs into near shore environments (Kaldy et al., 2015). Biotic factors include predation by green sea turtles (*Chelonia mydas*) and dugongs (*Dugong dugon*), which may assist in propagule release, competition and

potential facillitatory roles of existing seagrasses may aid in the ongoing productivity of populations (Holmgren et al., 1997; Orth et al., 2006).

Seagrasses are now known to utilise a number of strategies for reproduction, dispersal of propagules and recruitment in locations far removed from their origins. Thorough investigation into these facets will provide a critical tool in our ability to appropriately manage seagrass environments from naturally occurring or anthropogenic disturbance.

Seagrass taxonomy and evolution

Seagrasses are ecologically significant, highly specialised angiosperms (flowering plants) that have adapted to a marine existence throughout the world (den Hartog and Kuo, 2006). They flourish in near shore, temperate and tropical coastal regions with roughly 60 species occurring globally (Walker et al., 1999; Short et al., 2001). The limited number of plant families which are classified as seagrasses occur within the Alismatales, arguably the most primitive order of monocotyledons (Guerra, 2000). The taxonomy of some species provides much debate (e.g. Les et al., 2002; Jacobs et al., 2006), often leading to a lack of agreement within the literature on the true number of seagrass species. It is generally accepted that there are four families in which seagrasses occur. The Zosteraceae, Cymodoceaceae and Posidoniaceae consist exclusively of seagrass species while the Hydrocharitaceae has only three of the seventeen genera within this family considered as seagrasses. (Cook, 1998; Kuo and den Hartog, 2006). Although two additional families of marine plants, the Ruppiaceae and Zannichelliaceae, are on occasion, included in the seagrass literature due to similar growth forms and their aquatic

existence, their inclusion as seagrasses is often brought into question (Kuo and den Hartog, 2006).

Evolution of the seagrasses as marine species dates back to the Cretaceous (≈100mya) with fossil records of *Posidonia* (Zosteraceae) providing some of the oldest specimens (Hemminga and Duarte, 2000). More recent phylogenetic analysis using ribulose-biophosphate carboxylase gene sequences indicates evolution of seagrasses occurred from three distinct evolutionary origins (Les et al., 1997). The Zosteraceae evolved from a sister group of the primarily freshwater Potamogetonaceae / Zannichelliaceae clade that included a number of salt-tolerant species while these evolved from the freshwater families of the Scheuchzericeae and Aponogetonaceae (Les et al., 1997).

Zosteraceae distribution

There are now nine accepted species within the Zosteraceae with a broad distribution across much of the world's oceans (Les et al., 2002; Jacobs et al., 2006). They have the greatest latitudinal coverage of all the seagrasses and range from sub-Antarctic and Arctic to tropical waters (Short et al., 2001; Moore and Short, 2006). The northern hemisphere is often dominated by one species, *Zostera marina* L.*,* which forms extensive monospecific stands across much of its distribution, while species including *Z. muelleri* Irmisch ex Asch.*, Z. japonica* Asch. & Graebn. and *Z. capensis* Setch. are found growing in greater numbers within the southern hemisphere (Short et al., 2001) (Fig.1.1). *Zostera muelleri* is the dominant species found within Australian waters and inhabits much of the east coast from northern Queensland to Hobart near the southern most

Australian extent for the species. The species is also found in coastal waters of Western Australia and South Australia (ALA, 2015).

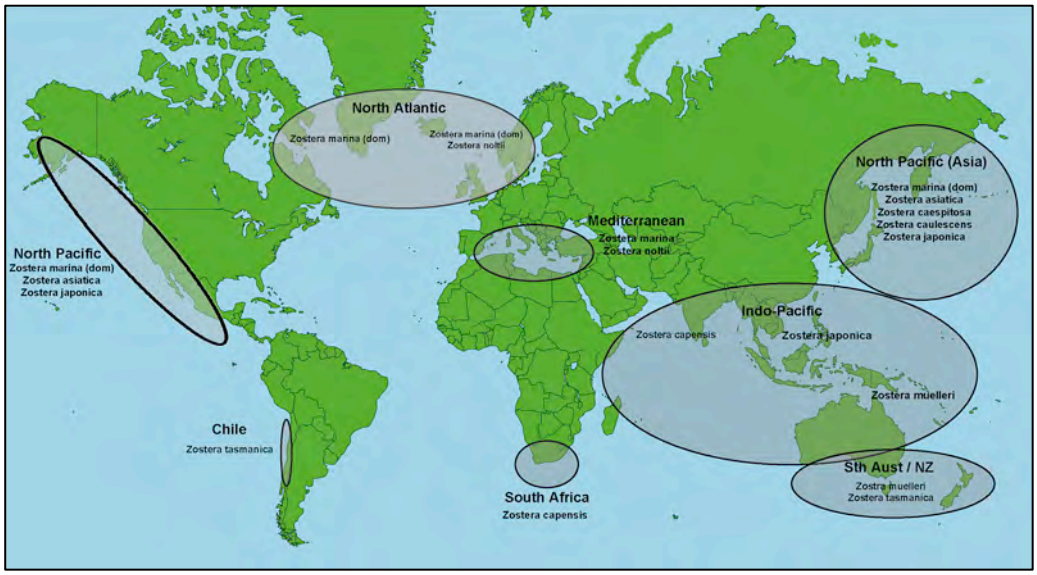

Fig.1.1: Global distribution of the Zosteraceae. Figure is adapted from Short et al. (2001)

Zostera muelleri

Zostera muelleri is predominantly an intertidal species though on occasion, it can be found in subtidal regions (to 4m) (Jones et al., 2008). Leaf blades can reach 60cm and up to 0.5cm in width with three longitudinal veins and a variable apex that can be truncate, obtuse or notched. Flowering occurs during the warmer months initiating hydrophilous pollination via the development of negatively buoyant filamentous pollen (Ackerman, 1997, 2006). The species is monoecious, having both female and male flowers on the one plant (Kuo and Den Hartog, 2001). In order to facilitate sexual reproduction between genetically different individuals and thereby reduce the likelihood of self-pollination, *Z. muelleri* utilises dichogamous pollination where the processes of pollen release

are separated from that of pollen reception by female flowers (Ackerman, 2006). Highly reduced, unisexual flowers appear on a conspicuous generative shoot (up to 50cm) in a spath/spadix formation that grows to above the canopy allowing for greater interaction with water currents (Kuo and den Hartog, 2001; Ackerman, 2006) (Fig. 1.2a). Pollen size within Zostera species is large (2700x7.5µm) when compared to the largest recorded terrestrial species, *Cymbopetalum odoratissimum* (350µm) (Ackerman, 2006; Shivanna and Tandon, 2014). Pollination of female flowers occurs via direct interception on stigmas whether this occurs immediately (direct release from male flower to female) or when water currents and localised eddies assist in the eventual interception. Measured pollen to ovule ratios in the order of 10^4:1 have previously been recorded for *Z. marina* (Ackerman, 2006) indicating a need for prolific pollen development.

Following pollination, the species is known to produce large numbers of small (≈2 mm), elongate ellipsoid seeds that are negatively buoyant. Seeds are either released directly into the water column or encased within a spathe on positively buoyant fragmented reproductive shoots, allowing for greater dispersal via marine currents (Moore and Short, 2006; Orth et al., 2006) (Fig. 1.2b).

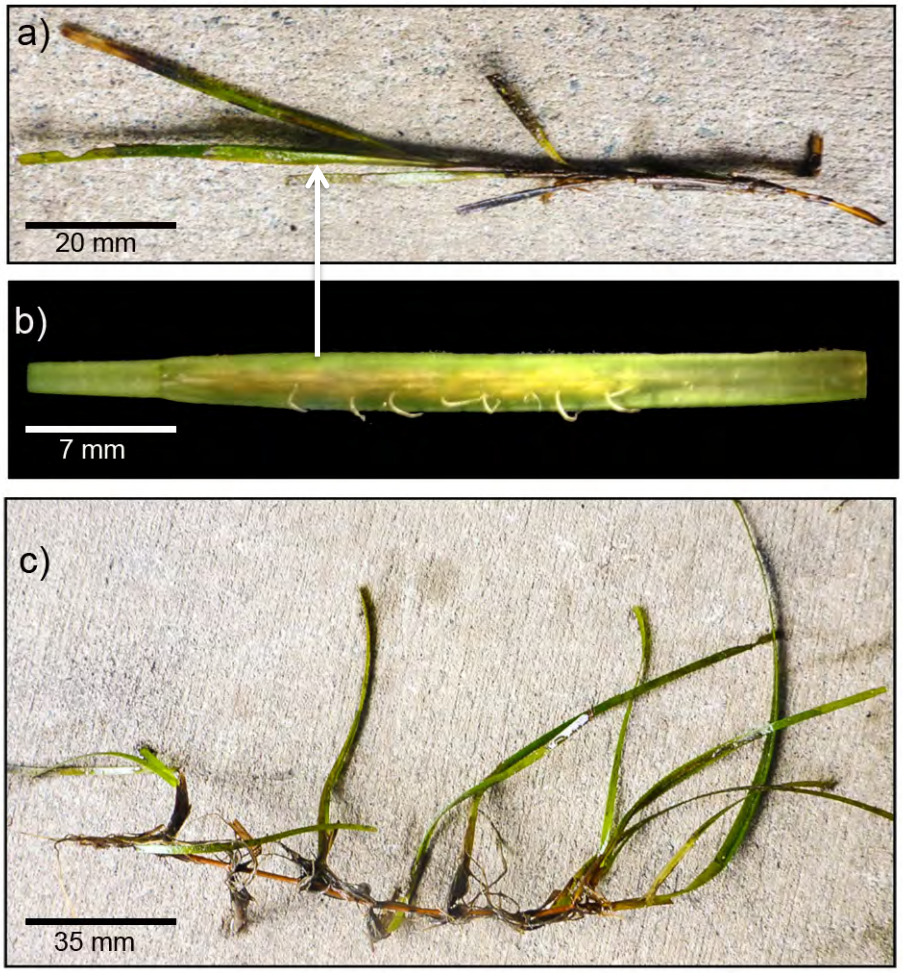

Fig. 1.2: Shoot forms of *Z. muelleri* showing: a) reproductive shoot; b) fragmented spathe containing flowers; and c) vegetative fragment containing rhizome, adventitious roots and shoots.

Embryonic development within mature seeds results in the resorbtion of the endosperm and the formation of the hypocotyl as an enlarged, curved structure (≈2 mm) that aids in positioning the developing germinant appropriately upon the substrate and also to provide stability (Cook, 1987). This feature is in direct contrast to some monocotyledonous species where the hypocotyl is an inconspicuous region which is sometimes only observable by the development of certain structures (Tillich, 2000; Bewley et al., 2013).

Zostera muelleri is a clonal plant and the establishment of extensive meadows of the species, in the order of square kilometres, is facilitated via vegetative reproduction where the horizontal extension of underground rhizomes develops new ramets (individual plants that are part of the clone) (Short et al., 2001) (Fig. 1.2c). While a mix between the two main reproductive strategies, sexual and vegetative, provides optimum growth opportunities, vegetative reproduction and rhizome encroachment increases the capacity for seagrasses to colonise bare substrates following disturbance events (Macreadie et al. (2014). Rhizomes are herbaceous and laterally compressed with a central steele, two opposite vascular bundles and cortical tissues with large lacunae (Kuo and den Hartog, 2006). The presence of lacunae (air-filled spaces within plant tissues) facilitates greater oxygen transport in species growing in waterlogged and anoxic conditions and aids in the dispersal of vegetative propagules (Coutts and Philipson, 1978; Justin and Armstrong, 1987; Stafford-Bell et al., 2015). Lacunae are continuous within *Z. muelleri* from the leaves to the roots and allow for the movement of gases throughout the entire plant. Of particular importance is the ability of the species to release oxygen, via lacunal spaces, directly into the sediment from the roots thereby creating an oxygen rich environment in very close proximity (<1mm) to roots and rhizomes (Moriarty and Boon, 1989).

Rhizomes provide four functions: anchorage to the sediment; mechanical support; nutrient storage (particularly starches); and ongoing vegetative growth (Kuo and den Hartog, 2006). Fragmentation of vegetative parts of *Z. muelleri* occurs through both natural (e.g. wave action, consumption by large herbivores) or anthropogenic (e.g. propeller scaring, dredging activities) processes

(Kenworthy et al., 2002; Greve and Binzer, 2004; Erftemeijer et al., 2006; Lanyon and Sanson, 2006). Once they become uprooted the fate of fragments is often varied and related to a number of species-specific traits or prevailing environmental conditions. For instance, fragments sink below the water column and decompose, re-establish in new environments or they can be carried ashore where they remain to decompose or are resuspended in response to local hydrological and meteorological conditions (Reusch, 2006; Oldham et al., 2010; Pattiaratchi et al., 2011).

Ecological role of *Zostera muelleri*

Seagrasses provide a multitude of benefits to estuarine systems, leading some authors to regard them as ecosystem engineers (Fig. 1.3) (Jones et al., 1994; Bos et al., 2007). *Zostera muelleri* has the ability to either directly or indirectly alter the physical state of the environment allowing for resources to become available for species that were otherwise not so (Jones et al., 1994). In particular this relates to the creation of habitats. In this role, *Z. muelleri* alters water flow and increases sedimentation, providing firm substrata for further colonisation by macroalgae and invertebrates (Bos et al. 2007). The ability of *Z. marina* leaves to eliminate water velocities has previously been identified with bare sediments having velocities up to 14cm/s greater than vegetated sites (Harlin et al., 1982). Experimental removal of mature seagrasses by the same authors also caused 25mm of the sediment to be quickly eroded due to increased water flows at the site (Harlin et al., 1982). The sediment accretion rate of a number of seagrass species has also been investigated with 7mm over

the course of three months and 2mm/yr being recorded for *Z. marina* and *Posidonia oceanica*, respectively (Gacia and Duarte, 2001; Bos et al., 2007).

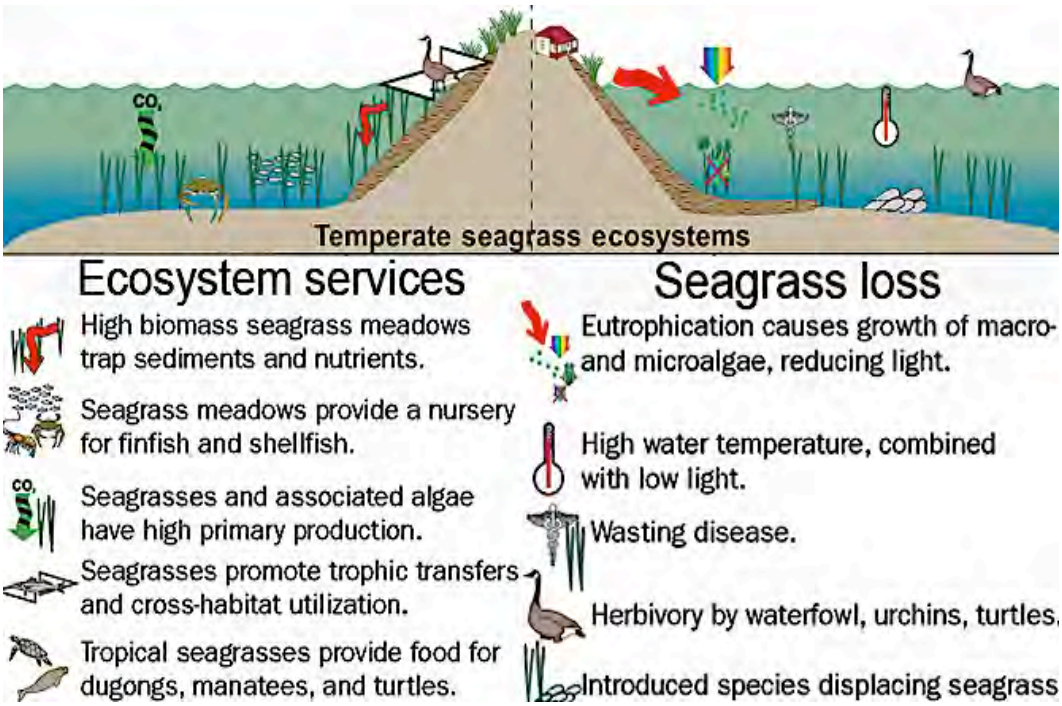

Fig.1.3: Overview of ecosystem services provided by temperate seagrasses and potential threats. Figure is adapted from Orth et al. (2006)

Although seagrasses may often be seen as the dominant ecosystem engineer within many intertidal systems, the highly complex ecological interactions between communities within these systems leads to the occurrence of cooperative ecosystem engineering (Passarelli et al., 2014). This interaction between multiple species or communities including those inhabiting the benthos, has a combined effect resulting in greater enhancement of the environmental habitat overall (Passarelli et al., 2014). Reduced water velocities as a result of the prevalence of high density growth of *Z. noltii*, can have a cascading influence on the trophic structure of the system (Short et al., 2001;

Widdows et al., 2008). Positive correlations have been identified between seagrass growth and concentrations of chlorophyll *a,* a situation indicative of increased microphytobenthic primary productivity within the substrate (Widdows et al., 2008).

Nutrient cycling services of seagrass meadows is another notable benefit and have been valued in the order of almost $2 trillion annually (Waycott et al., 2009). This primarily occurs through the acquisition of nutrients from the water column by leaves (mainly N) or from the sediment (mainly P) by rhizomes and roots (Duarte, 1995; Morris et al., 2007). Once acquired, nutrients eventually return to the environment through a number of processes including natural leaf loss or consumption by herbivores including green sea turtles (*Chelonia mydas*) and dugongs (*Dugong dugon*) (Costanza et al., 1998; Orth et al., 2006; Romero et al., 2006; Waycott et al., 2009). Export of seagrass detritus in the form of abscised leaves provides significant nutrient input into the marine system (Heck Jr et al., 2008). These inputs can occur near to the seagrass meadow potentially enriching the sediments and increasing food availability for other species (Shaw and Jenkins, 1992) or they are be carried great distances on water currents where detritus forms the basis of food webs within deep-sea canyons (Vetter, 1994).

Zostera muelleri as the foundation for primary production also supports a number of economically important marine species through provision of critical nursery habitat and protection from predators for many marine invertebrates (Walker et al. 1999; Edgar et al. 2001; Waycott et al. 2009). Numerous studies

have highlighted a preference for seagrass habitats for species such as yellowfin bream (*Acanthopagrus australis*), brown tiger prawn (*Penaeus esculentus*), blue rock whiting (*Haletta semifasciata*) and the King George whiting (*Sillaginidae punctate*) to name a few (Wassenberg, 1990; Connolly, 1994; Edgar and Shaw, 1995; West and King, 1996). Considering 2013 estimates of wild-catch production for yellowfin bream, King George whiting and tiger prawn were $3m, $6.2m and $39m respectively, the function that seagrasses provide for these industries is significant (ABARES, 2014).

Seagrass growth requirements and anthropogenic influence

Similar to their terrestrial counterparts, seagrasses require light and carbon uptake for photosynthesis and nutrients for ongoing growth (Hemminga and Duarte, 2000). Photosynthesis within the marine environment provides a number of challenges for *Z. muelleri* due to the very nature of marine waters. The ability of light to penetrate marine waters is roughly a thousand times less than through air, with inshore environments having lower light transmission than the open ocean (Hemminga and Duarte, 2000). Scattering of light particles is greatest within inshore regions due to higher concentrations of larger particles such as resuspended sediment (Carder and Costello, 1994). Light attenuation is also greatly increased in deeper water due to an increase in the absorption of light particles (Kirk, 1984). Seagrasses have high light requirements, requiring a minimum of 10% of surface irradiance, and are therefore very susceptible to changes in light availability (Abal et al., 1994; Zimmerman, 2006). This ultimately limits the depth range of Z. *muelleri* to inshore areas with a maximum

depth of around 4m (Hemminga and Duarte, 2000; Moore and Short, 2006; Jones et al., 2008).

In order to live in relatively low light environments, *Z. muelleri* has undergone a number of adaptations. Most importantly is the altering of the epidermis to be the primary site for photosynthetic activity as well as a reduction in photosynthetic cells within the leaf. A loss of stomata and greatly reduced cuticle also aids in the movement of gasses between the plant and its surrounding environment (Larkum et al., 2006; Zimmerman, 2006).

Reduction in available light can be the result of both natural (reduced light during dormant periods) or anthropogenic causes such as sedimentation causing turbidity (Hemminga and Duarte, 2000). Sediment usually enters the marine environment as runoff from the land or through erosive processes leading to a smothering of estuarine sediments causing significant impacts on local benthic communities (Thrush et al., 2004). Increased turbidity may have a significantly negative effect on permanently subtidal populations of *Z. muelleri,* however, morphological adaptations including increased leaf growth could offset this issue (Vermaat et al., 1997). Furthermore, as intertidal populations of the species are commonly exposed to periods of emersion during low tide, the likelihood of reduced photosynthesis due to increased turbidity would be further attenuated (Vermaat et al., 1997).

The movement of carbon dioxide (CO_2) mimics the reduction in available light in marine waters, with greatly reduced diffusion rates of CO_2 in the order of roughly 10,000 fold less than in air (Touchette and Burkholder, 2000).

Concentration of CO_2 in marine waters is roughly 10 nmol/m^3 at 25°C and so *Z. muelleri* utilises bicarbonate HCO_3^- as the most readily available source of inorganic carbon (Larkum et al., 2006). Early work on the species highlights the use of an active proton pump whereby inward movement of HCO_3^- is facilitated through the creation of a H^+ gradient (Beer et al., 2002).

Nutrient fluxes within the marine environment also play a critical role in the ongoing survival of seagrasses. Of particular importance is the availability of nitrogen (N) and phosphorus (P) (Walker et al., 1999; Short et al., 2001), with the majority of the former being assimilated by above-ground tissues and below-ground tissues being the major source of P uptake (Abal and Dennison, 1996). The addition of N+P has been previously found to have a positive influence on the above-ground tissues of *Z. muelleri* (Udy and Dennison, 1997) although it is generally accepted that high nutrient loads within marine systems plays a negative role in seagrass survival (Touchette and Burkholder, 2000).

Eutrophication as a result of high N loads can cause inhibition of seagrass growth and in some cases localised die offs (Touchette and Burkholder, 2000). The process of oxygen release into sediments by seagrasses from lacunal spaces within the roots and rhizomes oxidises ammonia to readily soluble nitrates (Moriarty and Boon, 1989; Touchette and Burkholder, 2000). Nitrates have been found to be assimilated quickly in some species of seagrass and the lack of product feedback inhibition provides for the continuous uptake of this nutrient (Burkholder et al., 1994). This adaptation has been suggested to be of importance for seagrasses growing within oligotrophic or nutrient-poor

environments, however, when nutrient loads approach eutrophic conditions, such an adaptation would be highly detrimental (Burkholder et al., 1994). Phosphorus is believed to be far less toxic than N due to the rapid assimilation and release of P to the surrounding water by seagrasses (absorption, translocation to leaves and release ≈ 50h in *Z. marina*) (McRoy and Barsdate, 1970). Phosphorus is readily absorbed within particulate matter and seagrass biomass and can be converted into a persistent authigenic form or organic forms such as phosphonates (Fourqurean et al., 1993; Paytan and McLaughlin, 2007). At the sediment-water boundary of marine waters, P is taken up via absorption and mineralisation with sediments that are often rich in iron and manganese (Paytan and McLaughlin, 2007). The concentration of phosphorus therefore sharply increases across the water-sediment boundary with a tripling of the nutrient concentration between the water column and the top centimetre of sediment (Sundby et al., 1992). This high concentration within the uppermost sediment layers ultimately leads to the dominance of adventitious roots over rhizomatous root structures due to their enhanced ability to uptake P (Walk et al., 2006).

Within high-nutrient environments, clonal species often utilise the phalanx growth strategy and develop shorter internodes and profuse branching indicating a consolidation of position in the environment (Slade and Hutchings, 1987). In oligotrophic environments, the opportunistic guerrilla strategy requires species to actively seek out nutrient pools through the rapid expansion of underground tissues (Doust, 1981).

The major indirect effect of eutrophication on seagrasses involves high nutrient inputs leading to an increase in both micro- and macroalgae and direct epiphytic growth on leaves (Vermaat et al., 1997). Experimental enrichment of seagrass environments has been found to have a significantly positive influence on the growth of epiphytic algae (Hughes et al., 2004; Morris et al., 2007). The prevalence of epiphytic algal growth leads to a smothering of seagrass leaves and a reduction in the photosynthetic ability of the species (Fourqurean et al., 1992; Morris et al., 2007). Many marine epiphytes, such as the *Bostrychietum* algae found within mangrove-dominated environments are also able to thrive in low-light environments as a result of their low light saturated photosynthetic rates (Raven et al., 1995). As a result, there are potentially greater impacts of increased epiphyte loads on those sites with permanently subtidal seagrass populations.

Reproduction, recruitment and connectivity of *Zostera muelleri* populations

Flowering and the process of sexual reproduction in seagrasses is restricted to a small proportion of the population (≈ 10%) and seedling mortality can be high (roughly 2% of seedlings will survive past the first year) (Hemminga and Duarte, 2000). It is therefore generally accepted that vegetative reproduction is the primary means of population maintenance (Kaldy et al., 2015) however, it is now becoming apparent that sexual reproduction plays an greater role in maintaining populations than previously thought (Kendrick et al., 2012). Sexual reproduction and recruitment of *Z. muelleri* is limited by local environmental

conditions and the influences of salinity, temperature and seed burial (Stafford-Bell et al., 2016).

Seagrasses have evolved a number of traits that allow them to sustain an entirely marine existence such as submarine development of large grains of sticky, filamentous pollen, positively buoyant reproductive shoots and fruits, and low water and osmotic potentials (Touchette, 2007; Kendrick et al., 2012). However, freshwater still plays a definitive role in the early-stage development of *Z. muelleri*. Freshwater inflows into estuarine systems are a direct function of local precipitation and the nature of the surrounding catchment, which can have varying degrees of anthropogenic influence (Gillanders and Kingsford, 2003). These inflows can occur as press (long-term) and pulse (short-term) events and although seagrasses are tolerant to a wide range of salinities (2-55ppt), growth rates have been found to be limited at these extremes, particularly during freshwater pulse events (Gillanders and Kingsford, 2003). High salinity conditions and the influence of osmotic stress on seeds of marine and estuarine species may also result in a strong inhibition of germination under these conditions (Conacher et al., 1994; Strayer and Findlay, 2010; Stafford-Bell et al., 2016). The reduced germination of some species under higher salinity conditions highlights the occurrence of niche conservatism, whereby species may still exhibit traits of their freshwater ancestors (Les et al., 1997; Wiens and Graham, 2005). Lower temperatures have, however, been found to counteract the influence of higher salinity and it is likely that variations in a number of critical environmental and physiological cues may independently or in concert

influence the germination of *Z. muelleri* seeds (Orth et al., 2000; Koch et al., 2007).

Germination of seeds of *Z. muelleri* has previously been documented (Conacher et al., 1994; Brenchley and Probert, 1998). Early-stage development of structures, hypocotyl hairs, which may aid in attachment of germinants to the sediment have, however, been ignored. Hypocotyl hairs are short lived, single celled structures that occur prior to elongation of the radicle and have been found to directly create the necessary conditions for the development of both roots and root hairs (Matsuo and Shibyama, 2002).

The function of hypocotyl hairs are three fold: (i) they assist in anchorage to the sediment/soil; (ii) they allow for the initiation of geotropism; and (iii) they potentially facilitate water uptake prior to the development of true roots and root hairs (Kuo and den Hartog, 2006; Robinson et al., 2008). Their ability to provide anchorage has been described for a number of species including *Artemisia* where soon after the hypocotyl emerges from the testa, the short-lived ring of hairs spreads out and attaches to the substrate, ensuring the elongating radicle is in direct contact with the substrate (Young and Martens, 1991). Given the dynamic nature of the environment in which *Z. muelleri* exists, the ability of hypocotyl hairs to develop upon germinants of the species and attach them to the substrate is likely to be of significant importance to the survival of *Z. muelleri* seedlings (cf. Coolidge Churchill and Riner, 1978).

The dispersal of seagrass propagules within the marine environment and their eventual re-colonisation ensures connectivity between both local and regional

populations is maintained. Dispersal of seagrass vegetative propagules, which develop through asexual reproduction, promotes further colonisation by a particular genet while the movement of and eventual colonisation of areas by seeds can enhance the genetic diversity of seagrass meadows, potentially increasing their resilience to disturbances (Procaccini et al., 2007). Connectivity science aims to identify the dispersal of individuals (propagules) and their eventual role in developing the genetic structure of far removed populations (Kool et al., 2013). Additionally metapopulation ecology, which aims to understand the flow of genes between spatially removed populations, is gaining a growing interest amongst seagrass conservationists concerned with the long-term survival of populations (Bell, 2006). Although local seagrass populations may be negatively impacted upon by anthropogenic or natural disturbance leading to local extinctions, a group of populations within a given area (a metapopulation) will persist due to the supplemental movement of vegetative and reproductive propagules from surrounding populations (Bell, 2006).

Historically, studies on the dispersal of seagrass propagules have primarily focussed on positively buoyant seeds, fruits, seedlings or spathes (in which seeds are contained prior to seed maturation and eventual release) (Fig. 1.2b) or negatively buoyant propagules such as seeds or seedlings (Harwell and Orth, 2002; Kendrick et al., 2012). As a result, much weight has been given to the role of seeds and their potential for developing new seagrass populations far from source populations. This was highlighted in a study on *Z. marina* where reproductive shoots were found to remain buoyant for 14 days and new populations were able to establish over 100 km from the closest potential

founder population (Harwell and Orth, 2002). Although it is generally accepted that seagrass seed dispersal distance may be limited if not contained within detached infructescences (Ackerman, 2006), if suitable conditions exist, the dispersal potential of vegetative fragments, containing shoots with attached rhizomes and roots, may be much greater. For example, the dispersal potential for *Z.nigricaulis* and *Z. noltii* have been estimated between several hundred to 2300 km, respectively (Berković et al., 2014; Thomson et al., 2014). Similar trends have been observed in other marine and freshwater flora, including the invasive marine alga *Caulerpa taxifolia* (Smith and Walters, 1999), the giant kelp *Macrocystis pyrifera* (Hernández‐Carmona et al., 2006), the freshwater *Elodea Canadensis* (Riis et al. 2006) and a number of species of *Ranunculus* (Johansson et al. 1993).

Although seagrass vegetative fragments have been found to remain both buoyant and viable for extended periods of time following removal from the sediment, success of transplantation studies or natural reattachment has generally been low (Di Carlo et al., 2005; Thomson et al., 2014; Stafford-Bell et al., 2015). Survivorship is therefore critical for the reestablishment of new populations and may be directly influenced by species-specific traits or prevailing environmental conditions. For some seagrass species e.g. *Posidonia australis,* the depth at which recruitment of vegetative fragments occurs is a critical factor in their long-term survival with a high majority (78%) of fragments surviving when depths exceed 10m (Campbell, 2003). However, in some *Zostera* species, survivorship subsequent to transplanting has been shown to be low with Thomson et al. (2014) reporting 100% mortality of *Z. nigricaulis*

fragments within 100 days. Artificial transplanting techniques have had greater success for *Z. marina* with 95% of transplanting surviving for greater than 90 days (Zhou et al., 2014) and 68% survival following 243 days (Kenworthy and Fonseca, 1992).

Microphytobenthic communities within seagrass systems

Seagrasses are important primary producers and contribute a significant proportion of the net oceanic productivity (≈12%) even though they only cover 0.15% of the ocean surface (Duarte and Chiscano, 1999). The protective nature and the provision of habitat by seagrasses also supports complex food webs, which involve the transfer of energy from primary producers through to tertiary consumers, within estuarine environments (Edgar and Shaw, 1995). Microphytobenthic (MPB) communities, often incorporating numerous microscopic, eukaryotic species from the supergroup SAR (stramenopiles, alveolates and Rhizaria), form a significant proportion of the benthos and are an important component of the trophic structure within seagrass systems. This is highlighted in their role as primary producers, where MPB have been estimated to contribute roughly 40-50% of estuarine productivity (Kemp et al., 1999; Underwood and Kromkamp, 1999).

Microphytobenthos are ubiquitous within both marine and freshwater environments and are capable of tolerating a broad range of environmental conditions (Buosi et al., 2011; Seckbach and Kociolek, 2011). The distribution and abundance of MPB species is often directly related to the availability of light and nutrients within the water column as well as physical stressors that influence their ability to accumulate or disperse such as wave action or

ecological interactions (predation and competition) that may reduce their abundances (Villac and Kaczmarska, 2011). Many MPB taxa are highly sensitive and respond rapidly to anthropogenic nutrient inputs making them useful bio-indicators of changes in estuarine systems, including eutrophication (Johnston and Roberts, 2009; Chariton et al., 2015). Based on sensitivities of indices (richness, diversity and evenness), contamination has been shown to have the largest influence on the species richness and diversity of marine communities, accounting for a 30-50% reduction in these indices (Johnston and Roberts, 2009). The resulting reduction in diversity following anthropogenic nutrient inputs ultimately leads to a reduction in the evenness of the communities, which shift to ones dominated by species that have a greater tolerance of the anthropogenic pollutant (Grall and Chauvaud, 2002; Hillebrand et al., 2007; Johnston and Roberts, 2009). Studies that identify MPB species that are able to tolerate high nutrient conditions are therefore able to provide a better understanding of the true health of those systems.

Identification of MPB communities has often involved labour intensive processes that include collection, separation of species from sediments and finally identification via microscopy, which requires a strong taxonomic ability (Nagy, 2011). Metabarcoding utilises DNA extraction techniques and high-throughput next generation sequencing of standard DNA regions and can allow for the identification of multiple species from a single environmental sample (Taberlet et al., 2012; Chariton et al., 2015; Coissac et al., 2016). The process can provide significant data on community composition and structure, a process

that lends itself fully to the identification of numerous sediment-bound MPB species.

Aims of this research

For the past three decades, an average of 110 km^2 of seagrass meadows have been lost annually, with habitat modification due to nutrient enrichment and increased sedimentation being considered as major causes (Waycott et al., 2009). Although the benefits that seagrasses provide to global marine systems have received extensive attention within the literature (cf. Waycott et al., 2009), investigations into the population ecology of *Z. muelleri* have been comparatively lacking. We have a very limited understanding of the connectivity of populations and means by which the species is able to disperse within the marine environment and potentially develop new populations through recruitment events. There is a need to investigate the influence of nutrient enrichment on existing populations of *Z. muelleri* to determine whether this anthropogenic stressor is negatively impacting on their growth and productivity. Furthermore, gaining an understanding of the MPB communities within populations of *Z. muelleri,* which hold a significant place in the trophic structure of seagrass systems, will ensure these populations can be managed appropriately into the future. This thesis contains five chapters, which have been written according to the requirements of specific journals with an aim to have all chapters published. Two of the chapters contained within this thesis have been published in international peer-reviewed journals, one chapter is currently under review, one under revision and one will shortly be submitted for review that address the following knowledge gaps:

Determine environmental conditions suitable for germination and early-stage development of *Z. muelleri*: This study aimed to identify the influence of salinity, temperature and light on the germination of *Z. muelleri* seeds and the development of hypocotyl hairs in the species. We hypothesised that seed germination would increase under lower salinity, simulated burial and lower water temperature conditions. We also hypothesised that *Z. muelleri* would produce hypocotyl hairs and aimed to determine how the above-mentioned variables affected the development of these structures. To investigate this, we undertook a range of germination experiments with varying salinity, temperature and light regimes and determined the conditions suitable for greatest seed germination and development of hypocotyl hairs.

Identify the potential for dispersal and recruitment of vegetative fragments of *Z. muelleri*: This study aimed to identify the potential dispersal and viability of *Z. muelleri* vegetative fragments. We hypothesised that vegetative fragments of the species could remain both buoyant and viable for extended periods of time. This was investigated by, firstly, estimating the proportion of vegetative fragments found in beach wrack that were potentially capable of regrowth. Secondly, identifying the period of time that dislodged vegetative fragments remained buoyant. Thirdly, by examining the relationship between rhizome porosity and buoyancy. And finally, by measuring the viability of vegetative fragments following extended periods of flotation.

Determine the connectivity of *Z. muelleri* populations: This study aimed to identify the connectivity of *Z. muelleri* populations along the Victorian and

eastern Tasmanian coast. Connectivity is enabled through the dispersal and eventual colonisation of either vegetative or reproductive fragments and plays an important role in ensuring gene flow among distant populations. We hypothesised that the historical barrier to gene flow for some species in the region, the Bassian Isthmus, would be mirrored across the western and eastern Victorian and Tasmanian populations of *Z. muelleri.* We further hypothesised that gene flow would occur between local populations of the species, enabling their ongoing persistence due to populations being supplemented by immigrating seagrass propagules. We examined the connectivity of 22 populations of *Z. muelleri* along the Victorian and Tasmanian east coast via DNA analysis using nine polymorphic microsatellite loci.

Identify the role of nutrient enrichment and facilitation in the growth of *Z. muelleri*: This study aimed to determine the influence of nutrient enrichment and the facillatory role of existing seagrass on the morphological and physiological characteristics and thereby, phenotypic plasticity, of established *Z. muelleri* populations. We hypothesized that low levels of nutrient enrichment ($NH4^+$and P) would have a positive influence on the phenotypic plasticity of *Z. muelleri* populations. To investigate this we determined morphological and physiological characteristics of 18 populations of the species across six south-eastern Australian estuaries with varying degrees of nutrient enrichment.

Determine the influence of *Z. muelleri* presence and environmental variables on microphytobenthic communities: This study investigated the interaction of *Z. muelleri* populations and water column nutrients ($NH4^+$and P)

on determining the structure of microphytobenthic communities within seagrass systems. We hypothesized that the presence of *Z. muelleri* would influence the structure of these communities. We also hypothesised that the nutrient status of estuaries would influence the richness, evenness, diversity and composition of these communities. To investigate this we undertook metabarcoding analysis to assess the composition of the microphytobenthos within 15 populations of *Z. muelleri* across five south-eastern Australian estuaries.

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Chapter 2

Germination and early-stage development in the seagrass, Zostera muelleri Irmisch ex Asch. in response to multiple stressors

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Germination and early-stage development in the seagrass, *Zostera muelleri* Irmisch ex Asch. in response to multiple stressors

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ABSTRACT

Episodic freshwater events within coastal environments may influence germination and early-stage seedling development within the seagrass *Zostera muelleri*. Hypocotyl hairs have the potential to provide anchorage to sediments, initiate geotropism and facilitate water uptake. To this point, production of these structures in seagrasses has received little attention in the literature though they may significantly influence their ability to maintain populations within estuarine environments. Early-stage development of *Z. muelleri* was examined under various salinity, temperature and light conditions using a fully factorial design. We found that germination rates declined significantly with increasing salinity, with the greatest germination occurring in treatments subjected to 24-h darkness at either 15 or 20 °C. Hypocotyl hair initiation was influenced by both temperature and salinity. Seeds which germinated at 20 °C in 24-h darkness had significantly more germinants developing hypocotyl hairs than the other treatments. Although the initiation of hypocotyl hairs was generally greater under higher salinity concentrations, germinants subjected to lower salinity conditions had a greater likelihood of developing fully extended hypocotyl hairs. Based on our results, freshwater pulses that occur shortly after seed maturation could initiate year round germination in temperate regions. Furthermore, germinants stored at salinities of less than 16ppt showed greater elongation of hypocotyl hairs, indicating such pulses would also aid in the development of these structures. The timing of freshwater pulses may exert control over the recruitment and long-term survival of the species.

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Germination and early-stage development in the seagrass, *Zostera muelleri* Irmisch ex Asch. in response to multiple stressors

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Abstract

Episodic freshwater events within coastal environments may influence germination and early-stage seedling development within the seagrass *Zostera muelleri.* Hypocotyl hairs have the potential to provide anchorage to sediments, initiate geotropism and facilitate water uptake. To this point, production of these structures in seagrasses has received little attention in the literature though they may significantly influence their ability to maintain populations within estuarine environments. Early-stage development of *Z. muelleri* was examined under various salinity, temperature and light conditions using a fully factorial design. We found that germination rates declined significantly with increasing salinity, with the greatest germination occurring in treatments subjected to 24-h darkness at either 15 or 20°C. Hypocotyl hair initiation was influenced by both temperature and salinity. Seeds which germinated at 20°C in 24-h darkness had significantly more germinants developing hypocotyl hairs than the other treatments. Although the initiation of hypocotyl hairs was generally greater under higher salinity concentrations, germinants subjected to lower salinity

conditions had a greater likelihood of developing fully extended hypocotyl hairs. Based on our results, freshwater pulses that occur shortly after seed maturation could initiate year round germination in temperate regions. Furthermore, germinants stored at salinities of less than 16ppt showed greater elongation of hypocotyl hairs, indicating such pulses would also aid in the development of these structures. The timing of freshwater pulses may exert control over the recruitment and long-term survival of the species.

Keywords

Germination; hypocotyl; light; salinity; seedling morphology; temperature; seagrass

Introduction

Sexual reproduction and recruitment of seagrass germinants including those of *Zostera muelleri* is limited by local environmental conditions and the influences of salinity, temperature and seed burial. This limitation is exacerbated by the fact that flowering in seagrasses is restricted to a small proportion of the population (≈ 10%) and seedling mortality can be high (roughly 2% of seedlings will survive past the first year) (Hemminga and Duarte, 2000). It is therefore generally accepted that vegetative reproduction is the primary means of population maintenance (Kaldy et al., 2015). With the advancements in genetic markers including microsatellite loci, however, it is now becoming apparent that sexual reproduction in the seagrasses plays an greater role in maintaining populations than previously thought (Kendrick et al., 2012; Stafford-Bell et al. Unpublished results).

For the past three decades, an average of 110 km^2 of seagrass meadows have been lost annually, with habitat modification due to nutrient enrichment and increased sedimentation being considered as major causes (Waycott et al., 2009). Although the benefits that seagrasses provide to global marine systems have received extensive attention within the literature (cf. Waycott et al., 2009), investigation into the processes of seed germination and development of structures that aid in the recruitment and maintenance of *Z. muelleri* populations, have been comparatively lacking.

Freshwater inflows into estuarine systems are a direct function of local precipitation and the nature of the surrounding catchment, which can have varying degrees of anthropogenic influence (Gillanders and Kingsford, 2003). These inflows can occur as press (long-term) and pulse (short-term) events and although seagrasses are tolerant to a wide range of salinities (2-55ppt), growth rates have been found to be limited at these extremes, particularly during freshwater pulse events (Gillanders and Kingsford, 2003). High salinity conditions and the influence of osmotic stress on seeds of marine and estuarine species may also result in a strong inhibition of germination under these conditions (Strayer and Findlay, 2010). While lower temperatures have been found to counteract the influence of higher salinity, it is likely that variations in a number of critical environmental and physiological cues, including seed burial may independently or in concert influence the germination of *Z. muelleri* seeds (Orth et al., 2000; Koch et al., 2007).

Zostera muelleri produces large numbers of small (≈2mm) negatively buoyant seeds which are either released directly into the water column or encased within a spathe on fragmented reproductive shoots that are positively buoyant, allowing for greater dispersal via marine currents (Moore et al., 1993; Orth, Harwell, et al., 2006). Once seeds mature and drop from the plant or spathe, the high sedimentation rates and moderate to strong localised water velocities that occur within the shallow littoral zones results in rapid burial of seeds (Marba and Duarte, 1995). The lack of light resulting from seed burial and the reducing conditions within the sediment may increase the germination of seeds of some seagrass species however the strength of this influence on *Z. muelleri* seeds requires further investigation (Orth, Harwell, et al., 2006).

While germination of seeds of *Z. muelleri* has previously been documented (Conacher et al., 1994; Brenchley and Probert, 1998), to date, the occurrence of hypocotyl hairs in the species and the environmental variables that result in the initiation of these structures have been ignored. First documented in *Eucalyptus globulus*, there are now 21 known families of angiosperms that produce these structures (Parsons, 2009). Despite this, investigation into seagrass hypocotyl hair or ‘anchoring hair’ development has only focussed on *Halophila* species (Kuo and Kirkman, 1992; Kuo and den Hartog, 2006). The function of hypocotyl hairs are three fold: (i) they assist in anchorage to the sediment/soil; (ii) they allow for the initiation of geotropism; and (iii) they potentially facilitate water uptake prior to the development of true roots and root hairs (Kuo and den Hartog, 2006; Robinson et al., 2008). The emergence of hypocotyl hairs upon the germinant occurs prior to elongation of the radicle and has been found to

directly create the necessary conditions for the development of both roots and root hairs (Matsuo and Shibyama, 2002). Their ability to provide anchorage has been described for a number of species including *Artemisia* where soon after the hypocotyl emerges from the testa, the short-lived ring of hairs spreads out and attaches to the substrate, ensuring the elongating radicle is in direct contact with the substrate (Young and Martens, 1991). Given the dynamic nature of the environment in which *Z. muelleri* exists, the ability of hypocotyl hairs to attach germinants to the substrate is likely to be of significant importance to the survival of *Z. muelleri* seedlings (cf. Coolidge Churchill and Riner, 1978).

The aim of this study was to determine conditions suitable for the germination of *Z. muelleri* seeds and the potential for hypocotyl hair development. We hypothesised that seed germination would increase under lower salinity, simulated burial and lower water temperature conditions. We also hypothesised that *Z. muelleri* would produce hypocotyl hairs and aimed to determine how the influence of salinity, simulated burial and water temperature affected the development of these structures.

Materials and Methods

Seed collection

For this initial assessment of factors that influence both seed germination and the development of hypocotyl hairs, seeds were collected from one site only (Wynnum, Moreton Bay, Queensland, Australia 27°26'3.47"S; 153°10'26.43"E) in November 2013. Collection of seed from one site ensured that potential variation amongst sites was not a confounding variable in our analyses.

Harvesting coincided with the peak flowering/fruiting period for the species (Young and Kirkman, 1975), and at a time when fertilisation had occurred but spathes had not yet abscised from the host plant and seeds had yet to drop into the sediment. Reproductive shoots containing seagrass seeds were harvested directly from the plants and following return to Victoria, were placed into a 140 L outdoor aquarium filled with recirculated artificial salt water (450 L/h; Neomarine; Brightwell Aquatics). Salinity was maintained at 32 ppt for the duration of the seed maturation period as determined through salinity modelling for the site (CSIRO, 2013). Temperature and light reflected ambient conditions. As matured seeds dropped out of the spathes they were collected via a siphon and stored in aerated seawater at a temperature of 5°C for later use.

Seed surface sterilisation

A two-stage process was used to sterilise seed surfaces prior to germination trials. Initial sterilisation treatment involved soaking seeds in 70% ethanol for a period of 2 minutes, with the seeds subsequently pipetted onto Whatman No. 4 filter paper to remove excess fluid. Seeds were then transferred to a 10% w/v sodium hypochlorite solution with three drops of Tween 20 (Sigma-Aldrich, St Louis), a non-ionic detergent, acting as the adjuvant. The seeds were swirled in this solution to ensure the Tween 20 came into contact will all surfaces and then left for a period of 5 minutes. Following sterilisation in sodium hypochlorite, seeds were triple washed in sterile distilled water and then placed on Whatman No. 4 filter paper to remove excess fluid (Sigma-Aldrich, 2014).

Germination and hypocotyl hair trials

A full factorial design was used to examine the effects and interactions of temperature, light and salinity on the germination rate (germination T^{50}) and resulting physiological development of hypocotyl hairs, including the rate of hypocotyl hair development (hypocotyl hair T^{50}). Three constant temperatures were chosen based on the mean summer (25°C) and winter (20°C) temperatures for Moreton Bay, and the average summer sea surface temperature (15°C) of Hobart, Tasmania, a site near the southernmost Australian limit of *Z. muelleri* (METOC, 2014). Two light regimes featuring a 12-h light/dark cycle or constant darkness (24-h) were selected to determine whether burial plays a role in the development of hypocotyl hairs on germinants. Although salinity has been shown to influence the germination and growth of a number of seagrass species (Conacher et al., 1994; Lirman and Cropper, 2003; Koch et al., 2007), concentration in overlying waters can vary greatly. To address this, six salinities were used ranging from fresh to marine (0, 2, 4, 8, 16 and 32 ppt).

For each treatment, 100 surface sterilised seeds were randomly placed in four replicate Petri dishes (25 seeds per replicate) on two sheets of Whatman No. 4 filter paper and covered with 5 ml of the appropriate saline solution as per the International Rules for Seed Testing (ISTA, 2011). Seeds were then incubated at the appropriate temperature and light regime and initial germination rates and development of hypocotyl hairs were recorded after 1, 2, 4, 7, and 14 d (ISTA, 2011). After two weeks of incubation, germination of seeds was documented

every 7 d, as was the presence of hypocotyl hairs. To ensure those seeds held in 24h darkness were not subjected to extended periods of light, germination and development of hypocotyl hairs was recoded on individual petri dishes under low light conditions and returned immediately to the incubators. Germination was deemed to have occurred once the hypocotyl had emerged from the shed testa and the germination rate was determined as the number of days for 50% of seeds that germinated to germinate (germination T^{50}).

Development of hypocotyl hairs

Growth of hypocotyl hairs was initially identified using an Olympus SZ3060 stereo microscope (0.9-4x). Once identified an Olympus (200x magnification) CHT 4N0031 4-100x zoom was used with images being recorded on a Panasonic Lumix DMC-FT3 digital camera for later processing. The Braun-Blanquet cover abundance scale has been widely used since its development as a means of determining the proportion of species composition within a given area (Ellenberg and Mueller-Dombois, 1974). The scale is particularly useful for providing an indicative measurement of percentage cover and abundance. The use of ranges (i.e. 50-75% cover) reduces the likelihood of unreported observer under- or overestimation in data collection. Images were assessed using the Braun-Blanquet cover abundance scale as a means of determining the extent to which the hypocotyl hairs covered the hypocotyl as well as the following developmental classes: fully extended; partially extended where the hair cell would swell but the hair would not completely elongate; and evidence of a primordia indicated by a darkening of the hair cell but with no elongation.

Development classes were given a score from the modified Braun-Blanquet cover abundance scale (+=<5% cover with few individuals, 1=<5% numerous individuals, 2=5-25%, 3=25-50%, 4=50-75%, 5=75-100%). For statistical analyses, the midpoint coverage value (0.1%, 2.5%, 15.0%, 37.5%, 62.5% or 87.5%) as descried by Wikum and Shanholtzer (1978) was used. Similar to the germination T^{50}, hypocotyl hair T^{50} was determined as the number of days taken for germinants that produced hypocotyl hairs to do so.

Influence of a freshwater pulse on seed germination

Following 20 weeks of the experiment, seeds that were yet to germinate under those treatments deemed to provide the most suitable conditions for germination of *Z. muelleri* seeds (32 ppt, 15°C, 12-h light/dark; 32 ppt, 15°C, 24-h dark; and 32 ppt, 20°C; 24-h dark) were removed from the petri dishes and underwent an experimental freshwater pulse. The freshwater pulse was simulated by placing the seeds into 200 ml glass bottles filled with distilled water (salinity of 0 ppt) for a period of 48 h. Glass bottles were wrapped in aluminium foil where necessary to continue the influence of 24-h darkness and all bottles were placed on a laboratory tube roller at 10 rpm for the duration of the simulated freshwater pulse. This process ensured the entire seed surface came into contact with the distilled water during freshwater immersion. Following the 48 h freshwater treatment, seeds were returned to replicate Petri dishes on two sheets of Whatman No. 4 filter paper and covered with 5ml of the appropriate saline solution (ISTA, 2011). The number of seeds that exhibited germination conditions was determined using the methods described.

Statistical analyses

Prior to analysis, data were tested for normality based on their symmetrical distribution using IBM SPSS version 20 (IBM, 2014). Where appropriate, data were log-transformed to meet normality requirements. Data were then analysed using univariate general linear models (GLM) with a Bonferroni correction for multiple comparisons, enabling the influence of light, temperature, salinity and potential interaction effects to be investigated on the germination of seeds, germination T^{50}, hypocotyl hair development and hypocotyl hair T^{50}; as well as the proportion of germinants with fully extended hypocotyl hairs and the extent of hypocotyl hair coverage on the hypocotyl. Tukey's honestly significant difference (HSD) post-hoc tests were used to evaluate significant differences among means (Zar, 2005).

Results

Germination and development of hypocotyl hairs

The germination of *Z. muelleri* seeds was influenced by light, temperature and salinity regimes. Interactive effects between variables were also observed with the greatest interaction occurring between salinity and temperature (Table 2.1). While there was a decreasing trend in germination under higher salinity conditions across all treatments, lower temperatures were shown to somewhat counteract this influence. There was a 14% increase (n=132) in germination within those treatments subjected to 24-h darkness as opposed to the 12-h light/dark cycle, while seeds germinated under the cooler temperature regimes, i.e. 15 and 20°C, had higher total germination rates accounting for 40 and 36%

of the total germination across all three temperatures, respectively. Post hoc tests clearly defined the three temperatures as separate subsets with a negative correlation between germination and increasing temperature. Overall, the treatment with the highest average germination (54% ±2.6%) was stored at 15°C in 24-h darkness. This treatment also had the highest individual germination of any light/temperature/salinity treatment with an average germination of 15% ±2.1% for seeds stored at a salinity of 2 ppt germinating (Fig. 2.1b). Salinity was shown to profoundly influence germination, with germination rates declining with increasing salinity (Figs. 2.1a and b). Post hoc tests also identified clearly defined subsets in regards to salinity with greatest germination occurring within those treatments stored at 0 and 2ppt, which had similar results. Germination then declined somewhat under the 4 and 8ppt treatments then progressively more so under 16ppt and 32ppt, which had the lowest germination for all salinity treatments. Overall 90% of all germination occurred in treatments of salinities of 8 ppt or less.

Table 2.1: Summary of general linear model (GLM) indicating significant influence of light, temperature and salinity on germination and development of hypocotyl hairs in *Z. muelleri.* Full model outputs are provided as supplementary material in Appendix 1.

Process	Variable	Type III Sum of squares	df	Mean square	F	Sig.
Germination	Light	484	1	484	22.4	<0.001
	Temp	984	2	492	22.8	<0.001
	Salinity	7325	5	1465	67.8	<0.001
	Light*Temp	204	2	102	4.7	0.36
	Temp*Salinity	729	10	73	3.4	0.34
Germination T^{50}	Temp	17149	2	8575	69.3	<0.001
	Temp*Salinity	5064	10	506	4.1	0.18
Hypocotyl hair (HH)	Temp	0.6	2	0.300	12.1	0.002
	Salinity	6.48	5	1.29	52.1	<0.001
HH T^{50}	Light	544	1	544	6.43	0.030
	Temp	852	2	426	5.03	0.031
	Salinity	3114	5	623	7.36	0.004
HH extent	Light	2108	1	2108	21.1	0.001
	Temp	1277	2	639	6.40	0.016
	Salinity	15688	5	3138	31.4	<0.001
Freshwater pulse	Salinity	4506	5	901	5.5	0.002

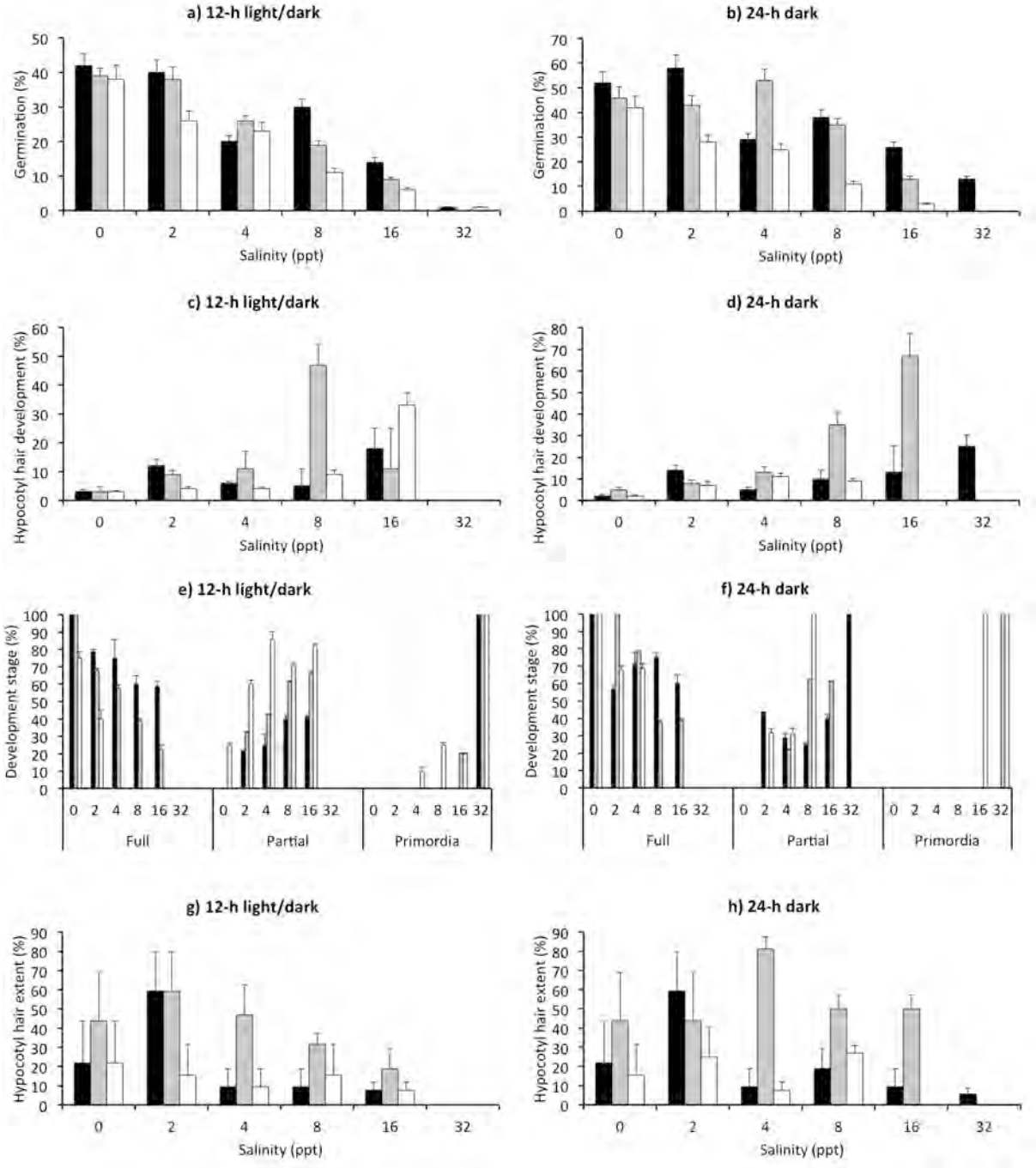

Fig. 2.1: Seedling development in *Z. muelleri* subjected to six salinity regimes (0, 2, 4, 8, 16 and 32 ppt), three temperatures (15 (black bars), 20 (grey bars) and 25°C (white bars)) and either 12-h light dark cycle or 24-h darkness featuring: mean percentage germination (Figs. a and b); mean percentage hypocotyl hair production (Figs. c and d); development stage of hypocotyl hairs (Figs. e and f); and the extent to which the hypocotyl was covered in hypocotyl hairs (Figs. g and h).

Initiation of hypocotyl hairs occurred across the majority of the convex surface of the hypocotyl in a region surrounding the site of radicle extension (Figs. 2.2a and c) and was influenced by both temperature and salinity regimes (Table 2.1). Interactive effects between temperature and salinity (Table 2.1) led to germinants held at 20°C in 24-h darkness having greater initiation of hypocotyl hairs under higher salinity concentrations. Post hoc tests indicated that the seeds which germinated at 20°C had more germinants developing hypocotyl hairs (range: 0-67%) than those held at 15°C (range 2-25%) and 25°C (range 0-33%) (Figs. 2.1c and d). Post hoc tests did not identify clearly defined subsets in regards to salinity, however the greatest development occurred under the 8 and 16ppt treatments.

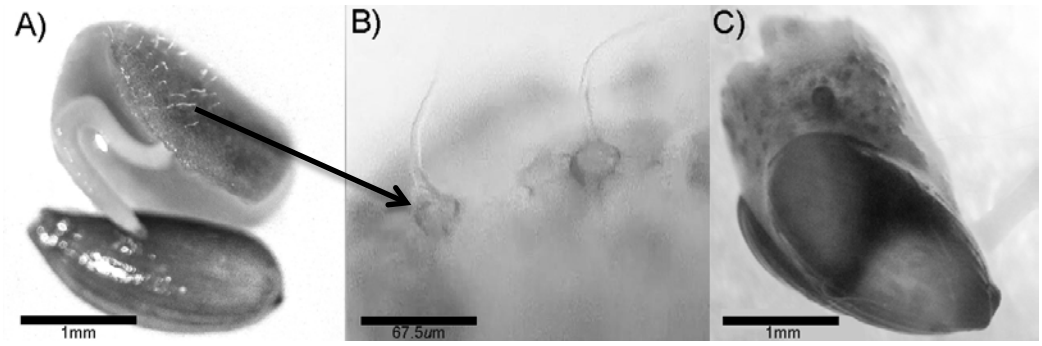

Fig. 2.2: Development of hypocotyl hairs on convex surface of the hypocotyl of *Z. muelleri* showing fully extended hairs at a) 40x and b) 200x magnification and primordia indicated by the darkening and swelling of the hair cells at c) 40x magnification.

The average number of germinants initiating hypocotyl hairs was generally greater under higher salinity concentrations (Figs. 2.1c and d). However, these

structures were not considered to be fully functioning with either primordia development (Fig. 2.2c) or partial extension occurring in roughly 40% of cases. Development of hypocotyl hairs in the 8 and 16ppt treatments accounted for 42% of all germinants developing these structures across all treatments. In common with the germination trials, the reduced number of seeds that germinated under the 32 ppt salinity concentration influenced the results pertaining to hypocotyl hair development within those treatments (Figs. 2.1c and d).

Development of fully extended hypocotyl hairs was strongly influenced by temperature and salinity regime (Table 2.1). The influence of temperature saw greater hypocotyl hair elongation under the 20°C treatment, although post hoc tests showed germinants stored at 15°C had similar elongation. Salinity was identified as having a dichotomous influence on the development of fully extended hypocotyl hairs with germinants stored between 2 and 16ppt having a greater likelihood of developing fully extended hypocotyl hairs than those at 0 and 32ppt (Figs. 2.1e and f). This relationship was, however, not linear and post hoc tests failed to identify clear subsets.

The extent of the hypocotyl over which hair development occurred was also influenced by temperature and salinity (Table 2.1). In common with the conditions suitable for elongation of hypocotyl hairs, salinity was deemed to have the greatest influence, with germinants stored under lower salinity conditions having a greater extent of hypocotyl hair covering. Post hoc tests showed germinants stored between 0 and 8ppt had the greatest coverage of

hypocotyl hairs, with the greatest development occurring within the 2ppt treatment. In contrast to the elongation results, germinants stored at 20°C had the greatest extent of hypocotyl hair coverage (Figs. 2.1h and i) and post hoc tests confirmed this.

Germination T^{50} and hypocotyl hair T^{50}

Germination rate was predominately influenced by light, temperature and salinity with seeds held at 25°C and lower salinities (0ppt and 2ppt) having a higher initial germination rate but lower overall germination. In contrast, seeds held at 15°C had a slower germination rate but a greater number of seeds germinating throughout the study. Seed germination was however, not synchronous with 50% of all germination occurring within the initial four weeks.

The number of days taken for the germination of 50% of the seeds (germination T^{50}) was influenced by light, temperature and salinity, however, there were also interactions between the variables with the greatest interaction occurring between salinity and temperature (Table 2.1). Overall, seeds stored at the higher temperatures (25°C and 20°C) reached germination T^{50}s faster than seeds stored at 15°C, with post hoc tests identifying a clear relationship. Average germination T^{50}s for the 25, 20 and 15°C treatments were 5, 22 and 58 d respectively (Fig. 2.3a and b). There was a general trend of increasing time to reach germination T^{50} under higher salinity conditions regardless of the temperature at which seeds were stored, indicating osmotic stress was an influencing factor. Post-hoc tests identified clear differences among some treatments with highest germination T^{50} s occurring under the 4, 8 and 16ppt

treatments. The average germination T^{50} for 0-16 ppt treatments ranged from 18 to 31 d with a maximum of 35 d for the 8 ppt treatment. Results of the 32 ppt treatment found average germination T^{50}s of 98 d, however, three of the treatments failed to achieve any germination (20°C, 12-h light/dark; 20°C, 24-h dark; 25°C, 24-h dark), which confounded the resulting germination T^{50} for this salinity concentration.

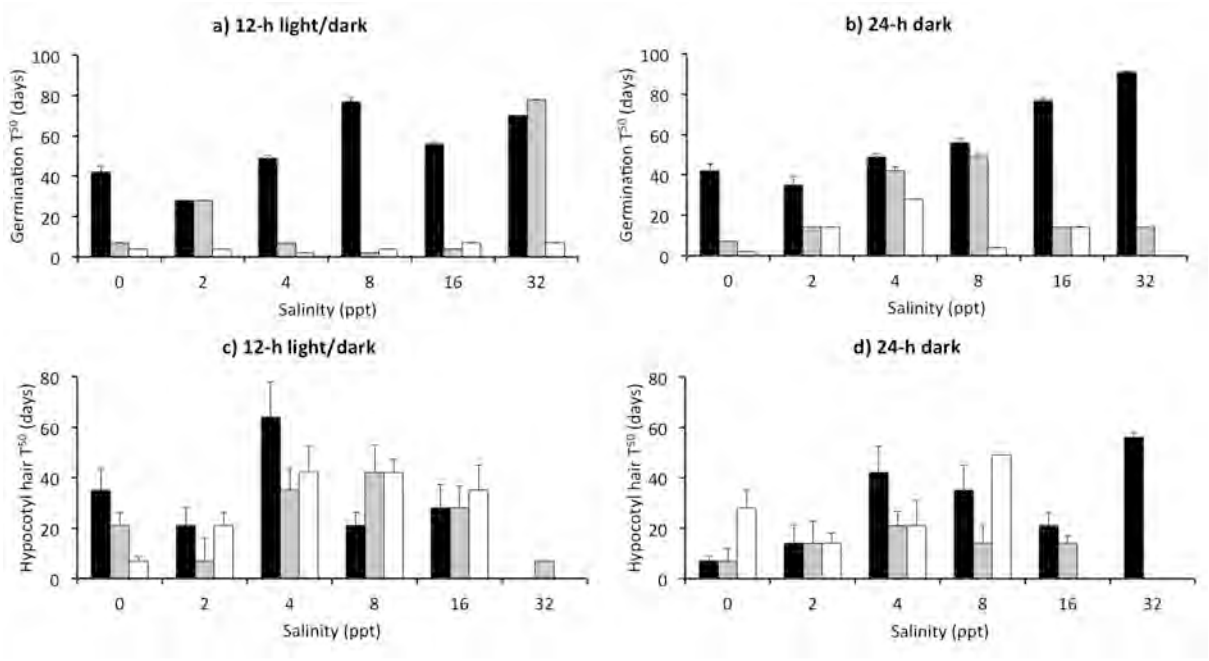

Fig. 2.3: Mean number of days to germination of 50% of all germinated seeds (germination T^{50}) (Figs. a and b) and number of days to hypocotyl hair development of 50% of all germinants with these structures (hypocotyl hair T^{50}) (Figs. c and d) subjected to six salinity regimes (0, 2, 4, 8, 16 and 32 ppt), three temperatures (15 (black bars), 20 (grey bars) and 25°C (white bars)) and either 12-h light dark cycle or 24-h darkness.

The number of days taken to develop hypocotyl hairs of 50% of all germinants with these structures (hypocotyl hair T^{50}) was influenced by temperature. However, there was significant interaction between the variables with the

greatest interaction occurring between salinity and temperature (Table 2.1). Hypocotyl hair T^{50} was reached marginally faster (17 versus 19 d) (Table 2.1) in treatments that simulated sediment burial (24-h darkness) as opposed to the treatments that simulated natural light exposure (12-h light/dark cycles). As 20°C was deemed to be the optimum temperature for hypocotyl hair development (Figs. 2.1c and d), germinants held at this temperature also produced hypocotyl hairs faster (12 days) than those held at 15°C (28 d) and 25°C (18 d) (Figs. 2.3 c and d). When the influence of salinity was taken into account, hypocotyl hair development and therefore hypocotyl hair T^{50} were directly influenced by the germination rate of the seeds themselves (Figs. 2.1a and b). The general trend of delayed germination under increasing salinity concentrations therefore had a negative influence on the speed of hypocotyl hair development in higher salinity concentration treatments.

Influence of a freshwater pulse on seed germination

The simulated 48-h freshwater pulse had a strong influence on the germination of *Z. muelleri* seeds previously stored at a salinity of 32 ppt (Table 2.1) (Fig. 2.4). This was most evident in the treatments held at 15°C which saw a 37-fold and three-fold increase in the number of germinated seeds for the 15°C, 12-h light/dark and 15°C, 24-h dark treatments respectively. Subsequent to the freshwater pulse, germination of seeds within the 20°C, 24-h dark treatment increased by 15 fold (Fig. 2.4).

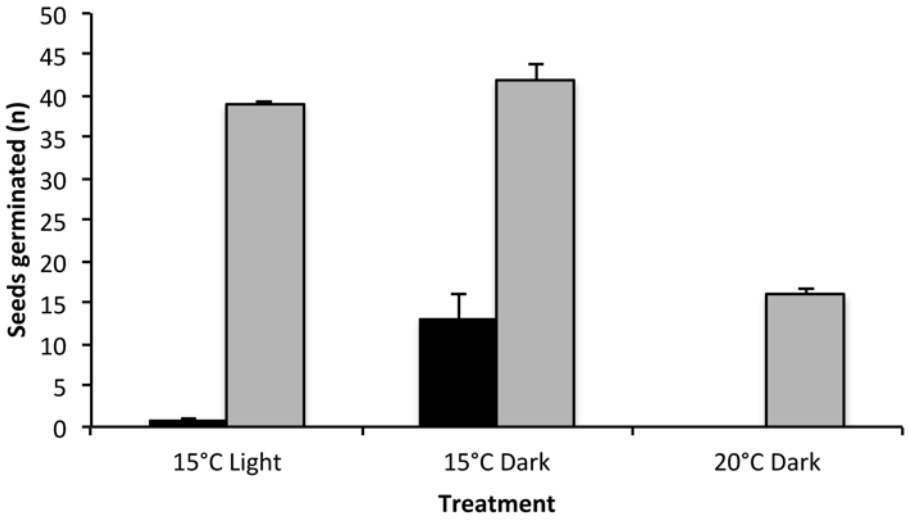

Fig. 2.4: Number of *Z. muelleri* seeds initially stored at a salinity of 32 ppt under the two temperature (15 and 20°C) and light (12-h light/dark, 24-h dark) regimes before and after an initiated freshwater (0 ppt) pulse for a period of 48 h. The black bars represent germination prior to the freshwater pulse and the grey bars represent germination post freshwater pulse.

Discussion

The duration and frequency of freshwater pulses, lower water temperatures and seed burial within the marine environment may have a profound influence on the germination and early-stage development of seeds of *Z. muelleri.* We found that germination declined significantly with increasing salinity, with the greatest germination occurring at lower salinity concentrations (<8 ppt) in treatments subjected to simulated burial (24-h darkness) and at temperatures of either 15 or 20°C. Development of fully extended hypocotyl hairs upon the hypocotyl was similarly influenced by salinity, simulated burial and temperatures with seeds germinating at salinity concentrations of 2-16ppt, in 24-h darkness and at a

temperature of 20°C having a greater likelihood of developing fully extended hypocotyl hairs.

Development of hypocotyl hairs in Z. muelleri

The hypocotyl and root collar or collet is an inconspicuous region in most monocotyledonous species, sometimes only observable by the development of hypocotyl hairs (Tillich, 2000). *Zostera muelleri* germinants, however, produce an enlarged, curved structure (≈2 mm) that would provide stability while positioning the developing germinant appropriately upon the substrate (Cook, 1987). In studies of the majority of species that produce hypocotyl hairs, it has been found that these short-lived, single celled structures usually develop in a ring formation at the base of the hypocotyl (Robinson et al., 2008; Parsons, 2009). When fully formed, hypocotyl hairs in *Z. muelleri* developed across the majority of the convex surface of the hypocotyl in a region surrounding the point of radicle extension (Tillich, 2000) (Fig. 2.2a and c). Similar development has been found to occur in *Halophila ovalis* (Kuo and Kirkman, 1992).

The influence of salinity

The *Zosteraceae,* which evolved as a marine species between 70 and 100 million years ago, is descended from a sister taxon of primarily freshwater species within the Potamogetonaceae / Zannichelliaceae clade of monocotyledons (Les et al., 1997; Orth, Carruthers, et al., 2006). While seagrasses have evolved a number of traits that allow them to sustain an entirely marine existence such as submarine development of large grains of sticky, filamentous pollen, positively buoyant reproductive shoots and fruits and

low water and osmotic potentials (Touchette, 2007; Kendrick et al., 2012), freshwater still plays a definitive role in the early-stage development of *Z. muelleri*.

The influence of reduced salinity conditions on germination can be clearly seen in our results with 90% of *Z. muelleri* germinants occurring in treatments of salinities of 8 ppt or less. Results of the experimental freshwater pulse provide further support for this argument with all treatments having a minimum three-fold increase in germination following the 0 ppt 'pulse' treatment. This significant increase in germination following a freshwater pulse has also been observed in *Z. marina* (60% increase) and *Z. japonica* (55% increase) (Yamaki et al., 2006; Kaldy et al., 2015).

In contrast to our germination results, we found that under the optimum conditions for hypocotyl hair initiation (20°C; 24-h darkness), higher salinity concentration actually increased hypocotyl hair production (Figs. 2.1c and d). However, this greater proportion of germinants displaying hypocotyl hairs was confounded by the fact that, under higher salinity conditions, the hypocotyl hair cells not only displayed stunted growth, (Fig. 2.2c), but the extent to which hypocotyl hairs developed across the surface of the hypocotyl was also greatly reduced (Figs. 2.1g and h). Lack of hypocotyl hair extension is likely a result of osmotic stress occurring within the hair cell, whereby higher salt ion concentrations in the surrounding environment leads to drawing out of water from the cell; ultimately resulting in the reduced elongation or even destruction of the hypocotyl hair (Kozlowski, 1997; Touchette, 2007).

While it is unlikely for *Z. muelleri* to be subjected to long-term conditions where salinity levels drop as low as 0 ppt, the highly dynamic nature of estuaries, rainfall events and floods may lead to local populations being subjected to a salinity range as great as 0.5-35 ppt (Eyre and Ferguson, 2006; Lee et al., 2006; Jordan, 2012). The influence of rainfall on reducing estuarine sediment salinity concentrations has been previously identified with measured salinities of <10ppt being recorded following periods of inundation (Coles et al., 1989; Dunton et al., 2001; Noe and Zedler, 2001). *Zostera muelleri* is able to grow both intertidally and subtidally with freshwater inputs arising from different sources having similar influences. Within the intertidal it is likely that simple rainfall events during periods of emersion would be sufficient for germination to be initiated, in fact, our results suggest that at lower salinities (0-16ppt), germination is initiated within 1 day. Conversely, subtidal populations of *Z. muelleri* would be more affected by ground water discharge into the pore waters of near-shore estuarine sediments which has been found to dramatically reduce salinity concentrations in the upper sediments (Miller and Ullman, 2004). The reduced salinity conditions following either of these events (rainfall or ground water seepage) would also aid in the development of fully extended hypocotyl hairs that can aid in the attachment of germinants to the sediment (Kuo and den Hartog, 2006).

The influence of temperature

Temperature was found to have both positive and negative influences on the germination of *Z. muelleri* seeds. Seeds stored at 25°C germinated several

times faster than those stored under cooler conditions (15 and 20°C) and it may be hypothesised that higher water temperatures may have a direct influence on the rapid exhaustion of seed banks. However, while seeds germinated faster at 25°C, the number of germinants across the 20 w period was lower at this temperature in comparison to the 15 and 20°C treatments. These results are supported by research on the germination of *Z. marina* and *Z. japonica* seeds whereby germination initiation and number of seeds germinating was greatest under low temperature conditions of roughly 15 to 20°C (Abe et al., 2008; Kaldy et al., 2015).

The influence of temperature on the development of hypocotyl hairs followed a similar pattern to our germination results. Seeds stored at 25°C developed hypocotyl hairs faster than those stored at 15 and 20°C but had a lower number of germinants developed these structures overall.

Within tropical or sub-tropical estuaries it is therefore likely that germination of *Z. muelleri* would primarily occur during the winter months due to cooler water temperatures (Brenchley and Probert, 1998). Towards the southern Australian limit of the species where average sea surface temperatures range from 11.5 - 18.9°C (METOC, 2014), year round germination is likely. The ideal temperature for hypocotyl hair development identified in the current study was 20°C. It is therefore likely that seeds, which germinate in the summer months in temperate regions, would have a greater opportunity to develop hypocotyl hairs.

The influence of light

Given the potential deposition and burial rates of some estuaries can be as high as 2.4mm/day, it is generally accepted that the negatively buoyant seeds of *Z. muelleri* would be exposed to sunlight for only brief periods once they drop from the spathes due to rapid burial (Marba and Duarte, 1995). Our results show that simulated burial (24-h darkness) increased germination by 14%. However, seeds were also able to germinate rapidly under 12hr light/dark treatments provided that suitable salinity concentrations and temperature ranges existed (Moore et al., 1993).

Similar to the influence of simulated burial on germination, the optimum conditions for *Z. muelleri* germinants to develop hypocotyl hairs within the current study was found to be when germinants underwent simulated burial (24-h darkness) and were stored at 20°C. While investigation of ideal conditions for hypocotyl hair development in seagrasses has received little attention in the literature, hypocotyl hairs have been found to develop well in *H. ovalis* under the same conditions found in our study (Kuo and Kirkman, 1992). Similar optimum conditions have also been found in *M. ericifolia* (Robinson et al., 2008).

Zostera muelleri inhabits both intertidal and subtidal zones where high sedimentation rates and moderate to strong localised water velocities occur (Bianchi, 2013; Day Jr. et al., 2013). The process of developing both enlarged hypocotyls and extensive coverage of hypocotyl hairs would provide significant opportunities for germinants to attach to the substrate potentially resulting in increased germinant survival.

The ability of seeds to germinate and remain attached to ever-changing sediments within the marine environment is a critical factor in the establishment of new populations and the ongoing survival of pre-existing genetically diverse populations. Periods of emersion during freshwater pulses that coincide with or occur shortly after seed maturation would initiate germination. The reduced salinity conditions would also aid in the development of fully formed hypocotyl hairs.

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Chapter 3

Prolonged buoyancy and viability of Zostera muelleri Irmisch ex Asch. vegetative fragments indicate a strong dispersal potential

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Prolonged buoyancy and viability of *Zostera muelleri* Irmisch ex Asch. vegetative fragments indicate a strong dispersal potential

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ABSTRACT

The establishment of clonal marine plant populations, including the seagrass *Zostera muelleri* (Zosteraceae), may be more dependent on the availability of viable vegetative fragments rather than seed. New populations may establish through long-distance dispersal of viable vegetative fragments, potentially increasing genetic diversity and resilience to anthropogenic or naturally occurring disturbance. A number of activities can dislodge vegetative fragments of *Z. muelleri* (leaves, rhizomes and roots) from the sediment. These fragments can remain positively buoyant, floating on the surface of the water. As the time since dislodgement increases, buoyancy may become reduced, causing fragments to move lower into the water column. However, what is not known is how long these fragments remain buoyant and potentially viable for recolonization. To address this knowledge gap, we collected wrack samples ($n = 125$) of *Z. muelleri* from four Victorian estuaries. Fragments were floated in outside aquaria for up to ten weeks, with subsamples tested for metabolic activity using tetrazolium violet. Porosity of seagrass rhizomes was also investigated to understand the influence of lacunae (large air filled spaces within plant tissues) on the flotation of vegetative fragments. The average proportion of potentially viable fragments collected in wrack ranged from 3.6% (SD = 2.23) to 11.2% (SD = 5.9). While there was a steady decline in the buoyancy of fragments across the ten-week period, initial buoyancy was relatively high, with approximately 50% of the fragments remaining positively buoyant for the initial five weeks. The viability of fragments following flotation was high. One hundred percent of fragments ($n = 25$ per assay) remained viable after floating for three weeks, with only a marginal decline (=96% viability) occurring after five weeks. When considered in conjunction with the highly porous nature of seagrass rhizomes (lacunae accounted for 45.2% of total volume), our findings indicate that the species may be capable of prolonged periods of transport dispersal within the marine environment.

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Prolonged buoyancy and viability of *Zostera muelleri* Irmisch ex Asch. vegetative fragments indicate a strong dispersal potential.

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Abstract

The establishment of clonal marine plant populations, including the seagrass *Zostera muelleri* (Zosteraceae), may be more dependent on the availability of viable vegetative fragments rather than seed. New populations may establish through long-distance dispersal of viable vegetative fragments, potentially increasing genetic diversity and resilience to anthropogenic or naturally occurring disturbance. A number of activities can dislodge vegetative fragments of *Z. muelleri* (leaves, rhizomes and roots) from the sediment. These fragments can remain positively buoyant, floating on the surface of the water. As the time since dislodgement increases, buoyancy may become reduced, causing fragments to move lower into the water column. However, what is not known is how long these fragments remain buoyant and potentially viable for recolonization. To address this knowledge gap, we collected wrack samples (n=125) of *Z. muelleri* from four Victorian estuaries. Fragments were floated in outside aquaria for up to ten weeks, with subsamples tested for metabolic activity using tetrazolium violet. Porosity of seagrass rhizomes was also investigated to understand the influence of lacunae (large air filled spaces within

plant tissues) on the flotation of vegetative fragments. The average proportion of potentially viable fragments collected in wrack ranged from 3.6% (SD=2.23) to 11.2% (SD=5.9). While there was a steady decline in the buoyancy of fragments across the ten-week period, initial buoyancy was relatively high, with approximately 50% of the fragments remaining positively buoyant for the initial five weeks. The viability of fragments following flotation was high. One hundred percent of fragments (n=25 per assay) remained viable after floating for three weeks, with only a marginal decline (=96% viability) occurring after five weeks. When considered in conjunction with the highly porous nature of seagrass rhizomes (lacunae accounted for 45.2% of total volume), our findings indicate that the species may be capable of prolonged periods of transport dispersal within the marine environment.

Highlights:

- *Zostera muelleri* vegetative fragments were tested for buoyancy and longevity
- Fragments remained positively buoyant for up to five weeks
- Cellular activity was identified after five weeks of flotation
- Vegetative fragments of *Z. muelleri* have strong transport dispersal potential

Keywords: *Zostera muelleri,* recruitment, disturbance, rhizome porosity, lacunae, tetrazolium violet

Introduction

Seagrasses, including *Zostera muelleri* Irmisch ex Asch. (Zosteraceae), flourish in near shore coastal environments in both temperate and tropical waters (Walker et al., 1999; Short et al., 2001) where they provide a multitude of benefits to coastal ecosystems, with some authors regarding them as 'ecosystem engineers' (Jones et al., 1994; Bos et al., 2007). In this role, seagrasses alter water flows and increase sedimentation, providing firm substrata for further colonisation by macroalgae and invertebrates (Bos et al. 2007). Other notable benefits include important nutrient cycling services, provision of critical nursery habitat for economically significant fish and prawn species, and protection from predators for many marine invertebrates (Waycott et al., 2009; Zhou et al., 2014). In *Z. muelleri*, anchorage to the sediment is achieved by the development of herbaceous, laterally compressed rhizomes. These structures are also important for mechanical support, storage of nutrients and vegetative propagation, which is an important process in the spread and ongoing survival of seagrass meadows (Tomlinson, 1974; Kuo and den Hartog, 2006). Vegetative growth, through the development and horizontal extension of rhizomatous biomass, has provided seagrasses with the ability to thrive on all continents with the exception of Antarctica (Short et al., 2001).

Once established from seed, individual genets may form large clones that rely heavily on vegetative growth for ongoing survival (Jarvis and Moore, 2010; Arnaud-Haond et al., 2012; Kendrick et al., 2012). While a mix between the two main reproductive strategies, sexual and vegetative, may provide optimum

growth opportunities, vegetative reproduction and rhizome encroachment increases the capacity for seagrasses to colonise bare substrates following disturbance events (Macreadie et al., 2014).

The dispersal of propagules of aquatic species within marine and freshwater environments plays a critical role in the establishment of new populations. Colonisation of new environments by seagrasses, including *Z. muelleri,* is important for the exchange of genetic material between populations, potentially increasing their resilience to natural and anthropogenic disturbances (Procaccini et al., 2007). While it is generally accepted that seagrass seed dispersal distance may be limited (<100m if not contained within detached infructescences) (Ackerman, 2006), if suitable conditions exist, the dispersal potential of vegetative fragments may be much greater. For example, for the seagrasses, *Z.nigricaulis* J. Kuo and *Z. noltii* Hornem, dispersal potential has been estimated between several hundred to 2300 km respectively (Berković et al., 2014; Thomson et al., 2014). Similar trends have been observed in other marine and freshwater flora, including the invasive marine alga *Caulerpa taxifolia* (Vahl) C. Agardh (Smith and Walters, 1999), the giant kelp *Macrocystis pyrifera* (L.) C. Agardh (Hernández-Carmona et al. (2006), the freshwater *Elodea canadensis* Michaux (Riis and Sand-Jensen, 2006) and a number of species of *Ranunculus* (Johansson and Nilsson, 1993).

Lacunae, or air filled spaces within plant tissues, facilitate greater oxygen transport in species growing in waterlogged and anoxic conditions and aid in the dispersal of vegetative propagules (Coutts and Philipson, 1978; Justin and

Armstrong, 1987). Lacunae are continuous within *Z. muelleri* from the leaves to the roots and allow for the movement of gases throughout the entire plant. Of particular importance is the ability of the species to release oxygen, via lacunal spaces, directly into the sediment from the roots thereby creating an oxygen rich environment in very close proximity (<1mm) to roots and rhizomes (Moriarty and Boon, 1989). A high proportion of lacunae within leaf tissue also provides greater buoyancy to both submerged and emergent aquatic species including seagrasses (Hemminga and Duarte, 2000) the water lily, *Nymphaea odorata* Ait. (Etnier and Villani, 2007) and species of marine macroalgae (Stewart, 2004). The increased buoyancy that lacunal spaces provide also facilitates dispersal of detached fragments via water currents. It is this dispersal potential and the ability to retain long-term viability which are critical factors in the establishment of new populations. Long term viability and effective dispersal potential of detached fragments following removal from the sediment has been reported for the seagrasses *Z. noltii,* (55 days) (Berković et al., 2014) and *Posidonia oceanica* (L.) Delile (408 days) as well as the marine alga *Hormosira banksii* (Turner) Decaisne (96 days) (McKenzie and Bellgrove, 2008).

Zostera muelleri has come to populate much of the eastern and southern coasts of Australia and is the dominant seagrass species in New Zealand (Jones et al., 2008; Waycott et al., 2014). This wide distribution may be attributable to the species' capacity for long-distance dispersal. Fragments of *Z. muelleri* can be dislodged from the sediment through both natural (e.g. wave action, consumption by large herbivores) or anthropogenic (e.g. propeller scarring, dredging activities) processes (Kenworthy et al., 2002; Greve and Binzer, 2004;

Erftemeijer et al., 2006; Lanyon and Sanson, 2006). Once dislodged, their fate is often varied and related to a number of species-specific traits or prevailing environmental conditions. For instance, fragments may sink out of the water column and decompose, re-establish in new environments or they can be carried ashore where they remain to decompose or are resuspended in response to local hydrological and meteorological conditions (Oldham et al., 2010; Pattiaratchi et al., 2011).

Although the movement of reproductive seagrass propagules has been well documented, what is less understood is the potential for vegetative fragments of *Z. muelleri* (leaves, rhizomes and roots) to remain viable subsequent to being dislodged from the sediment. This research was undertaken to identify the potential dispersal and viability of *Z. muelleri* vegetative fragments. This was performed by, firstly, estimating the proportion of vegetative fragments found in beach wrack that were potentially capable of regrowth. Secondly, identifying the period of time that dislodged vegetative fragments remained buoyant. Thirdly, by examining the relationship between rhizome porosity and buoyancy. And finally, by measuring the viability of vegetative fragments following extended periods of flotation.

Material and methods

Determining proportion of viable seagrass in wrack

Collection of fresh beach wrack that had been deposited during the previous high tide (<6hrs prior) occurred in January 2014 at the following five sites within Victoria, Australia: Shallow Inlet (two sites: 38°49'8.89"S, 146°10'13.62"E and

38°50'9.99"S, 146° 9'6.76"E); Corner Inlet (two sites: 38°48'37.98"S, 146°16'7.13"E and 38°41'47.26"S, 146°14'51.46"E) and Westernport (one site: 38°31'41.21"S, 145°22'11.19"E). Enough beach wrack to fill a 25 cm x 35 cm sealed freezer bag was collected from the high tide mark at twenty metre intervals along the beach. Twenty samples were collected from each site providing 100 samples in total.

Sealed samples were returned to the laboratory on ice within 48 hrs. Fragments of seagrass found in wrack were determined to be potentially capable of regrowth if: a) they contained rhizome with adventitious roots attached; b) the rhizome was fresh and turgid; and c) leaf shoots were fresh and in good condition. Seagrass leaves from fragments that were deemed potentially capable of regrowth were scraped with a razor blade to remove epiphytic algae and oven dried at 60 °C for 24 hrs. All samples were weighed to determine proportion of potentially viable seagrass within the total wrack sample.

Buoyancy of vegetative seagrass fragments

One hundred vegetative fragments of *Z. muelleri* were hand collected from Corio Bay, Victoria, Australia (38°52'30.17"S, 144°24'25.98"E). Samples were returned to the laboratory where they were sorted into fragments that were potentially capable of regrowth (refer above) and those with adventitious roots only, which were discarded. The length of the leaves, rhizomes and adventitious roots were measured in remaining samples (n = 40) using a tape measure and digital callipers prior to being placed into a 140 L outdoor aquarium filled with recirculated (450 L/hr) artificial salt water (NeoMarine, Brightwell Aquatics).

Salinity was maintained at 32 for the duration of the experiment to imitate conditions at the time of sampling and light and temperature reflected ambient conditions over the course of the experiment. As a means of minimizing entanglement, the samples were agitated daily by hand. To estimate the buoyancy of fragments, a scale was placed on the outside of the tank with the uppermost 10 cm being deemed positively buoyant, 11-30 cm deemed somewhat buoyant, and the lower 10 cm negatively buoyant. Buoyancy of fragments was determined on a weekly basis by counting the number of fragments whose majority was inside each of the buoyancy levels (positively, somewhat, negatively) calculated as a percentage of the total sample size.

Longevity of vegetative seagrass fragments

A total of 100 vegetative fragments of *Z. muelleri* were collected by hand from Altona Beach, Victoria, Australia (37°52'30.17"S, 144°48'53.84"E). Harvesting incorporated the use of a PVC corer with an internal diameter of 100mm placed into the sediment to a depth of ten centimetres. This depth was determined to be appropriate for this location as it ensured removal of all below ground biomass with the sample. Ten core samples were placed into 1 L sealed containers and covered with seawater for transport. Samples were returned to the laboratory where they were again sorted into fragments that were potentially capable of regrowth (refer above) and those with adventitious roots only. Samples with predominantly adventitious roots were again discarded and the remainder were washed in seawater to remove sediment before being placed

into a 140 L outdoor aquarium filled with recirculated (450 L/hr) artificial salt water (NeoMarine, Brightwell Aquatics).

Samples were floated in aquariums for one, three and five weeks, and agitated daily. At the end of each flotation period, 25 samples were removed and placed through small holes in a sheet of Styrofoam (10mm thickness) wedged into 3.5 L plastic aquaria containing 500 mL of Murashige and Skoog growth medium (1/4 strength (0.55 g/L)) dissolved in artificial salt water with a salinity of 32. The Styrofoam allowed below water-level biomass to remain submerged and the growing shoots to remain above water-level. The lower (submerged) portions of the aquaria were covered in aluminium foil, ensuring the rhizomes and roots remained in darkness. Aquaria with fragment samples were incubated for three days within a refrigerated incubator (constant 25 °C and 12 hr day/night cycles) to stimulate growth prior to the medium being supplemented with 0.005 % tetrazolium violet solution to assess for cell viability.

Tetrazolium salts have been widely used to identify the vitality of cells. Enzymatic activity alters the form of tetrazolium structures through reduction processes resulting in a change from a generally colourless or lightly coloured salt into brightly coloured and easily recognisable formazan dye products (Coolidge Churchill and Riner, 1978; Berridge et al., 2005; Jarvis and Moore, 2010). Viability of seagrass samples was determined through the presence or absence of purple colouration within the roots due to reduced formazan formation. Particular attention was paid to the root tip, which has been found by to be the site of greatest dehydrogenase activity (Kurzbaum et al. 2010). To

exclude the possibility of false staining, four control assays were undertaken. False-positive controls used the same growth medium and tetrazolium violet supplementation as described above with the addition of the following respiration inhibitors within two of the control assays, 0.2 % sodium azide or 200 mg/L cycloheximide. The third false-positive control used dead seagrass fragments, which had been boiled for ten minutes and in order to determine potential false-positive staining. A fourth control used seagrass fragments placed in growth medium only with no supplementation of tetrazolium violet. All control treatments were incubated for 72 hours within a refrigerated incubator (constant 25 °C and 12 hr day/night cycles) before being checked for formazan formation.

Porosity of seagrass rhizomes

Using the methods previously described, in January 2014, twenty-five vegetative fragments of *Z. muelleri* were hand collected from the Altona Beach site. Adventitious roots or leaf remnants were removed from rhizomes prior to the rhizomes being divided at each internode. The internode length and diameter of 100 rhizome fragments (4 internodes per sample) was determined using digital callipers; however, as rhizomes of *Z. muelleri* are laterally compressed, the diameter was measured at the longer side of each sample to ensure consistency.

Porosity of rhizomes (percentage of gas space as a factor of total tissue volume) was determined by calculation of rhizome weight in water before and after infiltration of lacunae and intracellular spaces with seawater using methods

described by Raskin (1983) and equations as presented by Thomson et al. (1990). In calculations of porosity of *Triticum aestivum* (L.) em Thell roots, Thomson et al. (1990) indicated that the volume of roots in air may have been overestimated as a result of solution adhering to the root surface and suggest calculations to remove this over-estimation. As adventitious roots were removed from seagrass rhizomes prior to fresh weight being calculated, the amount of solution adhering was minimal and excess solution was removed by drying with paper towel. The calculation of rhizome porosity was undertaken using the following equations as taken from Thomson et al (1990):

$$Volume\ of\ rhizome = \frac{(fresh\ weight\ of\ rhizome) - (weight\ of\ rhizome\ displaced\ in\ water)}{density\ of\ sea\ water}$$

Equation 1. Volume of rhizome samples was determined by investigating the difference between the fresh weight of samples and the weight of samples once they were displaced in seawater (Thomson et al., 1990).

$$Volume\ of\ gas\ in\ rhizome = \frac{(weight\ of\ infiltrated\ rhizome\ in\ water) - (weight\ of\ rhizome\ displaced\ in\ water)}{density\ of\ sea\ water}$$

Equation 2. Volume of gas within rhizomes was calculated using the weight of rhizomes following seawater infiltration and the weight of samples once displaced in seawater, prior to infiltration (Thomson et al., 1990).

$$\%\ Porosity = \frac{(volume\ of\ gas\ in\ rhizome)(100)}{volume\ of\ rhizome}$$

Equation 3. Final porosity of rhizomes was determined utilising results of equations 1 and 2 (Thomson et al., 1990).

Results

Proportion of potentially viable seagrass in wrack

The average proportion of seagrass fragments collected in beach wrack that was deemed to be potentially capable of regrowth ranged from 3.6 % (SD = 2.23) at Westernport to 11.2 % (SD = 5.9) at Corner Inlet site 1. Results of the remaining sites were as follows: Corner Inlet site 2‑10 % (SD = 4.94); Shallow Inlet site 1 – 5.88 % (SD = 7.58); Shallow Inlet site 2 – 4.61 % (SD = 2.42).

Buoyancy of vegetative seagrass fragments

The average length of *Z. muelleri* leaves, rhizomes and roots was 316.5 mm (min = 200 mm, max = 523.5, SD = 94.28), 25.4 mm (min = 9.9 mm, max = 82.2 mm, SD = 15.05) and 13.41 mm (min = 0.9, max = 60.3, SD = 9.57) respectively.

While there was a steady decline in the buoyancy of fragments during the ten weeks of the study, initial buoyancy was relatively high with nearly 50 % (n = 19) of fragments remaining positively buoyant for a period of five weeks (Fig. 3.1). However, once surviving fragments lost buoyancy and became only somewhat buoyant, i.e. above the bottom 10 cm but below the top 10 cm of the aquarium, they remained in this intermediate state for the majority of the study. There was a 20 % increase in the number of fragments deemed no longer viable after four weeks, however, we believe this result was likely confounded by the marked increase in water temperature which occurred when ambient temperature exceeded 35 °C for more than a week.

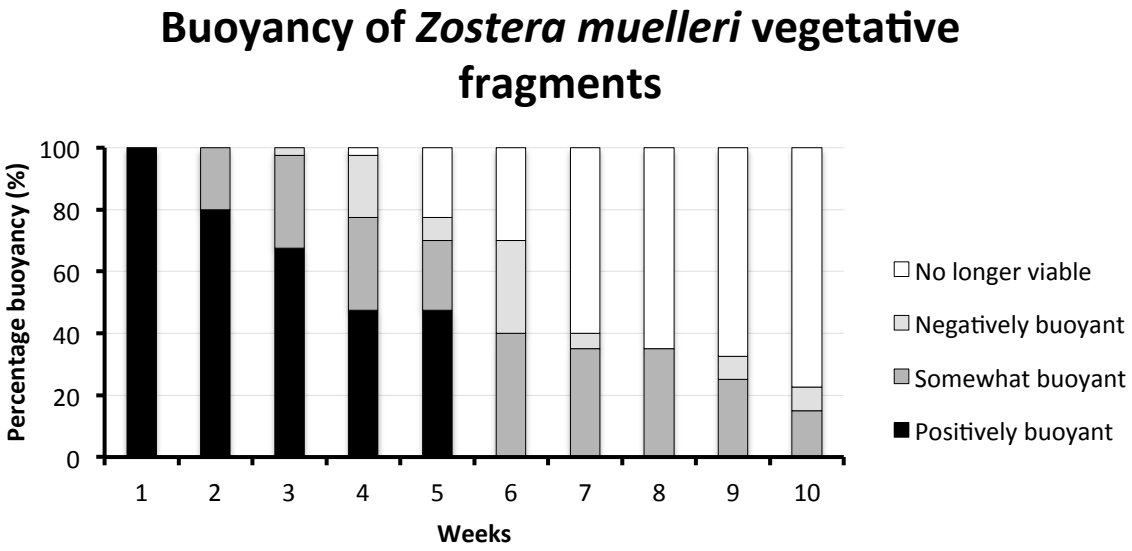

Fig. 3.1: Buoyancy of *Z. muelleri* vegetative fragments over a ten-week period.

Longevity of vegetative seagrass fragments

The viability of fragments of *Z. muelleri* following flotation was high. One hundred percent of fragments (n = 25 per assay) expressed formazan development within roots and rhizomes following treatment with tetrazolium violet, indicating cellular activity, for each of the studies lasting for one and three weeks respectively. There was only a minor decrease in viability (96 % showing cellular activity) after a flotation period of five weeks with a significantly greater proportion of root hairs expressing formazan development when compared to the shorter flotation periods (Fig. 3.2).

No formazan formation occurred for each of the controls using sodium azide or boiled plants, indicating that cellular activity within roots had been completely inhibited. However, in findings similar to Kurzbaum et al. (2010), greatly reduced colouration of roots occurred within the cycloheximide treatment

indicating that the protein synthesis inhibitor had greatly reduced formazan formation.

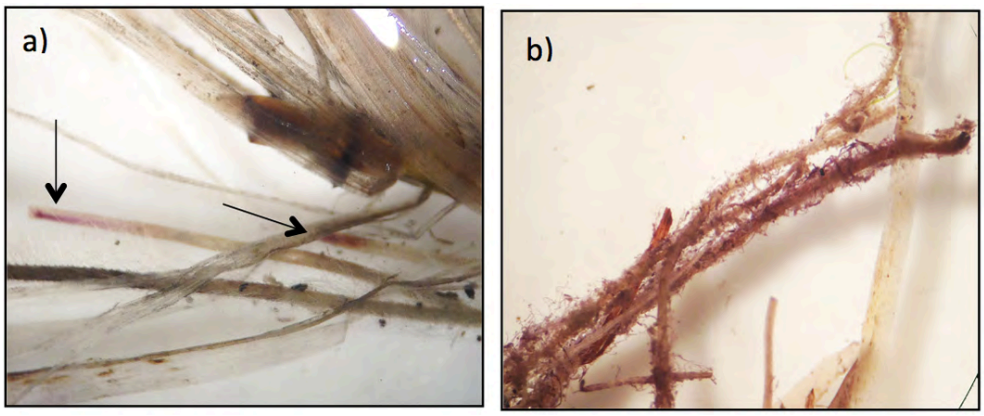

Fig. 3.2: Evidence of cellular activity within root tissue of *Z. muelleri* tested with tetrazolium violet after three (a) and five (b) weeks flotation.

Porosity of seagrass rhizomes

The average length and diameter of internodes for the 100 samples was 18.6 mm (min = 9.5 mm, max = 29.6mm, SD = 4.2) and 2.1 (min = 1.5mm, max = 2.6 mm, SD = 0.2) respectively. Rhizome porosity was found to be high (45.2 %) indicating lacunae accounted for a large proportion of total volume within rhizomes. Transverse sections of rhizomes (Fig. 3.3) identify the main components of *Z. muelleri* in particular, a central stele (S), two opposite vascular bundles (V) and extensive cortical tissues with significant lacunal spaces (C).

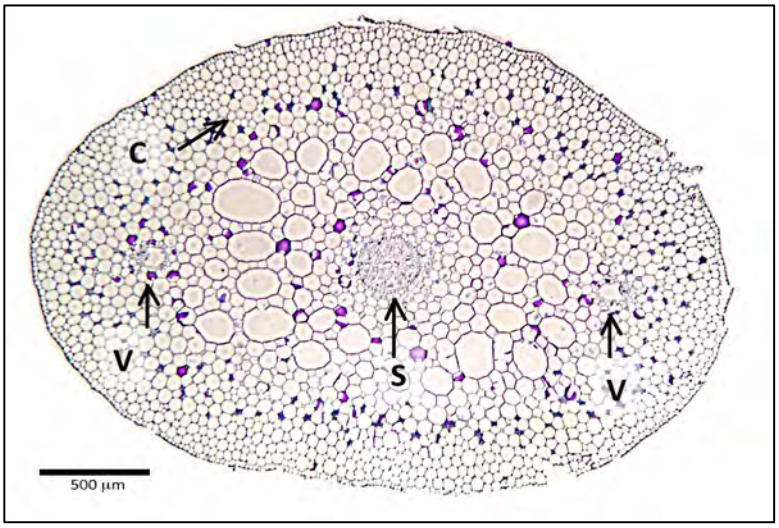

Fig. 3.3: Transverse section (4 µm thickness) of *Z. muelleri* rhizome under 40x magnification showing vascular bundles (V), central stele (S) and extensive cortical tissue with high proportion of lacunae (C).

Discussion

We have found vegetative fragments of *Z. muelleri* are able to remain buoyant for extended periods of time, indicating a high potential for long-distance dispersal. While this subject has received some recent positive attention in the literature e.g.(Di Carlo et al., 2005; Hall et al., 2006; Berković et al., 2014; Thomson et al., 2014), the potential for vegetative seagrass fragments to effectively develop new populations through hydrochoric dispersal has received varied results.

In our study, nearly 50% of vegetative fragments of *Z muelleri* remained positively buoyant for a five-week period. Although 15% of fragments were deemed to be somewhat buoyant after ten weeks, we posit that this is likely a conservative estimate due to the effect of a pronounced period of high ambient

temperature. Porosity of rhizomes was found to be high in the current study (45.2%) when compared to similar work on the seagrass, *Halophila ovalis* (R.Br.) Hook.f. (lacunae accounted for 27% of rhizome volume) (Connell et al., 1999). A high proportion of lacunae within leaf tissue has been found to provide greater buoyancy to both submerged and emergent aquatic species (Hemminga and Duarte, 2000; Stewart, 2004; Etnier and Villani, 2007). Therefore, considering the high proportion of lacunae within both the leaves (24.8% of total leaf area) (Grice et al., 1996), and rhizomes of *Z. muelleri*, these air spaces are likely to play a critical role in the buoyancy of the species, enabling for greater dispersal of vegetative fragments.

While fragments of *Z. muelleri* were found to remain buoyant for extended periods in the current study, what is of greater importance is their ability to remain viable during dispersal periods, potentially allowing them to develop new populations. In common with some terrestrial relatives (e.g. *Tillandsia usneoides* (L.)) (Martin and Peters, 1984), seagrasses have the ability to acquire nutrients via both root and leaf tissues, allowing some species to inhabit rocky substrates devoid of substantial sediment deposits (Terrados and Williams, 1997). Localised flow velocities within the water column also significantly influence acquisition of nutrients by leaves of seagrass species. Higher flow velocities increase nutrient uptake by leaves due to greater vertical exchange (Thomas et al., 2000), but under low flow velocity conditions a closing of seagrass canopies may result in a reduction of this process. Under conditions described in the current study where floating vegetative fragments would be

continually exposed to flow velocities within the water column, the issue of vertical exchange is negated (Koch and Gust, 1999).

In addition to nutrient uptake by seagrass leaves, their ability to store carbohydrate reserves as non-structural carbohydrates (NSC) within leaves and rhizomatous tissues may provide further opportunities for long-term viability of vegetative fragments (Kraemer and Alberte, 1995; Longstaff et al., 1999). Carbohydrate storage plays a critical role in the resilience of seagrasses to environmental stresses such as light deprivation. Studies by Kraemer and Alberte (1995) and Burke et al. (1996) show that a decrease in light saturation time is associated with a decrease in both growth rate and levels of soluble NSCs within tissues of *Z. marina*. In general, the species could withstand short periods of stress before the rate of NSC use in protein synthesis would lead to carbohydrate starvation. The acquisition of nutrients by leaves and the ability to store NSCs as accessible sugars would therefore significantly influence the viability of *Z. muelleri* vegetative fragments. Results indicate that after five weeks of flotation following removal from the sediment, 96% (n=24) of vegetative fragments of *Z. muelleri* were still capable of cellular activity based on the results of tetrazolium violet supplementation. It is therefore highly likely, although not tested, that these fragments were capable of surviving for such an extended period as a direct result of their ability to both acquire nutrients from the water column and store NSCs.

Although seagrass fragments have been found to remain both buoyant and viable for extended periods of time (Ewanchuk and Williams, 1996), success of

transplantation studies or natural reattachment has generally been low (Di Carlo et al., 2005; Thomson et al., 2014). Studies by Campbell (2003) and Hall et al. (2006) have identified effective dispersal of *Posidonia australis* Hook. f. and *Halodule wrightii* Asch. respectively, with fragments remaining viable for up to four weeks and able to successfully reattach. More recently, Thomson et al. (2014) investigated the potential of viviparous propagules of *Z. nigricaulis* to disperse and develop new populations. While a majority of propagules remained buoyant for a shorter period of time when compared with the current study (roughly 50 % losing buoyancy within 3 weeks), there were significant increases in seagrass biomass for the duration of the study, indicating long-term viability of the propagules (Thomson et al., 2014).

In addition to viability, survivorship is also critical for the reestablishment of new populations, which may be directly influenced by requirements of the species in question, or the environment in which recolonisation events occur. For some seagrass species e.g. *P. australis,* the depth at which recruitment of vegetative fragments occurs is a critical factor in their long-term survival with a high majority (78%) of fragments surviving when depths exceed 10 m (Campbell, 2003). However in other *Zostera* species, survivorship subsequent to transplanting has been shown to be low with Thomson et al. (2014) reporting 100 % mortality of *Z. nigricaulis* fragments within 100 days. While artificial transplanting techniques have had greater success for *Z. marina* (L.) with 95 % of transplants surviving for more than 90 days (Zhou et al., 2014) and 68 % survival following 243 days (Kenworthy and Fonseca, 1992), these varied

findings raise the question as to how effective vegetative fragments of *Z. muelleri* are at developing new populations?

Conclusions

Our research has identified a high potential for transport dispersal of seagrass vegetative fragments. The physical and biological attributes of *Z. muelleri* enable the species to persist in the water column for many weeks and also to retain their viability. Collectively, these attributes may help explain the wide distribution of the taxa within Australian and New Zealand waters. However, what it yet to be determined is the rate of success for fragments to re-establish into viable populations. Given the increasing pressures these systems are facing and their ecological importance, additional research is required to understand the capacity for *Z. muelleri* fragments to successfully reattach to the sediment, generate new populations and inter-breed with currently established populations. If reattachment is successful following extended periods of movement within the marine environment, the establishment of new populations that are far removed from their origins, has important ecological consequences for the ongoing survival of the species. Such recruitment can introduce new genetic material into populations leading to greater resilience of populations to natural or anthropogenic disturbance.

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Chapter 4

Population connectivity in the seagrass, Zostera muelleri, within south-eastern Australia

Population connectivity in the seagrass, *Zostera muelleri,* within south-eastern Australia.

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Abstract

Connectivity of seagrass populations, which provide significant benefits to coastal ecosystems, is enabled through the dispersal and potential recruitment of either vegetative (asexual) or reproductive (sexual) fragments. Once established, immigrating genetic individuals supplement the genetic diversity within populations leading to an increased resilience of those populations to disturbance. While seagrass fragments may be dispersed via oceanic and near-shore currents, the influence of historical barriers can still be evident in the distribution of genetic variation between populations. The degree of connectivity between populations of *Zostera muelleri* in southeastern Australia is unknown. Identifying the key factors that drive seagrass population connectivity is important for managing these ecologically significant environments. We examined the connectivity of 22 sites containing *Z. muelleri* in southeastern Australia using nine polymorphic microsatellite DNA loci. We analysed these using analysis of molecular variance, determination of pairwise F_{ST}, Mantel tests, and Bayesian model-based clustering. Moderate genetic differentiation was observed between most sample sites (0.177 ± 0.016 (SE)). While two

distinct genetic population clusters were identified, which mirror the location of a historical land bridge between mainland Australia and Tasmania, sites of admixture between these clusters suggest that propagules are dispersing via contemporary currents between some sample sites. The use of genetic analyses to identify the degree of connectivity between the studied sites will provide restoration ecologists with an important understanding of gene flow in the region.

Introduction

The ability of propagules to disperse and colonise marine and estuarine waters of south-eastern Australian has led to the identification of the Peronian, Maugean and Flindersian marine biogeographical provinces within the area (Bennett and Pope, 1953, 1960) (Fig. 4.1). These provinces are categorised based on a number of factors, including, the lack of shallow reef habitat along the Great Australian Bight and coastal zones east of Wilsons Promontory, temperature gradients, and the influence of oceanic currents (Bennett and Pope, 1953, 1960; Wilson and Allen, 1987; O'Hara and Poore, 2000; York et al., 2008). The Peronian province extends from southern Queensland into Bass Strait taking in north-eastern Tasmania and the east coast of Victoria to roughly Western Port. The Maugean province includes all of Victoria and Tasmania and the Flindersian province encompasses western Victoria (O'Hara and Poore, 2000) (Fig. 4.1). Biotic distinctions between the Flindersian and Peronian provinces include low kelp-forest cover within the Peronian and highly heterogenic kelp populations occurring in the Flindersian (Connell and Irving,

2008). Clearly defined phylogeographic gaps between the provinces have been identified, most notably between jellyfish populations (*Catostylus mosaicus*) (Dawson, 2005) and the intertidal gastropod *Nerita* (Waters, 2008; Waters et al., 2010).

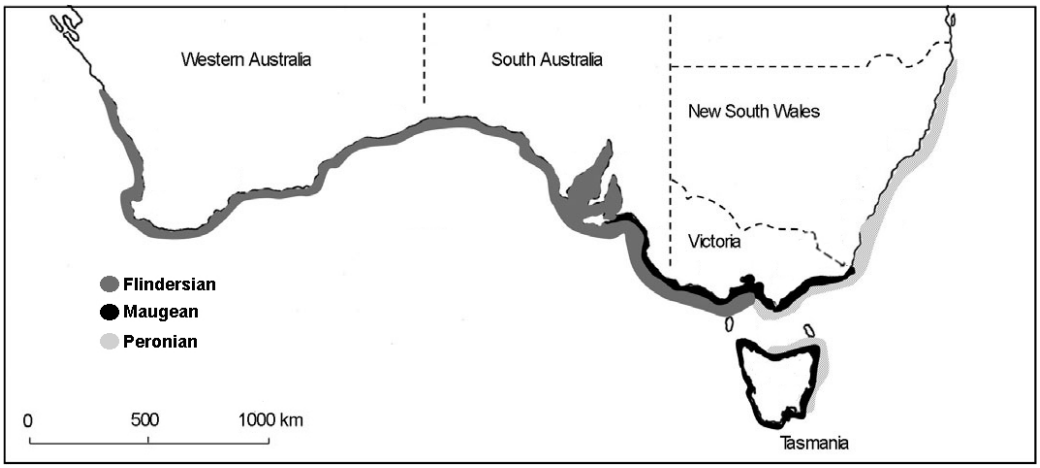

Fig. 4.1. Marine biogeographic provinces in southern Australia (Bennett and Pope, 1953). Figure is adapted from Waters et al. (2010).

The phylogeographic differences between the marine provinces are consistent with the location of the Bassian Isthmus that connected Tasmania to mainland Australia during the last glacial maximum in the Pleistocene epoch (Lambeck and Chappell, 2001). Deglaciation associated with the breaking of the last glacial maximum resulted in the valley between the two land masses being flooded by water entering from the west (≈17,500 years ago), a process that continued until the isolation of Tasmania some 14,000 years ago (Lambeck and Chappell, 2001).

The isolating influence of the Bassian Isthmus and the subsequent reduction on gene flow between populations located on the western and eastern Victorian

and Tasmania coasts has previously been identified for many species including marine algae and barnacles (*Catomerus polymerus*) (cf. Waters, 2008; York et al., 2008).

The Victorian coastline is characterised by strong wave action from the Southern Ocean with swells predominantly moving in a south-westerly direction towards King Island in the west of Bass Strait (Bird, 2010). These swells are refracted around King Island causing a south-easterly movement of water in towards Corner Inlet and Ninety Mile Beach at the Gippsland Lakes (Bird, 2010). Oceanic swells in western Victoria occur from the south-west leading to long-shore drift in an easterly direction for up to nine months of the year (BIrd, 2010). Hydrodynamic modelling (Fig. 4.2) suggests that these influences have the potential to transport propagules of marine species along the western coast of Victoria and Tasmania and by following the Victorian coastline into eastern Victoria (Greer et al., 2008). Reverse modelling undertaken to determine potential spawning grounds of King George Whiting (*Sillaginodes punctata)* has also identified the influence of these currents with spawning locations occurring some 400km to the west of the eventual recruitment site of Port Phillip Bay (Jenkins et al., 2000). Genetic similarities between populations of Southern bull-kelp (*Durvillaea potatorum)* near the Victorian site of Curdies Inlet and Bruny Island on the eastern coast of Tasmania provide further evidence of the role of oceanic currents in the transport of propagules within the region (Fraser et al., 2009).

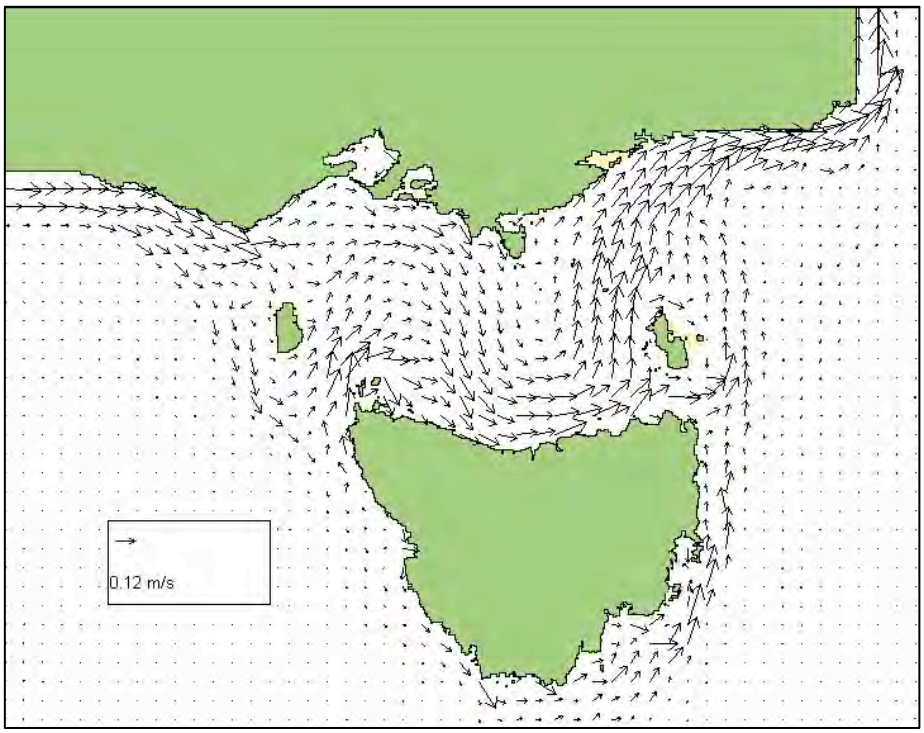

Fig. 4.2. Combined residual currents (non-tidal) around southeast Australia modelled over 25 days in Spring 2007 and 39 days in autumn 2008. Refer to Table 4.1 for population name abbreviations. Figure adapted from Greer et al. (2008).

Seagrasses are ecologically significant, highly specialised angiosperms (flowering plants) that provide a multitude of benefits to the systems they inhabit, and are considered to be ecosystem engineers (Jones et al., 1994; den Hartog and Kuo, 2006; Bos et al., 2007). In this role, seagrasses alter water flows and increase sedimentation, providing firm substrata for further colonisation by macroalgae and invertebrates (Bos et al., 2007). Other notable benefits include important nutrient cycling services and provision of critical nursery habitat for economically significant fish and prawn species and protection from predators for many marine invertebrates (Walker et al., 1999; Edgar et al., 2001; Waycott et al., 2009). The dispersal of seagrass propagules within the marine

environment and their eventual re-colonisation ensures connectivity between local and regional populations is maintained. Metapopulation ecology aims to understand the maintenance of populations through the dynamic processes of the movement of individuals either into or out of a population, whether by birth, death, immigration or emigration (Bell, 2006). Based on a metapopulation approach, the flow of genes between spatially removed populations, is gaining interest amongst seagrass conservationists concerned with the long-term survival and genetic composition of populations (Bell, 2006). Although local seagrass populations may be impacted by anthropogenic or natural disturbance leading to local extinctions (Orth et al., 2006), a group of populations within a given area (a metapopulation) can persist due to the supplement of local populations by vegetative and reproductive propagules arriving from surrounding populations (Hanski and Simberloff, 1997). There is, however, an inherent difficulty in establishing the dispersal of plants due to their often heterogeneous spatial arrangement and localised dispersal (Bell, 2006). By understanding the gene flow between populations, based on historical and contemporary dispersal of propagules, managers of seagrass systems are better equipped to maintain population viability even when those population may be negatively impacted upon by anthropogenic disturbance or habitat fragmentation (Allendorf and Luikart, 2009).

Seagrass propagules can be dispersed by both asexual and sexual modes, through fragmentation or the dispersal of seeds respectively. Asexual vegetative fragments of seagrasses including *Zostera muelleri* Irmisch ex Asch. have high dispersal potential due to large lacunal spaces within rhizomatous

tissues that provide long-term buoyancy (Stafford-Bell et al., 2015). Estimates of dispersal for vegetative propagules of other *Zostera* species including, *Z. nigricaulis* and *Z. noltii*, range from several hundred to 2300 km respectively (Berković et al., 2014; Thomson et al., 2014). While survivorship of vegetative fragments is generally low, they can recolonise should suitable local environmental conditions occur (Kenworthy et al., 2002; Campbell, 2003; Thomson et al., 2014; Zhou et al., 2014). Once establishment has occurred, *Z. muelleri* utilises predominantly vegetative growth to recolonise sediments following disturbance events while sexual reproduction and recruitment influences the genetic composition of populations (Macreadie et al., 2014).

The movement of seeds can more rapidly enhance the genetic diversity of seagrass meadows than asexual reproduction, potentially increasing their resilience to disturbances (Procaccini et al., 2007). Seed dispersal within seagrass species follows two main processes. Primary dispersal (Phase I) occurs when seeds (negatively buoyant) are released into the water column encased within positively buoyant reproductive shoots or fruits that enhance their dispersal potential (Nathan and Muller-Landau, 2000; Orth et al., 2006). Secondary dispersal (Phase II) occurs when seeds settle out of the water column and come into contact with the sediment in a process known as seed rain (Nathan and Muller-Landau, 2000). While it is generally accepted that seagrass seed dispersal distance may be limited (<100m if not contained within detached infructescences) (Ackerman, 2006), the dispersal potential of *Z. marina* seeds encased within floating reproductive shoots has been estimated

at more than 100km in a manner similar to vegetative (asexual) fragments (Harwell and Orth, 2002; Källström et al., 2008; Stafford-Bell et al., 2015).

The disjunction of populations across the Bassian Isthmus and the connectivity between populations of marine species located in Victoria and Tasmania has previously been identified (c.f. Waters, 2008; Fraser et al., 2009). What has not previously been investigated is whether this disjunction, or connectivity between populations of the seagrass *Z. muelleri* in south eastern Australia exists. Furthermore, we are yet to understand the connectivity of these populations and the likelihood of them being supplemented by the movement of seagrass propagules that originate from local, regional or more distant populations.

Microsatellites are one of the most commonly used DNA marker in population genetics and their highly polymorphic nature can provide insights into contemporary gene flow and the resulting connectivity, or lack thereof, between far removed seagrasses populations (Kendrick et al., 2012). We obtained multi-locus microsatellite DNA genotypes for 22 populations of *Z. muelleri* to determine the extent of connectivity between the populations. We hypothesised that the historical barrier to gene flow for some species in the region, the Bassian Isthmus, would be mirrored across the western and eastern Victorian and Tasmanian populations of *Z. muelleri.* We further hypothesised that gene flow would occur between local populations of the species.

Materials and Methods

Study species

Z. muelleri (Zosteraceae) is a monoecious seagrass that inhabits the intertidal zone of southern and eastern Australian and New Zealand estuaries (Kuo and den Hartog, 2001; Short et al., 2001). Flowering occurs during the warmer months and germination of seeds increases under cooler sea surface temperature (15-20 °C) and reduced salinity conditions (<16ppt) (Walker et al., 2001; Stafford-Bell et al., 2016). The species produces large numbers of small (≈2mm) negatively buoyant seeds that are either released directly into the water column or encased within a spathe on positively buoyant reproductive shoots (Ackerman, 1997, 2006). Vegetative fragments dislodged from the sediment through both natural (e.g. wave action, consumption by large herbivores) or anthropogenic (e.g. propeller scarring, dredging activities) processes can remain both buoyant and viable for extended periods (>5w) indicating a strong dispersal potential for these tissues (Erftemeijer et al., 2006; Lanyon and Sanson, 2006; Stafford-Bell et al., 2015).

Study sites and sampling protocols

Samples were collected along 686km of the Victorian coastline (22 sites in eight locations) and roughly 40km of the east Tasmanian coast (four sites in three locations) (Fig. 4.2). The majority of sampling occurred within estuaries and embayments with the exception of Maria Island (TAS) where samples were collected from exposed beaches. Estuaries and beaches were predominantly wave dominated and semi diurnal with a southerly exposure and entrance

widths ranging from 60m at Orford (TAS) to 4.8km at Western Port (VIC) (Table 4.1).

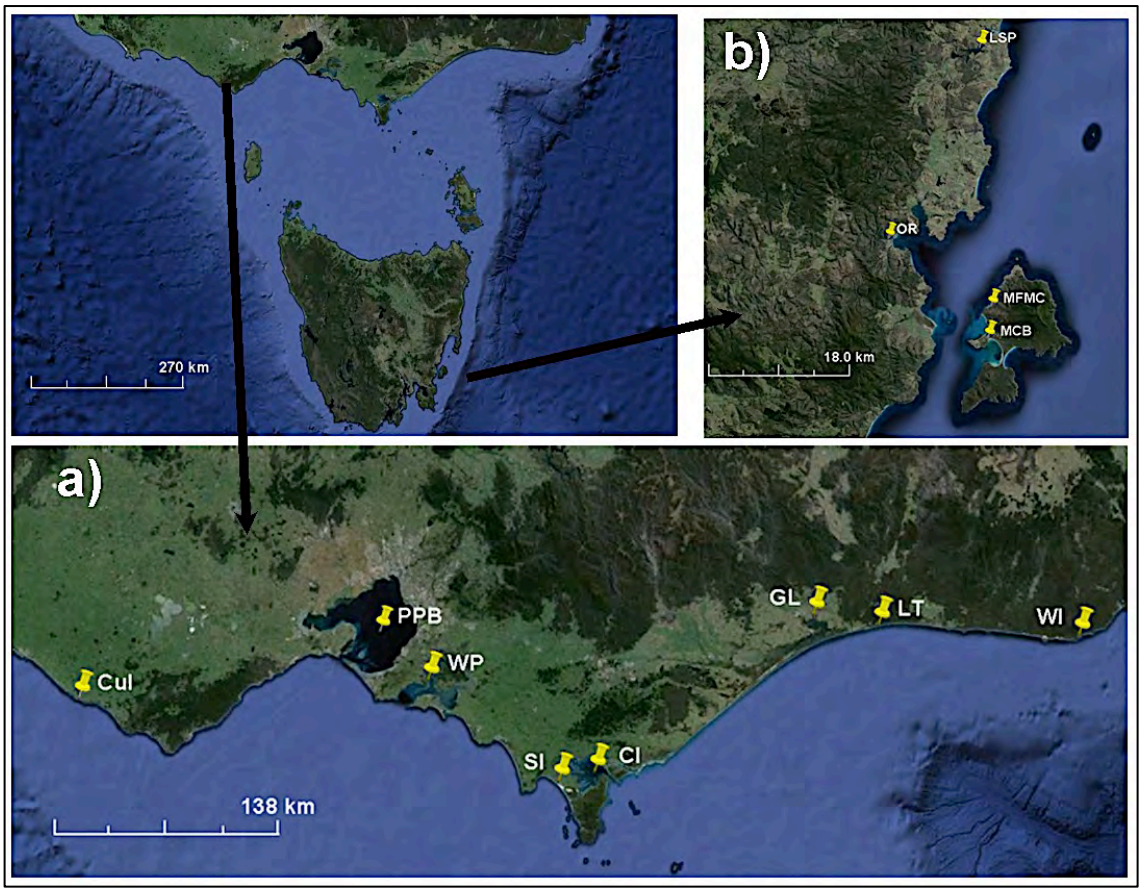

Fig. 4.3. Location of *Zostera muelleri* populations sampled within south eastern Australia. Sites were located within a) Victoria (CuI, PPB, WP, SI, CI, GL, LT, WI) and b) Tasmania (OR, MFMC, MCB, LSP). Refer to Table 4.1 for population name abbreviations.

Table 4.1. Sampled populations of *Z. muelleri* for microsatellite analysis. Samples sites are located within Victoria (CuI, PPB, WP, SI, CI, GL, LT, WI) and Tasmania (OR, MFMC, MCB, LSP). Victorian sample sites are ordered from west coast to east coast populations. For estuary classification: WD = Wave dominated; TD = Tide Dominated

Site	Abb.	Form	Classification	Entrance orientation	Intertidal area (km^2)	Water area (km^2)	Entrance width (km)	Mean wave height (m)	Tidal range (m)	Tide type
Curdies Inlet	CuI	Estuary	WD	S	0.24	2.94	0.13	2.3	0.9	Diurnal
Port Phillip Bay	PPB	Estuary	TD	SW	14.1	1897	3.46	0.61	1.2	Semi Diurnal
Western Port	WP	Estuary	TD	SW/SE	90.6	469	4.87	1.4	2.3	Semi Diurnal
Shallow Inlet	SI	Estuary	WD	SW	7.05	5.03	0.29	1.6	2.1	Semi Diurnal
Corner Inlet	CI	Estuary	TD	SE	387	378	1.89	0.34	2.3	Semi Diurnal
Gippsland Lakes	GL	Estuary	WD	SE	0	486	0.36	0.52	0.9	Semi Diurnal
Lake Tyers	LT	Estuary	WD	S	1.29	13.1	0.14	0.91	0.9	Diurnal
Wingan Inlet	WI	Estuary	WD	SSE	0.38	1.5	0.12	1.6	1.1	Diurnal
Orford	OR	Estuary	WD	SE	0.29	0.19	0.06	0.61	1.1	Diurnal
Maria Island Four Mile Creek	MFMC	Beach	WD	NW	0	NA	0.4	0.5	1	Semi Diurnal
Maria Island Chinaman's Bay	MCB	Beach	WD	SW	0	NA	0.25	0.1	1	Semi Diurnal
Little Swanport	LSP	Estuary	WD	E	0.14	4.28	0.39	0.5	1.2	Diurnal

Sampling of *Z. muelleri* occurred at low tide with collection of nine samples across a 10m x 10m grid from three meadows within each estuary where possible (Inglis and Waycott, 2001; Arnaud-Haond et al., 2007). Volunteers collected Tasmanian samples opportunistically and due to a small population occurring within Wingan Inlet (VIC) only nine samples in total were collected for that estuary. Meristematic material containing an upright shoot with attached rhizome were removed from the sediment by hand, flushed with fresh water, pat

dried with paper towel and placed in 50ml centrifuge containers with silica crystals for later analysis. Genomic DNA was extracted from each sample using DNeasy Plant Kits (QIAGEN) following the manufacturer's instructions.

Genetic analyses

We characterised the polymorphism of eleven microsatellite DNA loci using primers previously developed for *Z. muelleri* (ZosNSW02, ZosNSW18, ZosNSW19, ZosNSW20, ZosNSW23, ZosNSW28, ZosNSW34, ZosNSW38, ZosNSW43, ZosNSW45 and ZosNSW46) (Sherman et al., 2012). The forward primer of each pair was labelled with an M13 tag (5' CACGACGTTGTAAAACGAC) on the 5' end for later use in the universal dye labelling process (Boutin-Ganache et al., 2001). Polymerase chain reactions (PCR) (20µL) were undertaken using HotStar Plus PCR Master Mix (10µL) (QIAGEN) following manufacturer's instructions. Final concentrations of 2.4µM of the M13 tag 5' labelled with an Applied Biosystems (ABI) dye (NED, FAM, VIC or PET), the locus-specific tailed (0.6µM) and untailed (2.4µM) primers, approximately 10ng of genomic DNA and 10were used in each PCR. PCR products were amplified in a Biorad MyCycler thermocycler using the following conditions: an initial denaturation step of 95°C for 60s followed by 35 cycles of 94°C for 45s, 53°C for 60s, 72°C for 60s with final elongation at 72°C for 5 min.

PCR product sizes were scored commercially (Australian Genome Research Facility AGRF) on the GeneMapper software (Applied Biosystems) using the GeneScan 500 Liz size standard. Samples that produced poor results or failed to amplify were re-run following the process described previously Loci were

then tested for deviation from Hardy-Weinberg equilibrium (HWE) using CERVUS 3.0.7 (Marshall et al., 1998). To determine the proportion of variation within the total genetic variation that could be attributed to within and among sampled populations, analysis of molecular variance (AMOVA) was performed using ARLEQUIN 3.5 (Excoffier and Lischer, 2010).

Variation among sampled sites was estimated using pairwise F_{ST} where genotypes were permutated between sites 16,000 times with significance values of $P<0.05$. Sample sites were deemed to have: limited differentiation (F_{ST} <0.05); little differentiation (F_{ST} 0.05-0.15); moderate differentiation (F_{ST} 0.15-0.25) or significant differentiation (F_{ST} >0.25) (Weir and Cockerham, 1984). Analysis of isolation by distance (IBD) was undertaken through a Mantel test to identify correlations between genetic distance (F_{ST} /1- F_{ST}) and the oceanographic distance between populations (km) for all sample sites, western and central Victoria and eastern Victoria and Tasmania using GENALEX 6.5 (Peakall and Smouse, 2006, 2012; Sinclair et al., 2014). Oceanographic distance was calculated in QGIS 2.8.1 as the shortest distance between sampled sites (QGIS, 2015).

To identify the presence of distinct genetic clusters, assign individuals to populations and identify sites of admixture, Bayesian model-based clustering incorporated in the program STRUCTURE 2.3.4 (Pritchard et al., 2000) was used. We performed a 100,000 burnin length and 500,000 Markov Chain Monte Carlo (MCMC) simulations for K = 1-10 with 10 iterations for each K to ensure consistency across all runs. This program assumes a fixed number of

populations (*K*) using the Dirichlet distribution to model allele frequencies for each population and provides an estimation of the probability that an allele belongs to a particular population (Pr(X*K*) (Pritchard et al., 2000; Frankham et al., 2002; Hartl and Clark, 2007). To determine the appropriate value for *K*, we used the methods described by Evanno et al. (2005). A Principal Coordinate Analysis (PCoA) was also performed in order to provide further insight into the relationships between each multi-locus genotype (MLG) and the population means based on geographic distance. Geographic distance was calculated as the shortest possible distance over water between sampled populations (oceanographic distance) using GENALEX 6.5 (Peakall and Smouse, 2006, 2012; Putman and Carbone, 2014).

Results

Amplification of PCR products and microsatellite loci

Of the 11 polymorphic loci initially amplified, nine yielded consistent results. ZosNSW18 was found to consistently amplify with more than two peaks and ZosNSW28 seemed to be fixed for one allele across all sampled sites. Following re-checking of allele scoring these loci were omitted from further analyses. The number of alleles at a locus ranged from two to 14 (mean = 8, SD = 4) with a total of 76 alleles detected across all loci. Observed and expected heterozygosity ranged from 0.05-0.90 and 0.08-0.90 respectively (Table 4.2). Significant departures from HWE were observed for two loci (ZosNSW23 and ZosNSW43) due to heterozygote deficiency and as a result of the low number of alleles scored in ZosNSW02 and ZosNSW38, CERVUS 3.07

(Marshall et al., 1998), failed to complete HWE tests for these loci. All further analyses were tested with and without inclusion of ZosNSW02 and ZosNSW38. Inclusion of the loci did not significantly influence the results of the analyses and so the data presented here include analyses with the inclusion of ZosNSW02 and ZosNSW38.

Table 4.2: CERVUS output indicating departure from Hardy-Weinberg Equilibrium (HWE) with: the number of alleles per locus (A); the number of samples (N); the observed heterozygosity (Ho); the expected heterozygosity (He); and the inbreeding coefficient (F). *** and ** indicate highly significant (P<0.00001) and significant (P<0.0001) departures from HWE respectively.

Locus	A	N	Ho	He	F
ZosNSW02	2	213	0.056	0.155	0.4482
ZosNSW19	14	218	0.844	0.870	0.0146
ZosNSW20	11	192	0.880	0.881	-0.0014
ZosNSW23	9	218	0.624	0.792	0.1252***
ZosNSW34	7	218	0.665	0.593	-0.0660
ZosNSW38	3	218	0.073	0.080	0.0380
ZosNSW43	12	218	0.642	0.741	0.0751**
ZosNSW45	6	218	0.298	0.370	0.0944
ZosNSW46	12	218	0.849	0.866	0.0063

Population structure

Moderate genetic variation among most sample sites was observed with an overall F_{ST} value for all sample sites of 0.177 ± 0.016 (SE). Results of the AMOVA indicate that variation among individuals within sample sites accounted for 82% of the total variation and 18% occurred among sample sites (P<0.001). All pairwise comparisons of F_{ST} between pairs of sample sites were significantly different from zero (P<0.05). Although little or moderate differentiation was observed among many of the Victorian sample sites, both of the Maria Island sample sites (MFMC and MCB) showed significant differentiation from all other sample sites (Table 4.3). This differentiation is probably due to the occurrence

of one site-specific allele identified at the Maria Island sample sites. Results of the Mantel test (R^2=0.001, P=0.411) for all sites indicated no relationship existed between genetic distance (F_{ST} /1- F_{ST}) and the oceanographic distance between sample sites (km). Separate Mantel tests across the western and central Victorian and eastern Victoria and Tasmania sites showed positive (R^2=0.1434, P=0.040) and weak positive relationships (R^2=0.098, P=0.090) respectively.

Table 4.3: Pairwise F_{ST} values between the 12 sampled *Z. muelleri* sample sites. Figures in bold represent significantly greater than zero (P<0.001). Refer to Figure 4.2 for the location of each population and Table 4.1 for population name abbreviations

	CI	GL	CuI	LT	LSP	MFMC	MCB	OR	PPB	SI	WI	WP
CI	-											
GL	**0.207**	-										
CuI	**0.204**	**0.088**	-									
LT	**0.212**	0.016	**0.112**	-								
LSP	0.156	0.118	0.127	0.111	-							
MFMC	**0.345**	0.332	**0.368**	**0.316**	0.392	-						
MCB	**0.356**	**0.359**	**0.364**	**0.365**	0.533	0.681	-					
OR	0.174	0.123	**0.181**	0.111	0.131	0.383	0.413	-				
PPB	**0.109**	**0.174**	**0.173**	**0.211**	**0.206**	**0.431**	**0.379**	0.192	-			
SI	**0.134**	**0.192**	**0.148**	**0.199**	**0.213**	**0.403**	0.304	**0.164**	**0.151**	-		
WI	**0.238**	0.065	**0.162**	0.08	0.122	0.419	0.461	0.133	**0.176**	**0.247**	-	
WP	**0.148**	**0.095**	**0.078**	**0.128**	0.124	0.376	**0.333**	0.139	**0.061**	**0.125**	**0.13**	-

Assignment of individuals using STRUCTURE 2.3.4 (Pritchard et al., 2000) clearly identified that K=2 had the greatest support i.e. two distinct population clusters across all sampled sites (Fig. 4.3, supplementary material). When individual sample sites were taken into account a distinction between the central Victorian sample sites (PPB, WP, SI, CI) and those of eastern Victoria and Tasmania (GL, LT, LSP, MFMC, MCB, OR, WI) was identified. There were, however, a number of individuals placed within the eastern Victorian genetic cluster (yellow lines Fig. 4.3) that showed similarities with those of western Victorian (blue lines Fig. 4.3) indicating recent dispersal or admixture. This is also apparent when taking into account the placement of Curdies Inlet (far west Victoria) within the eastern cluster and Western Port and Wingan Inlet were sites of admixture between the two clusters.

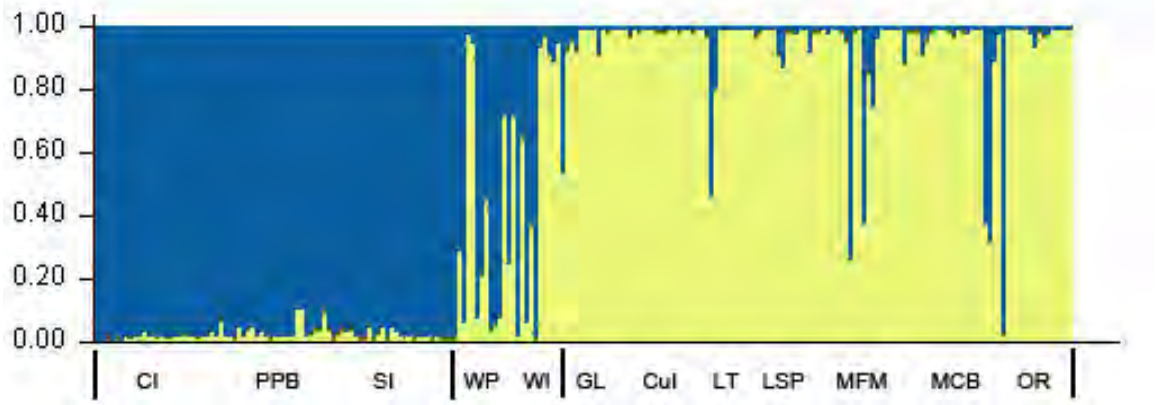

Fig. 4.4. Population clusters within the twelve *Zostera muelleri* sample sites as defined by STRUCTURE 2.3.4. Individual samples are represented by a single vertical line, broken into coloured segments for each *K.* Lengths of each colour are proportional to each of the *K* inferred clusters. Numbers on the y-axis refer to Q values. Refer to Table 4.1 for population name abbreviations.

Differentiation of sample sites via PCoA showed similar results to the STRUCTURE analysis (Fig. 4.4) with reasonable grouping of sample sites based on their location. Corner Inlet (CI), which is situated on the eastern side of Wilsons Promontory, was the only site with a higher number of MLGs less similar to other sites based on the spread of clustering in the PCoA.

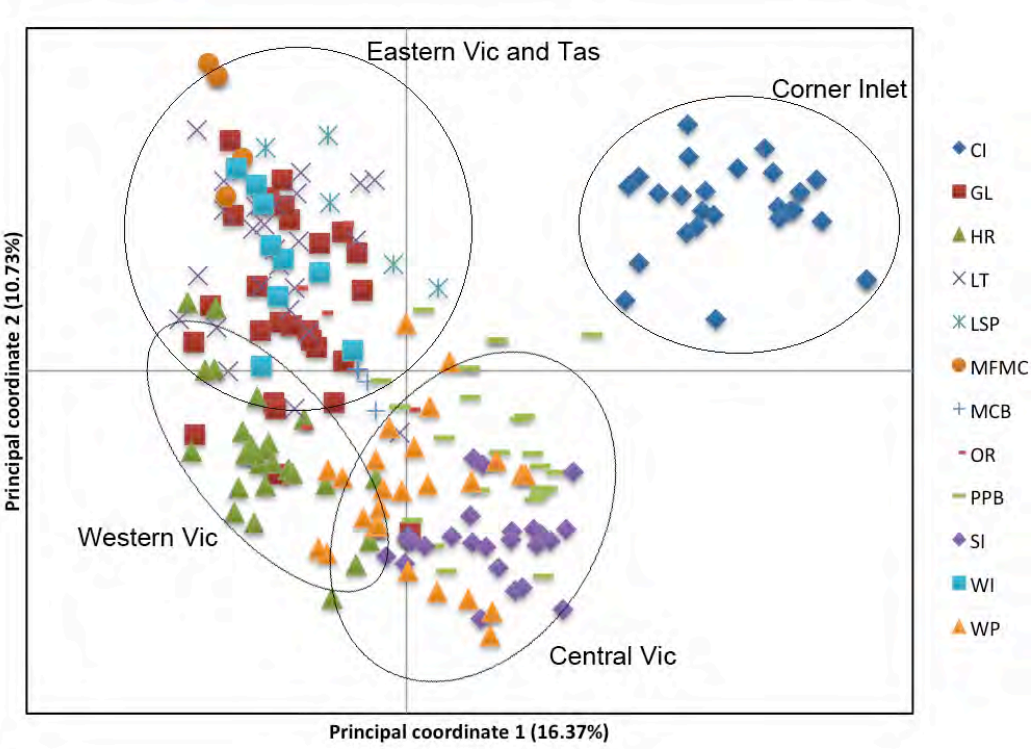

Fig. 4.5. Principal coordinates analysis (PCoA) indicating the spatial separation of MLGs of the twelve *Zostera muelleri* sample sites. Refer to Table 4.1 for population name abbreviations.

Discussion

We found moderate differentiation between the examined *Z. muelleri* sample sites. However, the differentiation between Victorian sample sites located on the western and eastern sides of Wilsons Promontory indicates a strong degree

of connectivity between some sites (Table 4.3, Fig. 4.2). Patterns of genetic differentiation between seagrass populations follows the general trend of limited differentiation (F_{ST} <0.1) occurring among local populations which are separated by less than 100 km which then increases when regional sampling is undertaken (Kendrick et al., 2012). When considered in conjunction with isolation by distance (IBD) relationships which reduce with increased distance between populations, a high F_{ST} value and no, or very weak IBD relationship, is a strong indicator of reduced gene flow among far removed populations (Muñiz-Salazar et al., 2005; Kendrick et al., 2012). The moderate differentiation identified between some sample sites in the current study was expected and our findings are similar to other regional studies on *Z. muelleri, Z. marina* and *Z. noltii* (Diekmann et al., 2005; Muñiz‐Salazar et al., 2005; Becheler et al., 2010; Sherman et al., 2016). Research by Jones et al. (2008) on populations of *Z. muelleri* within New Zealand waters found that sites with a high degree of connectivity (i.e no impedance to gene flow) were sites of genotypic admixture, while far removed sites were considered to be genetically isolated from one another. A more recent study by (Sherman et al., 2016) identified that while a high level of genetic differentiation (overall F_{ST}= 0.278) was observed between sample sites of *Z. muelleri,* significant structuring between populations show that gene flow between the populations was occurring. Given the low degree of differentiation on the western and eastern sides of Wilsons Promontory in the current study, supplementation of *Z. muelleri* sample sites by propagules from surrounding populations is likely and, while not tested in the current study, may influence the ongoing survival of these populations when considered in the

context of metapopulation ecology. Identifying the occurrence of supplementation from surrounding sites could be achieved through further genetic analysis and more in-depth, localised hydrological modelling being undertaken. Determining potential source and sink populations would facilitate more targeted genetic analysis of populations.

Historical barriers and contemporary currents

The two distinct population clusters identified across the studied sample sites, while not specifically examined, indicate the dispersal of *Z. muelleri* propagules, whether sexual or asexual, may be influenced by a number of physiological and environmental factors including the buoyancy of the fragments themselves, currents created by waves, swell and wind and barriers which may reduce dispersal potential. Seeds of *Z. muelleri* are negatively buoyant leading to a reduced likelihood of long distance dispersal, however, they can be enclosed in a positively buoyant reproductive shoot or spathe which detaches from the host plant and aid in the dispersal of seeds (Moore et al., 1993; Orth et al., 2006). Vegetative propagules of *Z. muelleri* have comparatively greater dispersal potential than seeds due to long term viability (>5w) and large lacunal spaces within rhizomatous tissues, which account for 45% of the internal volume (Stafford-Bell et al., 2015). The dispersal of such propagules in the order of hundreds to thousands of kilometres has previously been suggested for some *Zostera* species (Berković et al., 2014; Thomson et al., 2014). The majority of sample sites in the current study showed moderate differentiation but our

results suggest that historical barriers and contemporary oceanic and near-shore currents play a role in the dispersal of *Z. muelleri* propagules.

The historical barrier to the movement of marine species based on the location of the Bassian Isthmus has been well documented and may explain the divergence of sample sites of *Z. muelleri* within the current study (Dawson, 2005; Connell and Irving, 2008; Waters et al., 2010). However, we found three anomalies that call into question a simple binary explanation, namely the inclusion of Curdies Inlet (western Victoria) within the eastern Victorian and Tasmanian population cluster; the significant differentiation of the Corner Inlet site to other sample sites; and Western Port being a site of admixture.

Corner Inlet is characterised by large, shallow mudflats and sandbanks with more than 40% of the tidal flats being exposed during low tide (WGCMA, 2013). Flushing of waters within Corner Inlet takes a number of tidal cycles to occur due to the flat slope of the intertidal flats providing insufficient time for water to drain prior to being inundated again (Molloy et al., 2005). Our results suggest that the population within Corner Inlet showed moderate to significant differentiation to the remaining study sites. The occurrence of the gyre within central Bass Strait (Fig. 4.5) and the slow flushing of the Inlet are likely reducing the movement of propagules both into and out of the Inlet, leading to the differentiation observed between Corner Inlet and the remaining sample sites. Understanding how gene flow can influence *Z. muelleri* populations within Corner Inlet is an important step in conserving them. The moderate to significant differentiation observed in the current study indicates that while

sexual reproduction is occurring at the site, the exchange of genetic material between Corner Inlet and surrounding sample sites has historically been low (Reusch, 2001).

When considered in the light of metapopulation ecology, Corner Inlet may be deemed to be a somewhat fragmented population with little to no exchange of propagules to or from surrounding sites. Fragmentation, and therefore isolation of this population may lead to a number of negative impacts on the species itself and the biota which inhabit seagrass ecosystems including habitat loss, reduced populations sizes and increased genetic isolation (Aguilar et al., 2008). Given the complexity of habitat fragmentation processes, it is often difficult to identify clear species response patterns, however the majority of studies have identified habitat fragmentation to be a major cause of reduced genetic diversity (Aguilar et al., 2008). Should the low level of immigration from surrounding populations continue, potentially exacerbating heterozygosity erosion within Corner Inlet, resilience of those populations to disturbance may be greatly reduced (Procaccini et al., 2007; Aguilar et al., 2008).

Significant research into the hydrodynamics of Western Port shows that an overall clockwise circulation of currents exists with the majority of water entering and exiting via the Western Arm (Melbourne Water, 2011). Modelling does however, indicate that water movement into Western Port also occurs to some degree via the eastern arm (Melbourne Water, 2011). Our results show that Western Port was a site of admixture between the western Victorian population of Curdies Inlet, the central Victorian sample sites and the eastern Victorian and

Tasmanian sample sites (Fig. 4.3). Further analyses indicate that the population sampled on the western shore of the embayment was more closely related to the Curdies Inlet population than those sampled on the eastern arm of Western Port which were more closely related to the eastern Victorian sample sites (Fig. 4.3).

Based on these anomalies, it seems that contemporary currents are ameliorating the historical influence of the Bassian Isthmus as a barrier to the movement of gene flow. The marine waters of southern Australia, particularly within Bass Strait, are subject to a range of tidal, wind-driven and oceanic currents. Tidal currents occur simultaneously from both the west and east creating a region of reduced tidal current within central Bass Strait at the confluence of the westerly and easterly tides (Keough and Black, 1996). Similar to the tidal currents in the region, there is a reduction in wind-driven circulation around Port Phillip Bay and Western Port (Harrison et al., 2008).

Seasonal climatic differences, particularly between summer and winter, directly affect oceanic circulation and therefore may provide a temporal influence on dispersal of seagrass propagules. During winter, propagule movement would occur in an easterly direction as the South Australian Current, which originates as the Leeuwin Current in Western Australia, extends around the southern coast of Tasmania as it forms the Zeehan Current (Harrison et al., 2008). The combination of the Zeehan Current with the Flinders Current results in waters moving in an easterly direction, known as the Bass Strait Cascade (Harrison et al., 2008; Colton and Swearer, 2012). Given *Z. muelleri* flowers during the

warmer months in temperate regions (Walker et al., 2001), it can be hypothesised that winter-based propagule movement would consist primarily of vegetative propagules. Conversely, the summer months are dominated with water moving in a westerly direction as the East Australian Current enters Bass Strait (Harrison et al., 2008) resulting in an westerly movement of propagules that may include reproductive fragments containing seeds.

Results of our study suggest that populations of *Z. muelleri* within south-eastern Australia show varying degrees of connectivity. While the Bassian Isthmus, which once connected Tasmania to mainland Australia, seems to have some influence over the historical distribution of the species, contemporary oceanic and near-shore currents are allowing for the movement of propagules between sample sites of *Z. muelleri* within the region. Differentiation between sample sites highlights the importance of sexual reproduction for the species, however the fact that Corner Inlet showed moderate to significant differentiation to all remaining sites indicates a reduced flow of genes both into and out of the Inlet due to the reduced flushing. Appropriate management of Corner Inlet should identify means to ensure the ongoing survival of those populations, which are unlikely to be supplemented by propagules arriving from distant populations.

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Chapter 5

Phenotypic plasticity of the seagrass, Zostera muelleri Irmisch ex Asch., in south-eastern Australia

Phenotypic plasticity of the seagrass *Zostera muelleri* Irmisch ex Asch, in south-eastern Australia.

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Abstract

The growth and productivity of *Zostera muelleri* and the species ability to display phenotypic plasticity is influenced by the availability of nutrients within some oligotrophic estuaries. To investigate this, data were collected on canopy height, seagrass cover, shoot density, epiphytic algae cover, chlorophyll *a* concentration and seagrass biomass across eighteen populations in southern Australia. Estuaries with higher indicative nutrient loads were found to have reduced above-ground biomass although greater cover, proportion of below- to above-ground tissue and reduced internode lengths were also found under these conditions. Positive correlations were identified between many variables including cover abundance and shoot density (r=0.65, P<0.001), shoot density and internode length (r=0.41, P<0.001) and canopy height was positively correlated with both above-ground biomass (r=0.41, P<0.001) and chlorophyll *a* concentrations (r=0.32, P<0.001). Negative relationships were identified between some variables including canopy height and indicative $NH4^+$ concentrations (r=-0.54, P<0.001) and internode length and the proportion of below- to above-ground biomass (r=-0.32, P<0.001). Our results indicate that the influence of available nutrients within the oligotrophic estuaries had only

moderate effects on the morphological and physiological characteristics of *Z. muelleri*, a species that is capable of displaying phenotypic plasticity and utilises the guerrilla and phalanx growth strategies of clonal plants.

Additional Keywords: facilitation, *Zostera muelleri,* seagrass, epiphyte, phalanx, guerrilla, nutrients

Introduction

Seagrasses, including *Zostera muelleri* Irmisch ex Asch. (Zosteraceae), are ecologically significant, highly specialised angiosperms that have adapted to a marine existence (den Hartog and Kuo, 2006), inhabiting near shore, coastal environments within both temperate and tropical regions (Short et al., 2001). Establishment of extensive seagrass meadows is facilitated through vegetative reproduction and the resulting horizontal extension of underground rhizomes (Kendrick et al., 2012). Fragmentation of vegetative parts that are transported through the water column can enable development of new colonies of independent ramets (Reusch, 2006; Stafford-Bell et al., 2015). Should colonisation events occur following fragmentation, sexual reproduction between genetically different individuals would increase genetic diversity and resilience of populations to both naturally occurring and anthropogenic disturbance (Procaccini et al., 2007). Ultimately, the ways in which seagrass meadows and associated communities develop are a consequence of the environment in which recruitment events occur.

Human-induced alterations within marine environments, whether they result from physical or chemical disturbance, have a negative influence on those

systems (Ralph, 2000; Skilleter and Warren, 2000; Eklöf et al., 2009). Seagrasses are susceptible to marked changes in the water column, including altered salinity regimes, increased turbidity and changes in temperature (Walker et al., 1999; Short et al., 2001). Of particular importance is the availability of nutrients, mainly nitrogen and phosphorus (Walker et al., 1999; Short et al., 2001). Nitrogen (N) is primarily assimilated by above-ground tissues, with below-ground tissues being the major source of phosphorus (P) uptake (Abal and Dennison, 1996). While it is generally accepted that high nutrient loads within marine systems play a negative role in seagrass survival (Touchette and Burkholder, 2000), the addition of N+P has previously been found to significantly increase above-ground biomass and tissue nutrient content (%N and %P) of *Zostera muelleri subsp. capricorni* (Udy and Dennison, 1997). Furthermore, availability of P within sediment pore waters influences phenotypic plasticity and growth strategies used by seagrasses in heterogeneous environments (Slade and Hutchings, 1987a; Maxwell et al., 2014). Phenotypic plasticity allows seagrasses to utilise both the guerrilla and phalanx growth strategies of clonal plants. The opportunistic guerrilla strategy of clonal plants requires them to actively seek out nutrient pools through the rapid expansion of underground tissues within the low nutrient estuaries (Doust, 1981). This foraging behaviour has been identified in a number of clonal perennial species including *Ranunculus repens* (Doust, 1981) and *Glechoma hederacea,* (Slade and Hutchings, 1987b). Within high-nutrient environments, clonal species often develop shorter internodes and profuse branching indicating a consolidation of position in the environment (Slade and Hutchings, 1987b).

Within oligotrophic environments, seagrasses may therefore actively seek out nutrients through the underground extension of rhizomes, or conversely, simply consolidate their position through an abundant growth of adventitious roots when nutrients are readily available (Slade and Hutchings, 1987b).

The estuarine environments of Australia are diverse in their management and display varying levels of modification. In its initial assessment of Australian catchments, rivers and estuaries, the National Land and Water Resources Audit (NLWRA, 2002) identified a number of Victorian estuaries where populations of *Z. muelleri* exist. These included those located in environments, which were considered to be *largely unmodified* and *extensively modified* by anthropogenic activities. *Largely unmodified* estuaries were defined as having between 60% and 90% of their catchment under natural cover, limited infrastructure or anthropogenic influence on water flows and tidal regimes and an ecological system that is largely intact. Conversely, *extensively modified* estuaries had as little as 35% of their catchment under natural cover and had debilitating anthropogenic influences on the ecological system as a whole; including an increased area of tidal sandbanks as a result of greater sediment input from the disturbed catchment (Radke et al., 2006).

We aimed to determine whether the morphological and physiological characteristics of *Z. muelleri* would differ across the six studied estuaries, indicating the species is capable of phenotypic plasticity. We hypothesized that low levels of nutrient enrichment ($NH4^+$ and P) would provide conditions for greater growth in the above and below-ground tissues of the species. To

investigate this we determined *in situ* morphological and physiological characteristics of 18 sample sites across six southern Australian estuaries with varying degrees of nutrient enrichment.

Materials and methods

Site selection and classification

Six Victorian estuaries (Fig. 5.1, Appendix 2) containing populations of *Z. muelleri* were ranked as having low, medium, medium high or high indicative nutrient loading based on historical water quality data for each estuary (Hindell et al., 2009; EPA, 2012; Melbourne Water, 2012; EPA, 2013; Waterwatch, 2014). Where no water quality data existed for an estuary, data were supplemented using standard methods for $NH4^+$ and P determination (van de Wiel, 2003; Duncan et al., 2007; Riddellova, 2012). The appropriateness of each rank was measured against whether combined NH_4^+ and P concentrations for each estuary met, exceeded or greatly exceeded the Australian Water Quality Guidelines for Fresh and Marine Waters or the appropriate State Environmental Protection Policies (SEPP) for the Gippsland Lakes, Port Phillip Bay and Western Port (ANZECC and ARMCANZ, 2000; EPA, 2003) (Table 5.1).

Fig. 5.1. Location of *Zostera muelleri* populations sampled within Victoria. Sites were Port Phillip Bay (PPB), Western Port (WP), Shallow Inlet (SI), Corner Inlet (CI), Gippsland Lakes (GL) and Lake Tyers (LT).

Table 5.1. Water quality data and trigger values for the Gippsland Lakes (GL); Port Phillip Bay (PPB); Western Port (WP); Corner Inlet (CI); Lake Tyers (LT): and Shallow Inlet (SI). Figures in bold are those that do not meet trigger values for that variable. NDA indicates where no data were available Trigger values are taken from the following sources:

(A): Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000) – default trigger values for slightly disturbed ecosystems in South-East Australia

(S): Figures taken from the State Environment Protection Policy (Waters of Victoria) (EPA, 2003). Schedule 6: Waters of Port Phillip Bay; Schedule 3: Waters of Gippsland Lakes; and Schedule 8: Waters of Western Port

Site	Indicative nutrients	Chl a (µg/L-1)		Total P (µg/L-1)		Total N (µg/L-1)		DO (% Sat)		pH		NH4+ (µg/L-1)	
		Obs	Trigger value	Obs	Trigger value	Obs	Trigger value	Obs	Trigger value	Obs	Trigger value	Obs	Trigger value
LT	Low	NDA	4 (A)	30	30 (A)	250	300 (A)	85	>80% (A)	7.4	7.5-8.5 (A)	8	15 (A)
PPB	Med	**2**	1.0 (S)	**59**	30 (A)	185	300 (A)	95	>90% (S)	NDA	7.5-8.5 (A)	10	15 (A)
GL	Med	**22**	4 (A)	**81**	30 (A)	**594**	300 (A)	88	>73% (S)	7.8	6.5-8.5 (S)	3	15 (A)
SI	Med High	NDA	4 (A)	**80**	30 (A)	NDA	300 (A)	NDA	>80% (A)	NDA	7.5-8.5 (A)	**38**	15 (A)
CI	Med High	**10**	4 (A)	**69**	30 (A)	250	300 (A)	95	>80% (A)	NDA	7.5-8.5 (A)	**56**	15 (A)
WP	High	NDA	2.05 (S)	**210**	46 (S)	**2110**	520 (S)	**82**	>85% (S)	7.2	7.5-8.5 (S)	**49**	15 (A)

Source: (Hindell et al., 2009; EPA, 2012; Melbourne Water, 2012; EPA, 2013; Waterwatch, 2014)

Field sampling

Transect design

To determine seagrass morphological and physiological characteristics and thereby phenotypic plasticity within the six estuaries, a nested sampling design was used, with three seagrass meadows being sampled within each estuary type (low, medium or high nutrient). Sampling occurred along three interrupted belt transects running perpendicular to the shore, from the shoreward edge to the deepest extent for the species (Burdick and Kendrick, 2001). Four 1 m^2 sampling units (SU) were placed along each transect. To determine seagrass characteristics at the leading edge of the meadow, a SU was placed on each of the shallow and deep edges of the meadow, ensuring the entire quadrat was within the meadow. The remaining two SUs were placed equidistant between the shallow and deep edge quadrats (Fig. 5.2a).

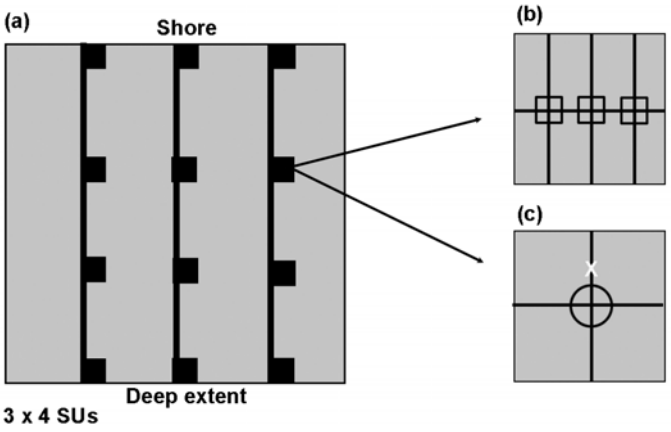

Fig. 5.2. Experimental sampling design displaying: (a) transect design with three interrupted belt transects running perpendicular to shore, each with four 1 m^2 sampling units; (b) position of canopy height measurements and placement of 20 cm^2 quadrats within each 1 m^2 sampling unit for collection of shoot density measurements; and (c) location of above and below ground sample point using 100 mm internal diameter corer and location of sampling point for chlorophyll *a* analysis (x). Note: diagrams not to scale.

Cover abundance of seagrass

The Braun-Blanquet cover abundance scale was used as a means of determining the percentage cover of seagrasses within each sampling unit (Ellenberg and Mueller-Dombois, 1974). Data were collected from each of the SUs (Fig. 5.2a) and the percentage of sediment with seagrass leaves present was determined and given a score from the modified Braun-Blanquet cover abundance scale. For statistical analyses, the midpoint coverage value as described by Wikum and Shanholtzer (1978) was used.

Canopy height

Average canopy height measurements for each SU were calculated by taking three measurements within each SU (Fig. 5.2b). For each measurement, a

large quantity of rooted *Z. muelleri* was extended to its full length with the bottom 80% of the canopy measured from the sediment, i.e. the tallest 20% of leaves was ignored (Duarte and Kirkman, 2001).

Shoot density

The total number of shoots of *Z. muelleri* was counted from three sampling points within each SU using a 20 cm^2 quadrat (Duarte and Kirkman, 2001) (Fig. 5.2b). Sampling points were placed parallel to the shore to remove any potential variation within a given SU as a result of depth. The average number of shoots from the three 20 cm^2 quadrat measurements was re-expressed as shoots/m^2.

Above- and below-ground biomass

From each SU, above- and below-ground biomass samples were collected to a sediment depth of 200 mm using a corer (100 mm diameter) (Fig. 5.2c). Before coring, all above ground shoots originating within the 100 mm diameter core sample were harvested using scissors ensuring the collection of the all above-ground biomass. Following removal of the core, below-ground samples were sieved to remove additional sediment and invertebrates and stored on ice (Duarte and Kirkman, 2001). All samples were then scraped with a razor blade to remove epiphytic algae, and oven dried at 60°C for 24 h to determine their dry weight. Following weighing, twenty random sub-samples for each estuary and the three longest rhizome internodes within each sample were measured to determine internode lengths and thereby growth forms of seagrasses within each estuary.

Cover abundance of epiphytic algae

Data were collected from each of the SUs (Fig. 5.2b) and the percentage of seagrass leaves that had epiphytic algae attached was determined and given a score from the modified Braun-Blanquet cover abundance scale. For statistical analyses, the midpoint coverage value as descried by Wikum and Shanholtzer (1978) was used.

Chlorophyll a

Seagrass samples were taken near the centre point of each SU prior to cores being removed for collection of above and below-ground biomass as described above (Fig. 5.2c). Five blades of actively growing seagrass were removed and stored on ice. For extraction of chlorophyll *a,* 150mg of plant material was submerged in 7ml of N, N-dimethylformamide (DMF) and stored in the dark at 4°C for 24 h. All plant samples were placed in DMF within 24 h of collection.

Following the extraction period, samples were analysed using a Biochrom Libra S12 UV/Vis spectrophotometer and 1cm quartz cuvette. Chlorophyll *a* concentrations (µg/g) were determined using equation 1 where A = absorbance in 1-cm cuvette (Inskeep and Bloom, 1985).

$$\text{Chl } a = 12.70A_{664} - 2.79A_{647}$$

Equation 1: determination of chlorophyll *a* concentration based on spectrophotometric analyses (Inskeep and Bloom, 1985).

Statistical analyses

Prior to analysis, data were tested for departure from normality based on their symmetrical distribution using IBM SPSS version 20 (IBM 2014). Where appropriate, data were log transformed to meet normality requirements. As the SUs could not be considered independent within each estuary (samples were taken from SUs within estuaries) we used a nested univariate general linear model in SPSS (IBM 2014) to identify differences in the morphological and physiological characteristics of *Z muelleri* among the six estuaries (samples were nested within SU nested within estuary). In this instance each SU was treated as a random effect while estuary was treated as a fixed effect. Where differences were identified between estuaries, Tukey's honest significant difference (HSD) post hoc tests were used to evaluate significant differences among means. To identify the presence and strength of any correlative relationships between the variables, data were analysed using Pearson's correlation coefficient (r). We deemed $r > 0.5$ to be a strong correlation, $0.3 > r > 0.5$ a moderate correlation and $r < 0.3$ a weak correlation (Cohen, 1988).

Results

Cover abundance of seagrass

The morphological and physiological characteristics of *Z. muelleri* were found to vary significantly across the six estuaries studied. We also found that these characteristics varied among the SUs within the estuaries. Full model outputs are provided as supplementary material in Appendix 2. Strong and moderate linear correlations were also identified between some variables. Cover

abundance was not found to vary between estuaries but did vary among SUs within the estuaries ($F_{18, 192}$=5.30, *P*<0.001) (Fig. 5.3). A strong positive correlation was also observed between cover abundance and shoot density (*r*=0.65, *P*<0.001). Average cover abundance of seagrass ranged from 34% (SD=20%) within the Gippsland Lakes to 48% (SD=23%) within Western Port. Seagrass growing at the uppermost section of the meadow had reduced abundance (mean=29%, SD=20%) as opposed to those growing in deeper water. Seagrass growing at the deepest extent was found to have slightly less cover (mean=39%, SD=19%) than the second deepest extent (mean=51%, SD=23%), as a result of the reduced shoot density within these SUs (refer below).

Canopy height

The height of the seagrass canopy was found to vary between estuaries ($F_{5, 192}$=9.70, *P*<0.001) and also among SUs within the estuaries ($F_{18, 192}$=6.95, *P*<0.001). There was a strong negative correlation between canopy height and $NH4^+$ concentrations (*r*=-0.54, *P*<0.001) and a moderate negative correlation with P concentrations (*r*=-0.32, *P*<0.001). A moderate positive correlation between canopy height and above-ground biomass (*r*=0.41, *P*<0.001) was also identified. Post hoc tests indicate the greatest growth was seen for those estuaries with permanently subtidal seagrass (Lake Tyers: mean depth=0.34m, SD=0.14m; Gippsland Lakes: mean depth=1.66m, SD=0.47m) (Fig 5.3). Average canopy heights ranged from 72mm (SD=23mm) for Corner Inlet (intertidal) to 285mm (SD=118mm) for the Gippsland Lakes (subtidal).

Seagrass growing at the upper edge of the meadow also had shorter canopies (mean=91mm, SD=73mm) than those growing in deeper water (mean=196mm, SD=131mm).

Shoot density

The density of shoots of *Z. muelleri* varied between the estuaries ($F_{5, 192}$=5.66, *P*=0.003) (Fig 5.3) and also among SUs within the estuaries ($F_{18, 192}$=2.31, *P*=0.003). A moderate positive correlation existed between shoot density and $NH4^+$ concentrations (*r*=0.34, *P*<0.001) and a strong positive relationship was found with the cover abundance of seagrass (r=0.65, *P*<0.001). Average shoot densities across all estuaries ranged from 520 shoots/m^2 (SD=227 shoots/m^2) in the Gippsland Lakes, to more than 1600 shoots/m^2 (SD=1059 shoots/m^2) in Port Phillip Bay (Fig. 5.3). The range of shoot densities across all SUs was large with the minimum density of 83 shoots/m^2 recorded at Western Port and a maximum of 3630 shoots/m^2 at Port Phillip Bay. However, it should be noted that those estuaries with permanently subtidal seagrass (Gippsland Lakes and Lake Tyers) had average shoot densities of 520 shoots/m^2 (SD=227 shoots/m^2) and 942 shoots/m^2 (SD=541 shoots/m^2) respectively, with both estuaries well below the overall average across all estuaries (1190 shoots/m^2, SD=796.31) as determined through post hoc tests.

Shoot density at the upper edge of seagrass meadows was sparser than for all other depths (mean=935 shoots/m^2, SD=755 shoots/m^2). The remaining three depths had similar average shoot densities, which ranged from 1270 shoots/m^2

(SD=783 shoots/m^2) at the deep extent to 1290 shoots/m^2 (SD=806 shoots/m^2) within the lower interior.

Above- and below-ground biomass

Above-ground biomass varied between the estuaries ($F_{5, 192}$=16.25, *P*<0.001) with a moderate negative relationship occurring between above-ground biomass and $NH4^+$ concentrations (*r*=-0.39, *P*<0.001). As expected the amount of above-ground biomass was positively correlated with the canopy height of sampled seagrass (*r*=0.45, *P*<0.001) and the amount of below-ground biomass (*r*=0.58, *P*<0.001). Results of post hoc tests clearly defined Corner Inlet as having the lowest average dry weight above-ground biomass measurement of 23 g/m^2 (SD=20 g/m^2). Lake Tyers and Shallow Inlet had below average (104 g/m^2) biomass measurements of 61g/m^2 (SD=68 g/m^2), and 75g/m^2 (SD=71 g/m^2) respectively. Biomass across all sampling units ranged from1g/m^2 recorded at Corner Inlet to a maximum of 1110g/m^2 at Port Phillip Bay (Fig. 5.3). There was no observed variation among SUs within the six estuaries studied.

Below-ground biomass also varied between the estuaries ($F_{5, 192}$=32.52, *P*<0.001) (Fig. 5.3) and showed a moderate and strong positive correlation to P concentrations (*r*=0.36, *P*<0.001) and above-ground biomass (*r*=0.58, *P*<0.001) respectively. Western Port had the highest P concentration of all estuaries and also had the highest dry weight below-ground biomass measurement (mean=783 g/m^2, SD=737 g/m^2). Post hoc tests clearly defined Lake Tyers and Shallow and Corner Inlets as being clearly defined subsets. The range of below-ground biomass across all SUs was large with the minimum weight of 12

g/m^2 recorded at Lake Tyers and a maximum of 2540 g/m^2 at Port Phillip Bay (Fig. 5.3). Similar to our above-ground biomass results, we found no variation among SUs within the estuaries.

Internode lengths were influenced by the estuary in which sampling occurred ($F_{5, 96}$=22.18, *P*<0.001) and had strong negative and moderate positive correlations to $NH4^+$ concentrations (*r*=-0.55, *P*<0.001) and the density of shoots (*r*=0.41, *P*<0.001) respectively. A moderate negative relationship between the ratio of below- to above-ground biomass and internode lengths was also observed (*r*=-0.32, *P*<0.001). Average internode lengths ranged from 9mm (SD=4mm) at Western Port to 20mm (SD=6mm) at Lake Tyers, with Lake Tyers and Shallow Inlet being identified as clearly defined subsets in our post hoc tests.

Below- to above-ground biomass ratio

The ratio of below- to above-ground biomass was influenced by the estuary in which sampling occurred ($F_{5, 192}$=12.33, *P*<0.001). We also found variation among SUs within the six estuaries ($F_{18, 192}$=1.81, *P*=0.026). There were moderate and weak negative correlations with $NH4^+$ concentrations (*r*=-0.48, *P*<0.001) and P concentrations (*r*=-0.24, *P*<0.001) respectively. Corner Inlet was found to have the highest average ratio of below- to above-ground biomass of 88% (SD=10%) while Lake Tyers had the lowest average ratio of 55% (SD=20%). The remaining estuaries ranged from 68% (SD=19%) in Shallow Inlet to 86% (SD=7%) at Western Port (Fig. 5.3). A moderate negative relationship was found between the below- to above-ground biomass ratio and

internode length (r=-0.32, P<0.001); as internode length decreased the ratio of below- to above-ground biomass ratio increased. Post hoc tests, however, failed to identify clear differences between the means.

Cover abundance of epiphytic algae

Variation in the cover abundance of epiphytic algae was observed between the estuaries ($F_{5, 192}$=11.21, P<0.001) (Fig. 5.3) although post hoc tests failed to identify clearly defined subsets. A moderate positive relationship was observed between epiphytic algae and canopy height of seagrasses (r= 0.45, P<0.001). Coverage of seagrass leaves by epiphytic growth ranged from 35% (SD=29%) at Lake Tyers to 78% (SD=17%) within the Gippsland Lakes (Fig. 5.3).

Epiphytic algal growth at the upper edge of seagrass meadows had on average 33% (SD=28%) of leaves with attached epiphytic algae, a result that was lower than the overall average of 43% (SD=30%) for all sampling units. The highest epiphytic algal coverage occurred at the deep extent of the species (mean=51%; SD=32%).

Chlorophyll a

Chlorophyll *a* concentration was found to vary between estuaries ($F_{5, 192}$=20.20, P<0.001) with an average concentration across all estuaries of 15 µg/g (SD=5 µg/g). A moderate positive correlation between chlorophyll *a* concentration and the canopy height of seagrasses was also observed (r=0.37, P<0.001). Post hoc tests identified Shallow Inlet (medium high nutrient) and Corner Inlet (medium high nutrient) and Lake Tyers (low nutrient) as being separate subsets.

All three sites had below average chlorophyll *a* concentrations of 10 µg/g (SD=4.1 µg/g), 13 µg/g (SD=3.4 µg/g) and 14 µg/g (SD=3.7 µg/g) respectively. Port Phillip Bay (medium nutrient) had the highest chlorophyll *a* concentrations across the six estuaries of 18 µg/g (SD= 4.5µg/g) (Fig. 5.3). Average concentrations of chlorophyll *a* increased slightly with depth at which seagrass was sampled with a mean concentration at the deepest extent of 16 µg/g (SD=4.9 µg/g) compared to 14 µg/g (SD=4 µg/g) at the uppermost section of the meadow.

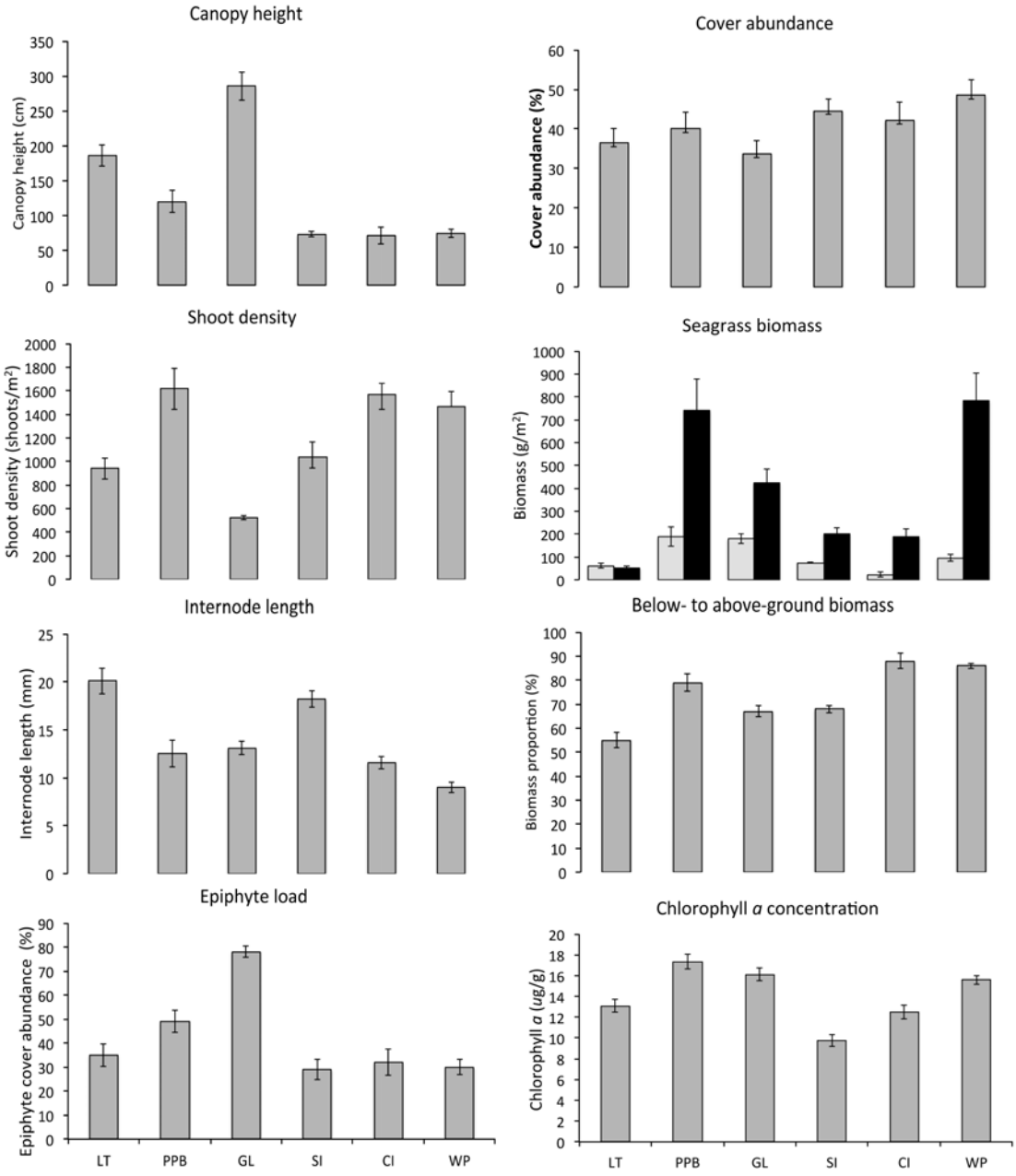

Fig. 5.3. Morphological and physiological characteristics of *Z. muelleri* across six Victorian estuaries (Lake Tyers (LT); Port Phillip Bay (PPB); Gippsland Lakes (GL); Shallow Inlet (SI); Corner Inlet (CI) and Western Port (WP). Indicative estuarine nutrients increase from LT to WP. For seagrass biomass graph, the grey bars represent above-ground biomass while the black bars represent below-ground biomass. Error bars indicate standard error of the mean.

Discussion

Anthropogenic nutrient enrichment within the marine environment has received much attention in the literature and negative effects on flora and faunal assemblages are common (e.g. Ralph, 2000; Skilleter and Warren, 2000; Eklöf et al., 2009). While we have identified a number of relationships, further investigation is required in order to determine direct effects.. Low level nutrient enrichment was found to have a positive influence on the cover abundance of seagrass and the development of greater stores of below-ground compared to above-ground tissues in the studied populations. Those estuaries with higher indicative nutrient loads (Western Port, Corner Inlet and Shallow Inlet), however, showed decreasing trends in a number of the morphological characteristics studied including seagrass canopy height, development of above- and below-ground tissues and chlorophyll *a* concentration. Our results identified strong and moderate correlative relationships that also had positive and negative influences on seagrass growth characteristics. Relationships that existed between the above-ground biomass variables of canopy height, cover abundance of seagrass, shoot density, chlorophyll *a* concentrations and epiphytic algal growth indicate that ongoing persistence and productivity may, in fact, be density dependent.

Given the positive correlations between P concentrations and the development of below-ground tissues identified in the current study, it could therefore be expected that a similar increase in internode length would occur. Our results, however, were contrary to this expectation where an increase in below-ground

tissues resulted in reduced elongation of rhizomatous tissues and estuaries with higher indicative P concentrations had significantly shorter internode lengths with an abundance of adventitious roots. Internode lengths, were comparable to those reported for *Z. muelleri* subsp*. capricorni* within modified New Zealand estuaries (Turner and Schwarz, 2006). However, within two of our estuaries internode lengths were greater (Lake Tyers 20.1 mm; SD=5.9 mm; Shallow Inlet 18.2mm, SD=2.9mm) than those reported for New Zealand (≈10 mm). Albeit correlative, these findings suggest that *Z. muelleri* is displaying phenotypic plasticity as a means of surviving within heterogeneous environments. This phenotypic response has previously been identified within a number of seagrass species including *Halodule wrightii* (Raniello et al., 2004), *Z. noltii* (Cabaço et al., 2009) and *Z. muelleri* (Maxwell et al., 2014). The highly adaptive capacity of the invasive alga *Caulerpa racemosa* has also been attributed to the species' plasticity (Raniello et al., 2004). Seagrasses are commonly found within nutrient poor environments, and exhibit the ability to utilise sediment bound nutrients as well as those within the water column itself (Duarte, 1995; Morris et al., 2007). Of particular importance for below-ground biomass growth is the concentration of P, which is readily absorbed within particulate matter and seagrass biomass and can be converted into a persistent authigenic form or organic forms such as phosphonates (Fourqurean et al., 1993; Paytan and McLaughlin, 2007). At the sediment-water boundary of marine waters, P is taken up via absorption and mineralisation with sediments that are often rich in iron and manganese (Paytan and McLaughlin, 2007). The concentration of phosphorus therefore sharply increases across the water-sediment boundary

with a tripling of the nutrient concentration between the water column and the top centimetre of sediment (Sundby et al., 1992). This high concentration within the uppermost sediment layers ultimately leads to the dominance of adventitious roots over rhizomatous root structures due to their enhanced ability to uptake P (Walk et al., 2006). The inverse relationship between internode length and the proportion of resource allocation to the development of below-ground biomass may therefore be important to the development of spatially large seagrass meadows.

Vegetative reproduction, facilitated through the horizontal extension of rhizomes, allows *Z. muelleri* to establish spatially extensive meadows which can be in the order of square kilometres (Turner and Schwarz, 2006). This reproductive strategy also provides seagrasses with a means of colonising bare substrates following disturbance events, with rates of recolonisation ranging from weeks to many years depending on the species in question (Kenworthy et al., 2002; Macreadie et al., 2014). Should localised disturbance events or a significant increase in P concentrations occur within one of the studied estuaries, it is highly likely that this would have a profound effect on the ability of *Z. muelleri* to recolonise and establish new populations (Cabaço et al., 2008).

The ability of seagrasses to alter water flows has long been recognised. Following recruitment, seagrass canopies can reduce the internal water flow (within-canopy) from 2 to more than 10 times less than that of unvegetated sediment (Koch et al., 2006). This is particularly the case within shallower waters where the ratio of water depth to seagrass canopy height is less than 10

(Fonseca and Cahalan, 1992; Nepf and Vivoni, 2000; Koch et al., 2006). Previous research within *Z. marina* meadows has shown reduced water flows can enhance seedling recruitment due to the reduced scouring effect of local water currents thereby facilitating the long-term persistence of populations (Marion and Orth, 2011). The strong correlations observed between shoot density, canopy height and cover abundance of *Z. muelleri* require further investigation to determine whether such correlations are providing suitable conditions for the long-term maintenance of the studied populations. These may be undertaken through experimental disturbance studies aimed at determining how a reduction in shoot density in particular influences the long-term maintenance of sample populations. Similarly, reduced water flows have been shown to allow a greater abundance of herbivorous grazers which may ameliorate the negative influence of epiphytic algae upon seagrass leaves (Schanz et al., 2002). Further research should investigate the presence of herbivores within high and low shoot density seagrass populations to determine whether the reduced flow within high density patches can lead to increased herbivore abundance.

Marine epiphytes, such as the *Bostrychietum* algae found within mangrove-dominated environments are able to thrive in low-light environments as a result of their low light saturated photosynthetic rates (Raven et al., 1995). As a result, there are potentially greater impacts of increased epiphyte loads on those estuaries with permanently subtidal seagrasses (Gippsland Lakes and Lake Tyers). While these estuaries had higher canopy heights, a morphological adaptation to low light environments (Vermaat et al., 1997), they also had shoot

densities well below the overall average of 1190 shoots/m^2. Lower shoot densities would provide limited habitat for herbivorous grazers potentially leading to higher epiphyte loads that would significantly influence the photosynthetic ability of seagrasses within these estuaries.

Seagrasses have high light requirements and are susceptible to changes in light availability (Abal et al., 1994). Reduction in available light may be the result of both natural (reduced light during dormant periods) or anthropogenic causes such as sedimentation causing turbidity or high nutrient inputs leading to an increase in both micro- and macroalgae and direct epiphytic growth on leaves (Hemminga and Duarte, 2000). Experimental enrichment of seagrass environments has been found to have a significantly positive influence on the growth of epiphytic algae (Hughes et al., 2004; Morris et al., 2007). Our results show that the growth of epiphytic algae was greatest within subtidal populations and was positively correlated with the canopy height of seagrass as opposed to the indicative nutrient status of the estuary. While chlorophyll *a* concentrations were also found to remain comparatively high within our higher nutrient estuaries, indicating that these populations remain productive, excess epiphytic growth may ultimately hinder the species ongoing survival ability (Morris et al., 2007).

Collectively, we have been able to identify a number of relationships within this descriptive study, however to fully assess the influence of available nutrients within the oligotrophic estuaries will require further investigation to determine direct effects. Further research is also recommended to determine whether the

strong and moderate positive correlations identified within the current study may be providing the necessary conditions for the long-term persistence of these populations and their ability to provide critical ecosystem services.

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Chapter 6

Composition of microphytobenthic communities within seagrass systems reflects environmental condition

Composition of microphytobenthic communities within seagrass systems reflects environmental condition.

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Abstract

Microphytobenthic (MPB) communities within seagrass systems provide a range of crucial ecosystem services including the provision of considerable amounts of primary production. MPB hold a significant place in the trophic structure of seagrass systems and their ability to transfer energy to higher tropic levels, often through complex food chains, can sustain a number of economically important fish species. The highly sensitive nature and rapid response of MPB to anthropogenic nutrient inputs, has led them to be considered as useful bio-indicators. However, because of their size, diversity and morphological characteristics, obtaining MPB community data can be challenging and time consuming. Here we used metabarcoding, a DNA profile technique, to examine MPB communities within populations of the seagrass *Zostera muelleri* from five Victorian estuaries of varying condition. We found each estuary contained a unique MBP community (PERMANOVA: F = 12.23, *P* = 0.001), with MOTU richness (ANOVA F=19.22, *P*<0.001), evenness (ANOVA: F = 5.4, *P* = 0.001) and diversity (ANOVA: F = 12.18, *P*<0.001) also varying among the estuaries. MOTU richness and diversity were generally

greater within the higher indicative nutrient estuaries while Lake Tyers (low nutrient) was identified as a having the lowest evenness of all of the estuaries studied. Interestingly, we found that positioning within the seagrass meadow had no influenced on MPB community structure, even in areas where there was no seagrass cover (PERMANOVA F = 1.03, *P* = 0.358). The presence of both nutrient-sensitive and nutrient-tolerant species, within the estuaries provides an indication of water quality at the time of sampling. The ability to identify and use multiple taxa in determining the condition of estuarine water quality is a novel approach that can facilitate improved management outcomes within seagrass systems.

Introduction

Microphytobenthic (MPB) species, within the supergroup SAR (stramenopiles, alveolates and Rhizaria), are ubiquitous within both marine and freshwater environments, tolerating a broad range of environmental conditions (Buosi et al., 2011; Seckbach and Kociolek, 2011). These microscopic, eukaryotic species form a significant proportion of the benthos and are often characterised and taxonomically identified via genetic analyses combined with morphological features or in the case of the diatoms, by their siliceous frustule, or outer ‘shell’ (Fig. 6.1) (Duff et al., 2008; Smol and Stoermer, 2010). The ability of MPB, in particular diatoms and dinoflagellates, to remain preserved over extended periods has also provided researchers with an ideal means of determining historical shifts in climate based on changes in the composition of MPB communities (Fritz et al., 1991; Bradbury, 2004; Falkowski et al., 2004).

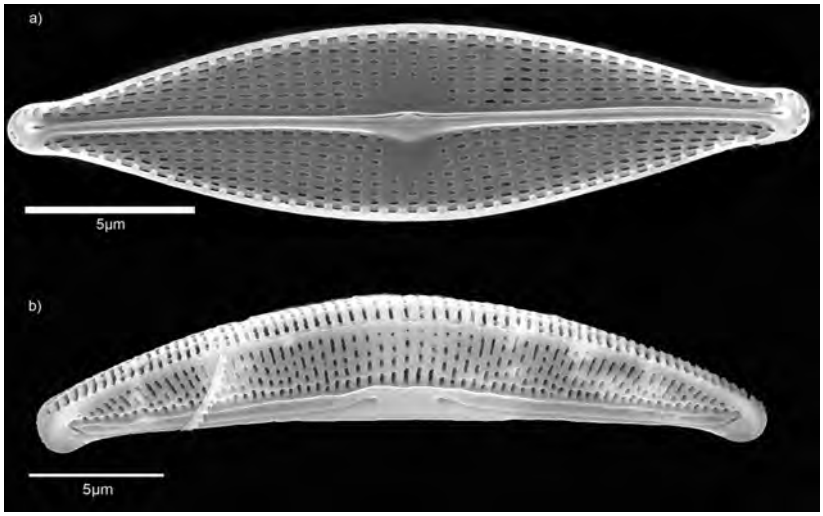

Fig. 6.1: Electron microscope imagery of a) *Navicula gregaria* and b) *Amphora veneta.* (Potapova, 2011; Stepanek and Kociolek, 2011).

The supergroup SAR forms a monophyletic clade of numerous MPB organisms with varying ecology and morphology (Burki et al., 2007). The stramenopiles (e.g. class Bacillariophyceae and Eustigmatophyceae) are the most widely studied and incorporate a diverse range of heterotrophic lineages that are often characterised by the presence of flagellum of differing morphology (Brown and Sorhannus, 2010). Ancestry of the photosynthetic stramenopiles can be traced back some 1000 Ma, based on the occurrence of the Xanthophyceae within the fossil record (Berney and Pawlowski, 2006). Within the Bacillariophyceae alone there are more than 1,200 known genera that form the basis of many marine food chains, sustaining economically important fisheries including those of the South American anchoveta (Castro et al., 2000; Villac and Kaczmarska, 2011).

The alveolates include the three lineages of ciliates, dinoflagellates and apicomplexans (McGrath and Katz, 2004) with ciliates and dinoflagellates

dating back 750 Ma and 1500 Ma respectively (Porter, 2004). Ciliates predate on both diatoms and bacteria and therefore play an important role in the transfer of energy from primary producer to secondary consumers (Lowe, 2011). The dinoflagellates are responsible for considerable primary production in tropical waters, particularly within coral reef systems, however they have also been identified as the cause of toxic red tides which may occur as a result of increased nutrient loading into marine systems (Anderson et al., 2002; McGrath and Katz, 2004).

The Rhizaria are largely amoeboid forms and include the divisions of Foraminifera, Cercozoa and the Radiolaria (Porter, 2004; Baldauf, 2008). The development of internal skeletal features within some of the Rhizaria, composed of silica and calcium carbonate, have provided important additions of these species within the fossil record which date back some 540 Ma (Baldauf, 2008; Caron et al., 2012). Similar to the alveolates, the Rhizaria have an important role in the transfer of energy through to upper trophic levels and predate on a range of organisms including bacteria and diatoms as well as small faunal species of copepods (Burki and Keeling, 2014; Brouwer et al., 2015).

The distribution and abundance of MPB species is often directly related to the availability of light and nutrients within the water column as well as physical stressors that influence their ability to accumulate or disperse such as wave action or ecological interactions (predation and competition) that may reduce their abundances (Villac and Kaczmarska, 2011). Many MPB taxa are highly

sensitive and respond rapidly to anthropogenic nutrient inputs making them useful bio-indicators of changes in estuarine systems, including eutrophication (Johnston and Roberts, 2009; Chariton et al., 2015). Furthermore, based on their extensive study within the literature, ideal nutrient states and tolerances of increased nutrients have been identified for many taxa allowing for conclusive investigation into estuarine nutrient status to be undertaken (Hall and Smol, 2004).

Identification of MPB communities has often involved labour intensive processes that include collection, separation of species from sediments and finally identification via microscopy, which requires a strong taxonomic ability (Nagy, 2011). Considering these communities are generally species-rich, with abundant taxa being represented in a single sediment sample, rapid means for identifying multiple taxa is important for their use in identifying short-term changes in estuarine water quality (Hall and Smol, 2004). Advances in genetic methods may provide an opportunity for the rapid identification of MPB communities that can be used to highlight their ecological importance within seagrass systems. Metabarcoding utilises DNA extraction techniques and high-throughput next generation DNA sequencing and can allow for the identification of multiple species from a single environmental sample (Taberlet et al., 2012; Chariton et al., 2015). The process can provide significant data on community composition and structure, a process that lends itself fully to the identification of numerous sediment-bound MPB species.

Here we used high-throughput next generation sequencing of a region of the 18S rRNA gene to determine the composition of MPB communities associated with populations of *Z. muelleri* within five Victorian estuaries. This was achieved by firstly determining whether DNA sequencing could identify differences in the MPB communities between the five studied estuaries. We then aimed to identify whether morphological characteristics and presence of *Z. muelleri* influenced the expression of those communities and finally we aimed to determine the extent to which the MPB communities could be used as bio-indicators of the water quality within *Z. muelleri* populations.

Materials and methods

Study sites

Five Victorian estuaries (Fig. 6.2) containing populations of *Z. muelleri* were previously ranked as having low, medium or medium-high indicative nutrient loading based on historical water quality data for each estuary (Hindell et al., 2009; Melbourne Water, 2012; EPA, 2013; Waterwatch, 2014; Stafford-Bell et al., 2016). The appropriateness of each rank was previously measured against whether combined NH_4^+ and P concentrations for each estuary met, exceeded or greatly exceeded low-risk guideline trigger values for slightly disturbed ecosystems within south-east Australia (Stafford-Bell et al., 2016) Trigger values were taken from the Australian Water Quality Guidelines for Fresh and Marine Waters or the appropriate State Environmental Protection Policies (SEPP) for the Gippsland Lakes, Port Phillip Bay and Western Port (ANZECC and ARMCANZ, 2000; EPA, 2003) (Table 6.1).

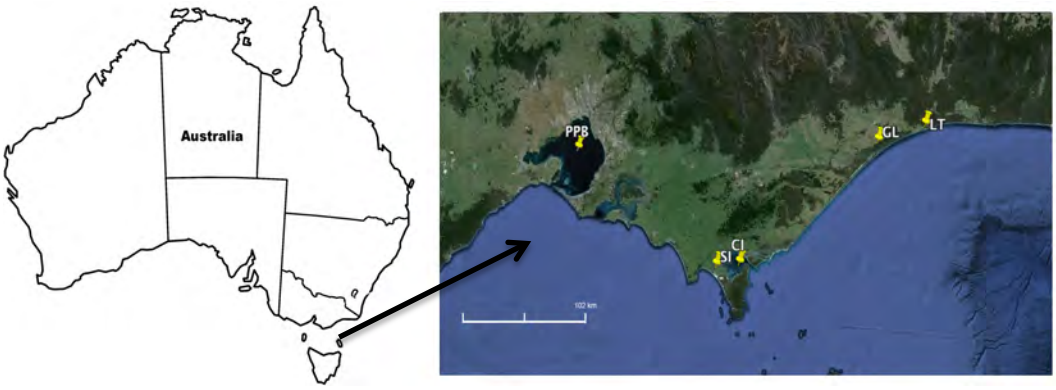

Fig. 6.2. Location of benthic MPB sampling within *Zostera muelleri* populations. Sites were Port Phillip Bay (PPB), Shallow Inlet (SI), Corner Inlet (CI), Gippsland Lakes (GL) and Lake Tyers (LT).

Table 6.1: Form and environmental condition of the five estuaries. Figures in bold are those that do not meet trigger values of 15 µg/L^{-1} ($NH4^{+}$) and 30 µg/L^{-1} (P).

Site	Nutrient status	$NH4^{+}$ (µg/L^{-1})	Tot P (µg/L^{-1})	Catchment area (km^2)	Intertidal area (km^2)	Water area (km^2)	Entrance width (km)	Condition modifiers
Lake Tyers	Low	8	30	572	1.29	13.1	0.14	Periodic algal boom/eutrophication, forestry, urban use, modified entrance
Gippsland Lakes	Med	10	**59**	20449	0	486	0.36	Altered freshwater flow, dam, groundwater extraction, stormwater / increased runoff, minor clearing, agriculture use, urban use, dredging, modified entrance
Port Phillip Bay	Med	3	**81**	15709	14.1	1897	3.46	Dam, effluent pollution, port/port works, multiple land use, dredging, 65% cleared natural cover, urban use
Shallow Inlet	Med high	**38**	**80**	83	7.05	5.03	0.29	Minor clearing, altered freshwater flow
Corner Inlet	Med high	**56**	**69**	2106	387	378	1.89	Minor clearing, sewage treatment plant, port/port works, grazing

Source: (Hindell et al., 2009; EPA, 2012; Melbourne Water, 2012; EPA, 2013; Waterwatch, 2014; OzCoasts, 2016)

Sample collection

To determine whether seagrass morphological characteristics and position within meadow influenced the structure and expression of MPB communities, a nested sampling design was used with three seagrass meadows being sampled within each of five estuaries. Sampling occurred along three interrupted belt transects running perpendicular to the shore, from the centre point of the meadow to two metres past the deepest extent for the species (Burdick and Kendrick, 2001). Three 1 m^2 sampling units (SU) were placed along each transect allowing the influence of meadow position (outside, leading edge and within meadow) on MPB assemblages to be investigated. To determine the composition and structure of MPB communities at the leading edge of the meadow, a SU was placed at the deep edge of the meadow, ensuring the entire quadrat was within the meadow. The remaining two SUs were placed equidistant from the deep edge quadrat with one quadrat placed within the meadow and the other outside the meadow (Fig. 6.3). Seven samples were taken from randomly selected SUs within each of the three meadow positions for each for the five estuaries resulting in 7 samples from each of the outside, leading edge and within meadow positions (total of 21 samples per estuary). The sample of approximately 100g was taken from the surficial layer (1.5-2cm) within each SU and stored on ice for return to the laboratory. Prior to DNA extraction, samples were stored at -80ºC to ensure degradation of the MPB was kept to a minimum.

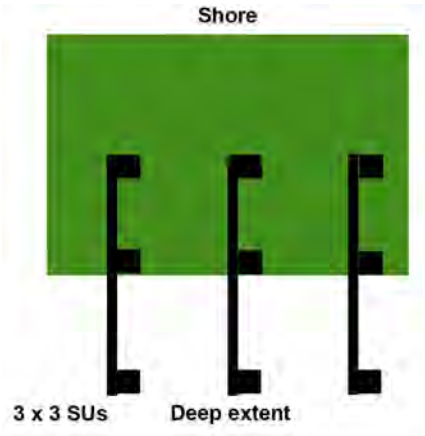

Fig. 6.3. Experimental sampling design displaying transect design with three interrupted belt transects running perpendicular to shore, each with three 1 m^2 sampling units (SU). Sediment samples were taken from the centre point of each SU.

Seagrass morphological characteristics

Data collection on seagrass morphological characteristics as described in Stafford-Bell et al. (2016) occurred concurrently with the collection and analysis of MPB DNA as described here. To determine whether the morphological characteristics and therefore presence of *Z. muelleri* influenced the benthic communities, the following characteristics were used in the statistical analyses: cover abundance of seagrass, shoot density and above- and below-ground biomass.

Metabarcoding

We used primers designed by Dr. Pierre Taberlet (University Of Joseph Fourier, France) which targeted MPB (DiaF/DiaR following page, unpublished), with the primers designed and tested *in silico* using the bioinformatic packages ecoPrimer and ecoPCR software packages (Riaz et al., 2011). EcoPrimer uses the quality indices of mismatch (B_c) and specificity (B_s) to

scan entire genomes to identify suitable markers from reference sequences (Riaz et al., 2011). ecoPCR utilises a computer-based analysis to select sequences similar to a chosen pair of primers (Ficetola et al., 2010). Due to the *in silico* nature of the process, there is an inherent chance that rare species within a sediment sample with high similarity to the primer pairs may be over amplified at the expense of species exhibiting less similarity with the primer pairs (Ficetola et al., 2010). As a result, ecoPCR provides for less rigorous PCR conditions to ensure the latter are not underestimated in the composition of communities within each sample (Ficetola et al., 2010).

DNA extractions were performed on a 10g sub-sample of each sediment sample using PowerMax® Soil DNA Isolation kits (Mo Bio Laboratories Inc., CA) following the manufacturer's protocols with extracted DNA stored at -20ºC for later use. The DNA template was then diluted (1:6) to reduce inhibition of the polymerase chain reaction amplification (PCR) (50µL) of a 100-200-bp fragment of the 18S rRNA gene. PCR amplifications were performed using Phusion High Fidelity Master Mix with HF Buffer (Thermo Fisher Scientific, PA) following manufactures protocols with 0.5µM of each of the primer pairs DiaF (5'- TCCAGCTCCAATAGCGTA -3') and DiaR (5'-AACACTCTAATT TTTTCACAGTA-3'). Each sample was provided a unique barcode sequence attached to the target sequence to allow for differentiation between samples. Amplifications were performed on a Biorad MyCycler Personal Thermal Cycler (Bio-Rad Laboratories Hercules, CA) under the following conditions: initial heating to 98°C for 60s followed by 35 cycles of 98°C for 30s, 58°C for 30s, 72°C for 90s with final elongation at 72°C for 5 min. All PCR products were

then purified using an Agencourt AMPure XP purification kit (Beckman Coulter Inc. Brea, CA) prior to pooling. Samples were sequenced on the Illumnia MISeq platform by the Australian Genome Research Facility (Melbourne, Victoria).

Bioinformatics was performed using CSIRO's custom amplicon pipeline, GHAP. Briefly, GHAP demultiplexes, merges the pair reads, uses USearch to dereplicate and cluster MOTUs (Molecular Operational Taxonomic Units)(Edgar, 2010). Identification of taxons using unique sequences, MOTUs, to the species level and higher was deduced using the Ribosomal Database Project (RDP) classifier with the SILVA 18S rRNA database (release 123) (www.arb-silva.de). Following removal of inaccurate sequences the dataset was rarefacted to 6248 reads. Only MPB associated MOTUs from the supergroup SAR (stramenopiles, alveolates and Rhizaria) were included in the analyses.

Statistical analyses

Prior to statistical analyses, date were tested for normality based on their symmetrical distribution and log transformed where appropriate. Non-metric multidimensional scaling (nMDS), using the Bray-Curtis dissimilarity to determine the differences in species composition between the five estuaries (BRAY CURTIS 1957) allowed ordination of MOTUs in Primer 6+ (Plymouth Marine Laboratory, UK). A nested two-factor permutational multivariate analysis of variance (PERMANOVA) was performed to determine the location of an estuary and the position samples within a meadow influenced MPB

composition. Pairwise post hoc tests based on 999 random permutations identified where these differences lay. Two-factor analysis of variance (ANOVA), again using position and estuary, were performed to identify where there differences in MOTU richness, evenness (Pielou's evenness index) and diversity (Shannon Wiener diversity index).

To identify which MOTUs were potentially characteristic of each estuary and combinations of estuaries we used the *Indispecies* package in R. Indicator Values incorporated the probability of each MOTU as an indicator of each estuary and the probability of finding that MOTU within each estuary. The *signassoc* function determined whether occurrence of a MOTU previously identified in the *multiplatt* analysis was random allowing us to correct for multiple testing.

To examine the correlative relationships between the composition of microphytobenthic communities, seagrass morphological variables, and environmental variables (indicative $NH4^+$ and P and depth), distance-based linear models (DISTLM) (Legendre and Anderson, 1999) using forward selection of all variables were performed with examination of statistical model quality using Akaike's information criterion corrected for finite samples (AICc) (Bellchambers et al., 2011; Chariton et al., 2015). Following variable selection for this model, we re-ran the DISTLM and visualised the influence of the selected variables using distance-based redundancy analysis (dbRDA).

Results

Sequencing

The three largest proportions of MOTUs that could be assigned to an Order were the Naviculales (24%), Thalassiophysales (12%) and the Bacillariales (10%).

Comparison of microphytobenthic communities between estuaries

Results of the nMDS (Fig. 6.4) provide a visual representation of the communities based on the estuary in which they were found and their position within meadow (outside, leading edge and within). Composition of MPB communities was found to vary between the five estuaries (PERMANOVA: F=12.23, *P*=0.001) and results of pairwise post hoc tests identified that each estuary contained significantly different assemblages (*P*=0.001). Position within meadow (outside, leading edge and within) was not found to influence the composition of the MPB (PERMANOVA F=1.15, *P*=0.201) nor was there any interactive effects between estuary type and position within meadow (PERMANOVA F=1.02, *P*=0.358). The two estuaries with the greatest similarity in MPB communities were those of Corner Inlet and Shallow Inlet (38.8%) while the lowest similarity was identified between Shallow Inlet and Lake Tyers (30.9%) (Table 6.2; Fig. 6.4).

Table 6.2: Average percentage similarity of MPB communities both between and within estuary.

Estuary	Corner Inlet	Shallow Inlet	Port Phillip Bay	Gippsland Lakes	Lake Tyers
Corner Inlet	55.7				
Shallow Inlet	38.8	42.0			
Port Phillip Bay	35.1	33.1	44.3		
Gippsland Lakes	34.5	31.6	31.8	43.9	
Lake Tyers	33.9	30.9	32.4	36.7	51.0

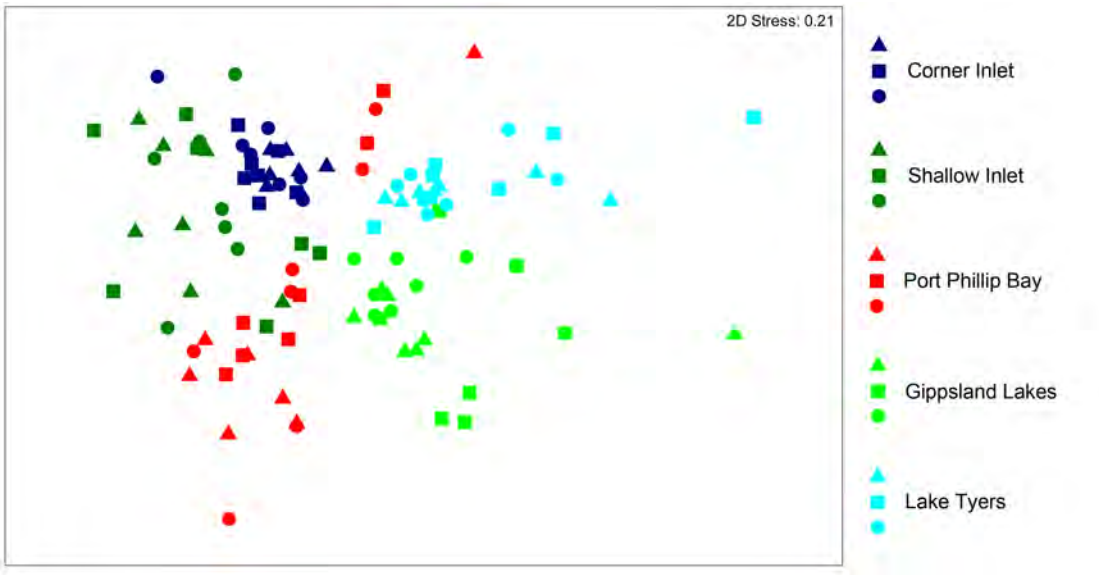

Fig. 6.4. nMDS plot visualising differences in MPB communities from the five sampled estuaries. Darker colours indicate the higher indicative nutrient estuaries and marker shapes indicate the position at which sampling occurred (triangle = outside meadow; square = leading edge; circle = within meadow).

The number of species that could be confidently identified in each of the five estuaries (MOTU richness) was found to vary significantly (ANOVA $F=19.22$, $P<0.001$). Corner Inlet was found to be the richest estuary (mean 262 ± 12 SE), with the remaining four estuaries having similar levels of MOTU richness (Gippsland Lakes 151 ± 14; Lake Tyers 148 ± 11; Port Phillip Bay 146 ± 11; Shallow Inlet 157 ± 11).

Evenness (Pielou's J') of the MPB communities was found to vary between the five estuaries (ANOVA: F=5.4, *P*=0.001). Lake Tyers had the lowest evenness (mean 0.75 ± 0.01) and was dominated by members from the Chaetocerotaceae Family (Fig. 6.5). The remaining four estuaries had similar evenness (Port Phillip Bay 0.79 ± 0.01; Shallow Inlet 0.80 ± 0.01; Gippsland Lakes 0.83 ± 0.01; Corner Inlet 0.83 ± 0.01).

Diversity (H') of MPB communities, also varied between the five estuaries (ANOVA: F=12.18, *P*<0.001). Corner Inlet had the greatest species diversity (ANOVA: F = 11.2, *P*<0.001) (mean 4.6 ± 0.1) when compared to the remaining estuaries, which had similar levels of MOTU diversity (Gippsland Lakes 4.1 ± 0.1; Lake Tyers 3.7 ± 0.1; Port Phillip Bay 3.9 ± 0.1; Shallow Inlet 4.1 ± 0.1).

Indicator analysis identified 229 MOTUs, which were characteristic of the five estuaries at the time of sampling (Tables 6.3 and 6.4, Fig. 6.5). Corner Inlet was found to have the largest proportion (39%). The lowest proportions were found within the Gippsland Lakes (12%) and Shallow Inlet (14%) and Port Phillip Bay and Lake Tyers had similar proportions of 17 and 18% respectively. Corner Inlet was found to contain four indicator Families of MPB (Achnanthidiaceae, Mastogloiaceae and Ochromonadaceae, Peronsporaceae), which were unique to that estuary. Both Corner Inlet and Shallow Inlet (medium-high nutrients) (Table 6.1) contained the highest proportions of the Families Catenulaceae (*Amphora* spp.), Triceratiaceae (*Odontella* spp.) and Plagiogrammaceae (*Plagiogramma* spp.). Lake Tyers

was dominated by mesohalobes of the Chaetocerotaceae Family (*Chaetoceros* spp.) while the Naviculaceae Family (*Fistulifera* spp. and *Navicula* spp.) were ubiquitous across all estuaries (Fig. 6.5).

Table 6.3: Largest proportion of indicator MOTUs that could be confidently assigned for each estuary where N is the number of occurrences for that indicator MOTU.

Corner Inlet		Shallow Inlet		Port Phillip Bay		Gippsland Lakes		Lake Tyers	
Indicator MOTU	Proportion (%) and (N)	Indicator MOTU	Proportion (%) and (N)	Indicator MOTU	Proportion (%) and (N)	Indicator MOTU	Proportion (%) and (N)	Indicator MOTU	Proportion (%) and (N)
Naviculales	15 (14)	Naviculales	19 (6)	Naviculales	31 (12)	Naviculales	30 (8)	Chaetocerotanae incertae sedis	29 (12)
Thalassiophysales	14 (13)	Thalassiophysales	16 (5)	Bacillariales	13 (5)	Bacillariales	11 (3)	Naviculales	20 (8)
Triceratiales	10 (9)	Triceratiales	13 (4)	Thalassiophysales	8 (3)	Rhipidiales	7 (2)	Bacillariales	10 (4)

Table 6.4: Five MOTUs with the highest potential indicator MOTUs for the five estuaries based on indicator analysis where: A is the probability that the identified MOTU is an indicator within the estuary and B is the probability of finding the MOTU within the estuary.

Estuary	A	B	Indicator value	p.value	Genus	Species	Estuary	A	B	Indicator value	p.value	Genus	Species
Corner Inlet	0.99	1.00	0.99	0.001	*Amphora*		Port Phillip Bay	0.95	0.52	0.7	0.001	*Pseudo-nitzschia*	
Corner Inlet	0.98	0.95	0.96	0.001	*Odontella*	*aurita*	Port Phillip Bay	0.96	0.48	0.68	0.001	*Bellerochea*	*malleus*
Corner Inlet	0.98	0.95	0.96	0.001	*Odontella*	*aurita*	Gippsland Lakes	0.88	0.81	0.89	0.001	*Monodus*	
Corner Inlet	0.98	0.95	0.96	0.001	*Odontella*	*aurita*	Gippsland Lakes	0.89	0.86	0.86	0.001	*Paralia*	
Corner Inlet	1.00	0.91	0.95	0.001	*Pleurosigma*	*planctonicum*	Gippsland Lakes	0.81	0.91	0.85	0.001	*Navicula*	*cryptocephala*
Shallow Inlet	0.99	0.91	0.94	0.001	*Triceratium*	*pentacrinus*	Gippsland Lakes	0.73	0.95	0.83	0.001	*Navicula*	*cryptocephala*
Shallow Inlet	0.92	0.81	0.86	0.001	*Trieres*		Gippsland Lakes	0.77	0.91	0.83	0.001	*Nitzschia*	*draveillensis*
Shallow Inlet	0.95	0.52	0.71	0.001	*Navicula*	*reinhardtii*	Lake Tyers	0.98	0.86	0.91	0.001	*Pinnularia*	*altiplanensis*
Shallow Inlet	0.92	0.52	0.69	0.001	*Navicula*	*pulchripora*	Lake Tyers	0.83	1.00	0.91	0.001	*Navicula*	
Shallow Inlet	0.97	0.43	0.64	0.001	*Gyrosigma*	*acuminatum*	Lake Tyers	0.91	0.91	0.9	0.001	*Chaetoceros*	*muelleri*
Port Phillip Bay	0.99	0.72	0.83	0.001	*Leyanella*	*arenaria*	Lake Tyers	0.96	0.81	0.88	0.001	*Chaetoceros*	
Port Phillip Bay	0.78	0.81	0.79	0.001	*Extubocellulus*	*cribiger*	Lake Tyers	0.99	0.72	0.84	0.001	*Chaetoceros*	
Port Phillip Bay	1.00	0.57	0.75	0.001	*Eunotia*								

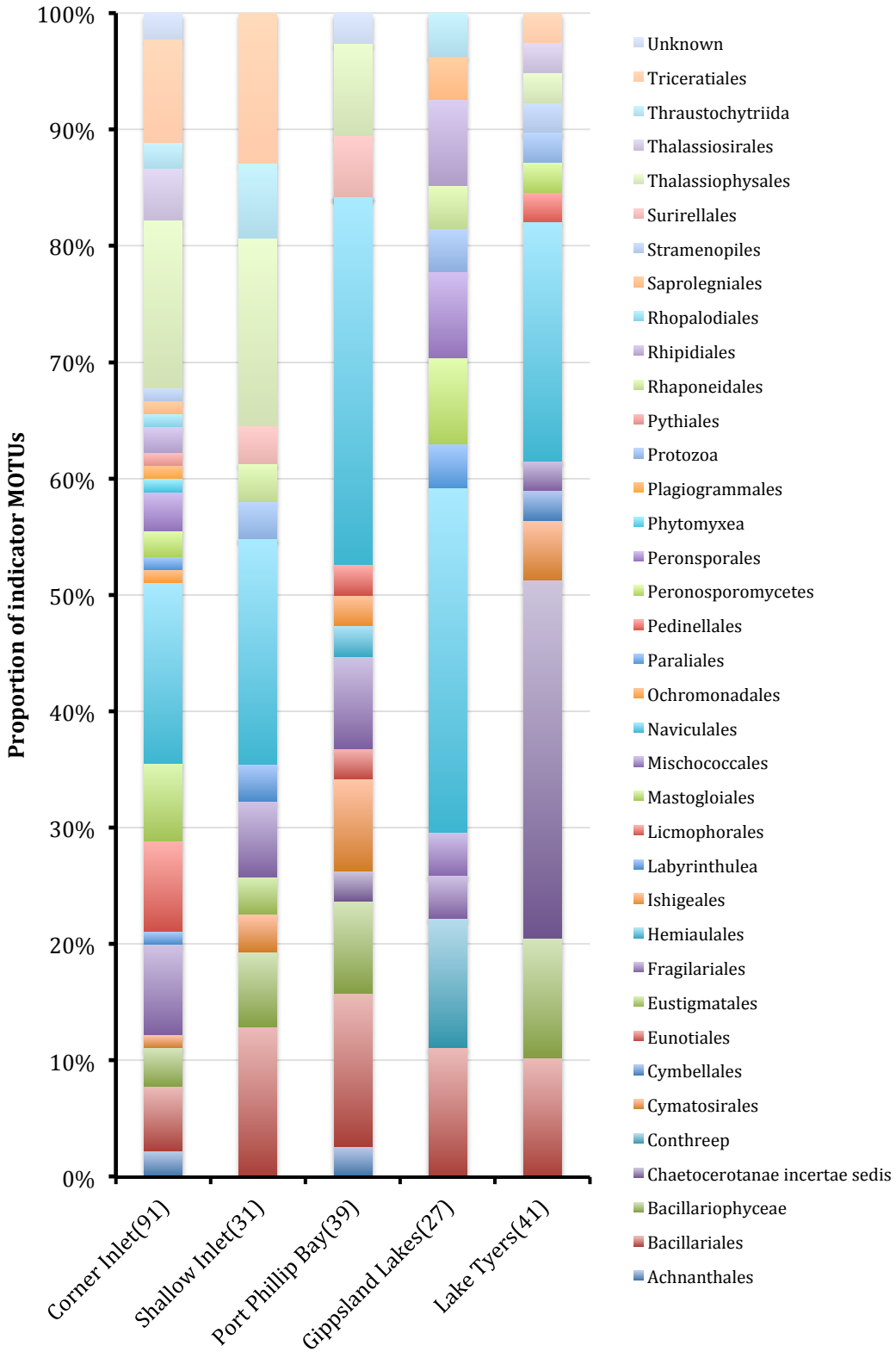

Fig. 6.5. Indicator analysis showing the proportion of MPB Orders identified at the time for sampling for each of the five estuaries. Numbers in brackets indicate the total number of potential indicator MOTUs for each estuary.

The influence of environmental variables on the expression of MPB communities

The distance-based linear regression model (DISTLM) found that there were significant correlations between MPB community structure and the environmental variables $NH4^+$ (F=13.05, *P*=0.001), P (F=9.77, *P*=0.001) and depth (F=2.63, *P*=0.001). With these three variables explaining 21% of the total variation within the MPB community structure. Seagrass presence and morphological characteristics were not found to influence MPB community structure within the estuaries. The first dbRDA coordinate axis explained 12.7% of the variation within these communities and clearly distinguished the medium-high indicative nutrient ($NH4^+$ and P) estuaries of Corner Inlet and Shallow Inlet from the remaining three sites. The second dbRDA coordinate axis explained 6.5% of the total variation and indicated that depth was a defining variable and separated the permanently subtidal communities of the Gippsland Lakes from the other four estuaries (Fig. 6.6).

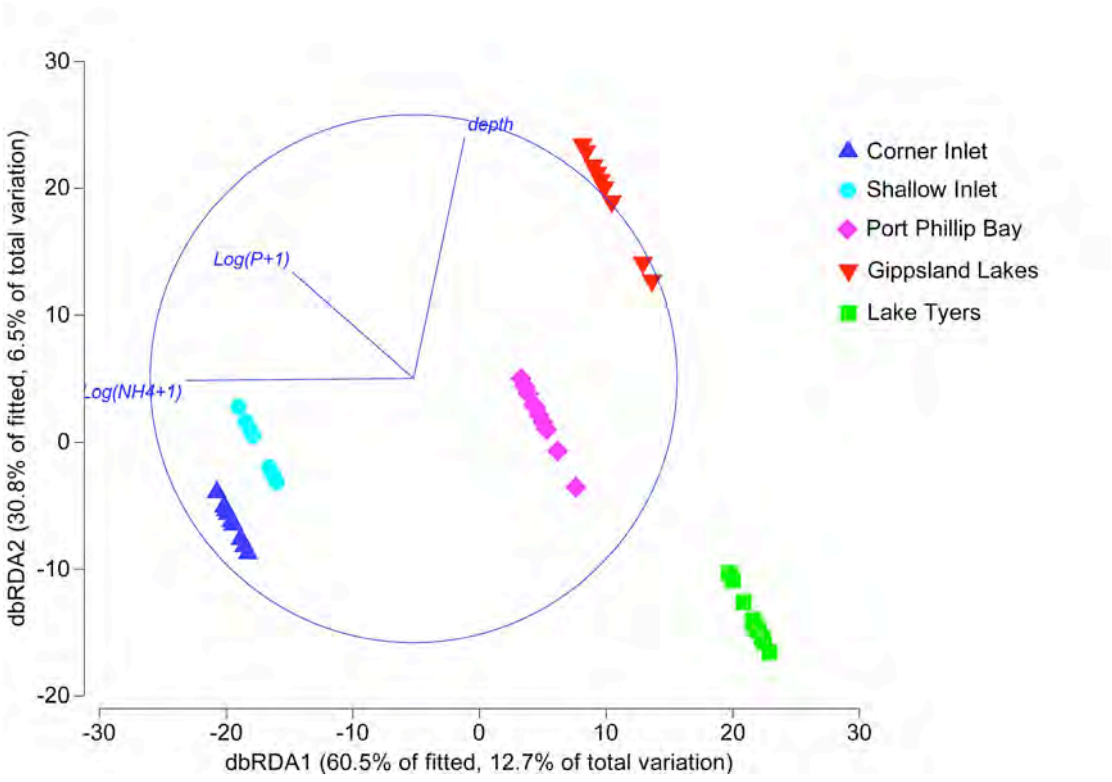

Fig. 6.6. dbRDA ordination plot showing the relationship between the environmental variables of indicative $NH4^{+,}$ P and depth at which sampling occurred.

Discussion

In this study we show that the composition of MPB communities within intertidal and shallow subtidal populations of *Z. muelleri* are influenced by the estuary in which they were sampled and their corresponding environmental conditions. While we hypothesised that the morphological characteristics and presence of *Z. muelleri* would have an influence on the structure of the MPB communities we found that MOTU richness, evenness and diversity were in fact, more clearly influenced by the indicative nutrient status of the estuaries and the depth at which sampling occurred (Fig. 6.6). Identifying key MPB

communities within seagrass systems can therefore provide an important assessment of the ecological condition of the studied estuaries.

The ecological role of MPB in seagrass systems

Microphytobenthic communities, found within the surficial layer of estuarine sediments, are comprised of highly abundant (10^5 - 10^7 cells cm^{-3}) unicellular eukaryotic algae and cyanobacteria (MacIntyre et al., 1996). Distribution of MPB species is often characteristic of the sediments in which they are found with diatoms (Bacillariophyceae) often dominating sandy or muddy substrates and cyanobacteria and the flagellates (e.g. Bicoeceae) surviving within less exposed environments (Yallop et al., 1994; Stal, 2003; Sullivan, 2010). MPB are significant contributors to the highly productive nature of estuarine systems, accounting for roughly 40-50% of total estuarine production (Underwood and Kromkamp, 1999). However, MPB also play a number of important roles in the development of estuarine ecological communities including stabilisation of sediments and provision of a food source for higher trophic levels (Kemp et al., 1999; Underwood and Kromkamp, 1999).

The ability of species to alter an environment making resources available for other species has been termed ecosystem engineering (Bos et al., 2007). Seagrasses are often referred to as ecosystem engineers as they alter local hydrodynamics, thereby reducing currents that stabilises and increases sedimentation and the seagrass itself provides habitat and protection for numerous epibiont species (Passarelli et al., 2014). Seagrasses may often be seen as the dominant ecosystem engineer within many intertidal systems, however the highly complex ecological interactions between communities

within these systems leads to the occurrence of cooperative ecosystem engineering (Passarelli et al., 2014). This interaction between multiple species or communities has a combined effect resulting in greater enhancement of the environmental habitat overall (Passarelli et al., 2014). The ecosystem engineering effect of seagrasses has been long understood (cf.Jones et al., 1994; Bos et al., 2007), however, the role that MPB have in this process has received comparatively less attention.

While our results identified that the presence of *Z. muelleri* had no significant influence on the structure of the MPB communities within the five studied estuaries, previous research has shown that seagrass presence has a strong influence on MPB community development. Research into the occurrence of MPB communities within meadows of *Z. noltii,* a species found within European and northwest African waters, using both experimental flumes and transplant studies found that when compared to unvegetated or sites of low-density seagrass growth, the prevalence of high density growth of *Z. noltii*, had a cascading influence on the trophic structure of the system (Short et al., 2001; Widdows et al., 2008). High density growth of *Z. noltii,* led to a significant increase in the presence of MPB identified through greater chlorophyll *a* concentration in the surficial layer which was positively correlated to the presence of extracellular polymeric substances (EPS) (Widdows et al., 2008). The ability of *Z. noltii* to stabilise the sediments by reducing hydrodynamic pressure was identified as providing conditions suitable for the development of persistent biostabilising biofilms developed through the secretion of EPS within the MPB communities (Widdows et al., 2008). Prevalence of an herbivorous grazing gastropod was also found to be

significantly lower within high density sites indicating the protective nature of the seagrass for MPB community development (Widdows et al., 2008). When considered in light of our research, these discrepancies are likely the result of an historical dependence on the analysis of chlorophyll *a* concentrations as a means of determining MPB biomass within estuarine systems (Anderson et al., 2002; Widdows et al., 2008). The strength of metabarcoding, therefore, is its ability to determine fine-scale community composition within MPB communities, providing a more holistic understanding of the complexity of these communities that may be utilised as an indicator of ecosystem health.

The protective nature and the provision of habitat by seagrasses also supports complex food webs, which involve the transfer of energy from primary producers through to tertiary consumers, within estuarine environments (Edgar and Shaw, 1995). *Zostera muelleri* is often part of simple and short food chains involving the transfer of energy directly from the primary producer (*Z. muelleri*) to tertiary consumers including large herbivorous species such as the black swan (*Cygnus* atratus) and dugong (*Dugong dugon*) (Preen, 1995; Eklöf et al., 2009). Phytoplankton and MPB communities however are often involved in food webs of far greater complexity. Species within MPB communities identified in the current study, including *Skeletonema* spp. and *Navicula* spp. are significant food sources for a number of crustacean species (Deason, 1980; Wylie and Currie, 1991; Sommer, 1997). As a result of greater MPB biomass occurring within some seagrass habitats, production of macrobenthic communities, particularly benthic crustaceans can also be significantly greater within *Z. muelleri* meadows when compared to unvegetated habitats (Edgar and Shaw, 1995;

Widdows et al., 2008). High macrobenthos production has a flow on effect to higher trophic levels with crustaceans providing a significant proportion of the diets of demersal fish highlighting the many levels of energy transfer in some systems where MPB are often the foundational trophic level (Edgar and Shaw, 1995). Within complex estuarine food chains, *Z. muelleri* therefore plays an important role in the provision of habitat for foundational MPB communities.

Influence of available nutrients on MPB communities

Seagrasses play a number of important roles within the marine environment and have been estimated to contribute a significant proportion of the net oceanic productivity (≈12%) even though they only cover 0.15% of the ocean surface (Duarte and Chiscano, 1999). Over the past three decades an average of 110km^2 of seagrass has been lost with anthropogenic nutrient enrichment being considered a major cause for seagrass loss (Orth et al., 2006). In low nutrient environments slow growing seagrasses have been identified as strong competitors due to their low nutrient requirements and ability to utilise sediment-bound nutrients as well as those in the water column (Duarte, 1995). As nutrient concentrations increase, however, the vegetation community shifts from one dominated by slow growing seagrasses to one dominated by fast growing macroalgal species that smother the former and reduces their photosynthetic ability. (Duarte, 1995; Matheson and Schwarz, 2007).

MPB composition and productivity is also governed by the availability of nutrients in oligotrophic estuaries and our results indicate that the communities were influenced by the indicative nutrient status of the five

estuaries. The transport of nutrients, into estuarine systems from creeks or rivers provides a significant source of nutrients for MPB and estuarine productivity (Mozetic et al., 2012). When nutrients are abundant, a proliferation of both phytoplankton and MPB occurs resulting in considerable increases in concentrations of chlorophyll *a* and shifts to a larger size structure within the communities (Giani et al., 2012; Mozetic et al., 2012). This increase in size is often attributed to a bottom-up control mechanism whereby eutrophication as a result of anthropogenic inputs leads to increased availability of resources allowing for greater growth as the influence of grazer control is diminished (Irwin et al., 2006). Given the increase in cell size within the MPB communities under eutrophic conditions, it could therefore be expected that the relative density of cells would be reduced (Irwin et al., 2006). Phytoplankton and MPB of larger size are consumed by larger zooplankton resulting in shorter food webs due to a reduction in the number of trophic levels required to transfer energy to secondary and tertiary consumers (Ryther, 1969).

Conversely, when deliberate attempts to reduce nutrient inputs into estuarine systems occurs, through a process known as oligotrophication, a shift to top-down control prevails (Irwin et al., 2006). In this instance, a reduction in phytoplankton and MPB communities results in a reduction of algal blooms and therefore chlorophyll *a* concentrations and a return to smaller size structure within MPB communities (Mozetic et al., 2012). Reduced size in this case aids in improved rates of nutrient uptake by phytoplankton and MPB due to increased surface area to volume ratios allowing for greater efficiencies in nutrient poor environments (Irwin et al., 2006; Mozetic et al., 2012). Within the

estuaries studied, the presence of both picoplankton (*Skeletonema* spp.) and nanoplankton (*Navicula* spp.*, Thalassiosira* spp*.* and *Chaetoceros* spp.) species indicates top-down control conditions may be occurring with a dominance of small celled species. This fact supports the concept of these estuaries being in a largely oligotrophic condition.

Our results have identified important MPB communites within populations of *Z. muelleri,* each of which deliver critical ecosystem services in their roles as cooperative ecosystem engineers. By gaining a greater understanding of the the ecological condition of these seagrass meadows through identification of MPB community structure, we can provide estuarine managers with a timely baseline from which to manage these systems into the future.

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Chapter 7

Synthesis and future directions

This research aimed to investigate the processes by which *Zostera muelleri* is able to disperse, recruit and grow in south-eastern Australia. We also aimed to examine the population dynamics of the species and its role in the development of microphytobenthic (MPB) communities that inhabit seagrass systems. This was achieved through a multi-faceted approach using: 1) laboratory experiments to investigate germination, early-stage development of germinants and the dispersal potential of the species; 2) *in situ* collection of morphological and physiological characteristics to determine the influence of nutrients and the facillitatory role of the species; and 3) genetic and sequence-based analyses of both *Z. muelleri* and the MPB communities that inhabit seagrass systems to identify connectivity between populations and MPB community composition respectively.

There is growing global concern, clearly identified in the literature and by marine managers, for seagrasses due to significant population declines that have occurred over the past three decades (Orth et al., 2006). Particular attention has been paid in a general sense to some aspects of seagrass ecology investigated in this current study including reproduction (e.g. Conacher et al., 1994; Brenchley and Probert, 1998), dispersal (e.g. Harwell and Orth, 2002), the influence of nutrients (e.g. Kenworthy and Fonseca, 1992; Romero et al., 2006) and the development of MPB communities (Widdows et al., 2008). Specific studies into these factors, however, have been comparatively lacking for *Z. muelleri.* This has resulted in significant knowledge gaps that may have hindered the management of populations of this important seagrass species found within eastern Australian, Papua New Guinean and New Zealand waters.

Germination in *Z. muelleri* has previously been identified to increase under lower salinity conditions (Conacher et al., 1994). The negative influence of high salinity concentrations on germination can, however, be lessened by lower water temperatures (Brenchley and Probert, 1998). While our results provide further support for these finding we have also identified that not only does *Z. muelleri* produce hypocotyl hairs, structures previously undocumented in the species, we have also identified the ideal environmental conditions that influence their development (Chapter 2). Extensive coverage of hypocotyl hairs would provide significant opportunities for germinants to attach to the substrate, potentially resulting in increased germinant survival. We found significant germination occurring following a 48 hr fresh water pulse and development of hypocotyl hairs was found to occur after twelve days when seeds were stored at the optimum temperature for the development of these structures (20ºC). These are important findings for managers of these systems, particularly in revegetation projects utilising broadcast of seed, which have historically showed poor results (Marion and Orth, 2008). A delay in seed broadcasting that incorporates the conditions identified in the current study would greatly improve seed germination and the development of hypocotyl hairs. A significant increase in the likelihood of germinant survival leading to improved management and rehabilitation outcomes would result from sowing seed under the comparatively narrow set of conditions identified as ideal in this study. Our work clearly identifies the importance and role of freshwater pulses to the successful recruitment and establishment of *Z. muelleri* under naturally-occurring situations.

Once germinants, and thereby populations, have established, the ability of *Z. muelleri* to disperse within the marine environment provides an important opportunity to develop or supplement far removed populations with new genetic material. Dispersal of seagrass fragments either derived from vegetative (asexual) or reproductive (sexual) sources has been identified for a number of *Zostera* species including *Z. noltii* (Berković et al., 2014), *Z. nigricaulis* (Thomson et al., 2014) and *Z. marina* (Harwell and Orth, 2002). Our research has identified the means by which *Z. muelleri* vegetative fragments may potentially provide a propagule source for recruitment and maintenance of seagrass populations, namely their long-term buoyancy and viability (Chapter 3). The porosity of rhizomes in *Z. muelleri* is high with lacunae accounting for over 45% of the internal volume of rhizomatous tissues. Fragments are also able to remain viable for extended periods (≈ 5wk). These biological factors provide an opportunity for a high dispersal potential that may explain the wide distribution of the taxa within Australian and New Zealand waters. The environmental conditions required for the successful re-attachment of seagrass vegetative propagules have, however, been difficult to quantify and recruitment of these tissues is generally low. Given the increasing pressures these systems are facing and their ecological importance, there is a need to understand the capacity for such fragments to successfully reattach to the sediment, generate new populations and inter-breed with currently established populations.

Supplementation of populations of *Z. muelleri* can ensure their ongoing survival even though localised impacts may have negative consequences for seagrass systems (Hanski and Simberloff, 1997). Our research into the connectivity of *Z.*

muelleri populations (Chapter 4) has identified the degree to which this occurs. Populations of *Z. muelleri* are often viewed and managed as discreet parcels inhabiting the estuarine systems of south-eastern Australia. This is confounded by the fact that many estuaries may also be under individual management regimes. *Zostera muelleri,* however*,* has strong dispersal ability and connectivity between some populations in the region was high indicating a high potential for population supplementation. However, our results also suggest that the population within Corner Inlet may be deemed to be somewhat fragmented with little to no exchange of propagules to or from surrounding sites. Fragmentation, and therefore isolation of this population may lead to a number of negative impacts on the species itself and the biota which inhabit seagrass ecosystems including habitat loss, reduced populations sizes and increased genetic isolation. This has important consequences for the management of *Z. muelleri* across the studied region and based on our findings, managers must take a more holistic view of these populations, one based on metapopulation ecology.

Anthropogenic disturbance via nutrient inputs into seagrass systems has been identified as a major source of seagrass decline globally (Orth et al., 2006). Our research into the phenotypic plasticity of *Z. muelleri* (Chapter 5) within six estuaries of relatively good water quality identified that both the guerrilla and phalanx growth strategies of clonal plants are utilised by the species. We found that the indicative nutrient status of the six estuaries had a clear influence on some of the below-ground morphological characteristics with higher indicative nutrient sites having an increase in below-ground tissues. This being said, the

ongoing survival and productivity of *Z. muelleri* in the region may be density dependent. This has significant implications for the management of populations that may exhibit a patchy distribution and further understanding of the processes identified is warranted.

The microphytobenthic (MPB) communities identified in our research (Chapter 6) have provided an important indicator of the environmental status of the estuaries, which can be considered to be in a largely oligotrophic condition. These analyses have furthered the understanding of MPB communities that inhabit seagrass systems. Metabarcoding of MPB communities is a novel approach to understanding the condition of seagrass systems which has historically relied on determining MPB biomass and their related chlorophyll *a* concentrations. We identified the ecological structure of MPB communities, in particular the richness, evenness and diversity of separately identified taxon sequences or Molecular Operational Taxonomic Units (MOTU) that may be used as a baseline for the ongoing management of these important populations of *Z. muelleri.* Further work in this field has the potential to provide significantly greater understanding of estuarine and therefore seagrass systems as a whole.

While we may often consider plants to be solitary sentinels, only dispersing through reproductive propagules when conditions are favourable; *Z. muelleri* has shown itself to participate in complex interactions with the abiotic and biotic environment in which it lives. Connectivity between populations of the species shows an important ability to disperse in the marine environment, supplementing populations with new genetic information that aids in survival of

metapopulations that may be far removed from one another. It is a significant contributor to estuarine environments and management of the species in south-eastern Australia must consider the species' interactions in the environment and the population dynamics of the species from germination of seeds under low salinity conditions to their eventual recruitment, growth and potential decline. It is hoped that through gaining a greater understanding of this species we may ensure its ongoing survival and, like the Yunyawa people, perhaps we might one day hold seagrasses in as high regard.

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Chapter 8

Appendices

Appendix 1: Supplementary material to Chapter 2

Full model outputs of univariate general linear models (GLM) determining the influence of salinity, temperature and light and potential interaction effects on the germination of *Zostera muelleri* seeds, germination T^{50}, hypocotyl hair development and hypocotyl hair T^{50}; as well as the proportion of germinants with fully extended hypocotyl hairs and the extent of hypocotyl hair coverage on the hypocotyl.

Dependent Variable: Germination

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Corrected Model	9803.722[a]	25	392.149	18.141	<0.001
Intercept	22400.111	1	22400.111	1036.243	<0.001
Light	484.000	1	484.000	22.390	.001
Temp	983.722	2	491.861	22.754	<0.001
Salinity	7324.556	5	1464.911	67.768	<0.001
Light * Temp	204.167	2	102.083	4.722	.036
Temp * Salinity	728.611	10	72.861	3.371	.034
Light * Salinity	78.667	5	15.733	.728	.618
Error	216.167	10	21.617		
Total	32420.000	36			
Corrected Total	10019.889	35			

Dependent Variable: Germination T^{50}

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Corrected Model	24003.947[a]	25	960.158	7.759	.001
Intercept	28242.483	1	28242.483	228.217	<0.001
Light	48.651	1	48.651	.393	.545
Temp	17149.283	2	8574.641	69.288	<0.001
Salinity	1575.791	5	315.158	2.547	.098
Light * Temp	55.651	2	27.826	.225	.803
Temp * Salinity	5064.006	10	506.401	4.092	.018
Light * Salinity	110.565	5	22.113	.179	.964
Error	1237.530	10	123.753		
Total	53483.960	36			
Corrected Total	25241.477	35			

Dependent Variable: Hypocotyl hairs

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Corrected Model	7.614[a]	25	.305	12.241	<0.001
Intercept	24.386	1	24.386	980.140	<0.001
Light	.056	1	.056	2.231	.166
Temp	.600	2	.300	12.057	.002
Salinity	6.484	5	1.297	52.120	<0.001
Light * Temp	.020	2	.010	.393	.685
Temp * Salinity	.375	10	.037	1.507	.264
Light * Salinity	.080	5	.016	.644	.672
Error	.249	10	.025		
Total	32.249	36			
Corrected Total	7.863	35			

Dependent Variable: Hypocotyl hair T^{50}

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Corrected Model	6448.944[a]	25	257.958	3.047	.035
Intercept	10540.444	1	10540.444	124.502	<0.001
Light	544.444	1	544.444	6.431	.030
Temp	852.056	2	426.028	5.032	.031
Salinity	3114.222	5	622.844	7.357	.004
Light * Temp	264.056	2	132.028	1.559	.257
Temp * Salinity	1271.278	10	127.128	1.502	.266
Light * Salinity	402.889	5	80.578	.952	.490
Error	846.611	10	84.661		
Total	17836.000	36			
Corrected Total	7295.556	35			

Dependent Variable: Fully extended hypocotyl hairs

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Corrected Model	16.000[a]	35	.457	1.543	.047
Intercept	16.000	1	16.000	54.000	<0.001
Light	.250	1	.250	.844	.360
Temp	5.167	2	2.583	8.719	<0.001
Salinity	4.583	5	.917	3.094	.012
Light * Temp	.000	2	.000	.000	1.000
Light * Salinity	.333	5	.067	.225	.951
Temp * Salinity	5.250	10	.525	1.772	.074
Light * Temp * Salinity	.417	10	.042	.141	.999
Error	32.000	108	.296		
Total	64.000	144			
Corrected Total	48.000	143			

Dependent Variable: Extent

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Corrected Model	21853.424[a]	25	874.137	8.759	.001
Intercept	86093.340	1	86093.340	862.671	<0.001
Light	2108.340	1	2108.340	21.126	.001
Temp	1277.097	2	638.549	6.398	.016
Salinity	15688.701	5	3137.740	31.441	<0.001
Light * Temp	239.597	2	119.799	1.200	.341
Temp * Salinity	1210.486	10	121.049	1.213	.383
Light * Salinity	1329.201	5	265.840	2.664	.088
Error	997.986	10	99.799		
Total	108944.750	36			
Corrected Total	22851.410	35			

Appendix 2: Supplementary material to Chapter 5

Detail of location and average depths of eighteen sampled populations of *Zostera muelleri* in Victoria Australia

Site name	Lat	Long	Mean depth (m)	SD (m)
Lake Tyers 1	-37.834957°	148.071554°	0.62	0.18
Lake Tyers 2	-37.833679°	148.077257°	0.22	0.14
Lake Tyers 3	-37.851332°	148.069996°	0.18	0.12
Port Phillip Bay 1	-37.876294°	144.815728°	0.47	0.43
Port Phillip Bay 2	-38.071200°	144.407217°	0.7	0.3
Port Phillip Bay 3	-37.943162°	144.998100°	1.1	0.45
Gippsland Lakes 1	-37.890684°	147.952230°	1.08	0.1
Gippsland Lakes 2	-37.826265°	147.715609°	1.83	0.61
Gippsland Lakes 3	-37.895377°	147.931333°	2.1	0.7
Shallow Inlet 1	-38.819136°	146.170451°	0.54	0.35
Shallow Inlet 2	-38.836108°	146.151877°	0.52	0.33
Shallow Inlet 3	-38.814876°	146.165142°	0.58	0.34
Corner Inlet 1	-38.835229°	146.268379°	0.39	0.21
Corner Inlet 2	-38.696461°	146.247629°	0.3	0.19
Corner Inlet 3	-38.690607°	146.337043°	0.26	0.17
Western Port 1	-38.375945°	145.223467°	1.08	0.16
Western Port 2	-38.525394°	145.343320°	1.3	0.23
Western Port 3	-38.527538°	145.370022°	1.24	0.19

Full model outputs of nested univariate general linear models (GLM) identifying variation between estuaries and among SUs located within estuaries on morphological and physiological characteristics of *Zostera muelleri*

Dependent Variable: Canopy height

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	4584.776	1	4584.776	3441.783	<0.001
	Error	23.978	18	1.332[a]		
Estuary	Hypothesis	64.535	5	12.907	9.689	<0.001
	Error	23.978	18	1.332[a]		
depth(Estuary)	Hypothesis	23.978	18	1.332	6.948	<0.001
	Error	36.810	192	.192[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Cover abundance

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	2668.835	1	2668.835	1462.874	<0.001
	Error	32.839	18	1.824[a]		
Estuary	Hypothesis	4.418	5	.884	.484	.783
	Error	32.839	18	1.824[a]		
depth(Estuary)	Hypothesis	32.839	18	1.824	5.296	<0.001
	Error	66.147	192	.345[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Shoot density

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	10069.632	1	10069.632	10157.390	<0.001
	Error	17.844	18	.991[a]		
Estuary	Hypothesis	28.044	5	5.609	5.658	.003
	Error	17.844	18	.991[a]		
depth(Estuary)	Hypothesis	17.844	18	.991	2.311	.003
	Error	82.350	192	.429[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Above-ground biomass

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	3365.117	1	3365.117	2183.237	<0.001
	Error	27.744	18	1.541[a]		
Estuary	Hypothesis	125.203	5	25.041	16.246	<0.001
	Error	27.744	18	1.541[a]		
depth(Estuary)	Hypothesis	27.744	18	1.541	1.437	.118
	Error	205.992	192	1.073[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Below-ground biomass

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	1117.422	1	1117.422	6504.885	<0.001
	Error	3.092	18	.172[a]		
Estuary	Hypothesis	27.932	5	5.586	32.520	<0.001
	Error	3.092	18	.172[a]		
depth(Estuary)	Hypothesis	3.092	18	.172	.998	.464
	Error	33.049	192	.172[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Biomass proportion

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	1777.408	1	1777.408	1618.068	<0.001
	Error	19.773	18	1.098[a]		
Estuary	Hypothesis	67.692	5	13.538	12.325	<0.001
	Error	19.773	18	1.098[a]		
depth(Estuary)	Hypothesis	19.773	18	1.098	1.810	.026
	Error	116.525	192	.607[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Epiphyte load

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	2334.231	1	2334.231	1614.293	<0.001
	Error	26.028	18	1.446[a]		
Estuary	Hypothesis	81.015	5	16.203	11.206	<0.001
	Error	26.028	18	1.446[a]		
depth(Estuary)	Hypothesis	26.028	18	1.446	1.443	.116
	Error	192.451	192	1.002[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Chlorophyll *a* concentration

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	45322.117	1	45322.117	3032.863	<0.001
	Error	268.986	18	14.944[a]		
Estuary	Hypothesis	1509.284	5	301.857	20.200	<0.001
	Error	268.986	18	14.944[a]		
depth(Estuary)	Hypothesis	268.986	18	14.944	1.111	.344
	Error	2581.686	192	13.446[b]		

a. MS(depth(Estuary)) b. MS(Error)

Dependent Variable: Internode length

Source		Type III Sum of Squares	df	Mean Square	F	*P*-value
Intercept	Hypothesis	23789.568	1	23789.568	1482.072	<0.001
	Error	288.928	18	16.052[a]		
Estuary	Hypothesis	1779.784	5	355.957	22.176	<0.001
	Error	288.928	18	16.052[a]		
Depth(Estuary)	Hypothesis	288.928	18	16.052	.829	.662
	Error	1857.720	96	19.351[b]		

a. MS(Depth(Estuary)) b. MS(Error)