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The Long-term Effects of *Sargassum muticum* (Yendo) Fensholt Invasion on *Zostera marina* L. and its Associated Epibiota

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Abstract

The Long-term Effects of *Sargassum muticum* (Yendo) Fensholt Invasion on *Zostera marina* L. and its Associated Epibiota

STACEY LYNN DEAMICIS

In this thesis I review how *Sargassum muticum* (Yendo) Fensholt, an invasive alga from Asia, has spread globally due to human activities and describe how this species can affect seagrass ecosystems. Abiotic factors such as nutrient and substratum availability may facilitate the spread of *S. muticum* into *Zostera marina* L. meadows, but analyses of seawater nutrients, and sediment particle size and % organic content revealed no significant differences between experimental quadrats in seagrass meadows either with, or without the presence of *S. muticum*. Phenolic compounds were examined because they form the basis of defensive mechanisms in plants and algae, therefore any change in phenolic content may affect the ability of *Z. marina* to protect itself from disease, herbivory and invasive species through allelopathic interactions. Results from a four year field study and multiple annual laboratory experiments showed significant reductions ($p = 0.034$ and $p = 0.002$, respectively) in the caffeic and tannic acids equivalents content of *Z. marina* when in the presence of *S. muticum*.

As the abundance of *S. muticum* increases, other changes in the physiology of *Z. marina* may occur including variations in growth rates, nutrient partitioning and chlorophyll fluorescence, but data from multiple laboratory experiments illustrated no significant differences in growth. Chlorophyll fluorescence analyses revealed significant differences between treatments with and without *S. muticum* ($p = 0.008$), but pairwise comparisons indicated these differences only occurred in 2008 ($p < 0.001$). Significant differences were also found in nutrient partitioning amongst functional regions of the shoots ($p = 0.024$), but pairwise comparisons detected these differences between a biomass control treatment (ZZ: *Zostera* + *Zostera*) and the ZS (with *S. muticum*) and ZM (*Z. marina* on its own at a lower biomass per replicate) treatments ($p = 0.013$ and $p = 0.019$, respectively), but not between ZS and ZM. Previous *in situ* research has found negative effects of *S. muticum* presence on densities of kelp and other algae. Results from the long-term field study indicated significantly lower mean *in situ Z. marina* densities within the ZS treatment ($p < 0.001$).

Epibiota found living on the blades of *Z. marina* provide food for organisms within seagrass ecosystems and also create microhabitats for other species to occupy. Alterations in the abundances of epibiota and microhabitats formed could further modify seagrass ecosystems

through shifts in timing of food availability, food preferences and microhabitats created. The long-term field study data revealed significantly lower epibiota abundances within the ZS treatment (p = 0.019), but differences in biomass between treatments were not detected. Changes in the biochemistry, physiology, vegetative physiognomy and epibiota assemblages of *Z. marina* revealed during experimental manipulations are presented and considered within the context of long-term seagrass survival in light of increasing *S. muticum* invasion.

Table of Contents

List of Figures

List of Tables

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Authors declaration

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A programme of advanced study was undertaken that included a variety of postgraduate taught modules within the Faculty of Science, the 6th Annual Postgraduate International Conservation Biology Short Course held in Rovinj, Croatia, the Graduate Teaching Associate Course, and the Learning and Teaching in Higher Education (LTHE) 300 module, from which I gained recognition as a Registered Associate Practitioner of the Higher Education Academy. I was invited to attend a NERC, BES and ERFF Science-Policy Interactions Workshop in Reading and was also selected to represent the University of Plymouth at "The Value of PhD Research" postgraduate conference held Cumberland Lodge within The Great Park in Windsor. I successfully completed the HSE Part IV Professional Diver qualification and attended many professional and skills development short courses and workshops throughout the course of my degree. In 2007, I used my diving qualifications as a volunteer for Lucy Woodall, a PhD student from University College London, working on European seahorse genetics at two locations in Greece.

Relevant school and departmental scientific seminars and conferences and interdisciplinary seminars were regularly attended.

Presentations Given and Conferences Attended:

2007:

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Marine Information: Issues and Responses, Coastal Management for Sustainability, London, UK.

2008:

Talking Science: The Cycle of Learning, European Dana Alliance for the Brain & King's College, London, UK.

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University of Plymouth Vice Chancellor's Research and Innovation Conference, University of Plymouth, Plymouth, UK.

International Seagrass Biology Workshop 8 (ISBW8), Bamfield Marine Station, Vancouver Island, Canada.
Poster presentation: The effects of *Sargassum muticum* invasion on *Zostera marina*

The Salcombe-Kingsbridge Estuary Forum, Kingsbridge, UK.
Oral presentation: The effects of *Sargassum muticum* invasion on *Zostera marina*

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Poster presentation: The effects of *Sargassum muticum* invasion on *Zostera marina*

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Chapter 1

General Introduction

1.1 Invasive species in marine systems

In a rapidly changing world, invasive species are considered one of the major components of global change (Elton, 1958; Vitousek *et al.*, 1997; Ruiz *et al.*, 2000; Mooney & Cleland, 2001; Lodge *et al.*, 2006) and biodiversity loss (Walker & Kendrick, 1998; Bax *et al.*, 2003; Schaffelke *et al.*, 2006; Galil, 2007) due to alterations in ecological processes (Ruesink *et al.*, 2006) and disruptions of ecosystem services (Vitousek *et al.*, 1996). Opportunities abound for non-native species introductions to coastal ecosystems (Ashton & Mitchell, 1989) with burgeoning human populations along the world's coastlines (von Bodungen & Turner, 2001) and a continued rise in global transportation (Bax *et al.*, 2003; Keller *et al.*, 2011), both in terms of human travel and cargo shipping. In North America, for example, there has been an exponential increase in reported invasions in marine systems over the past 200 years (Ruiz *et al.*, 2000; Pascal *et al.*, 2010). With significantly more invasions occurring in polyhaline and euhaline zones than lower salinity zones (Ruiz *et al.*, 2000), the threat and consequences of introduced and potentially invasive species in coastal ecosystems may have severe consequences such as habitat modification and displacement of native species if left unmonitored or unstudied (Elton, 1958; Ruiz *et al.*, 1999; Wonham & Carlton, 2005).

Invasions within coastal systems are not as well studied compared to terrestrial or freshwater ecosystems (Ruiz *et al.*, 2000), but this is slowly changing. Worldwide, at least 56 non-native species, consisting mostly of invertebrates and seaweeds, have been introduced to seagrass beds (Williams, 2007) through vectors such as shipping via hull-fouling and ballast water and aquaculture (Carlton, 1989; Bax *et al.*, 2003; Keller *et al.*, 2011). Ecologists are now challenged to understand how and why certain introduced species become successful invaders and try to determine any long-term effects they may

have on ecosystems (Schaffelke *et al.*, 2006).

1.1.1 Introduced *vs.* Invasive

Although used interchangeably, the words 'introduced' and 'invasive' differ in their definitions. Introduced species can colonise an area where previously not present (Carlton, 1989; Boudouresque & Verlaque, 2002), have their range extension linked, directly or indirectly, to human activity (Ashton & Mitchell, 1989; Carlton, 1989; di Castri, 1989; Mack, 1996; Boudouresque & Verlaque, 2002; Bax *et al.*, 2003; Williams, 2007) and show geographic discontinuity between their native ranges and newly colonised areas (Ashton & Mitchell, 1989; Ruiz *et al.*, 2000; Boudouresque & Verlaque, 2002). Invasive species, by contrast, are a subset of introduced species that are ecologically and/or economically harmful, potentially influencing ecological relationships among native species (Boudouresque & Verlaque, 2002) and altering ecosystem functioning over a short period of time (Mack, 1996; Williams, 2007). These issues, coupled with the potential to adversely affect the economic value of ecosystems and even human health in certain circumstances (Bax *et al.*, 2003), gives rise to great concern amongst scientists, environmental managers and policy makers alike (Williams, 2007).

1.1.2 What makes a successful invader?

Invasive species, considered 'biological pollution' by Boudouresque & Verlaque (2002), exhibit a number of traits that facilitate their successful establishment. Although initial introductions may have been through direct or indirect human activity (Ashton & Mitchell, 1989; Carlton, 1989; Mack, 1996; Boudouresque & Verlaque, 2002), invasive species may be capable of establishing a breeding population without further intervention by humans through high reproductive output (Ashton & Mitchell, 1989; Mack, 1996; Ruiz *et al.*, 2000) and may also attain widespread distribution through highly effective dispersal mechanisms (Ashton & Mitchell, 1989; Carlton, 1989; di Castri, 1989; Boudouresque

& Verlaque, 2002; Van Riel *et al.*, 2011), enabling them to establish and rapidly build populations. Invasive species are often generalists able to tolerate a wide range of habitats and environmental conditions (Ashton & Mitchell, 1989; Mack, 1996; Boudouresque & Verlaque, 2002), which enables invaders to expand their range and often colonize recently disturbed areas (Mack, 1996; Williams, 2007; Kiparissis *et al.*, 2011), further facilitating their spread. In addition, invasive species may potentially have enough genetic variation to allow for greater adaptability to new environments (Mack, 1996), and once established, can be difficult to control and eradicate (Critchley *et al.*, 1986; Mack *et al.*, 2000; Williams, 2007).

1.1.3 Impacts of invasive species

Extensive homogenisation of the earth's biota is occurring with an estimated 10–35 % of introduced species becoming invasive (Lodge, 1993). In the United States, most invasive species were once introduced to new habitats for food, fibre, and/or ornamental purposes (Pimentel *et al.*, 2001), but by the late 1990s it was estimated that 5–25 % of all vascular plants within the US were non-native species (Vitousek *et al.*, 1996). At the same time, 1,623 native species and 442 non-native species were documented within the UK (Vitousek *et al.*, 1996). The number of non-native species was equivalent to over 21 % of the total number of species found within Great Britain (Vitousek *et al.*, 1996). These figures are alarming.

Negative consequences of uncontrolled invasive species proliferation may include major economic losses (Vitousek *et al.*, 1996; Parker *et al.*, 1999), habitat destruction (Vitousek *et al.*, 1996; Pimentel *et al.*, 2001), displacement of native species (Lodge, 1993; Vinther *et al.*, 2008), biodiversity loss (Schaffelke *et al.*, 2006), facilitation of other non-native species (Simberloff & Von Holle, 1999; Grosholz, 2005), and adverse effects on human health (Vitousek *et al.*, 1996; Bax *et al.*, 2003). For example, the estimated costs associated with the invasion of *Dreissena polymorpha* (Pallas, 1771) (zebra mussel) into North

America was ~$3.1 billion USD over a 10-year period to clear blocked intake valves and waterways (Vitousek *et al.*, 1996). This expenditure does not include costs affiliated with the reduction of native algal populations, decreased biological productivity and increased nutrient concentrations. With respect to facilitation, the "invasional meltdown" theory states that well established invasive species may harbour and facilitate the spread of other introduced, and potentially invasive, species to settle and establish themselves, further altering ecosystems (Simberloff & Von Holle, 1999; Grosholz, 2005). One example can be found in Willapa Bay in the NE Pacific. The Asian hornsnail (*Batillaria attramentaria* (G. B. Sowerby II, 1855)) introduced with *Crassostrea gigas* (Thunberg, 1793) (the giant Pacific oyster) enhances the abundance and growth of *Zostera japonica* Aschers. & Graebn., an invasive seagrass from Japan (Wonham & Carlton, 2005; Williams, 2007). Newly introduced species may also be carriers of viral pathogens. *Aedes albopictus* (Skuse, 1895), the Asian tiger mosquito, is host to dengue fever and other human arboviruses (Vitousek *et al.*, 1996). Currently, control and/or suppression of the invasive mosquito is not possible as they are highly adaptable to new environments. It is clear from the above examples that the detrimental effects of invasive species establishment in 'natural' ecosystems can be extensive and expensive (Vitousek *et al.*, 1996).

1.2 Biology, ecology and species interactions

1.2.1 *Zostera marina*: A native under threat?

Zostera marina L., a marine angiosperm in the class Monocotyledoneae and one of only 72 described seagrass species (Short *et al.*, 2011), has long been considered an ecosystem engineer (Hemminga & Duarte, 2000; Koch, 2001). The blades of the plant can slow water flow (Fonseca & Cahalan, 1992), dissipating kinetic energy that encourages settlement of fine sediments, detritus, and larvae under their canopy (Posey, 1988; Hemminga & Duarte, 2000). Its roots and rhizomes can stabilize sediments and protect areas against wave disturbance (Fonseca & Cahalan, 1992), which creates more suitable habitat for sedentary

and infaunal organisms (Harrison & Bigley, 1982; Hemminga & Duarte, 2000; Jackson *et al.*, 2006). In addition, the rhizomes can sequester nutrients as well as transport oxygen deeper into the sediment, creating microhabitats of oxygenated substrata (Hemminga & Duarte, 2000; DiCarlo & Kenworthy, 2008). *Zostera marina* enhances species richness directly through a provision of complex structure (Attrill *et al.*, 2000), providing shelter for larval fish and egg deposition for many different organisms (Bell & Pollard, 1989; Hemminga & Duarte, 2000; Jackson *et al.*, 2006) and substrata for epibiota (Jacobs *et al.*, 1983; Saunders *et al.*, 2003; Johnson *et al.*, 2005), periphyton (Hemminga & Duarte, 2000; Spivak *et al.*, 2009), and food for waterfowl (Harrison & Bigley, 1982; Lewis III, 1982; Hemminga & Duarte, 2000). However, with a median Redfield ratio (C:N:P) of 435:20:1, which is greater than the empirically developed stoichiometric ratio of 108: 15.5:1 (Miller, 2004), relatively few herbivores rely primarily on *Z. marina* and other seagrasses as their preferred food sources due to the large percentage of carbon within its tissues (~30–40 % dry weight) (Hemminga *et al.*, 1991; Hemminga & Duarte, 2000).

Z. marina is found ubiquitously in the temperate regions of the north Pacific and Atlantic basins and is the dominant seagrass species found along Britain's southwest coast. Capable of growing up to an average of 1–2 cm d^{-1} (Hemminga & Duarte, 2000) and in exceptional cases 3 cm d^{-1} (Borum *et al.*, 2004), the mean *Z. marina* blade length is ~20-50 cm (Pihl *et al.*, 2006; Tyler-Walters, 2008) and at the height of summer may exceed 1 m or more (author's own observation), with lengths of up to 2 m reported in the Salcombe-Kingsbridge Estuary (E. Jackson, personal communication, 2006). *Z. marina* can reproduce asexually via creeping rhizomes that produce clonal ramets and/or sexually through inflorescence pollination (Hemminga & Duarte, 2000). However, flowering in many seagrass species is rare (Hemminga & Duarte, 2000) except under stressful conditions (Phillips *et al.*, 1983) such as low light or high temperatures (Diaz-Almela *et al.*, 2007), and contributes < 10 % of annual reproduction per year (Phillips & Backman, 1983; Olesen, 1999; Hemminga & Duarte, 2000).

Seagrasses typically occur in low flow areas, can tolerate a broad range of salinities (2–46 ‰) (Gillanders & Kingsford, 2002) and have a wide range of daily light requirements to achieve growth. *Z. marina* becomes light saturated at 78–100 μmol m^{-2} d^{-1} (Dennison & Alberte, 1985; Dennison, 1987), but the light saturation point can vary (~55-400+ μmol m^{-2} s^{-1}) depending upon photoperiod, location, depth and water quality (Dennison & Alberte, 1982; Marsh Jr. *et al.*, 1986; Moore & Wetzel, 2000; Lee *et al.*, 2007; Thom *et al.*, 2008). This species may also be able to tolerate wide-ranging changes in pH. Invers *et al.* (1997) found that increasing pH from 8.2 to pH 8.8 had little affect on photosynthesis, although a further increase to pH 9.0 produced a significant reduction in photosynthesis in the congener *Z. noltii* Hornemann. Under decreasing pH resulting from ocean acidification, all seagrass species may flourish due to an increase in diffuse CO_2 within the water column (Zimmerman *et al.*, 1997; Palacios & Zimmerman, 2007; Hall-Spencer *et al.*, 2008; Martin *et al.*, 2008; Fabricius *et al.*, 2011). This would ultimately reduce inorganic carbon limitation and enable seagrasses to increase photosynthate production in high irradiance conditions (Beardall *et al.*, 1998).

Even with low sexual reproductive success, seagrass meadows are highly productive coastal communities (Hemminga & Duarte, 2000) and it has been estimated that their gross financial benefits to society amount to ~$34,000 USD ha^{-1} yr^{-1} (Costanza *et al.*, 1997; Short *et al.*, 2011, recalculated here to 2010 US dollars). Although seagrasses represent only 1 % of the total oceanic primary production, they, together with saltmarshes and mangroves, store ~5% of the total carbon within the oceans (Duarte *et al.*, 2005), with seagrasses specifically storing between 48–112 Tg C yr^{-1} globally (Mcleod *et al.*, 2011). This amount is equal to or greater than the amount of carbon stored within temperate, tropical and boreal forests annually (Mcleod *et al.*, 2011). The highly beneficial ecosystem service of CO_2 sequestration provided by vegetaged coastal habitats may therefore lead to enhanced protection of seagrasses by policymakers, as they can function as part of the 'blue carbon' solution, acting as natural carbon sinks to remove increasing CO_2 from the atmosphere (United Nations Environment Programme & GRID-Arendal, 2009; IUCN &

Conservation International, 2011).

Despite its listing as a UK Biodiversity Action Plan (BAP) species (Foden & Brazier, 2007), *Z. marina* and other species of seagrass face an uncertain future (Short *et al.*, 2011) due to natural and anthropogenic disturbances (Short & Burdick, 1996). Numerous studies have illustrated the deleterious effects of eutrophication (Burkholder *et al.*, 1992; Short & Wyllie-Echeverria, 1996; Billen *et al.*, 1999; Hemminga & Duarte, 2000; Howarth *et al.*, 2002), sedimentation (Short & Wyllie-Echeverria, 1996; Erftemeijer & Lewis, 2006), direct mechanical damage (Short & Wyllie-Echeverria, 1996; Reed & Hovel, 2006) and invasive species (den Hartog, 1997; Ruesink *et al.*, 2006; Williams, 2007) on seagrass growth and survival. These detrimental impacts led to the loss of 110 km^2 yr^{-1} of seagrass habitat between 1980 and 2006 (Waycott *et al.*, 2009). Wasting disease has also had a direct effect on seagrass species survival (Short *et al.*, 1988; Vergeer *et al.*, 1995; Ralph & Short, 2002), especially on *Z. marina*. An epidemic outbreak of seagrass wasting disease in the 1930s led to approximately 90 % loss of all *Z. marina* meadows worldwide (Muehlstein, 1989), with some populations failing to return to the eastern seaboard of the US and the Wadden Sea (Short *et al.*, 1988; Burdick *et al.*, 1993) even 75 years later (Steele *et al.*, 2005). Although the debate still continues as to the exact mechanism of the outbreak (Vergeer *et al.*, 1995; Bull *et al.*, 2011), it is widely believed that infection by a slime-mould protist, *Labyrinthula zosterae* Porter & Muehlstein, coupled with higher than normal sea surface temperatures (SSTs) (Short *et al.*, 1988) and/or increases in salinity (McKone & Tanner, 2009) were the underlying causes of the mass die-off. In light of increasing global temperatures, *L. zosterae* prevelance within seagrass ecosystems may increase, thus irreversibly tipping the balance out of favour for seagrasses.

1.2.2 *Sargassum muticum*: An invasive opportunist

Sargassum muticum (Yendo) Fensholt is an invasive macroalga from Asia, believed to have been introduced to the Northeast Pacific region of North America and the Northeast

Atlantic coasts in Western Europe with *C. gigas* spat (Boalch & Potts, 1977), another non-native species used in aquaculture. It has spread prolifically across two continents and was first recorded on British shores in 1973 on the Isle of Wight (Farnham *et al.*, 1973) and in the Plymouth area in 1976 (Boalch & Potts, 1977). Since its first sighting in Great Britain, it has spread extensively along the southern British coast and into other European coastal areas at a rate of ~30 (Farnham *et al.*, 1980) to 50+ km yr^{-1} (Strong *et al.*, 2006; Kraan, 2008). Even larger stands of *S. muticum* may be found in the future due to its ability to tolerate a wide range of light environments, temperatures and a moderate range of salinities (Norton, 1977a; Hales & Fletcher, 1989) along with its ability to colonize recently disturbed areas (den Hartog, 1997; Strong *et al.*, 2006). A fast growth rate (Jephson & Gray, 1976; Kane & Chamberlain, 1979; Hales & Fletcher, 1989), coupled with high sexual reproductive potential (Norton & Deysher, 1989) and fertile lateral fronds that can remain viable up to three months after detachment from the parental stipe (Norton, 1981), may also facilitate its spread.

In temperate waters, *S. muticum* is considered to be a pseudo-perennial (Buschbaum *et al.*, 2006) as the lateral fronds detach, leaving behind a short perennial stipe from which regeneration of fronds occurs the following spring (Jephson & Gray, 1976; Gorham & Lewey, 1984; Critchley *et al.*, 1987). Optimal growth conditions for *S. muticum* occur at 25°C, 44 μmol m^{-2} s^{-1} and 34‰ salinity (Hales & Fletcher, 1989), and although the species can tolerate a wide range of temperatures (5–30 °C), salinities and light conditions (Norton, 1977a; Hales & Fletcher, 1989), it favours higher temperatures (Norton, 1977a; Hales & Fletcher, 1989) and full oceanic salinity (Norton, 1977a). Its ability to withstand such a range of conditions has enabled its successful establishment outside its native range in eastern Asia, but with increasing freshwater inputs into estuarine systems as the number of storms and rainfall increase due to climate change (Burt *et al.*, 1998; Kaste *et al.*, 2006), the decreasing salinity may help to reduce or slow the spread of this invasive species, at least in true estuaries.

S. muticum is an invasive opportunist, establishing itself in any suitable empty space almost immediately upon the removal of other macrophytes (den Hartog, 1997; Strong *et al.*, 2006). It has an average spring/summer growth rate of 1.5–2 cm d^{-1} (Kane & Chamberlain, 1979), but was recorded growing up to a maximum of 4 cm d^{-1} by Jephson & Gray (1976). This maximum growth rate is several times faster than the maximum growth rates documented for indigenous brown macroalgae (Hales & Fletcher, 1989) or *Z. marina* (Hemminga & Duarte, 2000). In its native China, *S. muticum* rarely exceeds 0.5 m in length (European Commission Directorate-General XII for Science Research and Development, 1993), but in the English Channel under the right conditions, this species has the ability to exceed 5 m (Gorham & Lewey, 1984; den Hartog, 1997), displacing kelp species such as *Laminaria saccharina* (Linnaeus) Lamouroux on the French Atlantic coast (Joint Nature Conservation Committee, 2007) and affecting the relative abundance of two kelp species, *Laminaria bongardiana* Postels et Ruprecht and *Agarum fimbriatum* Harvey, in Washington State (Britton-Simmons, 2004).

Sargassum muticum may be slowly displacing seagrass meadows through shading (den Hartog, 1997) and by peripatetic dispersal (a.k.a. "stone-walking") facilitated by its large number of pneumatocysts (Critchley, 1983a; Strong *et al.*, 2006); as seagrass dies back, *S. muticum* attached to stones can move further up shore due to increased patchiness within the beds. In Strangford Lough, Northern Ireland, Strong *et al.* (2006) found that *S. muticum* can travel up to three meters in two months via peripatetic dispersal, thereby making up a significant portion of species re-establishment after clearing.

1.2.3 Species interactions

In the NE Pacific region of the US and Canada, the first recorded observation of *S. muticum* occurred in the mid-1950s (Norton, 1977a), some 20 years before it was first observed on British shores. In the Pacific Northwest (PNW) region and other global locations where the two species co-occur, *S. muticum* and *Z. marina* are not often found growing intermixed

(den Hartog, 1997). In fact, in Washington State, *S. muticum* has been found to occupy a very narrow band (0.3 m down to 1.5 m below Mean Lower Low Water (MLLW)) in the shallow subtidal on rocky substrata (Norton, 1977a), which is unsuitable for seagrass. This restriction may be due to the tidal cycle in the PNW as the lowest tides of the year occur during extreme temperature events (freezing temperatures in the winter months and high temperatures in the summer months) (Norton, 1977a), unlike those experienced along the English Channel where the lowest tides of the year occur near the vernal and autumnal equinoxes. Therefore, the tidal cycle in the PNW may restrict the ability of *S. muticum* to peripatetically disperse into the intertidal and shallow subtidal *Z. marina* meadows and persist as it is sensitive to desiccation and frost (Norton, 1977a).

S. muticum is typically found growing attached to submerged rocky reefs in lower littoral tide pools (den Hartog, 1997) or attached to small shells and stones within the lower intertidal (Norton, 1977b; den Hartog, 1997), but also has been reported to have its holdfast buried in soft sediment (Tweedley, 2006; Tweedley *et al.*, 2008) due to sediment accretion. With its ability to disperse peripatetically, increasingly high densities of *S. muticum* have been found lodged within the root-rhizome matrix of *Z. marina* over the last several years in the intertidal and shallow subtidal along the southern British coast, and in particular, within the Salcombe-Kingsbridge Estuary (Fig. 1.1) (Tweedley, 2006) where the substrata is a mixture of sand, gravel and stones (den Hartog, 1997). Although the biological and ecological implications of the intermixing of these two macrophytes are still unknown, interaction consequences may include allelopathy (Cuny *et al.*, 1995; Dumay *et al.*, 2004; Pergent *et al.*, 2008), substratum competition (den Hartog, 1997; Strong *et al.*, 2006; Tweedley, 2006; Kiparissis *et al.*, 2011) and competition for light (Hauxwell *et al.*, 2001; McGlathery, 2001; Britton-Simmons, 2004; Strong *et al.*, 2006) and nutrients (Valiela *et al.*, 1997; Rabalais, 2002). With the ability to colonise both hard and soft substrata (Critchley, 1983b; Strong *et al.*, 2006; Tweedley *et al.*, 2008), the potential for continued coastal modification by *S. muticum* remains high, pointing to significant potential implications of its further spread (Strong *et al.*, 2006). For example,

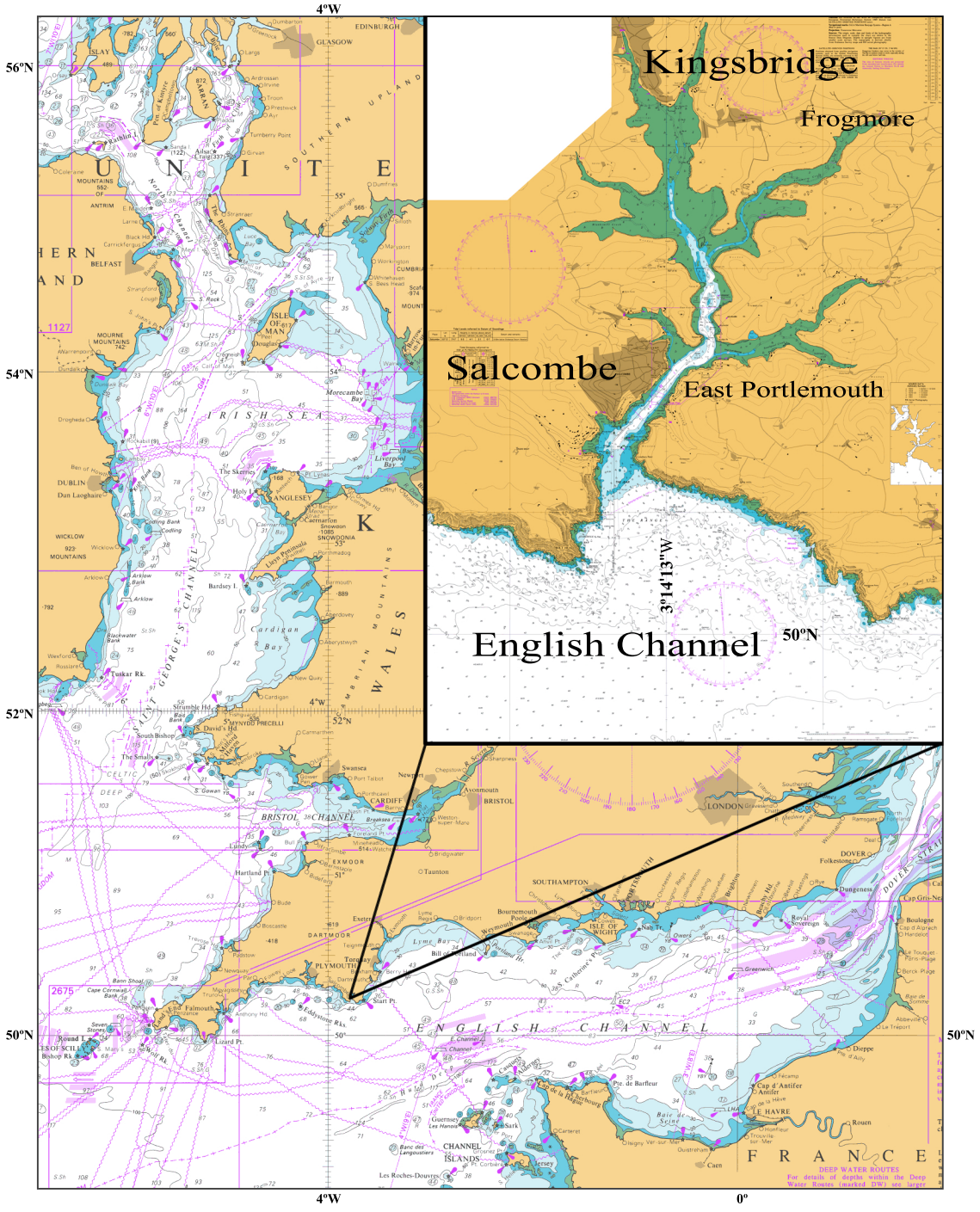


Figure 1.1: Study site: The Salcombe-Kingsbridge Estuary located in South Devon at the western edge of the English Channel (map credit: ©Crown Copyright / SeaZone Solutions Ltd (2008). All rights reserved. Not to be used for navigation.)

in previous studies, *S. muticum* and other invasive macroalgae have been found to have a negative effect on understory organisms by overgrowing and shading out the shorter, slower growing kelps and seagrasses, ultimately decreasing macrophytic diversity (Hauxwell *et al.*, 2001; McGlathery, 2001; Britton-Simmons, 2004; Strong *et al.*, 2006). Shading by larger macrophytes and/or increased phytoplankton blooms in eutrophic conditions, coupled with the much higher light requirements of *Z. marina* (Hemminga & Duarte, 2000), may result in unfavourable conditions for the long-term health and survival of *Z. marina*.

In addition to the potential competition for light, nutrients and substrata, *Z. marina* may also incur increased metabolic costs associated with the production of secondary metabolites (Dumay *et al.*, 2004). Previous investigations of the biochemical and physiological interactions between invasive seaweeds and seagrass in the Mediterranean Sea conducted by Dumay *et al.* (2004) have shown deleterious effects upon seagrass biochemistry and vigour. Dumay *et al.* (2004) found that secondary metabolite production increased in *Posidonia oceanica* (L.) Delile with increasing species interactions (i.e., density increases) between the seagrass and the rhizomatous invasive algae *Caulerpa taxifolia* (Vahl) C. Agardh and *C. racemosa* (Forsskål), perhaps as an allelopathic response to the invader. This was the first documented example of marine allelopathy between a seagrass and a macroalga.

1.2.4 Invasive species: Law and policy

With the strengthening of the EU as a governing body, the UK is not only bound by international agreements such as the Convention on Biological Diversity, the United Nations Convention on the Law of the Sea, the IMO Ballast Water Management Convention and the Convention on the Conservation of Migratory Species of Wild Animals, but must also adhere to EU legislation such as the The Convention on the Conservation of European Wildlife and Natural Habitat, the Habitats and Species Directive and the Water Framework Directive as well as the Wildlife and Countryside Act of 1981 and the Marine and Coastal

Access Act 2009 (both UK specific), all of which aim to protect biodiversity, endangered species, habitats and water quality. Provisions within these agreements require prevention of introductions and/or control of non-native species, with special focus on the invaders that threaten native or protected species (Joint Nature Conservation Committee, 2008). Within the UK, greater enforcement of such policies is needed, but local councils and government agencies lack the financial power and cohesiveness to effectively implement such wide-ranging, over-lapping policies (Carlton, 1989; McClanahan, 2001; Williams & Grosholz, 2008).

Globally, seagrasses play an important role in carbon sequestration as seagrass meadows and the sediment in which they are found may sequester between 48 and 112 Tg of carbon annually (Kennedy *et al.*, 2010) even though they represent only 1 % of total oceanic primary production (Hemminga & Duarte, 2000). Therefore seagrasses, in conjunction with saltmarsh and mangrove ecosystems, may ultimately function as part of the 'blue carbon' solution as seagrass meadows are natural carbon sinks (United Nations Environment Programme & GRID-Arendal, 2009; IUCN & Conservation International, 2011). By providing a natural ecological solution to the growing global problem of increaseing atmospheric CO_2, enhanced protection of seagrasses may become a priority (Duarte, 2002) within the UK.

1.3 Aims of the thesis

This thesis describes work investigating the effects of the invasion of *S. muticum* into *Z. marina* meadows within the Salcombe-Kingsbridge Estuary. The following chapters present the results from multiple short-term laboratory experiments and a long-term field study conducted to determine the biological and ecological consequences of the presence of *S. muticum* on the biochemistry, physiology, vegetative physiognomy and density of *Z. marina* and its associated epibiota assemblage abundance and biomass.

In **Chapter 2** I introduce my field site and present abiotic data for the ambient field

conditions in the Salcombe-Kingsbridge Estuary over the four-year field study. My findings are presented in the context of the potential effects that seawater nutrients and sediment type may have in coastal ecosystems if conditions are perturbed by anthropogenic activities such as farming, boating, dredging, and coastal development.

In **Chapter 3** I describe the effects of the presence of *S. muticum* on *Z. marina* in the context of changes in phlorotannins in the seagrass based on results from a four year field experiment and several lab experiments carried out in concurrent years. Previous studies using different species of seagrass and invasive algae have shown that increasing densities of invasive seaweeds negatively affect the amount of phenolics present in the seagrass (Dumay *et al.*, 2004; Pergent *et al.*, 2008). This chapter will elucidate my findings within a similar framework. Data are also presented from a supplementary laboratory experiment that tested the total alkalinity and carbonic components of seawater in addition to the nutrient levels for nitrogenous compounds (NH_4^+, NO_3^-, NO_2^-) and phosphates (PO_4^{3-}) within each treatment tank to contextualize possible nutrient limitation effects on the growth and biochemistry of the seagrass.

In **Chapter 4** I investigate how the presence of *S. muticum* affects the physiology of *Z. marina* including its growth, nutrient partitioning and photosynthetic output. Previous research has indicated that invasive marine macrophytes can compete for abiotic resources including nutrients, substrata and available light, adversely affecting native species' long-term survival. Results from multiple laboratory trials investigating the interactions between *Z. marina* and *S. muticum* are presented within the context of how changing abiotic conditions and the presence of *S. muticum* have the potential to negatively influence the physiology of *Z. marina*.

In **Chapter 5**, I address *in situ* density changes for *Z. marina* and *S. muticum* using data collected from permanent experimental quadrats and transects in my long-term field study. It has been widely accepted that *S. muticum* can negatively affect the densities of kelps and other algal species found growing below its canopy. This chapter will explore the affect of

S. muticum presence on the *in situ* densities and vegetative physiognomy of *Z. marina* and considers how increasing *S. muticum* density within the Salcombe-Kingsbridge Estuary can potentially alter this coastal ecosystem.

Chapter 6 investigates how the presence of *S. muticum* affects the associated epibiota of *Z. marina*, exploring potential effects on species assemblage composition, abundances and biomass. Previous research has found alterations in epiflora and epifauna in systems invaded by non-native marine macrophytes. These changes, nonetheless, may not always be negative as invasive algae may augment habitat complexity and increase available detritus, adding nutrients back to into the system. Results from samples collected over four years are presented and discussed in the context of increasing algal invasion.

In **Chapter 7** I bring together the discrete research that I have investigated over the duration of my PhD, consider some of the issues that arose during the project and discuss the main findings of each chapter in a broader context.

Chapter 2

Abiotic Conditions in the Salcombe-Kingsbridge Estuary

Abstract

Coastal waters throughout the UK have experienced increased *S. muticum* invasion over the last 40 years. In England, *Z. marina* meadows are found along the south and western coasts, with one of the largest beds located in the Salcombe-Kingsbridge Estuary at the western edge of the English Channel (50°13′53″N, 03°46′18″W), where the sediment is a mixture of fine silty-sand, pebbles and stones. With ideal conditions for establishment of both species, these sensitive beds have not escaped the invasion of *S. muticum*, therefore, a four year manipulative field study with permanent quadrats in two treatments (ZS: *Z. marina* + *S. muticum* and ZM: *Z. marina* only) was carried out to determine the consequences of potential interactions between these two species.

This chapter introduces the field site and presents the seasonal and annual abiotic data associated with the field site, including seawater nutrient concentrations, sediment particle size and % organic content. Nutrient data for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and SiO_2 indicated a seasonal draw-down in the late spring and summer. Pulse events, potentially related to winter storm run-off from the surrounding fields, were also evident. Sediment particle size analyses indicated no significant treatment effect between the ZS and ZM permanent quadrats, nor did these results vary over time. Loss on ignition analysis to determine the % organic content did not reveal any treatment or year effects, but seasonal differences within each year were found ($p = 0.027$). I conclude that these results provide no evidence that the presence of *S. muticum* significantly alters the sediment conditions found within its immediate vicinity in the Salcombe-Kingsbridge Estuary.

2.1 Introduction

Located on the southwest coast of England at the western edge of the English Channel (50°13′53″N, 03°46′18″W) (Fig. 1.1), the Salcombe-Kingsbridge Estuary has been designated as an Area of Outstanding Natural Beauty (AONB), Special Area of Conservation (SAC) and a Site of Special Scientific Interest (SSSI) (South Hams District Council, 2008). It is home to a suite of unusual and biodiverse marine habitats, flora and fauna (Salcombe-Kingsbridge Estuary Conservation Forum, 2005b) including some of the largest seagrass beds within southwestern England (Mortimer, 1997) in addition to invasive species including *S. muticum*. This estuary is a dendritic ria (a.k.a. a 'false' estuary) (Salcombe-Kingsbridge Estuary Conservation Forum, 2005a), so named due to very little fresh water flowing into the estuary from terrestrial sources such as streams or rivers. With very little freshwater input, the estuary experiences full oceanic salinity conditions (~33–35‰), ideal for *Z. marina* and *S. muticum* alike. This system, along with the rest of the southern coast of Britain, experiences semi-diurnal tides with two similar highs and lows daily (Laboratory & Council, 2008). During the equinoctial spring tides, tidal height fluctuations may reach nearly six meters (Salcombe-Kingsbridge Estuary Conservation Forum, 2005b) and current velocities of ~1.0 m s^{-1} at mid-flood/ebb (Kinetics Ltd., 1992), which can result in the estuary experiencing up to 79 % tidal flushing with each turning of the tide (Salcombe-Kingsbridge Estuary Conservation Forum, 2005c).

The gently rolling hills and valleys surrounding the estuary are mainly farmland used for crops and livestock (Salcombe-Kingsbridge Estuary Conservation Forum, 2005b). Although not as highly developed as many other estuarine systems within the UK, there are two small towns and two large villages situated on its banks: Salcombe and Kingsbridge, and East Portlemouth and Frogmore, respectively. Salcombe is a highly seasonal town that experiences a large population influx during the milder months (April to October) with a high percentage of the houses owned as second or summer homes (South Hams District Council, 2007). The seasonal influx of people to the area leads to increased boating activity

within the estuary that can directly affect the seagrass beds through increased pollution from run-off, boat traffic, anchorage and moorings (Hastings *et al.*, 1995), dredging navigation channels (Erftemeijer & Lewis, 2006) and trampling of the shoreline (Eckrich & Holmquist, 2000). These types of activities coupled with the annual scallop dredging fishery can decrease available light through resuspension of fine particles (Erftemeijer & Lewis, 2006) and may further facilitate the spread of *S. muticum* through an increased number of anchoring and mooring scars, opening up substrata for *S. muticum* to colonise (den Hartog, 1997; Strong *et al.*, 2006; Kiparissis *et al.*, 2011).

The seagrass beds within this estuary are particularly sensitive to the effects of climate change and increasing sea levels due to shoreline hardening that includes seawall defences, housing construction and harbour developments, which are a ubiquitous feature within the estuary around Salcombe and Kingsbridge. Sea level is predicted to rise 0.2–0.8 m by 2050 (Watson *et al.*, 1996; Short & Neckles, 1999), which may adversely affect the seagrass through sediment resuspension and decreased light availability. Between 1961 and 2003, the mean global sea level rose at a rate of ~1.8 mm (1.3 to 2.3) yr^{-1}, and between 1993 to 2003, the mean global rate increased to 3.1 mm (2.4 to 3.8) yr^{-1} (Intergovernmental Panel on Climate Change, 2007). This has severe implications for *Z. marina* as it will be unable to move up shore due to the impermeable blockade of the hardened coastline.

The predicted higher frequency and magnitude of storms and increase in annual precipitation during the winter months may lead to an increase in the number of coastal and estuarine flood events (Burt *et al.*, 1998; Salcombe-Kingsbridge Estuary Conservation Forum, 2005a; Kaste *et al.*, 2006). Increased storminess and precipitation can potentially threaten the structural viability of upland soils, leading to an increased influx of suspended sediment and excess nutrients from surface run-off into the estuary, especially from dairy and/or livestock farms identified in Nitrate Vulnerable Zones (NPZs) in the surrounding area (Department for Environment, Food and Rural Affairs, 2011). This may be one of the most significant environmental problems the Salcombe-Kingsbridge Estuary faces, which

may ultimately jeopardise the estuary's ability to continue to meet EU Water Quality and Bathing Waters standards (Salcombe-Kingsbridge Estuary Conservation Forum, 2005c) as well as threaten the ecosystems supported, potentially compounding the negative effects of other abiotic and biotic factors affecting seagrass growth (Short & Wyllie-Echeverria, 1996).

The invasive Asian seaweed *S. muticum* has been found intermixed with *Z. marina* (Foden & Brazier, 2007) at increasing densities within the intertidal and shallow subtidal over the last several years particularly within the Salcombe-Kingsbridge Estuary (Tweedley, 2006; Tweedley *et al.*, 2008). Although the biological and ecological implications of this intermixing are still unknown, interaction consequences between these two macrophytes may include substrata competition, competition for light (Hauxwell *et al.*, 2001; McGlathery, 2001; Britton-Simmons, 2004; Strong *et al.*, 2006) and competition for nutrients (Valiela *et al.*, 1997; Rabalais, 2002), with each species having different requirements. Therefore, the aim of this research was to assess the ambient abiotic conditions including seawater nutrient concentrations of NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and SiO_2 and to sample sediment within permanent quadrats to determine if sthe presence of *S. muticum* affected particle size distribution and the % organic content contained within the quadrats.

2.2 Methods

2.2.1 Field study

A long-term field study was conducted from March 2007 until March 2011 through the establishment of 20 permanent 1 m^2 quadrats, 10 for each treatment (with and without *S. muticum*, hereafter ZS and ZM, respectively) at a depth of 0.5 meters below chart datum, in addition to four permanent 70 m long transects parallel to the shore 12 m apart at increasing depths, starting at Mean Low Water Low (MLWL) for transect 1 down to 1.2 m below MLWL for transect 4. The quadrats and transects were established to investigate how the

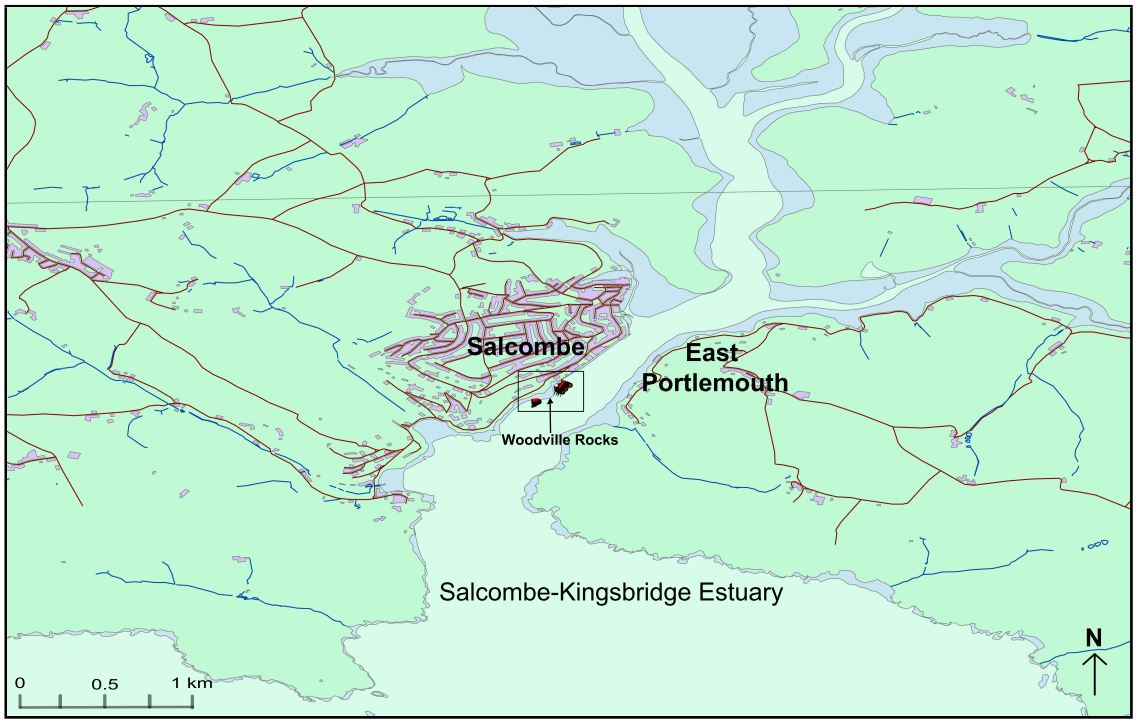


Figure 2.1: Small-scale GIS map of the Salcombe-Kingsbridge Estuary. The field site, located inside the black square, is indicted by the markers on the map located north and south of Woodville Rocks (rocky outcrop between the north and south quadrats). Kingsbridge is located just off the top of the map at the head of the estuary.

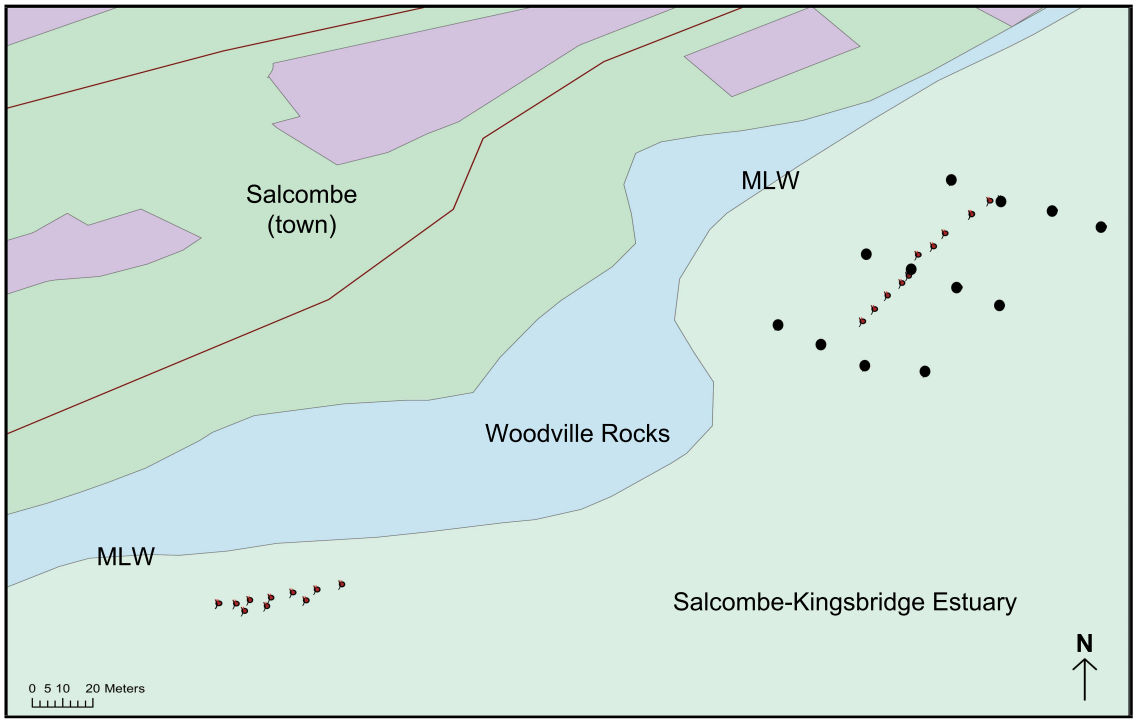


Figure 2.2: Large-scale GIS map (from within the black square, Fig. 2.1) of permanent quadrats and transects GPS points located within the Salcombe-Kingsbridge Estuary. The black circles indicate the end and mid-points of the transects and the red squares indicate the permanent quadrats located north and south of Woodville Rocks.

presence of *S. muticum* affects *Z. marina* within the Salcombe-Kingsbridge Estuary. Ten quadrats (five for each treatment) were located north of Woodville Rocks and ten to the south (Fig. 2.2). Similar-sized *S. muticum* thalli (~75–90 cm long) were harvested from other locations within the estuary and were attached to 25 cm x 25 cm plastic fencing grids using cable ties to secure the holdfasts in place. Two *S. muticum* thalli were attached to each grid and two plastic grids were secured within each of ten randomly selected quadrats using 10 mm wide reinforcing bar 'hooks', ~12-16 inches long, driven deep into the sediment to establish the ZS treatment. Two 'empty' plastic fencing grids with cable ties attached were anchored into the remaining 10 quadrats for the ZM treatment to account for any potential disturbance effect. Seagrass blades were pulled carefully through all fencing grids to remain upright within the water column and not trapped underneath. The areas around the ZM quadrats were 'gardened' at each sampling session to remove any unwanted *S. muticum* thalli from the vicinity of the control treatment plots.

2.2.2 Seawater samples

Every six to eight weeks from early spring until early autumn annually, two seawater samples were collected from the field site to determine ambient nutrient conditions. Two types of bottles were used to collect seawater samples on each sample date; one plastic bottle washed in a 2 % Neutracon (Decon Laboratories Limited, Hove) solution, soaked in a 10 % HCl bath for no less than 24 hours and rinsed with MilliQ water, and one glass bottle burned at 450 °C for 6 hours in a Carbolite AAF 1100 muffle furnace (Carbolite Limited, Hope) prior to collection. Samples were frozen at -20 °C until processed. Seawater samples collected in plastic bottles were analysed for silicates (SiO_2) using the 8186 Heteropoly Blue Method (Hach-Lange, Manchester) and the colourmetric results were read using a DR 2800 portable spectrophotometer (Hach-Lange). Samples collected in glass bottles were analysed for NH_4^+, NO_3^- and NO_2^- using the AQ2+ Automated Discrete Analyzer (Seal Analytical, Fareham) and PO_4^{3-} with LCK-349 test kits (Hach-Lange) read on the DR 2800 spectrophotometer.

2.2.3 Sediment samples

As sediment sampling occurred within the lower intertidal, one sediment core per permanent quadrat was taken twice annually for four years near the vernal and autumnal equinoxes to ensure that the permanent quadrats were fully emerged and accessible when sampled. Samples were collected using a 4 cm diameter x 14 cm PVC corer to a standard depth of 10 cm and were bagged at the site and frozen at -20 °C until processed.

Particle size

To determine the particle size for each sediment core, samples were defrosted and homogenised by thoroughly stirring the sediment. 45–60 mL of sediment was placed in a 300 mL glass basin and large biotic particles, such as *Z. marina* roots/rhizomes, blade tissue and animals, were removed. 6 % H_2O_2 (enough to cover the sample) was added to each sample to induce wet oxidation. After 24 hours, the liquid layer was skimmed to remove organic content that had bubbled to the surface. The samples were then heated over a boiling water bath to remove any other fine organic matter. Once wet oxidation was complete, samples were wet sieved through a 1 mm sieve. The fine fraction was caught in a pre-weighed 250 ml beaker placed below the funnel and the coarse fraction retained on the 1 mm sieve was rinsed into an unweighed 100 ml beaker, as the beaker was only used to hold the course fraction for drying purposes. All beakers were then placed in a 105 °C drying oven until dried completely. Once dried and weighed (sample + beaker) on a Satorius Genius analytic balance (Sartorius Mechatronics UK Ltd, Epsom) to determine the total weight of the fines, the fine fraction samples were rehydrated to a 'toothpaste' consistency and were subsampled five times for analysis by laser diffraction using a Malvern Mastersizer 2000 with wet sample unit Hydro-G (Malvern Instruments Ltd, Malvern). The coarse fraction was dry sieved using a series of sieve sizes (16.0 mm, 8.0 mm, 5.6 mm, 4.0 mm, 2.8 mm, 2.0 mm, 1.4mm, and 1 mm (pan)) and the particles retained on each sieve size were weighed on a Satorius LC 4800P pan balance (Sartorius Mechatronics UK Ltd). Percentage weights by particle size were calculated and data from

the two fractions were amalgamated for analysis in Gradistat 8.0 (Kenneth Pye Associates, Ltd., Crowthorne). Gradistat calcuated numerous means for each quadrat sample, but only the Method of Moments mean (logarithmic ϕ) was used for further analysis as it accounts for every grain in the distribution (U.S. Geological Survey, 2003).

% organic content

Loss on ignition (LOI) was used to determine the percentage of organic content present in each sediment core. Using clean, pre-weighed ceramic crucibles, approximately 5–10 mL of homogenised sediment was placed in the crucibles, oven-dried at 105 °C and then weighed 24 hours later. Crucibles were covered with lids and samples were then burned in a muffle furnace (Carbolite Limited, London) for 4 hours at 550 °C. After samples cooled, they were reweighed and the organic content was calculated by subtracting the final (burned) weight from the oven dried weight.

2.2.4 Data analysis

All data within this chapter and hereafter, followed a strict analytical protocol. Studies were primarily designed around analysis using General Linear Models (GLMs). First, raw, untransformed data were tested by univariate GLM in SPSS 19 (SPSS Inc., Chicago) using Levene's test for homogeneity of variances to verify data conformity to analytical assumptions. If conformity to assumptions of homogeneity of variances was indicated by Levene's Test, analysis of the raw data continued. If non-conformity to assumptions of homogeneity of variances were indicated by results of Levene's Test, data were transformed appropriately based on left- or right-skewedness. After transformation, the test was performed again, and if conformity to assumptions of variance homogeneity were met, analysis proceeded. If conformity was not met, residuals were visually inspected and if marked non-normality was evident, data were analysed using a non-parametric permutational approach in PRIMER (Plymouth Routines in Marine Ecological Research)

v6.1.13 (PRIMER-E, Plymouth, UK) with PERMANOVA+ v1.0.3 (Anderson *et al.*, 2008). Post-hoc tests were performed where explicit comparisons between treatment levels were not planned, otherwise planned comparisons were employed using Tukey's LSD tests based upon estimated marginal means.

Seawater samples

Only a single seawater sample was collected on each sample date between 2008 and 2011 (all 2007 samples were lost due to a refrigeration malfunction) to provide ambient nutrient concentrations for the duration of the long-term study, therefore no formal statistical analyses were intended.

Sediment samples

Effects of experimental 'treatments', 'year' and 'season' upon particle size in the sediment data from permanent quadrats were analysed using a mixed model univariate GLM in SPSS 19 with the Method of Moments mean (logarithmic ϕ), calculated in Gradistat 8.0 as the dependent variable. The GLM model had three factors, 'treatment' and 'year' were designated as fixed with two (ZS and ZM) and four (2007, 2008, 2009 and 2010) levels respectively and because of a lack of orthogonality 'season' was set as a random factor with two levels (spring and autumn), nested within 'year'. Type III Sums of Squares were used and S-N-K *post hoc* tests were performed for 'year'. Non-conformity to assumptions of homogeneity of variances were indicated by results of Levene's Test; examination of unstandardised analytical residuals indicated need for a power transformation (X^2). Equality of variances was improved by transformation, but remained marginal. Therefore, to confirm the univariate results, non-parametric analysis was also carried out using PRIMER v6.1.13 with PERMANOVA+ v1.0.3. Method of Moments mean (logarithmic ϕ) data were normalised and a Euclidean distance matrix was constructed. The same three-factor design described above was employed with type III Sums of Squares, unrestricted permutation of raw data and 9999 permutations. Pairwise tests of the factor 'treatment'

within the 'treatment * year' interaction term were also performed after inspection of initial results.

Effects of the experimental 'treatments', 'years' and 'season' upon % organic content (from LOI results) in the sediment data were analysed using a mixed-model univariate GLM in SPSS 19 after a routine angular (arcsine (p′ = arcsin $\sqrt{p}$)) transformation of the data expressed as proporations (Quinn & Keough, 2002). The GLM utlised the same three factors as described above. Type III Sums of Squares were used and S-N-K *post hoc* tests were performed for 'year'. Levene's Test indicated conformity to analytical assumptions.

To determine effects of the experimental 'treatments', 'years' and 'seasons' upon the combined % organic contents and particle sizes, data for both variables were normalised and a Euclidean distance matrix was constructed in PERMANOVA+ v1.0.3. Data were analysed with the same three-factor design as described above and S-N-K *post hoc* tests were performed for 'year'.

2.3 Results

2.3.1 Seawater nutrients

Results from the AQ2+ analyses for NH_4^+, NO_3^- and NO_2^- are presented in Figs. 2.3, 2.4 and 2.5. Results of the ambient PO_4^{3-} concentrations are shown in Fig. 2.6 and the colorimetric SiO_2 assay results are shown in Fig. 2.7. Patterns in NO_3^- concentrations emerged that may reflect the effect of autotrophic uptake of nitrogen during the late spring and summer months. Seasonal variability for all other nutrient concentrations were, in most cases, higher in the late summer/early spring sample months, perhaps as a result of a reflux of nutrients to the water column from phytoplankton due to light-limitation and a subsequent reduction in their growth.

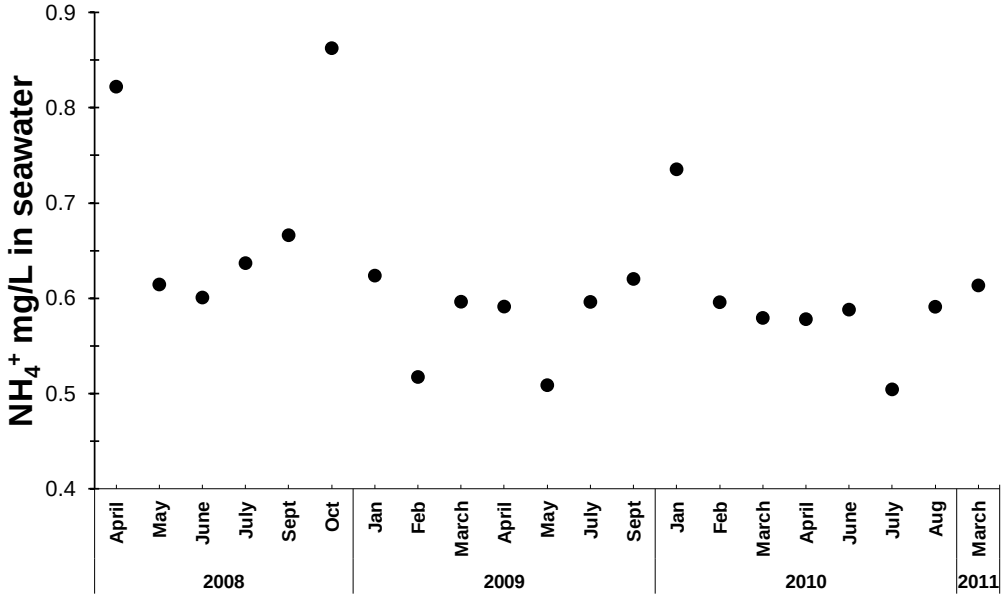


Figure 2.3: Results for ambient NH_4^+ mg/L from the long-term field study (2008–2011) in the Salcombe-Kingsbridge Estuary; n = 21.

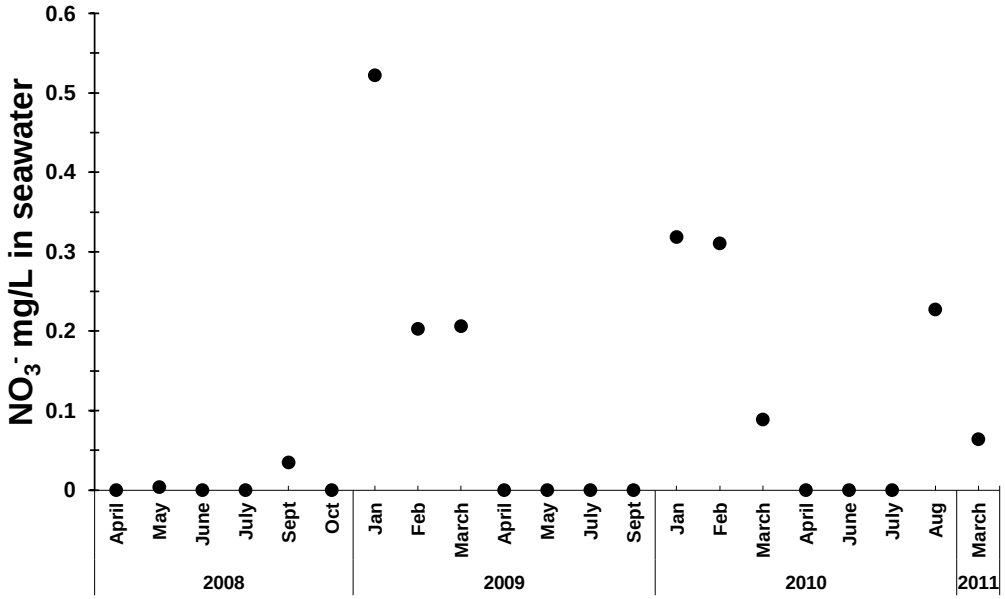


Figure 2.4: Results for ambient NO_3^- mg/L from the long-term field study (2008–2011) in the Salcombe-Kingsbridge Estuary; n = 21.

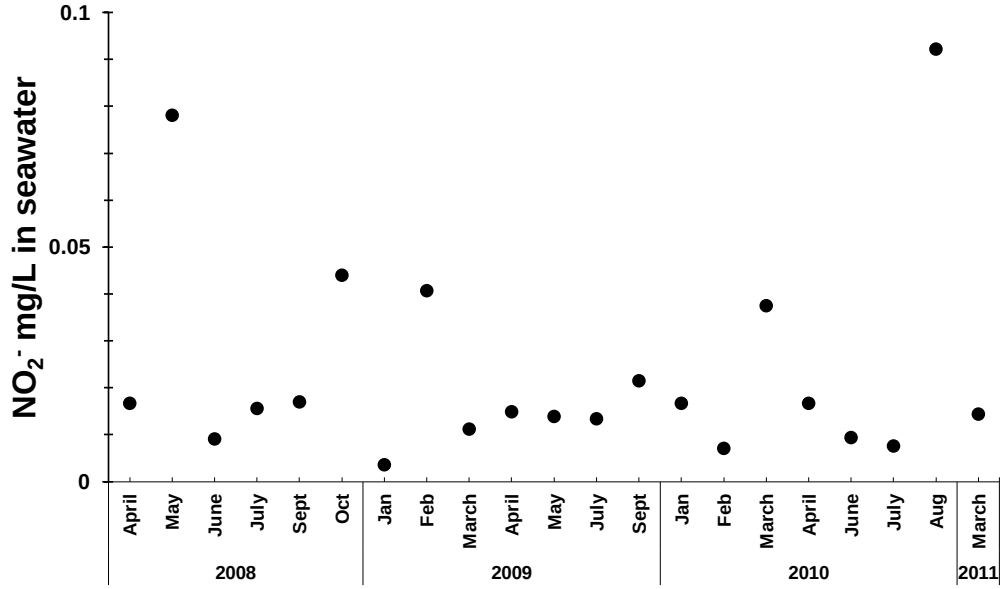


Figure 2.5: Results for ambient NO_2^- mg/L from the long-term field study (2008–2011) in the Salcombe-Kingsbridge Estuary; n = 21.

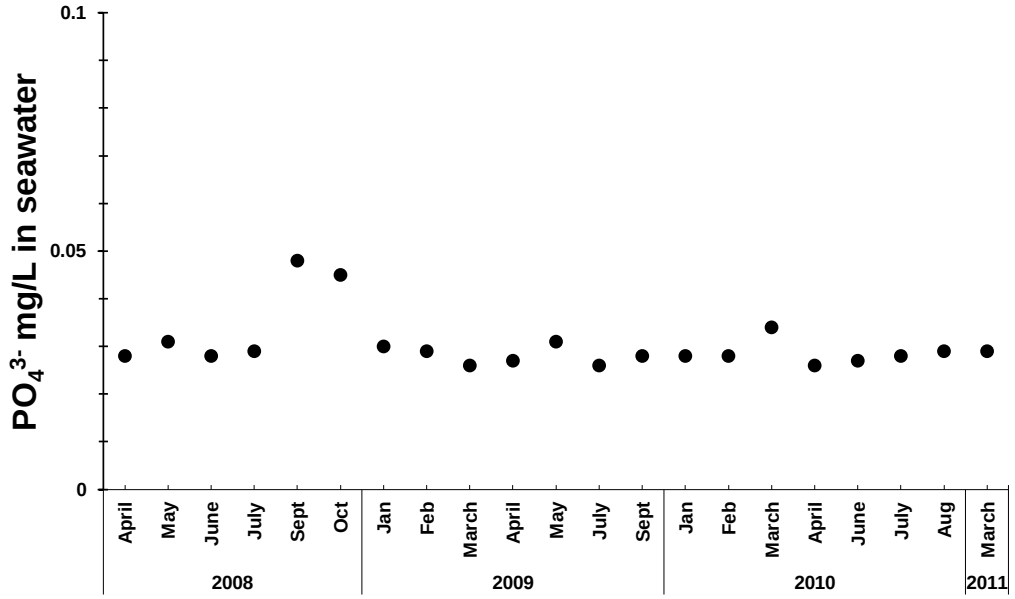


Figure 2.6: Results for ambient PO_4^{3-} mg/L from the long-term field study (2008–2011) in the Salcombe-Kingsbridge Estuary; n = 21.

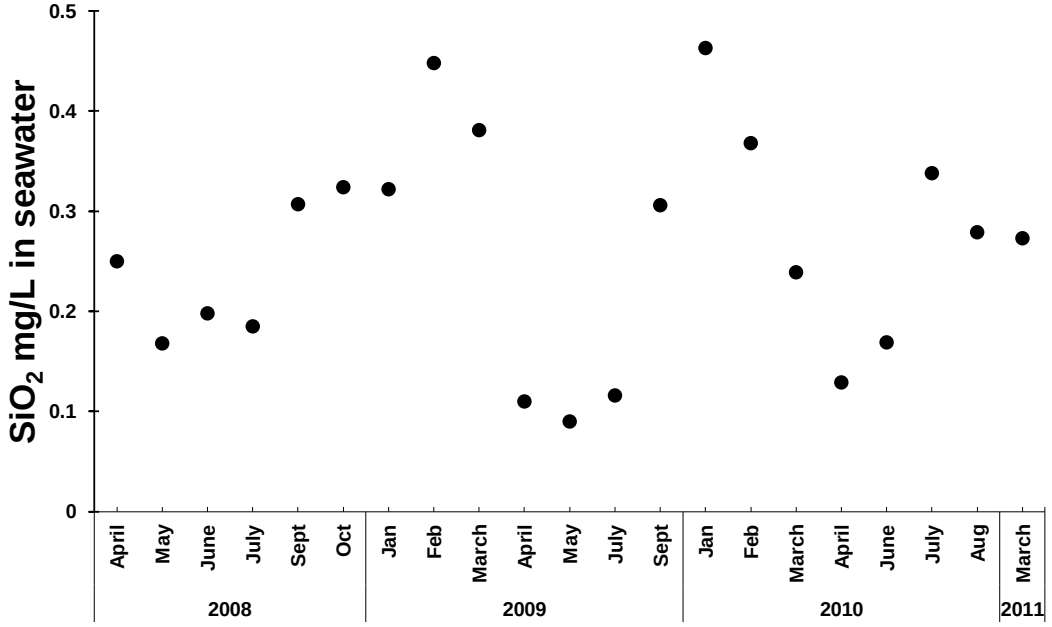


Figure 2.7: Results for ambient SiO_2 mg/L from the long-term field study (2008–2011) in the Salcombe-Kingsbridge Estuary; n = 21.

2.3.2 Sediment analyses

Particle size

Sediment sample analysis revealed the average particle size using the Method of Moments mean (logarithmic ϕ) was 1.4±0.08 ϕ (equivalent to ~250–500μm (Blott & Pye, 2001)). The sediment samples were classed as very poorly to moderately sorted, mainly composed of sandy gravel to sand, comprised of approximately 93.5 % sand, 6.5 % silt and 0.08 % clay, irrespective of treatment. Results of GLM indicated no significant differences between 'treatments', 'years' or 'seasons' nested within 'year' for particle size. PERMANOVA+ analyses confirmed no significant differences between 'treatment' and 'year', but a significant effect of 'seasons' nested within 'year' was revealed (Table 2.1).

% organic content

Sediment samples consisted of approximately 2.6% organic content, irrespective of treatment. Results from the univariate GLM for % organic content indicated no significant

'treatment' or 'year' effects, but significant differences were found in the random factor 'seasons' nested within 'year' (Table 2.2). Annual variation between 'treatments' and across the site as a whole was not significant (Fig. 2.8), but S-N-K *post hoc* tests indicated significant differences between 2007 and 2009.

Combined particle size and % organic content

Results from the PERMANOVA+ analyses indicated no significant differences between 'treatments' or 'year' for the combined particle size (Method of Moments mean (logarithmic ϕ)) and % organic content, but a significant difference was revealed for 'seasons' nested with 'year' (Table 2.3).

Table 2.1: PERMANOVA+ results for laser diffraction particle size data (Methods of Moments mean (logarithmic ϕ)) for two treatments (ZS and ZM) and four years (2007–2010); n = 70 for each treatment over 4 years with 2 seasons per year.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	1.786	1.786	1.966	0.260
Year	3	33.973	11.324	0.405	0.892
Season(Year)	3	83.974	27.991	17.514	**< 0.001**
Treatment * Year	3	2.367	0.789	0.868	0.557
Treatment * Season(Year)	3	2.726	0.909	0.569	0.645
Res	126	201.37	1.5982		
Total	139	325.39			

Table 2.2: Univariate GLM results for % organic content loss on ignition (LOI; arcsine transformed); n = 70 for each treatment over 4 years with 2 seasons per year.

Source		Type III SS	df	MS	F	Sig. (*p*)
Intercept	Hypothesis	0.091	1	0.091	469.503	**< 0.001**
	Error	0.001	3	0.000		
Year	Hypothesis	0.000	1	0.000	0.084	0.791
	Error	0.001	3	0.000		
Treatment	Hypothesis	0.000	3	0.000	0.605	0.655
	Error	0.001	3	0.000		
Season(Year)	Hypothesis	0.001	3	0.000	14.788	**0.027**
	Error	0.000	3	0.000		
Treatment * Year	Hypothesis	0.000	3	0.000	0.805	0.569
	Error	0.000	3	0.000		
Treatment * Season(Year)	Hypothesis	0.000	3	0.000	0.356	0.785
	Error	0.005	126	0.000		

Table 2.3: PERMANOVA+ results for the combined laser diffraction particle size (Method of Moments mean (logarithmic ϕ)) and loss on ignition % organic content for two treatments (ZS and ZM) and four years (2007–2010); n = 70 for each treatment for both particle size and % organic content over 4 years with 2 seasons per year.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	0.771	0.771	1.085	0.409
Year	3	23.166	7.722	0.462	0.922
Season(Year)	3	50.18	16.727	10.525	**< 0.001**
Treatment * Year	3	1.790	0.597	0.8392	0.585
Treatment * Season(Year)	3	2.132	0.711	0.447	0.847
Res	126	200.25	1.589		
Total	139	278			

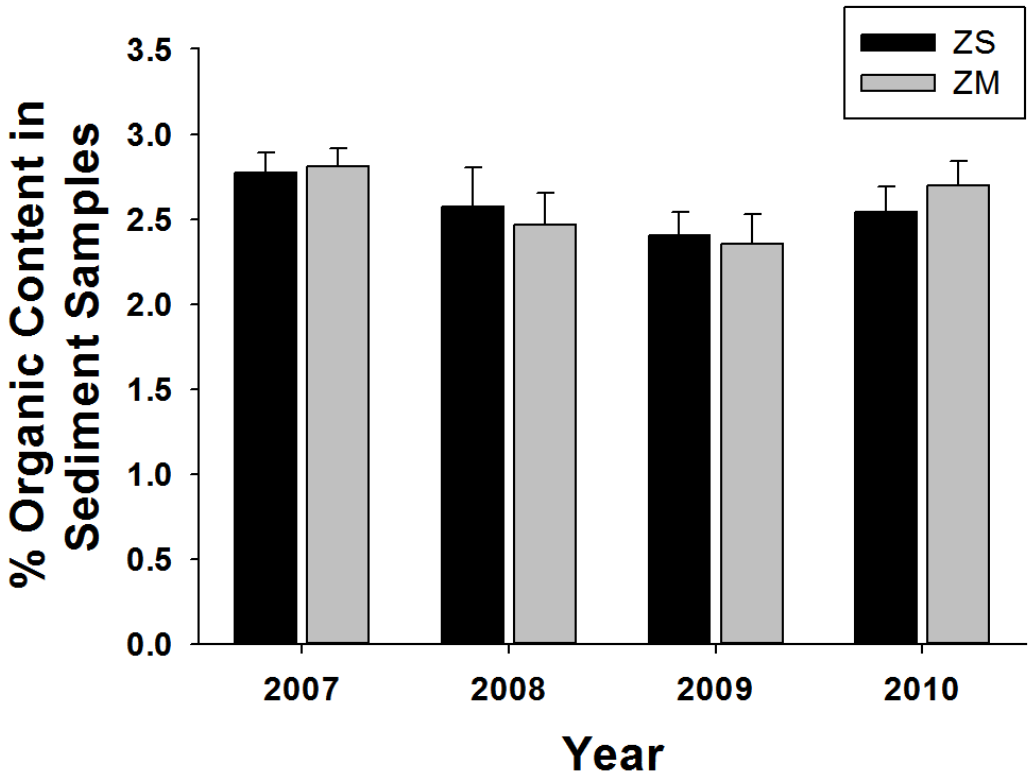


Figure 2.8: Mean % organic content (±SE) in sediment samples collected from 2007–2010; n = 70 for each treatment over 4 years with 2 seasons per year.

2.4 Discussion

2.4.1 Nutrient limitation and eutrophication

Seagrasses have the capacity to alter the habitats they colonise (Koch, 2001) through the reduction of resuspension of bottom sediments (Ward *et al.*, 1984; de Boer, 2007) as the blades act as wave attenuators (Fonseca & Cahalan, 1992), dissipating turbulent energy (Posey, 1988; Hemminga & Duarte, 2000), and the roots and rhizomes stabilise the sediments. Dominant primary producers, such as *S. muticum*, however, can increase organic matter and nutrient turnover (Cebrián *et al.*, 1998; Pedersen *et al.*, 2005) due to their rapid growth and annual senescence. With faster biomass turnover, herbivory can increase and carbon storage within the system can decrease (Cebrián *et al.*, 1998). In Denmark, Pedersen *et al.* (2005) found that *S. muticum* increased nutrient cycling through faster detritus decomposition compared to the native species, *Halidrys siliquosa* (Linnaeus) Lyngbye. With increasingly dense stands of *S. muticum*, nutrient cycling within the Salcombe-Kingsbridge estuary may experience similar effects.

Replicate seawater samples were not taken as variability within samples from a single date was not the focus of this study. However, replicate water samples may have eliminated the potential for sample error due to exceeding detection limits and/or processing. Patterns for NO_3^- concentrations in the seawater samples from the long-term field study indicated a seasonal draw-down of nitrogen in the late spring and summer, potentially inversely related to the annual autotrophic uptake. Ammonia, phosphates and silicates were consistently available during the sample months (Fig. 2.3), whereas nitrates and nitrites were more variable and low overall (Figs. 2.4, 2.5). Although nitrates may become limited at various times during the year as seen in Fig. 2.4, *Z. marina* is generally not nutrient-limited (Zimmerman *et al.*, 1987) due to the high nutrient supply from marine sediments to its roots and rhizomes (Short & McRoy, 1984; Touchette & Burkholder, 2000a).the root-rhizome matrix of *Z. marina* may confer a competitive advantage over other primary producers such as *S. muticum* due to the seagrass' ability to uptake particulate organic

nutrients from marine sediments (Evrard *et al.*, 2005). It is also unlikely that *S. muticum* was nutrient-limited at this site due to its ability to utilise both NH_4^+ and NO_3^- (Sánchez *et al.*, 2006).

With spring tide current velocities of ~1.0 m s^{-1} at mid-flood/ebb (Kinetics Ltd., 1992), the boundary layer of the blades is reduced, allowing nutrients to pass into the cell membranes. Although *Z. marina* can capture 30-90 % of its overall nitrogen requirement through its leaves (Short & McRoy, 1984; Evrard *et al.*, 2005), nutrient uptake from the water column is considered a secondary source of nutrient capture (Short & McRoy, 1984). Previous research has found that *Z. marina* preferentially takes up NH_4^+ as its nitrogen source from sediment (see reviews in Short, 1987; Touchette & Burkholder, 2000b), but high ammonia concentrations within the water column can be toxic to the leaves of *Z. marina* (van Katwijk *et al.*, 1997; Hauxwell *et al.*, 2001). With increasing densities of *S. muticum* (see Chapter 5), localised increases in ammonia concentrations due to the rapid return of nutrients to the water column as the algal tissues break down at the end of the summer (Morand & Merceron, 2005) may affect the health of the leaves of *Z. marina*.

Globally, coastal ecosystems have begun to suffer the negative effects of eutrophication, such as dead zones, more frequent phytoplankton blooms and increased turbidity (Nixon, 1995; Beer & Koch, 1996; Short & Wyllie-Echeverria, 1996; Udy & Dennison, 1997b; Rabalais, 2002). Pulse events found in the seawater samples collected during the field study indicate eutrophic conditions may arise, but not persist within the Salcombe-Kingsbridge Estuary. There was no indication that eutrophication occurred at any time sampled, which may be a result of large-scale tidal flushing. If eutrophic conditions increase in frequency and persist due to storm intensification and subsequent run-off from hillside farms, further changes within the benthic assemblage may occur including a shift from slow-growing macroalgae to fast-growing, turf-forming algae (Russell *et al.*, 2005). Algae are generally unable to fulfil similar ecosystem functions to seagrasses as they have no capacity for long-term retention of nutrients (i.e., below-ground storage) and have limited capacity to

uptake increased nutrient concentrations into their tissues after pulse or flood events except through increased frond production (Morand & Merceron, 2005).

2.4.2 Marine sediments

Sediment deposition is a positive feedback mechanism found within seagrass meadows (de Boer, 2007) as seagrass blades can capture phytodetritus and particulate organic matter (POM) through canopy friction, sustaining their own nutrient supply (Evrard *et al.*, 2005), reducing turbidity and thereby improving water clarity (de Boer, 2007). Currents also fulfil a critical role in the advection of organic matter into and out of marine sediments as particles have a differential settling velocity in relation to the upward current force based on particle size (de Boer, 2007) and factor into degradation processes (Huettel & Gust, 1992; Evrard *et al.*, 2005). However, the amount of suspended POM can vary markedly due to biological processes including bioturbation, consolidation by microbes and metazoan mucoidal substances (Ward *et al.*, 1984), and through mechanical processes including dredging, anchorage and moorings (Hastings *et al.*, 1995; Erftemeijer & Lewis, 2006; Collins *et al.*, 2010).

Findings by Ward *et al.* (1984) indicated that seagrass meadows are composed of finer, more poorly sorted sediments than adjacent, non-vegetated areas. Results from the long-term field study revealed significant seasonal differences for both particle size and % organic content, but no significant differences between treatments were found. Samples from both treatments were comprised of over 93 % sand, which is highly mobile especially during winter storms, 6.5 % silt and 0.08 % clay. The seasonal differences found in both particle size and % organic content may be attributed to seasonal differences in macrophytic biomass within the estuary, as densities for both *Z. marina* in the permanent quadrats and for both species along the transects were lower in the spring than later in the year (see Chapter 5, Figs. 5.1, 5.3).

2.5 Conclusions

This chapter reported the ambient field conditions for nutrients, sediment particle size and % organic content experienced by both *Z. marina* and *S. muticum* inter- and intra-annually within the Salcombe-Kingsbridge Estuary. Although no treatment effects due to *S. muticum* presence were found, seasonal effects were revealed in the ambient nutrient concentrations and for the sediment particle size and % organic content within the sediment. The seasonal variation found in the NO_3^-, NO_2^- and SiO_2 concentrations may be attributed to the annual autotrophic bloom and subsequent nutrient uptake. Significant seasonal differences within the sediment may indicate that lower macrophytic biomass in early spring affects sediment composition and the % organic content within the sediment.

Chapter 3

Invasion Impacts: Phenolic Compounds

Abstract

Polyphenolic compounds are found in seaweeds, seagrasses and other higher plants and are known for their defensive properties, which can inhibit growth, interrupt physiological processes and prevent herbivory. Through induced and constitutive defence strategies based on the production of phenolic compounds, plants and algae can rapidly respond to changing conditions. Detectable changes in production of these compounds have been linked to stresses such as intra- and interspecific competition, nutrient limitation, herbivory, pollution, inadequate light and temperature changes. This has allowed phenols to be used as exemplar biochemical indicators of plant and/or algal condition. Previous studies investigating the biochemical and physiological interactions between invasive seaweeds and a seagrass in the Mediterranean Sea have shown deleterious effects upon seagrass biochemistry and vigour. With reported elicitation of polyphenol upregulation in seagrasses and algae in the presence of invasive marine macrophytes, the rapid proliferation of *S. muticum* into seagrass beds within the UK gives rise to concern for the long-term health of *Z. marina*.

To determine how *S. muticum* may affect the phenolic production of *Z. marina*, a four year field study was conducted in conjunction with several short-term laboratory experiments using caffeic and tannic acids as indicators. Laboratory conditions were established to closely simulate environmental field conditions at the time of year the experiments were conducted (i.e., early spring). Wild-harvested *Z. marina* shoots were established at natural densities (~160 shoots m^{-2}) in aquaria under artificial daylight for four weeks. Three treatments (ZS: *Z. marina* + *S. muticum*, ZM: *Z. marina* only and ZZ: *Z. marina* + *Z. marina* (used as a biomass control and to account for any potential effects *S. muticum* may have had on available seawater nutrients in the ZS treatment)) were used in the laboratory experiments. The long-term field study utilised only two treatments (ZS and

ZM). These experiments were coupled with a short-term nutrient limitation experiment to determine if inorganic carbon (HCO_3^-, CO_3^{2-} and pCO_2), nitrogen (NH_4^+, NO_3^- and NO_2^-) and phosphate (PO_4^{3-}) limitation occurred within the treatment tanks due to the inherent biomass differences within each treatment (ZS, ZM and ZZ) or as a result of the length of time between water changes.

Results from both the field study and laboratory experiments indicated a significant difference between treatments, $p = 0.034$ and $p < 0.001$, respectively. The ZS treatment had significantly lower % dry weight (DW) caffeic and tannic acid contents compared to the ZM and ZZ treatments. The duration of the experiments, whether long- or short-term, and where they were conducted, whether in the field or laboratory, had no effect on the pattern that emerged. The nutrient limitation experiment revealed no significant differences between treatments either for the inorganic carbonic components HCO_3^-, CO_3^{2-} and pCO_2 or for the nutrients NH_4^+, NO_3^- and PO_4^{3-} in the seawater samples. These results indicated that treatment type (i.e., the variation in biomass and species complement within each tank) did not result in differences in nutrient limitation or photosythetic levels during the laboratory experiments. From these results I conclude that the invasion of *S. muticum* may be directly affecting the biochemistry of *Z. marina* as exemplified by phenol production and that this result is unlikely to be an artifact of nutrient limitation. Lower phenolics may secondarily weaken the natural defense mechanisms of *Z. marina*.

3.1 Introduction

Phlorotannin polyphenolic compounds, like tannins in higher plants, are well-known for their defensive properties in seaweeds (Lobban & Harrison, 1994; Strack, 1997; Dumay *et al.*, 2004). These properties include their ability to inhibit bacteria and epifaunal larval settlement (Lobban & Harrison, 1994; Lau & Qian, 1997), reduce growth of surrounding plants (Lambers *et al.*, 1998; Hopkins & Hüner, 2004), interfere with physiological and behavioural functions of herbivores (Karban & Baldwin, 1997; Borell *et al.*, 2004; Hopkins

& Hüner, 2004; Vergés *et al.*, 2007) and can even inhibit enzyme activity of cellular processes within humans (Liu *et al.*, 2011). Many seaweeds and marine macrophytes produce and accumulate phenolic compounds in response to stress (Sauvesty *et al.*, 1992; Dumay *et al.*, 2004), which can be induced by intra- and interspecific competition (allelopathy) (Agostini *et al.*, 1998; Lambers *et al.*, 1998), nutrient limitation (Lambers *et al.*, 1998), pollution (Lambers *et al.*, 1998), herbivory (Quackenbush *et al.*, 1986; Buchsbaum *et al.*, 1990; Agostini *et al.*, 1998; Buschbaum *et al.*, 2006), inadequate light (Lambers *et al.*, 1998) and temperature change (Strack, 1997; Dumay *et al.*, 2004). Plants and algae can respond rapidly to changing ambient conditions because phenolic compounds have a fast turnover time of only a few days (Buchsbaum *et al.*, 1990; Arnold & Targett, 2002; Amsler & Fairhead, 2006).

Changes in nitrogen availability can be a significant factor in the variation of phenolic content within plants (Mattson Jr., 1980). When prevailing environmental conditions are nitrogen-limited, but not light-limited, plants can turn excess fixed carbon into phenolics or other nitrogen-free compounds (Tuomi *et al.*, 1984). This process is facilitated by the common pathways used to make carbohydrates and phenols, both of which are mediated through allocation of phenylalanine as a precursor in the shikimic acid pathway (Herrmann, 1995; Strack, 1997; Arnold & Targett, 2002). Amazingly, both the production pathway and the signal transduction method by which predation is sensed are common to vascular plants and brown algae (Coleman *et al.*, 2007), albeit through the shikimic acid and phenylpropenoid pathways in vascular plants and through the polyketide synthase enzyme complexes in brown algae (Harborne, 1997; Strack, 1997; Arnold & Targett, 2002). Regardless of pathway, the production of secondary metabolites as defensive mechanisms have been highly conserved within the distantly related lineages.

Seagrasses contain phenolics (Zapata & McMillan, 1979; McMillan *et al.*, 1980) and exhibit a number of similarities to terrestrial plants and some marine algae with respect to the production and location as well as age (Harrison, 1982; Vergeer & Develi, 1997;

Agostini *et al.*, 1998; Dumay *et al.*, 2004) and seasonal variation (Harrison & Durance, 1989; Dumay *et al.*, 2004; Pergent *et al.*, 2008) of phenolics within their tissues. The primary allelopathic polyphenols found within seagrasses are produced at comparable levels to those found in land plants and some marine algae (Harrison & Durance, 1989). Phenolic compounds in seagrasses are concentrated in the areas of growth known as the subapices, where the metabolic rates are highest (Dumay *et al.*, 2004). Agostini *et al.* (1998) found that the phenolic content in the leaves of *Posidonia oceanica* (L.) Delile, a seagrass endemic to the Mediterranean, decreased with increasing age, a trait also common in longer lived terrestrial plants (Dey & Harborne, 1997); Harrison (1982) found a similar pattern in *Z. marina*. Harrison & Durance (1989) also found that *Z. marina* exhibits seasonal variation in phenolic levels, having the lowest concentrations at the start of the growing season and the highest levels in late summer to early autumn, again, a common trend found in *P. oceanica* (Dumay *et al.*, 2004) and terrestrial species such as oaks (Harborne, 1997).

With 45–80 % of nitrogen assimilation occurring within the blades (Zimmerman *et al.*, 1987; Lee & Dunton, 1999), nitrogen availability can also be a factor affecting the variability of blade tissue phenolic content (Buchsbaum *et al.*, 1990). Seagrasses uptake nitrogen (NH_4^+, NO_3^-) not only as an essential nutrient for primary metabolism, but also for the costly metabolic processes used in the biosynthesis of phenolics (Herms & Mattson, 1992; Harborne, 1997). Buchsbaum *et al.* (1990) found that as the percentage of available nitrogen increased, the percentage of phenolics within the blades of *Z. marina* decreased as they adapted to ambient nutrient conditions. Decreases in allelopathic compounds may weaken the natural defensive mechanisms within seagrasses, which could result in increased herbivory (Quackenbush *et al.*, 1986; Buchsbaum *et al.*, 1990; Agostini *et al.*, 1998), pathogen attacks (Short & Burdick, 1996) and effects of competition (Dumay *et al.*, 2004).

In brown seaweeds such as *S. muticum*, phenolic compounds can account for 10–30 % of

an alga's dry weight (Lobban & Harrison, 1994; Targett *et al.*, 1995; Amsler & Fairhead, 2006), giving them a particularly high phlorotannin content and therefore making them more unpalatable to grazers (Lobban & Harrison, 1994; Boyer *et al.*, 2004; Plouguerne *et al.*, 2006). The phenolic content of *S. muticum* can vary due to habitat type, light intensity and variability, increases in UV-B radiation, salinity, emersion, and nutrient availability (Plouguerne *et al.*, 2006) and handling and harvesting *S. muticum* can cause phenolic compound leakage, altering the final composition within the alga (Gorham & Lewey, 1984). Abiotic factors aside, *S. muticum* reaches its highest polyphenolic content during the summer months of June and July (Gorham & Lewey, 1984), tracking the same seasonal variation as found in *Z. marina* (Harrison & Durance, 1989).

Several phenolic compounds have been identified in *P. oceanica* and *Z. marina* (Cuny *et al.*, 1995; Arnold & Targett, 2002), with caffeic acid predominant in both species (Harrison, 1982; Buchsbaum *et al.*, 1990; Dumay *et al.*, 2004). Previous research in the Mediterranean found that *P. oceanica* increases its production of these secondary metabolites to limit the invasion of *Caulerpa taxifolia* (Vahl) C. Agardh into the seagrass beds (Dumay *et al.*, 2004), ultimately allocating more resources to production of defensive mechanisms than to growth (Pergent *et al.*, 2008). This was the first documented example of marine allelopathy between a seagrass and a macroalga. The rapid and uncontrolled proliferation of *S. muticum* within seagrass beds in the UK gives rise to concern for similar long-term implications for *Z. marina* and its associated fauna. As *S. muticum* densities continue to increase within the estuary, the physiology of *Z. marina* may be affected through a potential increase in metabolic costs associated with the production of secondary metabolites (Cuny *et al.*, 1995; Dumay *et al.*, 2004; Pergent *et al.*, 2008).

To test the effects of the presence of *S. muticum* on *Z. marina* over time, a long-term field study, in conjunction with multiple laboratory experiments, modelling the approach used in the Mediterranean Sea, were established. This research aimed to investigate the potential effects on the production of chemical defences (i.e., phenolics) within the

blade tissues of *Z. marina* in the presence of the invasive alga. A short-term laboratory experiment was also conducted to assess the ambient seawater nutrient concentrations for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and SiO_2 and the total alkalinity (composed of HCO_3^-, CO_3^{2-} and pCO_2) of the seawater to contextualize possible nutrient limitation effects on the growth and biochemistry of the seagrass due to the differences in biomass and species between treatments in these experiments. I hypothesised that the relative phenolic content of *Z. marina* would increase in the presence of *S. muticum*, following the same pattern found in *P. oceanica* in the previous research carried out in the Mediterranean.

3.2 Methods

3.2.1 Experimental design

To elucidate the effects of the presence of *S. muticum* upon *Z. marina*, a long-term field study was carried out over consecutive years between 2007-2011 (see Chapter 2 for field site and methodology), concurrent with multiple laboratory experiments.

Field study

Seagrass samples were collected within the established permanent quadrats every six to eight weeks over a four year period from three seasons (spring: March–May, summer: June–August and autumn: September–October) during which time *S. muticum* thalli were present and actively growing. Three randomly selected shoot samples from each quadrat were harvested by cutting the blades just above the basal meristem; these were bagged and brought to the lab, where they were processed immediately. All blades were measured (length and width) and the blades used for phenolics assay were gently scraped clean of epibiota (Parker *et al.*, 2001; Tomas *et al.*, 2005; Jaschinski & Sommer, 2008) and frozen at -20 °C.

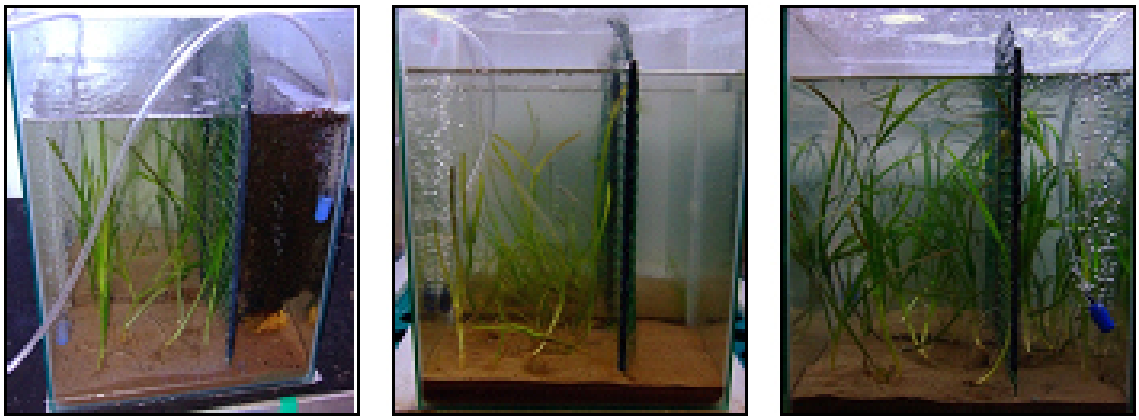


Figure 3.1: Laboratory experimental design using three treatments (L-R): *Z. marina* + *S. muticum* (ZS), *Z. marina* only (ZM) and *Z. marina* + *Z. marina* (ZZ).

Laboratory experiments

Seagrass shoots were hand-harvested to ensure collection of intact rhizomes and roots from Torre Abbey Sands, Torquay (50°27'38.45"N, 3°32'02.61"W) and the Salcombe-Kingsbridge Estuary (50°13'52.77"N, 3°46'23.80"W) in early spring in three successive years and were acclimated to laboratory conditions for two weeks in aerated tanks at early spring *in situ* densities of ~160 plants m^{-2} (see Chapter 5: Results) in 2008 and for one week for the 2009–10 experiments. Ten glass tanks, each measuring 29.5 x 23 x 39 cm with a capacity of 26.5 L of seawater, were partitioned into two, unequally-sized compartments (60 %:40 % size ratio) by 1 cm grid plastic fencing to allow for water exchange between the two sides while keeping algae or control seagrass shoots from physically interacting with the trial *Z. marina* plants.

Three treatments were established in 2008 and 2009: *Z. marina* + *S. muticum* (ZS), *Z. marina* only (ZM) and *Z. marina* + *Z. marina* (ZZ) (Fig. 3.1). In 2010, only the ZS and ZM treatments were tested as no significant differences were found between the ZS and ZZ treatment in 2008 or 2009 (Table 3.3). After epiphytes were gently removed by lightly scraping with a razor blade (Parker *et al.*, 2001; Tomas *et al.*, 2005; Jaschinski & Sommer, 2008), five *Z. marina* shoots (~16–18 g wet weight(WW)) were anchored into the larger compartment of each tank to maintain similar seagrass biomass and spring *in situ* densities (~160 shoots m^{-2}) for each tank. One *S. muticum* thallus (~60 g WW) attached to a

small stone was added to the smaller compartment of each tank for the ZS treatment. For the ZZ treatment, five to seven additional *Z. marina* shoots (~12 g WW total biomass) were added to the smaller compartment to act as a biomass control, used to account for any potential effects *S. muticum* may have had on available seawater nutrients in the ZS treatment. The ZM treatment consisted only of the five shoots anchored within the larger tank compartment. In 2008, the roots and rhizomes were covered with a thin layer of sediment collected from the same location to anchor the shoots in place. Despite artificial disturbance the sediment rapidly became anoxic, therefore in 2009 and 2010, shoots were secured onto plastic grids using loosely tightened cable ties around the rhizomes, and no sediment was used.

In 2008, the mid-water photosynthetic photon flux (PPF) was ~55–60 μmol m^{-2} s^{-1} on a 16L:8D cycle and plants were held at 15±3 °C in a controlled temperature (CT) room. In 2009 and 2010, infrastructural improvements allowed the PPF to be increased to ~95–110 μmol m^{-2} s^{-1} and the daylength shorted to 12L:12D as the experiments occurred earlier in the year; the temperature was lowered to 10±3 °C. Artificial daylight was supplied by 80W Giesemann Aquablue+ fluorescent lamps (Giesemann Aquaristic, Nettetal) used to mimic seawater light availability to a depth of one to twenty meters. The PPF was measured with a SKP 200 light meter and SKP 215 quantum sensor (Skye Instruments, Wales).

In 2008, the seawater in the tanks was changed every three days, at which time the sediment was disturbed to reduce anoxic conditions for the roots and rhizomes. In 2009 and 2010, the seawater was changed every second day. Seawater salinity was monitored using a REF201/211/201bb refractometer (Omega Portable Refractometer, Stamford) and maintained at near-full oceanic conditions (~32 ppm). Shoots were harvested after 28 days, any epiphytes were removed from the blade tissue by gentle scraping with a razor blade (Parker *et al.*, 2001; Tomas *et al.*, 2005; Jaschinski & Sommer, 2008) and samples were stored at -20 °C until phenolic assays and nutrient analyses were performed. Only two

treatments (ZS and ZM) with six tanks per treatment were tested in 2010, as previous years results indicated no significant differences between the ZM and ZZ treatments.

3.2.2 Nutrient limitation experiment

To determine if *Z. marina* shoots experienced carbon, nitrogen and phosphate limitation due to differences in biomass within the treatment tanks or from the length of time between water changes during previous laboratory experiments, a six day experiment was conducted in 2011. Treatment tanks were partitioned and set-up using the same laboratory conditions as described above for the 2009–10 experiments and four treatments were established using wild-harvested seagrass shoots and algae as before: ZS (six tanks), ZM (six tanks), ZZ (five tanks), and three seawater-only control tanks (C). Every second day, tanks were fully drained and seawater changed as previously described, consistent with the 2009 and 2010 experimental protocol. Seawater temperature, salinity and pH (using the NBS scale [National Bureau of Standards, now the National Institute of Standards and Technology]) were recorded daily and were used as parameters in calculation of total alkalinity.

Seawater samples were collected daily, which allowed data to be organised by time within the water cycle: i) START: fresh seawater, ii) MID: 24 hours after water change and iii) END: 48 hours after water change taken just before tanks were drained for the next water change. Two 2 mL and 1 100 mL seawater samples were taken from each tank at the start of the experiment (i.e., directly following a water change), the day between water changes and before a water change occurred. This sampling cycle was repeated three times. Samples were frozen at -20 °C until analysed for NH_4^+, NO_3^-, NO_2^- using an AQ2+ Automated Discrete Analyzer (Seal Analytical, Fareham) and with LCK-349 test kits (Hach-Lange, Manchester) read on the DR 2800 spectrophotometer for PO_4^{3-}.

The 100 mL seawater sample was collected in a glass bottle washed in 10 % HCl acid and was immediately innoculated with 0.02 % $HgCl_2$ to stop all biological activity. These water samples were stored at 10 °C in black plastic bags to block all light from the samples then

processed using an Alkalinity Titrator AS-ALK2 (Apollo SciTech Inc., Bogart) calibrated at 25 °C and a pH probe calibrated using three standard buffer solutions of pH 4.01, 7.00 and 9.32. 24 mL of each seawater sample were titrated to calculate total alkalinity following standardised EPOCA guidelines (Riebesell *et al.*, 2010). Bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and pCO_2 were calculated with the CO_2Sys programme (Lewis & Wallace, 1998) using the titrated total alkalinity results and the pH, temperature and salinity data collected during the experiment.

3.2.3 Polyphenolic compounds extraction

To quantify the equivalent % DW content of caffeic (CA) and tannic acids (TA) within the blade tissues, samples were dried at 65 °C for 24 hours, ground and ~150 mg of weighed sample was extracted in 50 % MeOH for 24 hours in a dark, 4 °C refrigerator. Phenols in blade tissue were assayed using an adapted Folin-Ciocalteu colorimetric assay (Harrison & Durance, 1989; Sauvesty *et al.*, 1992), processed in triplicate and read against caffeic and tannic acid standard dilution series at 765 nm using a Unicam Helios Epsilon spectrophotometer (Unicam Ltd, Cambridge).

3.2.4 Data analysis

Field data

Effects of experimental 'treatments', 'years' and 'seasons' on phenolics (% DW CA mg^{-1} and % DW TA mg^{-1} equivalents) were analysed in PRIMER v6.1.13 with PERMANOVA+ v1.0.3 due to extreme left skewedness that could not be corrected for by tranformation in SPSS 19.0. Data were averaged for each quadrat in each treatment. A PERMANOVA+ was calculated for each of the phenolic variables using a Euclidean distance resemblance matrix and a three-factor PERMANOVA+ design, where 'treatment' with two levels (ZS and ZM) and 'year' with four levels (years 2007–2010) were designated as fixed factors. 'Season' was set as a random factor with three levels (spring, summer and autumn), nested

within 'year' due to a lack of orthogonality. Unrestricted permutations of raw data, type III Sums of Squares and 9999 permutations were set as design parameters (Anderson *et al.*, 2008). A final PERMANOVA+ was calculated for the normalised combined % DW CA mg^{-1} and % DW TA mg^{-1} equivalents data using the same three factor design described above.

Laboratory data

Phenolic data (% DW CA mg^{-1} and % DW TA mg^{-1} equivalents) from the laboratory experiments were analysed using PRIMER v6.1.13 with PERMANOVA+ v1.0.3 as described above due to extreme left skewedness that could not be corrected for by tranformation in SPSS 19. Data were tested using a Euclidean distance resemblance matrix and a two-factor PERMANOVA+ design, where 'treatment' with three levels (ZS, ZM and ZZ) and 'year' with three levels (2008, 2009 and 2010) were fixed factors. Unrestricted permutations of raw data, type III Sums of Squares and 9999 permutations were set as design parameters. Multivariate data (% DW CA mg^{-1} and % DW TA mg^{-1}) were normalised then analysed for the effects of three treatments (ZS, ZM and ZZ for 2008–09 only) and for all three 'years' combined (2008–2010). Data for all years combined (2008–2010) with only two treatments (ZS and ZM) were analysed using the same three-factor design after removing the biomass control (ZZ) treatment from the design. A PERMANOVA+ was calculated for the 2009–2010 data using two treatments (ZS and ZM) due to changes in the experiment design between 2008 and the other two years. Annual means (±SE) for % DW CA and TA equivalents were calculated in SPSS 19.

Nutrient limitation experiment

All data ($HCO_3{}^-$, CO_3^{2-}, pCO_2, NH_4^+, NO_3^-, NO_2^- and PO_4) from the nutrient limitation experiment were divided into three levels (after water change (START), mid cycle (MID), before water change (END)) by time point sampled within the water cycle to create the factor 'cycle'. Effects of potential differential nutrient limitation (differences

in nutrient limitation effects between treatments) were analysed in PRIMER v6.1.13 with PERMANOVA+ v1.0.3 by comparing the nutrient concentrations of the seawater samples collected immediately before the water changes (END data) using one-way PERMANOVA+ analyses. PERMANOVA+ was employed because initial parametric analyses indicated significant deviation from homogeneity of variances; skew in the data could not be corrected by transformation. Each nutrient was analysed independently using PERMANOVA+ and one combined multivariate design was also conducted. Seawater only control tank (C) data for each of the carbon, nitrogen and phosphate components were analysed separately to check for any background nutrient cycling due to the presence of protists, algae and other microbes within the seawater using data from all three water 'cycle' time points (START, MID and END). No significant effects of water 'cycles' were revealed in these analyses, and subsequent regression analyses indicated a lack of significant slope of substrate concentrations plotted within the water 'cycles', therefore the C treatment was eliminated from the analytical design, and no correction for microbial action was deemed necessary in analyses of the other experimental treatments.

Tests for nutrient limitation were performed using one-way PERMANOVA+ to test for differences between the treatments at the end of the water 'cycle'. A separate one-way PERMANOVA+ was employed for each inorganic carbon component, inorganic carbon components combined, nutrients separately and combined and finally for all substrates and nutrients combined; all multivariate tests were performed using normalised data. Pairwise tests between treatments were employed in the event of a significant treatment effect in these analyses.

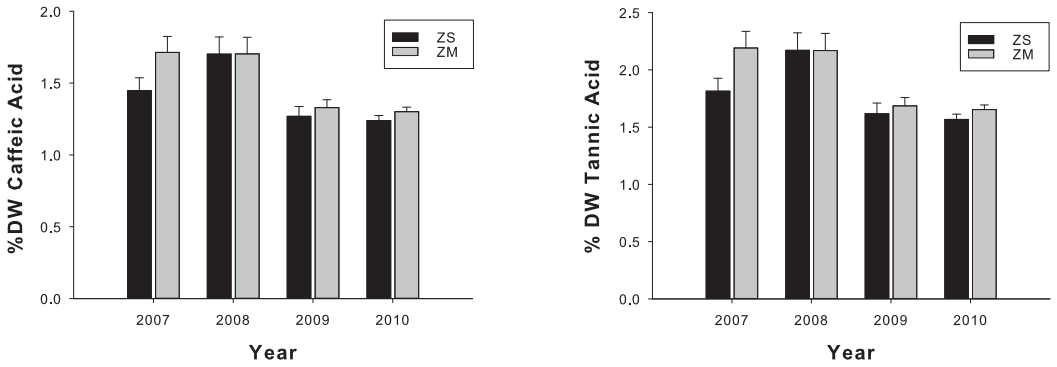
3.3 Results

3.3.1 Field phenolics

The overall mean phenolic content ranged from 1.39 % to 1.48 % DW CA and 1.76 % DW TA to 1.89 % DW TA (n = 1137 total samples) from the four year field study (Fig. 3.2). PERMANOVA+ analyses of the combined mean % DW CA and TA mg^{-1} equivalents revealed a significant difference between the ZS and ZM 'treatments' and 'seasons' within 'years', but no significant differences were found between 'years' (Table 3.1). *Z. marina* shoots in the ZS treatment had lower % DW phenolic content, for both CA and TA mg^{-1} equivalents, than shoots in the ZM treatment (Fig. 3.2) in all years of the long-term field study.

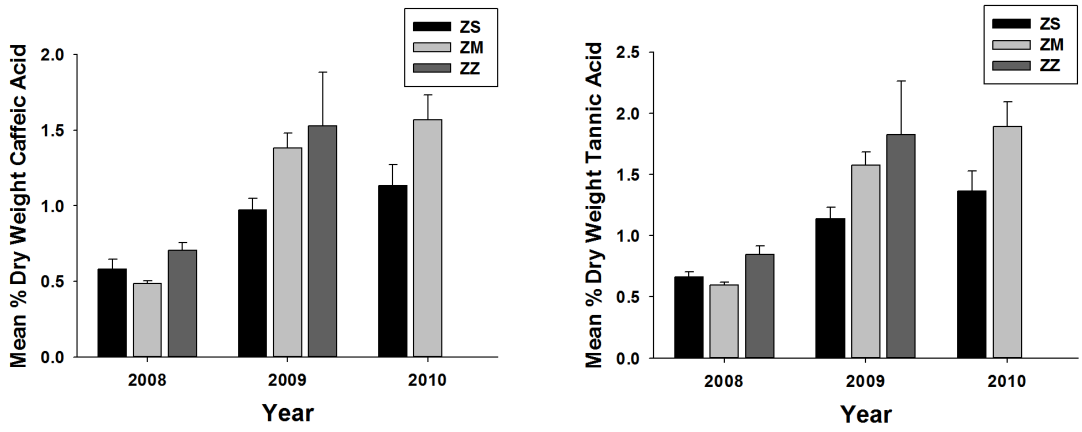
Table 3.1: PERMANOVA+ results from the 2007–2010 long-term field experiment for % DW combined caffeic and tannic acid equivalents for two treatments (ZS and ZM). Quadrat was used as the replicate; n = 190 for each treatment over 4 years with 3 seasons per year.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	5.349	5.349	7.314	**0.034**
Year	3	49.369	16.456	0.659	0.597
Season(Year)	7	168.21	24.03	17.52	**< 0.001**
Treatment * Year	3	7.021	2.3403	3.177	0.106
Treatment * Season(Year)	7	5.068	0.724	0.528	0.816
Res 357	489.65	1.372			
Total	378	756			



(a) *Caffeic Acid* **(b)** *Tannic Acid*

Figure 3.2: Mean (±SE) % DW for caffeic (a) and tannic (b) acid mg^{-1} equivalent contents from the 2007–2010 long-term field study for two treatments (ZS and ZM). Quadrat was used as the replicate; n = 190 for each treatment over 4 years with 3 seasons per year.



(a) *Caffeic Acid* **(b)** *Tannic Acid*

Figure 3.3: Mean (±SE) % DW for caffeic (a) and tannic (b) acid mg^{-1} equivalent contents for three treatments (ZS, ZM and ZZ) in 2008 and 2009 and from two treatments in 2010 from laboratory experiments. Tank was used as the replicate; n = 26 for the ZS and ZM treatments, n = 20 for the ZZ treatment over 3 years.

Table 3.2: PERMANOVA+ results from the 2008–09 laboratory experiments for % DW CA and TA mg^{-1} equivalents for three treatments (ZS, ZM and ZZ). Tank was used as the replicate; n = 20 for each treatment over 2 years.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Year	1	37.315	37.315	28.506	**< 0.001**
Treatment	2	6.532	3.266	2.495	0.078
Year * Treatment	2	3.4651	1.733	1.324	0.287
Res	54	70.688	1.309		
Total	59	118			

Table 3.3: PERMANOVA+ pairwise comparisons for 'treatment' within the interaction term 'year * treatment' the from 2008–09 laboratory experiments.

Within level '2008' of factor 'year'		
Groups	t	*p* (perm)
ZS, ZM	1.3835	0.173
ZS, ZZ	1.8714	0.074
ZM, ZZ	3.7446	**< 0.001**
Within level '2009' of factor 'year'		
ZS, ZM	3.187	**0.004**
ZS, ZZ	1.536	0.162
ZM, ZZ	0.486	0.608

Table 3.4: PERMANOVA+ results for the combined % DW CA and TA mg^{-1} equivalents for two treatments (ZS and ZM) from the 2008–10 laboratory experiments. Tank was used as the replicate; n = 26 for ZS and ZM treatment over 3 years, n = 20 for ZZ treatment over 2 years.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Year	2	60.332	30.166	48.164	**< 0.001**
Treatment	1	7.299	7.2988	11.653	**0.001**
Year * Treatment	2	6.799	3.400	5.428	**0.008**
Res	46	28.811	0.6263		
Total	51	102			

Table 3.5: PERMANOVA+ pairwise comparisons for 'treatment' within the interaction term 'year * treatment' for three years (2008–2010).

Year	Groups	t	*p* (perm)
2008	ZS, ZM	1.383	0.184
2009	ZS, ZM	3.186	**0.005**
2010	ZS, ZM	2.004	0.074

3.3.2 Lab phenolics

The mean phenolic content of the blades of *Z. marina* ranged from 0.86 % to 1.12 % DW CA mg^{-1} equivalent and from 1.01 % to 1.28 % DW TA mg^{-1} equivalent (n = 72) from the three laboratory experiments (Fig. 3.3). PERMANOVA+ analysis of the 2008 and 2009 data using all three treatments (ZS, ZM and ZZ) indicated no significant differences between 'treatments', but 'year' was highly significant (Table 3.2). Pairwise comparisons for the factor 'treatment' within the interaction term 'treatment * year' revealed significant differences only between the ZZ and the ZM treatment in 2008 and the ZS and ZM treatment in 2009 (Table 3.3).

As significant differences were not found in 2009 between the seagrass only treatments (ZM and ZZ) after laboratory conditions were amended, the ZZ treatment was therefore excluded from the final 2008–2010 analysis and entirely from the 2010 experiment. PERMANOVA+ results from the combined 2008, 2009 and 2010, two treatment (ZS and ZM) analysis indicated significant differences between 'treatments' and 'years' and a significant 'year * treatment' interaction (Table 3.4). Investigation of pairwise tests of the factor 'treatment' within the interaction term 'year * treatment' revealed these differences were due to highly significant differences between 'treatments' in 2009 (Table 3.5). The final PERMANOVA+ for the 2009–2010 data revealed significant differences between 'treatments', but not between 'years'; no significant 'year * treatment' interaction was found (Table 3.6). 2008 data were removed from the analytical design due to improvements in the experimental design in 2009 and 2010.

Using the % DW nitrogen content determined in Chapter 4 as the independent variable, data were graphed against the results from the same samples for % DW CA content. Variation within the nitrogen content may partially explain differences in the % DW CA mg^{-1} equivalent between treatments (Fig. 3.4).

Table 3.6: ERMANOVA+ results for the combined % DW CA and TA mg^{-1} equivalents from the 2009–10 laboratory experiments for two treatments (ZS and ZM). Tank was used as the replicate; n = 16 for the ZS and ZM treatment over 2 years, n = 10 for the ZZ treatment in 2009.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	6.244	6.244	12.925	**0.002**
Year	1	1.482	1.482	3.0675	0.088
Treatment * Year	1	2.7393E-2	2.7393E-2	5.6705E-2	0.845
Res	28	13.526	0.483		
Total	31	21.503			

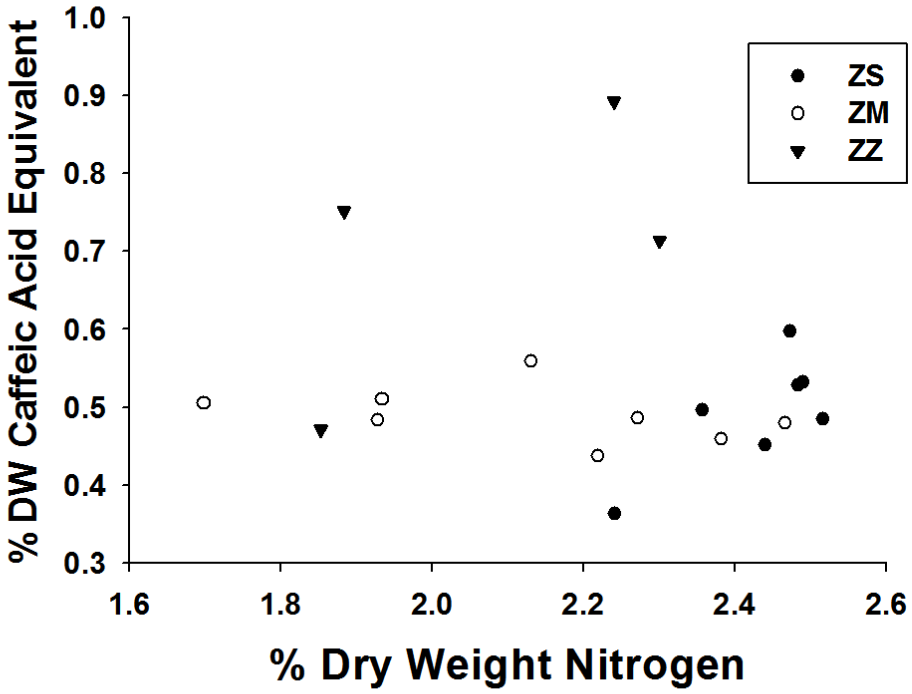


Figure 3.4: % DW nitrogen in the blade tissues of *Z. marina* vs. % DW CA mg^{-1} equivalents from 2008 laboratory experiment. Low sample size (n = 19) for all treatments was due to blade tissue lost to wasting disease.

3.3.3 Nutrient limitation experiment

Total alkalinity: Carbonic components

PERMANOVA+ analysis using only the END data for each dependent variable and for the combined carbonic components indicated no significant differences between 'treatments' at the end of the water 'cycle' (Fig. 3.5).

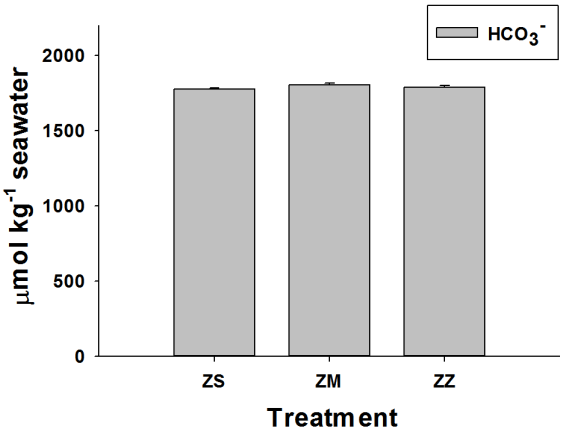
Nitrogen compounds and phosphates

PERMANOVA+ analysis using only the END data for each dependent variable and for the combined nutrients indicated no significant differences between 'treatments' with the exception of NO_2^- ($p = 0.001$; Table 3.7) at the end of the water 'cycle' (Fig. 3.6). Results from the one-factor design using only the END data for the combined inorganic carbonic components and nutrient revealed no significant differences between 'treatments'.

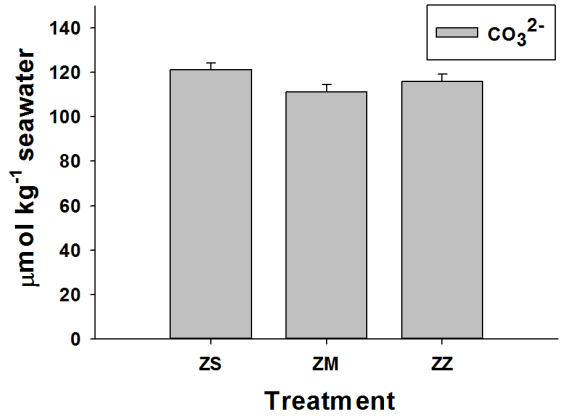
Because there was no evidence of differential substrate or nutrient limitation by the end of the water 'cycles', no analysis of the data from the samples taken at the MID cycle time point was deemed necessary.

Table 3.7: PERMANOVA+ results for NO_2^- in seawater for three treatments (ZS, ZM and ZZ) and one water cycle time point (END) from the short-term nutrient limitation experiment. Tank was used as the replicate; n = 18 for ZS and ZM treatment, n = 14 for ZZ treatment.

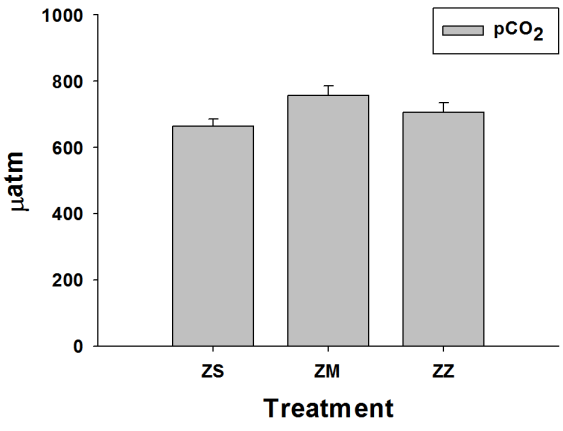
Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	2	6.6217E-5	3.3108E-5	8.9799	**0.001**
Res	34	1.2536E-4	3.687E-6		
Total	36	1.9157E-4			



(a) *Mean (±SE)* HCO_3^-

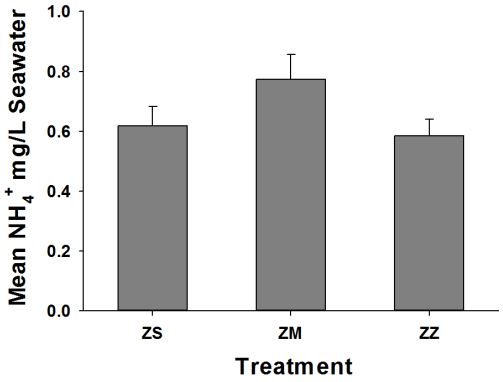


(b) *Mean (±SE)* CO_3^{2-}

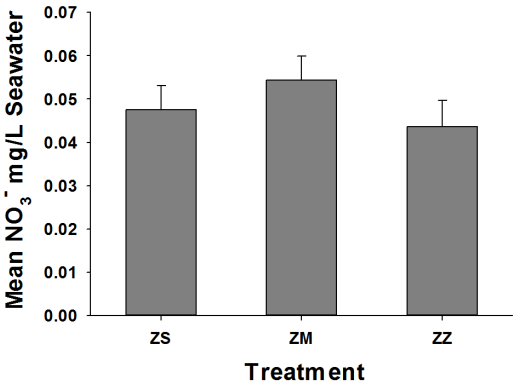


(c) *Mean (±SE)* pCO_2

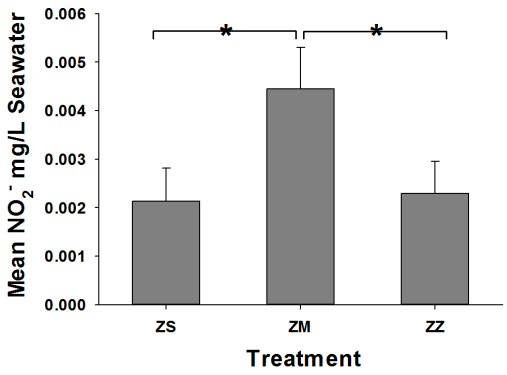
Figure 3.5: Mean (±SE) HCO_3^- (a), CO_3^{2-} (b), and pCO_2 (c) by treatment from 2011 nutrient limitation laboratory experiment. Tank was used as the replicate; n = 54 for the ZS and ZM treatments, n = 44 for the ZZ treatment.



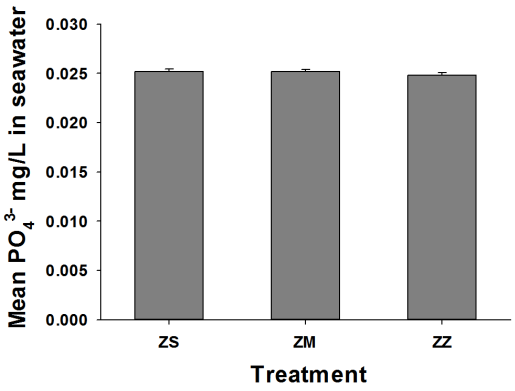
(a) *ZS: n = 38; ZM: n = 41; ZZ: n = 32.*



(b) *ZS: n = 21; ZM: n = 36; ZZ: n = 22.*



(c) *ZS and ZM: n = 44; ZZ: n = 36.*



(d) *ZS and ZM: n = 29; ZZ: n = 27.*

Figure 3.6: Mean (±SE) NH_4^+ mg/L (a), NO_3^- mg/L (b), NO_2^- mg/L (c), and PO_4^{3-} mg/L (d) (averaged across all water cycle time points) by treatment from the short-term nutrient limitation experiment. * indicates significant difference ($p < 0.05$). Tank was used as the replicate.

3.4 Discussion

3.4.1 Phenols

Phenolic content varies within seagrasses (Harrison & Durance, 1989), with simple and polymeric phenolic levels being both high and highly variable (Arnold & Targett, 2002). *Z. marina* has eight known phenolics (Agostini *et al.*, 1998), with CA cited as the dominant phenolic compound within the *Zostera* genus (Harrison, 1982; Quackenbush *et al.*, 1986). Although CA has been found to be the dominant phenolic compound, % DW TA equivalents content was consistently higher in the samples processed (Figs. 3.2, 3.3), but this may reflect the use of tannic acid as standard to represent total phenolics present. This result may also be due to the Folin colorimetric assay's inability to discriminate between the various classes of plant phenolics (Arnold & Targett, 2002). Phenolic results from both the field and laboratory experiments revealed similar ranges of CA and TA % DW mg^{-1} equivalent contents.

The short-term experiments conducted over a three to four week period allowed adequate time for a stress or allelopathic response to be generated and quantitatively evaluated. In 2008, % DW CA mg^{-1} equivalents determined by absorbance spectrophotometry were lower than the HPLC results reported by Harrison (1982) and the colorimetric assay results reported by Buchsbaum *et al.* (1990). The 2008 ZS and ZM results were nonetheless consistent with colorimetric assay results reported later by Harrison & Durance (1989) who used a similar Folin-Denis assay to the Folin-Ciocalteu assay used here, although the ZZ treatment was slightly elevated compared to the ZS and ZM results (Fig. 3.3). Results from the 2008 experiment with three treatments may have been a result of nutrient limitation and/or inadequate PPF, as the % DW CA and TA mg^{-1} equivalents content within shoots in the ZS treatment were of intermediate level (Fig. 3.3), therefore the experiment was repeated in 2009 with improved laboratory conditions.

Results from the long-term field study and multiple laboratory experiments showed a high degree of inter-year variability within phenolic content (Figs. 3.2, 3.3), which Cuny *et al.*

(1995) also found in *P. oceanica* with increasing interactions between *C. taxifolia* and *C. racemosa*. Dumay *et al.* (2004) and Pergent *et al.* (2008) found a positive correlation between increasing densities of the invasive seaweeds (*C. taxifolia* and *C. racemosa*) and a rise in phenol production within *P. oceanica*. Contrary to their findings, results from this long-term field study in conjunction with the laboratory experiments conducted in 2009 and 2010 and to a degree in 2008, showed decreased production of polyphenolic equivalents of both CA and TA in *Z. marina* cultured with *S. muticum* (Table 3.1, Fig. 3.2).

Young leaves act as a carbon sink and older leaves function as a carbon source (Zimmerman *et al.*, 1995). Maximum phenolic concentrations in *Z. marina* are found in young, growing leaves and minimum concentrations in older leaves (Harrison, 1982; Vergeer & Develi, 1997), similar to the pattern found in the leaves of *P. oceanica* (Cuny *et al.*, 1995; Agostini *et al.*, 1998; Dumay *et al.*, 2004; Pergent *et al.*, 2008). Previous research by Dumay *et al.* (2004) found that higher levels of phenolics were observed only in the intermediate leaves of *P. oceanica* compared to the amount found in the adult leaves with increasing levels of *C. taxifolia* interaction. As the leaves from each experiment and field study were homogenised by treatment tank or quadrat, no discrimination can be made regarding blade age and phenolic production.

Although no significant differences were found between the three treatments in the 2008 and 2009 laboratory experiments, significant differences were found between years, perhaps driven by the alteration in laboratory infrastructure (i.e., increased light and lowered temperature). Planned pairwise comparisons for the 2008–09 analysis revealed significant differences between the ZM and ZZ treatment in 2008 and the ZS and ZM treatment in 2009 (Fig. 3.3). The analyses indicated a recurrent pattern within the phenol response to the experimental treatments. Despite the potential infrastructural issues in 2008, which reduce the confidence that can be expressed regarding this year's results, the juxtaposition of these data and those of 2009 serves only to reinforce the results of the latter and those

of 2010.

Analysis of the combined years (2008–2010) using two treatments (ZS and ZM) revealed significant differences between treatments and years with a significant interaction between year and treatment, making it to difficult determine the phenolic response of *Z. marina* to the presence of *S. muticum*. Planned pairwise comparisons for treatment within the interaction term revealed highly significant differences between treatments in 2009, but no differences were revealed between treatments in 2008. Although the same pairwise comparison of the 2010 data indicated lack of statistical differences, they may be biologically relevant (Table 3.5). Removal of the 2008 data from the final PERMANOVA+ analysis due to the improvements in laboratory infrastructure in 2009, allowed two similar years (2009 and 2010) to be analysed for treatment effect. Results from the PERMANOVA+ analysis of the 2009 and 2010 data revealed highly significant differences in the % DW CA and TA equivalent contents between the two treatments, with phenolic levels more in concordance with other studies (Fig. 3.3) and were visibly indistinguishable from the field study results (Figs. 3.2, 3.3). Although phenolic results varied across years, viewed collectively they indicate a consistent treatment effect due to algal presence.

Release of water-soluble phenolic compounds into the water column from diseased and/or decaying seagrass tissue may not deter or limit competition from an invading alga (Zapata & McMillan, 1979; McMillan *et al.*, 1980), as phenolics can quickly dissipate within the water column. A more effective delivery method would be to release phenolic compounds into the sediment (Zapata & McMillan, 1979) via roots and rhizomes, but as *S. muticum* is a non-rhizomatous alga, any allelopathic defences produced by *Z. marina* may have little influence in directly deterring the continued spread of *S. muticum*.

3.4.2 Nutrient limitation

Seagrasses and macroalgae are sensitive indicators of nitrification (Hauxwell *et al.*, 2001). *Z. marina*, as with many seagrasses, help offset nutrient-loading through uptake and

retention of nutrients within their blades and complex root-rhizome matrix (Hemminga *et al.*, 1991; Hemminga & Duarte, 2000; Evrard *et al.*, 2005), but a decline in seagrasses, coupled with increased macroalgal blooms resulting from increased nutrient supply, has been observed worldwide (Short & Wyllie-Echeverria, 1996; Valiela *et al.*, 1997; McGlathery, 2001). Although an increase in nitrogen may initially lead to increased seagrass growth (Uku *et al.*, 2005), nitrogen accumulation in eutrophic conditions can eventually cause a severe imbalance in the Redfield (C:N) ratio of *Z. marina*, ultimately leading to growth deficiencies (Burkholder *et al.*, 1992; Gillanders & Kingsford, 2002; Uku *et al.*, 2005) as carbon resources within the shoot may be used to synthesize amino acids rather than carbohydrates to prevent internal accumulation of toxins such as ammonia (Touchette *et al.*, 2003; Burkholder *et al.*, 2007). This may be due to *Z. marina* blades lacking an inhibition mechanism for sustained NO_3^- uptake that may negatively affect this species' physiology, weakening meristematic regions (Burkholder *et al.*, 1992) and increasing the likelihood of pathogen attacks due to under-production of phenolics (Short & Burdick, 1996). However, ammonia (van der Heide *et al.*, 2008) and nitrate toxicity may only occur under very specific enrichment conditions as found by Burkholder *et al.* (1992) and may not be a considerable concern for *Z. marina* (Moore & Wetzel, 2000).

Inorganic carbon (C_i) is essential for autotrophic photosynthesis, with seagrasses obtaining most of their C_i supply from the water column rather than sediments (Sand-Jensen, 1977; Touchette & Burkholder, 2000a). As $HCO_3{}^-$ is the main dissolved inorganic carbon (DIC) source of C_i in seagrasses (Beer *et al.*, 1977; Beer, 1989), it was previously believed that seagrasses were C_i limited (Beer & Koch, 1996) as a result of the low diffuse concentrations of CO_2 in seawater and seagrasses subsequent reliance on the carbonic anhydrase-mediated conversion of HCO_3^- to dissolved CO_2 at the external cell membrane surface within the diffusion boundary layer of the leaves (Beer, 1989). Interestingly, recent studies have shown that seagrasses are often not C_i limited and can take up HCO_3^- directly (Beer & Rehnberg, 1997; Beer *et al.*, 2002).

Results from the short-term nutrient limitation experiment provided no evidence of uneven patterns of C_i limitation in either univariate or multivariate analyses; comparison with the seawater control tanks showed that nutrients were being taken up, but this pattern was not different for each treatment. These results suggest that *Z. marina* was not C_i limited during the laboratory experiments and that allocation of carbon resources to phenolics production was possible.

Production of phenolics and other secondary metabolites is a resource-intensive process that requires a constant stream of carbon building blocks, enzymes, and energy-rich compounds such as ATP and NADH to drive the shikimate/arogenate (SA) and phenylpropenoid pathways (Harborne, 1997; Arnold & Targett, 2002). Compared to the energetic costs associated with the production of alkaloids (i.e., nitrogen-rich toxins), production of phenolics requires only one half the energetic expenditure (Harborne, 1997). Plants and/or algae that produce these defensive compounds must balance their production with growth, but nitrogen availability plays a significant role in how seagrasses (Tuomi *et al.*, 1984; Buchsbaum *et al.*, 1990), and plants in general, achieve this balance.

The observed differences in the phenolic production in *P. oceanica* (increased phenolics) (Dumay *et al.*, 2004; Pergent *et al.*, 2008) and *Z. marina* (decreased phenolics) to the presence of invasive algae may be ecosystem driven, rather than solely as a result of differing species. The Mediterranean Sea is an oligotrophic system (Turley *et al.*, 2000), whereas the coastal waters of the UK are relatively nutrient replete (Butler, 1979; Widdicombe *et al.*, 2010). Localised increased nutrient deficiency as a result of increased *Caulerpa* densities in the Mediterranean may have therefore been the underlying mechanism affecting phenolic production within *P. oceanica* (Harborne, 1997; Lambers *et al.*, 1998). As nutrient limitation is of little concern within UK coastal waters, the suppressed phenolic production within the shoots in the ZS treatment was unlikely to be a result of nutrient limitation.

PERMANOVA+ results from the short-term nutrient limitation experiment indicated that

only NO_2^- differed significantly between treatments (Table 3.7, Fig. 3.6), but this effect may be attributable to a biomass effect as both treatments with additional biomass differed significantly from the ZM treatment. C:N ratio imbalances in *Z. marina* could potentially influence allocation of carbon reserves to phenolic defences through both direct and indirect consequences of stoichiometry, however, *Z. marina* does not utilise NO_2^- as its primary nitrogen source (Zimmerman *et al.*, 1987; Touchette & Burkholder, 2000b) so effects of this limitation are unlikely to elicit the observed responses in phenolic compounds.

When prevailing environmental conditions are nitrogen-limited, but not light-limited, plants can turn excess photosynthate (i.e., fixed carbon) into phenolics or other nitrogen-free compounds (Tuomi *et al.*, 1984) through condensation of carbohydrate intermediates (Arnold & Targett, 2002), helping to restore the Redfield C:N ratio and boosting internal defensive mechanisms (Amsler & Fairhead, 2006). However, if plants are light-limited, and in effect, carbon-limited, sucrose synthesis diverts carbon resources to the most essential plant cells (Touchette & Burkholder, 2000a). Nutrient limitation may have influenced phenolic production by the shoots over the duration of the laboratory experiments due to the nutrients used by the macrophytes within the treatment tanks over time, however the % DW CA and TA mg^{-1} equivalents were within previously reported ranges for caffeic acid (Quackenbush *et al.*, 1986; Harrison & Durance, 1989; Buchsbaum *et al.*, 1990; Vergeer & Develi, 1997). As the % DW CA and TA mg^{-1} equivalents from the long-term field study were similar to those from the laboratory experiments, it is unlikely that nutrient limitation affected the total phenolics produced.

Results from nutrient partitioning analyses in 2008 (see Chapter 4) indicated that the shoots within the experimental tanks were not nitrogen-limited even in suboptimal conditions. If the shoots had been nitrogen-limited, the C:N ratio within the *Z. marina* shoots would have been higher than was found (Fig. 3.4). There is some indication of nitrogen limitation within the blades of the ZZ treatment from the 2008 experiment, but due to the low sample size, these results must be viewed with caution. As *Z. marina* has the capacity to rapidly

respond to changing ambient nutrient conditions, changes in the phenolics content present within the blade tissue may vary greatly over time (Buchsbaum *et al.*, 1990).

3.4.3 Weakened defences

Labyrinthula zosterae, a slime-mould protist (Vergeer & den Hartog, 1994) and biological agent responsible for wasting disease outbreaks within *Z. marina* beds, remains omnipresent in seagrass meadows (Short *et al.*, 1988; Vergeer & den Hartog, 1994; Bull *et al.*, 2011). Although it is still unclear what the exact trigger mechanism was for the 1930s epidemic that killed 90 % of the temperate seagrasses worldwide (Short *et al.*, 1988), some link it to warmer-than-average sea surface temperatures (Short *et al.*, 1988). Others have reported that elevated salinities may influence the onset and severity of an outbreak of wasting disease (Muehlstein *et al.*, 1991; Short & Neckles, 1999; McKone & Tanner, 2009). In light of these two possible causative mechanisms for the proliferation and spread of *L. zosterae*, a notable result from the 2008 experiment was the interaction between the density, growth, chemistry of *Z. marina* and *L. zosterae* infection prevalence. In 2009, the experiment was ended after three weeks due to the emergence of *L. zosterae* infection evident within all treatment tanks. The experiment in 2010 did not suffer from *L. zosterae* infection.

Agostini *et al.* (1998) found that seagrass meadows with high densities (~500 shoots m^{-2}) have the highest phenolic compound concentrations. In 2008, much higher levels of % DW CA and TA mg^{-1} equivalents were produced in the ZZ treatment, which had the highest *Z. marina* density (Fig. 3.3). Although *L. zosterae's* presence induces increased phenolic production within the leaves of *Z. marina*, including a sharp rise in % DW CA mg^{-1} equivalent content within the blade tissue as was also found in previous studies by Vergeer *et al.* (1995) and Vergeer & Develi (1997), increased conspecific densities may also be a factor (Bull *et al.*, 2011). This could indicate that higher density seagrass beds have the potential for reduced growth due to increased phenolic production and higher *L. zosterae* transmission between the closer proximity shoots. From personal observations,

it appeared that increasing *Z. marina* densities in higher than normal temperatures (in 2008) may have increased *L. zosterae* transmission within tanks as the ZZ treatment tanks appeared to have higher wasting disease infection and subsequent shoot die-off than the ZS and ZM treatments. As some ZZ treatment shoots were severely infected with *L. zosterae*, the upregulation of TA, and possibly CA, production recorded for this treatment may have been a physiological response to wasting disease prevalence rather than in its natural state as the ZS and ZM treatments had lower, but similar % DW TA mg^{-1} equivalent content (Fig. 3.3).

Although statistical analysis of the 2008 data indicated no evidence of increased phenolic production within the blade tissues of *Z. marina*, it is difficult to know with certainty if this is a real result due to the prevalence of *L. zosterae*, as Vergeer *et al.* (1995) found that biosynthesis of phenolics dramatically increases within diseased shoots. Short *et al.* (1988) noted that in plants that are severely infected, even the young leaves developed black necrotic lesions typical of a wasting disease outbreak. This occurred in all treatment tanks during the latter half of the experiment in 2008, possibly as a result of thermal stress and/or full oceanic salinity. To try and manage the outbreak, blades were trimmed below any necrotic areas to potentially reduce the prevalence of the wasting disease within the tank to prevent further spread. Although trimming the blades in this manner may have led to changes in phenolic content within the remaining tissue, the trade-off for not doing so (i.e., the potential for entirely diseased or dead shoots), was necessary. With a number of possible triggers for wasting disease outbreaks, the delicate balance needed to maintain healthy seagrass ecosystems may be lost as sea temperatures continue to rise (Short & Neckles, 1999; Harley *et al.*, 2006; Waycott *et al.*, 2009).

3.5 Conclusion

This chapter developed a framework to analyse how *S. muticum* invasion into *Z. marina* meadows changes the seagrasses biochemistry, specifically the phenolic compounds of caffeic and tannic acids, when the invasive alga is in close proximity to the native seagrass.

The results from this long-term field study and multiple laboratory experiments indicate that this invasion is not benign as the production of phenolics is suppressed in the presence of *S. muticum* in both the field and laboratory experiments. Shading due to the presence of *S. muticum* may therefore indirectly lead to unforeseen consequences (den Hartog, 1997; Arnold & Targett, 2002) such as weakening the defensive barrier of *Z. marina* to wasting disease with increasing SSTs (Short *et al.*, 1988) as phenols form the foundation of their protection (Buchsbaum *et al.*, 1990; Arnold & Targett, 2002). Weakened plant defences may ultimately aid in the facilitation and spread of invasive species if *Z. marina* is lost due to an increase in the spread of wasting disease.

Effects of nutrient imbalances can be numerous and may be expressed through changes in the production of phenolic compounds (Dumay *et al.*, 2004; Amsler & Fairhead, 2006). Results from the short-term nutrient limitation indicated the uptake of nutrients over time within each treatment, but it is unlikely that differences in nutrient concentrations occurred between treatments. This suggests that phenolic compound synthesis within the *Z. marina* shoots was possible and not as a result of nutrient imbalances.

Chapter 4

Invasion Impacts: Seagrass Physiology

Abstract

Abiotic factors such as light, temperature, water quality and inorganic nutrient availability and biotic factors that include disease, invasive species and herbivory, can influence plant physiology, metabolism and subsequent growth. Multiple laboratory experiments were conducted over successive years to determine how *S. muticum* affects the photosynthesis, growth and nutrient partitioning within the tissues of *Z. marina*. The presence of *S. muticum* significantly affected the chlorophyll fluorescence output measured by F_v/F_m ($p = 0.008$), but this was due to a highly significant difference in 2008 only ($p < 0.001$). Growth measurements indicated no significant differences between treatments. Results from analyses of the carbon, hydrogen and nitrogen contents within various tissue types (root-rhizome, sheath and blade) indicated significant differences between treatments and between all tissue types in the case of hydrogen and nitrogen, but not for carbon, which showed no treatment effect. Results for PO_4 μg/L P and SiO_2 μg/L Si were highly variable and no significant differences were found. Although the presence of *S. muticum* can alter the biochemistry and nutrient partitioning within the various tissue types of *Z. marina*, results from the improved 2009–2010 experiments (i.e., increased irradiance and lower seawater temperatures) suggest that the physiology of *Z. marina*, in terms of its fluorescence and growth, is not adversely affected by the invasive alga in a laboratory environment.

4.1 Introduction

Abiotic factors such as photoperiod (Dennison & Alberte, 1985), irradiance levels (Markager & Sand-Jensen, 1994; Sand-Jensen *et al.*, 2007; Thom *et al.*, 2008), temperature (Marsh Jr. *et al.*, 1986), salinity (McKone & Tanner, 2009) and nutrient imbalances

(Tuomi *et al.*, 1984; Howarth, 1988) can induce stress in plants and algae (Short & Wyllie-Echeverria, 1996; Lambers *et al.*, 1998), leading to weakened plant defences (Vergeer *et al.*, 1995), and may ultimately aid in the facilitation and spread of invasive species (Ruiz *et al.*, 2000; McGlathery, 2001). The effects of suboptimal abiotic factors and ambient conditions can be numerous and may be expressed in various ways. Manifestations of such imbalances include decreased growth (Lee *et al.*, 2005) and increased flowering in *Z. marina* due to temperature stress (Setchell, 1929; de Cock, 1981; Short & Neckles, 1999), growth deficiencies and abnormalities due to nutrient (i.e., Redfield ratio) imbalances (Burkholder *et al.*, 1992; Gillanders & Kingsford, 2002; Uku *et al.*, 2005) and even premature death or reduced growth due to lack of light penetration (Sand-Jensen, 1977; Short & Burdick, 1996; Short & Neckles, 1999) and/or increased UV-B radiation (Larkum & Wood, 1993; Short & Neckles, 1999).

Light intensity and the duration of the daily light photoperiod at which the light equals or exceeds the photosynthetic light saturation point (H_{sat}) are two critical factors governing marine macrophyte distribution (Touchette & Burkholder, 2000a). *Z. marina* becomes light-saturated at ~100 μmol m^{-2} s^{-1} (equivalent to 4.32 mol quanta m^{-2} d^{-1}) (Dennison & Alberte, 1985; Dennison, 1987; Jaschinski & Sommer, 2008), but this value can vary widely (~55–400+ μmol m^{-2} s^{-1}) depending upon location, depth and water quality (Dennison & Alberte, 1982; Marsh Jr. *et al.*, 1986; Moore & Wetzel, 2000; Lee *et al.*, 2007; Thom *et al.*, 2008). To maintain a positive carbon balance and vigorous growth, *Z. marina* requires 5–6 hours of light-saturated photosynthesis per day (Alcoverro *et al.*, 1999), but this too can vary by location (Touchette & Burkholder, 2000a). To attain its required light levels, *Z. marina*, and seagrasses in general, have evolved morphological characteristics that maximise light absorption, while reducing light scattering. These include: i) chloroplast localisation almost exclusively within the epidermis and a reduced mesophyll layer (Mazzella *et al.*, 1981), ii) lacunae (i.e., internal gas spaces) that buoy the leaves, ensuring an upright blade arrangement that reduces self-shading (Dennison & Alberte, 1982), and iii) a lack of stomata as CO_2 and/or HCO_3^- can be directly absorbed

through the epidermal cells (Beer *et al.*, 1977; Beer & Rehnberg, 1997; Hellblom *et al.*, 2001; Beer *et al.*, 2002). With ~80 % less photosynthetic capacity than other marine macrophytes such as *Ulva lactuca* Linnaeus and *Macrocystis integrifolia* Bory de Saint-Vincent (Zimmerman *et al.*, 1995), modest changes in lighting conditions can affect the survival of *Z. marina* due to its high irradiance (Hemminga & Duarte, 2000) and photoperiod requirements (Touchette & Burkholder, 2000a). Therefore, habitat generalists such as the invasive *S. muticum* with much lower irradiance requirements (Hales & Fletcher, 1989), may be at a competitive advantage under low light conditions (den Hartog, 1997; Britton-Simmons, 2004).

With a maximum growth rate of just 1–2 cm d^{-1}, even with the above physiological and morphological features in its favour, *Z. marina* is unable to reach the 1–4 cm d^{-1} growth rate measured in *S. muticum* (Jephson & Gray, 1976), making it susceptible to over-shading by the much larger and faster growing invasive alga (den Hartog, 1997; Britton-Simmons, 2004). Coupled with inefficient light absorption by photosynthetic pigments in seagrasses when compared to microalgae and most macroalgae (Hemminga & Duarte, 2000), the photosynthetic capacity of *Z. marina* may be put at a further disadvantage and may suffer greatly with continued unchecked proliferation of this invasive alga as found by den Hartog (1997) in Roscoff, France.

HCO_3^- is the most abundant form of inorganic carbon (C_i) in the oceans, with concentrations 150 times greater than dissolved CO_2 and six times greater than CO_3^{2-} at pH 8.2 and 15 °C (Beer & Koch, 1996; Beer & Rehnberg, 1997). Seagrasses have the ability to utilise inorganic carbon from HCO_3^- and dissolved CO_2 to cope with the 104 times slower diffusivity of CO_2 in water compared to in air (Lewey & Gorham, 1984; Hemminga & Duarte, 2000). Previous research found ${HCO_3}^-$ to be the main source of C_i for seagrasses (Beer *et al.*, 1977; Beer, 1989) through the conversion of HCO_3^- to dissolved CO_2 at the external cell membrane surface within the diffusion boundary layer of the leaves, a process catalysed by carbonic anhydrase at normal seawater pH (Beer & Rehnberg, 1997; Beer

et al., 2002). Recent studies have also indicated that seagrasses such as *Z. marina* can take up HCO_3^- directly via H^+-driven exudation (Hellblom & Bjork, 1999; Beer *et al.*, 2002), but seagrasses are much less efficient at C_i uptake than many algae and are perhaps C_i limited in terms of photosynthesis (Beer *et al.*, 1977; Beer & Rehnberg, 1997). Macroalgae, conversely, are not C_i limited (Lewey & Gorham, 1984; Beer & Rehnberg, 1997) and therefore may be able to utilise additional nutrients (N and P) directly from seawater under eutrophic conditions (Beer & Koch, 1996). However, most marine macroalgae already range from 80–100 % C_i saturated in current oceanic pH conditions (Beer & Koch, 1996; Beardall *et al.*, 1998), therefore little to no increase in photosynthetic yields or growth rates are expected to occur as dissolved CO_2 concentrations continue to rise (Beer & Koch, 1996). In addition, species of macroalgae such as *S. muticum* may become C_i limited in deeper water as photosynthetic capacity decreases because, unlike seagrasses, there are no root structures to uptake additional nutrients from the sediment (Beardall *et al.*, 1998) in these light-limited environments.

Seagrasses, with nutrient requirements 50–100 times lower than phytoplankton and 1.5–1.8 times lower than macroalgae, have the capacity to sequester considerable amounts of available nutrients within their rhizomes (Duarte & Cebrian, 1996), preventing uptake by other primary producers (Short & Wyllie-Echeverria, 1996; Hemminga & Duarte, 2000). This ability may enable seagrasses to potentially out-compete other marine primary producers in nutrient-poor environments (Hemminga & Duarte, 2000). With a Redfield ratio of 435:20:1 (C:N:P), *Z. marina* is a low-quality food source for many organisms as its blades are carbon-rich (Hemminga *et al.*, 1991; Hemminga & Duarte, 2000), which leads to a lower biomass turnover due to reduced grazing pressure and a subsequent increase in standing biomass. Macroalgae, conversely, have limited capacity for long-term nutrient storage. According to Valiela *et al.* (1997), however, increases in nitrogen raise macroalgal nitrogen uptake and elevate nitrogen concentrations within macroalgal tissues, resulting in increased frond production (Morand & Merceron, 2005). Despite increased nitrogen uptake, algal tissues break down more quickly upon senescence and death, leading to a

rapid return of accumulated nutrients to the water column (Morand & Merceron, 2005), potentially altering nutrient concentrations further by creating high concentrations of ammonia, which can be toxic to the leaves of *Z. marina* (Hauxwell *et al.*, 2001).

Under the right conditions, abiotic factors may further facilitate the spread of *S. muticum* outside of its native range, leading to increased competition for resources between *Z. marina* and *S. muticum*. Therefore, multiple laboratory experiments were conducted in a controlled environment to determine how the presence of *S. muticum* affects the photosynthesis (chlorophyll fluorescence output (F_v/F_m)), growth (total production shoot^{-1} tank^{-1} d^{-1}) and nutrient (C, H, N, P and Si) partitioning and allocation within the various tissue types (root-rhizome, leaf sheath and blade) of *Z. marina*.

4.2 Methods

4.2.1 Experimental design

To investigate the nutrient and physiological responses of *Z. marina* to the presence of *S. muticum*, four, three to four week laboratory experiments using wild-harvested *Z. marina* were undertaken in 2008, 2009, 2010 and 2011 in a CT room using the laboratory experimental design previously described (see Chapter 3). The *Z. marina* shoots harvested had approximately the same number of blades per shoot. Three treatments (ZS, ZM and ZZ) were employed in 2008 and 2009, two treatments in 2010 (ZS and ZM) and four treatments in 2011 (ZS, ZM, ZZ and C (seawater-only control)).

4.2.2 Chlorophyll fluorescence

In 2008, shoots were held at 15±3 °C in a CT room and the PPF was ~55–60 μmol m^{-2} s^{-1} on a 16L:8D cycle (equivalent to 3.17-3.46 quanta mol m^{-2} d^{-1}). In 2009 and 2010, infrastructural improvements allowed the PPF to be increased to ~95–110 μmol m^{-2} s^{-1} (equivalent to 4.1-4.32 quanta mol m^{-2} d^{-1}); the duration of irradiance was shorted to

12L:12D and the experimental temperature was lowered to 10±1 °C as the experiments occurred earlier in the spring than in 2008. To determine the maximum photochemical efficiency of the PSII apparatus in dark-adapted seagrass blades, (F_v/F_m) measurements were recorded over a 5 sec period at 100 % light intensity on five dates (T = 0, and weekly thereafter) throughout the experiment using a MK2 Plant Efficiency Analyser (PEA meter) (Hansatech Instruments Ltd, King's Lynn). Once the tanks had been drained for a water change, five randomly selected green blades per tank were dark adapted using leaf clips for at least 15 min before readings were taken; readings were recorded on each of five dates in 2008 and 2010 and four dates in 2009. The mean F_v/F_m for each tank at each date was used for statistical analysis to avoid pseudo-replication.

4.2.3 Growth

All blades in five individual shoots were punctured with a fine needle at the blade-sheath interface (Tomasko *et al.*, 1996; Westera & Lavery, 2006) at the start of the experiment and again after ~14 days. Growth was measured as the distance the hole had grown away from the interface. Length measurements between the top of the sheath and the puncture holes were taken on two dates, once at approximately two weeks after the start of the experiment and again at the end of the experiment, approximately two weeks later in 2008 and one week later in 2009. In 2010, growth was measured on three separate occasions using the same puncture method; once at the end of the first week, again at the end of the second week and final measurements were taken at the end of the fourth week when the experiment concluded. Growth data from each shoot were summed to produce the total production per shoot; data from the five shoots measured in each tank were then averaged to give the mean total production shoot^{-1} tank^{-1}. The mean total production shoot^{-1} tank^{-1} d^{-1} was calculated by dividing the total production shoot^{-1} tank^{-1} by the number of days from the initial hole punch. The 2011 nutrient limitation experiment (see Chapter 3) did not test for the growth of *Z. marina*.

4.2.4 *Z. marina* tissue nutrients

Three tissue types (root-rhizome, sheath and blade) within *Z. marina* shoots harvested at the end of the 2008 laboratory experiment were analysed separately for carbon, hydrogen, nitrogen, phosphate and silicate content. These analyses were carried out to determine nutrient partitioning and allocation within each tissue type.

Carbon, hydrogen and nitrogen

Tissue samples were dried at 65 °C for 24 hours and ground to a fine powder using a mortar and pestle. Approximately 2 mg of ground tissue was used to determine % DW content of carbon, hydrogen and nitrogen (C-H-N) for each tissue type (in duplicate) using an EA1110 Elemental Analyser (CE Instruments, Wigan). Sample results were compared against the cyclohexanone-2,4-dinitrophenylhydrazone (CYC) standard and the National Institute of Standards (NIST) peat soil standard, in which exact quantities of carbon, hydrogen, nitrogen and sulphur were known. The EA1110 Elemental Analyser was calibrated every 20 samples using the CYC and NIST standards to prevent sample drift from occurring. Results from the C-H-N analysis were then used to determine if shoots were nitrogren limited, as the % DW nitrogen content of each blade was graphed against the C:N ratios from the same samples (Buchsbaum *et al.*, 1990).

Phosphates and silicates

Tissue samples were dried at 65 °C for 24 hours, ground using a mortar and pestle and ashed at 550 °C for 6 hours in a muffle furnace (Carbolite Ltd, London). 20 mg of ashed tissue was digested in 20 mL of 1 M HCl for 24 hours and filtered through a 25 μm filter (Fisher Scientific, Loughborough). 15 ml of filtered sample was used to determine the % DW content of total dissolved phosphates (TDP) and silicates for each tissue type (in triplicate) using a Skalar SAN Plus Flow Analyser (SA 4000) (Skalar UK Ltd., York). Every 20–30 samples, a tracer (a standard at the highest concentration of pure P and Si) and a drift (a standard of mid-range concentration of pure P and Si) were inserted into the

manifold to maintain the analyser's calibration throughout the run. Sample results were compared against standard dilution series of pure stock solution P and Si, respectively.

4.2.5 Data analysis

Chlorophyll fluorescence

Effects of experimental 'treatments', 'years' and 'dates' sampled on chlorophyll fluorescence data (F_v/F_m) were analysed in SPSS 19 using repeated measures type III Sums of Squares GLMs. Analysis of the 2008 data used 'treatment' with three levels (ZS, ZM and ZZ) for the single fixed-factor repeated measures GLM, with 'date' set as the within-subject factor with five levels, representing the five sample dates (T = 0, 1, 2, 3 and 4). The combined 2008 and 2009 analysis utilised a two-way design where both 'year' with two (2008 and 2009) and 'treatment' with three (ZS, ZM and ZZ) levels respectively were fixed factors; 'date' was designated the within-subject factor with four levels (T = 0, 1, 2, and 3). A final repeated measures GLM was calculated for the combined 2008–2010 data; this used the same two-factor design as described above, but 'treatment' only had two (ZS and ZM) and 'date' four (T = 0, 1, 2, and 3) levels respectively. Where differences in the 'date' level occurred between 'years' due to experiment duration differences, only four weekly corresponding measurements from each experiment were used in analyses. Box's Test of Equality of Covariance Matrices was employed to ensure that the observed covariance matrices of the dependent variables were consistent across groups prior to analyses using type III Sums of Squares. Mauchly's Test of Sphericity was also used to ensure that analytical assumptions were met, and where indicated, corrected degrees of freedom were employed using the Greenhouse-Geisser correction (Field, 2009). Conformity of data within each time class to assumptions of homogeneity of variances were confirmed using Levene's Test. Planned pairwise comparisons were used to identify significant differences between the ZS, ZM and ZZ treatments ($p < 0.05$) using Tukey's LSD based upon estimated marginal means.

Growth

Effects of experimental 'treatments', 'years' and 'dates' sampled on growth data (total production shoot^{-1} tank^{-1} d^{-1} (in mm)) were analysed in SPSS 19 using repeated measures GLMs with type III Sums of Squares and tests for conformity to analytical assumptions (as described in section 4.2.5: Chlorophyll fluorescence). To avoid pseudo-replication, multiple data per tank were amalgamated and a single datum per tank was used as the replicate. Analysis of the 2008 data used 'treatment' with three levels (ZS, ZM and ZZ) for the single fixed-factor repeated measures GLM, with 'date' set as the within-subject factor with two levels (mid and end experiment). Planned pairwise comparisons were used to identify significant differences between 'treatments' ($p < 0.05$) using Tukey's LSD based upon estimated marginal means. The combined 2008 and 2009 analysis used a two-way design where both 'year' with two (2008 and 2009) and 'treatment' with three (ZS, ZM and ZZ) levels respectively were fixed factors. 'Date' was set as the within-subject factor with two levels (mid and end experiment). S-N-K *post hoc* tests were performed for 'year' and planned pairwise comparisons were used to identify significant differences between 'treatments' ($p < 0.05$) using Tukey's LSD based upon estimated marginal means. In 2010, growth was measured three times, therefore a separate repeated measures GLM was calculated using the same one-factor design as described above. A final repeated measures GLM with 'date' set as the within-subject factor with two levels (mid and end experiment) was calculated for the combined 2008–2010 data using the same one-factor design as described above, but with only two 'treatment' levels (ZS and ZM).

Seagrass nutrients

Effects of 'treatment' and 'tissue' type on C-H-N data were analysed using type III Sums of Squares univariate GLMs in SPSS 19; data were tested using a two-factor design, with 'treatment' with three (ZS, ZM, and ZZ) and 'tissue' types with three (root-rhizome, sheath and blade) levels respectively as fixed factors. As reproductive tissue was not equally produced amongst treatments and perhaps was a stress response, reproductive

tissue was not included in the analysis. Non-homogeneity of variances was indicated by results of Levene's Test for carbon and hydrogen data; examination of unstandardised analytical residuals indicated need for a power transformation (X^2); Equality of variances was improved by transformation, but remained marginal. Therefore, to confirm the univariate results, non-parametric analysis was also carried out using PRIMER v6.1.13 with PERMANOVA+ v1.0.3. Data were tested individually using two fixed-factors in PERMANOVA+, with 'treatment' with three (ZS, ZM, and ZZ) and 'tissue' type with three (root-rhizome, sheath and blade) levels respectively and a Euclidean distance resemblance matrix with type III Sums of Squares, unrestricted permutation of raw data and 9999 permutations (Anderson *et al.*, 2008). An overall PERMANOVA+ for the combined normalised C-H-N data was also conducted, where 'treatment' with three (ZS, ZM and ZZ) and 'tissue' with three (root-rhizome, sheath and blade) levels respectively were set as factors. Pairwise tests of the factor 'treatment' within the 'treatment * tissue' interaction term were calculated for all analyses.

Due to issues of inequality of variances which could not be improved by transformation, PO_4 and SiO_2 data were analysed in PRIMER v6.1.13 with PERMANOVA+ v1.0.3. Data were tested individually using two fixed-factors in PERMANOVA+, with 'treatment' with three (ZS, ZM, and ZZ) and 'tissue' type with three (root-rhizome, sheath and blade) levels respectively and a Euclidean distance resemblance matrix with type III Sums of Squares, unrestricted permutation of raw data and 9999 permutations. An overall PERMANOVA+ for the combined normalised phosphate and silicate data was also conducted, where 'treatment' with three (ZS, ZM and ZZ) and 'tissue' with three (root-rhizome, sheath and blade) levels respectively were set as fixed-factors.

To complete the analytical hierarchy for all within-tissue nutrients, the combined normalised nutrient data (% DW C, % DW H, % DW N, PO_4 μg/L and SiO_2 μg/L) were tested with a two-factor PERMANOVA+ as above.

4.3 Results

4.3.1 Chlorophyll fluorescence

Repeated measures GLM results of the within-subjects test indicated that the interaction between 'date' and 'treatment' in 2008 (three treatments (ZS, ZM and ZZ) and five sample dates) was significant (Greenhouse-Geisser correction for non-sphericity; F = 3.305, d.f. = 6.496, 87.695, p = 0.004). The main effect 'date' was not significant (p = 0.242); this interaction confounds interpretation of the 'treatment' main effect, but indicates significant heterogeneity in response of 'treatments' across time. Repeated measures GLM tests of within-subject effects for the combined 2008–09 data (three treatments and four sample dates) revealed a non-significant interaction between 'date' and 'treatment' (Greenhouse-Geisser correction for non-sphericity; F = 5.445, d.f. = 2.189, 147.021, p = 0.053). The main effect 'date' was significant ($p < 0.001$); analysis progressed to consider the accompanying univariate GLM output. Univariate GLM results indicated significant differences between 'treatments' and 'years' as well as a significant 'year * treatment' interaction (Table 4.1, Fig. 4.1). Pairwise comparisons for the factor 'treatment' within the interaction term 'year * treatment' indicated significant differences between the ZS and ZM treatments and the ZM and ZZ treatments in 2008, but no significant differences were found between 'treatments' in 2009 (Table 4.2).

The final repeated measures GLM for all three years (2008–10) with four sample dates (T = 0, 1, 2 and 3) and two treatments (ZS and ZM) revealed a significant effect of the 'date * treatment' interaction (Greenhouse-Geisser correction for non-sphericity; F = 4.265, d.f. = 2.701, 124.250, p = 0.009). The main effect 'date' was also significant (Table 4.3). These results indicate that chlorophyll fluorescence output responded differently through the experiment in different 'treatments' and 'years' (Fig. 4.2). Planned pairwise comparisons for the factor 'treatment' within the interaction term 'year * treatment' in the 2008–10 repeated measures GLM revealed significant differences in 2008 ($p < 0.001$); no 'treatment' effect was found in 2009 or 2010. This result nonetheless, needs to be viewed with caution due to the heterogeneous response of F_v/F_m found over time.

Table 4.1: Repeated measures GLM results for mean F_v/F_m from the combined 2008 and 2009 laboratory experiments with three treatments (ZS, ZM and ZZ). Tank was used as the replicate; n = 90 for each treatment over 2 years.

Source	Type III SS	df	MS	F	Sig. (p)
Intercept	137.232	1.000	137.232	78955.032	**< 0.001**
Year	0.032	1.000	0.032	18.610	**< 0.001**
Treatment	0.036	2.000	0.018	10.416	**< 0.001**
Year * Treatment	0.013	2.000	0.007	3.862	**0.027**
Error	0.094	54.000	0.002		

Table 4.2: Univariate GLM planned pairwise comparisons for 'treatment' within the interaction term 'year * treatment' for the combined 2008 and 2009 F_v/F_m data. Tank was used as the replicate; n = 90 for each treatment over 2 years.

Year	(I) Treatment	(J) Treatment	Mean Diff. (I-J)	Sig. (p)
2008	ZS	ZM	0.045	**< 0.001**
		ZZ	0.014	0.141
	ZM	ZZ	-0.031	**0.001**
2009	ZS	ZM	0.015	0.113
		ZZ	0.017	0.076
	ZM	ZZ	0.002	0.841

Table 4.3: Repeated measures GLM results for the mean F_v/F_m from the combined 2008–2010 laboratory experiments with two treatments (ZS and ZM) and four sample dates. Tank was used as the replicate; n = 120 for the ZS and ZM treatments, n = 90 for the ZZ treatment over 3 years.

Source	Type III SS	df	MS	F	Sig. (p)
Date	0.069	2.701	0.026	16.745	**< 0.001**
Date * Year	0.031	5.402	0.006	3.680	**0.003**
Date * Treatment	0.018	2.701	0.007	4.265	**0.009**
Date * Year * Treatment	0.016	5.402	0.003	1.971	0.082
Error(Date)	0.191	124.250	0.002		

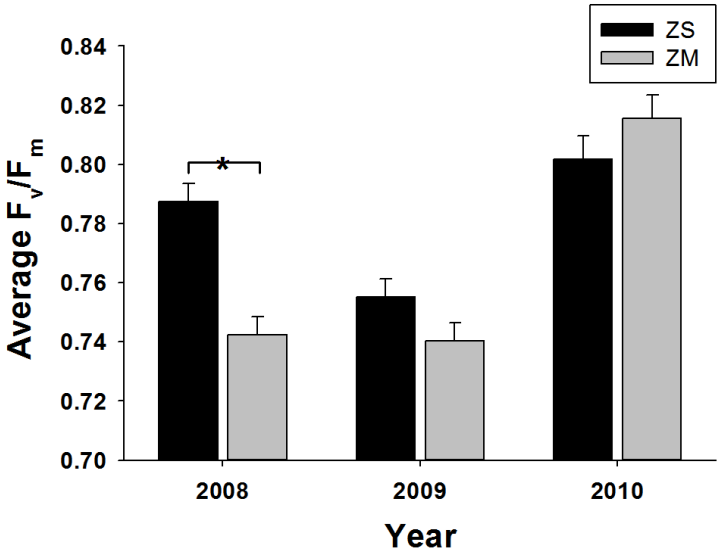
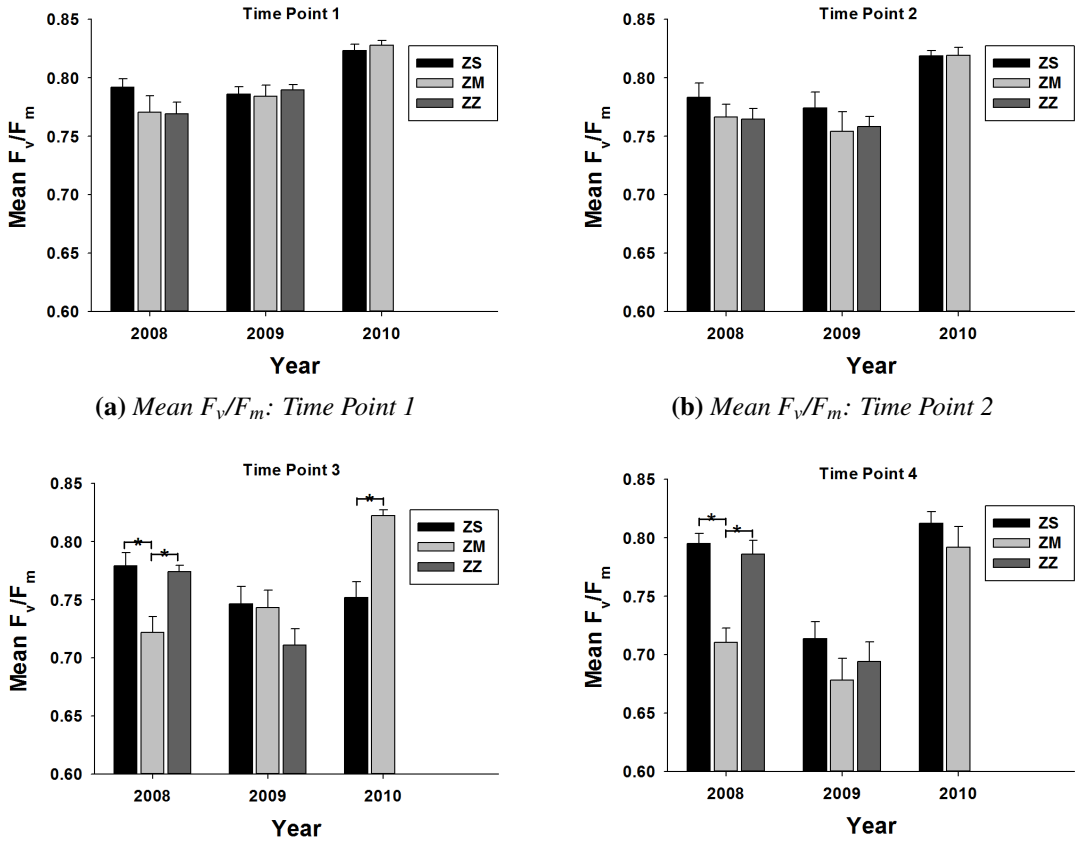


Figure 4.1: Mean (±SE) F_v/F_m from the combined 2008–2010 laboratory experiments for two treatments (ZS and ZM). * indicates significant difference ($p < 0.05$). Tank was used as the replicate; n = 120 for the ZS and ZM treatments, n = 90 for the ZZ treatment over 3 years.



(a) *Mean F_v/F_m: Time Point 1*

(b) *Mean F_v/F_m: Time Point 2*

(c) *Mean F_v/F_m: Time Point 3*

(d) *Mean F_v/F_m: Time Point 4*

Figure 4.2: Mean (±SE) F_v/F_m by treatment for all years at time points 1 (a), 2 (b), 3 (c), and 4 (d). * indicates significant difference ($p < 0.05$). Tank was used as the replicate; n = 120 for the ZS and ZM treatments, n = 90 for the ZZ treatment over 3 years.

4.3.2 Growth

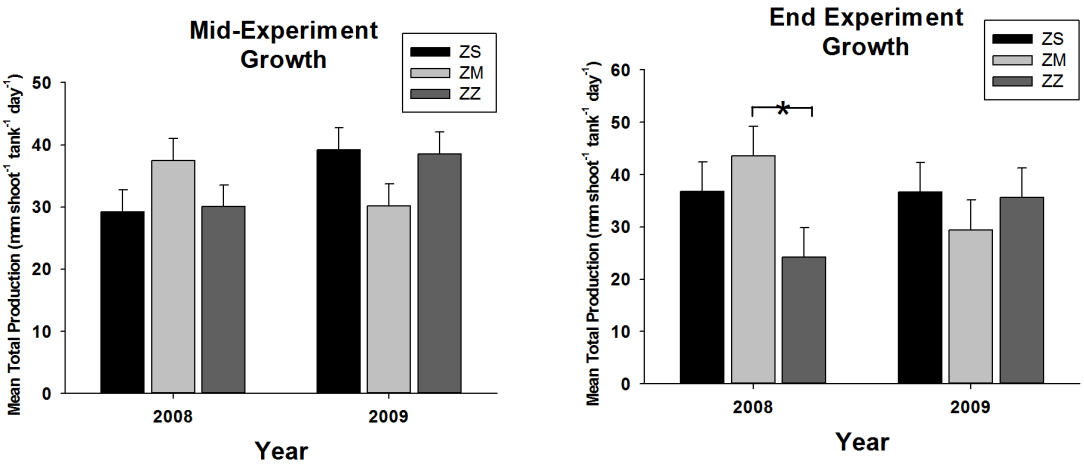
Repeated measures GLM tests of within-subject effects for the 2008 growth data (three treatments and two sample dates) revealed a non-significant interaction between 'date' and 'treatment' (Greenhouse-Geisser correction for non-sphericity; F = 2.422, d.f. = 2, 54, p = 0.108). The main effect 'date' was also not significant. Univariate GLM results indicated no significant differences between 'treatments'. Pairwise comparisons for the factor 'treatment' within the interaction term 'treatment * date' indicated significant differences between the ZM and ZZ treatments (Table 4.4).

Repeated measures GLM tests of within-subject effects for the combined 2008–09 growth data (three treatments and two sample dates) revealed a non-significant interaction between 'date' and 'treatment' (Greenhouse-Geisser correction for non-sphericity; F = 0.92, d.f. = 2, 54, p = 0.405). The main effect 'date' was also not significant. Univariate GLM results indicated no significant differences between 'treatments' or 'years', but a significant 'year * treatment' interaction was indicated (Table 4.5). Pairwise comparisons for the factor 'treatment' within the interaction term 'year * treatment' indicated significant differences between the ZM and ZZ treatments in 2008; no other significant differences were found (Table 4.6).

The repeated measures GLM for 2010 only with three sample dates and two treatments (ZS and ZM) revealed no significant differences for within-subject effects for the interaction term 'date * treatment' (Greenhouse-Geisser correction for non-sphericity; F = 0.369, d.f. = 1.684, 16.842, p = 0.662). The main effect 'date' was also non-significant. Univariate GLM results indicated no significant differences between 'treatments'.

The final repeated measures GLM for all three years (2008–10) with two sample dates and two treatments (ZS and ZM) revealed no significant effect of the 'date * treatment' interaction (Greenhouse-Geisser correction for non-sphericity; F = 0.346, d.f. = 1, 46, p = 0.549). The main effect 'date' was also not significant. Univariate GLM results indicated no significant differences between 'treatments' and no significant 'year * treatment' interaction

was revealed, but a significant difference between 'years' was found (Table 4.7). S-N-K *post hoc* tests for 'year' revealed that overall total production was significantly higher in 2010 than the previous two years (Fig. 4.4).



(a) *2008–09 mid-experiment growth* **(b)** *2008–09 end-experiment growth*

Figure 4.3: Mean (±SE) total production shoot^{-1} tank^{-1} d^{-1} (mm) for three treatments (2008 and 2009 data only). Data shown are mid-experiment growth measurements (a) and end of experiment growth (b). Significant pairwise differences ($p < 0.001$), indicated by *. Tank was used as the replicate; n = 20 for each treatment for both mid and end growth measurements for each year.

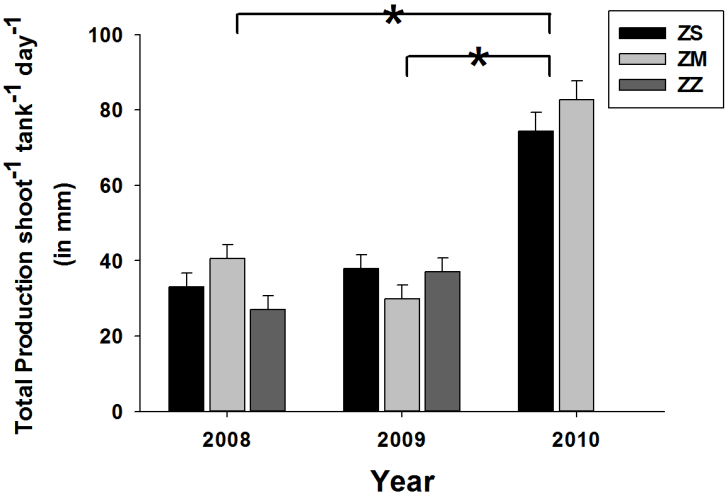


Figure 4.4: Mean (±SE) total production shoot^{-1} tank^{-1} d^{-1} (mm) for three treatments (ZS, ZM and ZZ) in 2008–09 and two treatments (ZS and ZM) in 2010 from 2008–2010 laboratory experiments. * indicates significant difference ($p < 0.05$). Tank was used as the replicate; n = 20 for each treatment in 2008 and 2009, n = 12 for each treatment in 2010.

Table 4.4: Univariate GLM planned pairwise comparisons for the factor 'treatment' within the interaction term 'treatment * date' for the 2008 total production shoot^{-1} tank^{-1} d^{-1} with three treatments (ZS, ZM and ZZ). Tank was used as the replicate; n = 30 for each treatment.

Year	(I) Treatment	(J) Treatment	Mean Diff. (I-J)	Sig. (p)
2008	ZS	ZM	-7.516	0.065
		ZZ	5.904	0.143
	ZM	ZZ	13.421	**0.002**

Table 4.5: Univariate GLM results for mean total production shoot^{-1} tank^{-1} d^{-1} from the 2008–2009 laboratory experiments with three treatments (ZS, ZM and ZZ) and two sample dates (mid and end). Tank was used as the replicate; n = 20 for each treatment for each year.

Source	Type III SS	df	MS	F	Sig. (p)
Intercept	140753.805	1.000	140753.805	526.296	**< 0.001**
Treatment	278.271	2.000	139.136	0.520	0.597
Year	58.363	1.000	58.363	0.218	0.642
Treatment * Year	2322.964	2.000	1161.482	4.343	**0.018**
Error	14441.877	54.000	267.442		

Table 4.6: Univariate GLM planned pairwise comparisons for 'treatment' within the interaction term 'year * treatment' for the 2008 and 2009 total production shoot^{-1} tank^{-1} d^{-1}. Tank was used as the replicate; n = 20 for each treatment for each year.

Year	(I) Treatment	(J) Treatment	Mean Diff. (I-J)	Sig. (p)
2008	ZS	ZM	-7.516	0.152
		ZZ	5.904	0.259
	ZM	ZZ	13.421	**0.012**
2009	ZS	ZM	8.082	0.124
		ZZ	0.820	0.875
	ZM	ZZ	-7.261	0.166

Table 4.7: Univariate GLM results for mean total production shoot^{-1} tank^{-1} d^{-1} from the 2008–2010 laboratory experiments with two treatments (ZS and ZM) and two sample dates (mid and end). Tank was used as the replicate; n = 20 for each treatment in 2008 and 2009, n = 12 for each treatment in 2010.

Source	Type III SS	df	MS	F	Sig. (p)
Intercept	242670.922	1.000	242670.922	778.670	**< 0.001**
Year	34591.433	2.000	17295.717	55.498	**< 0.001**
Treatment	164.828	1.000	164.828	0.529	0.471
Year * Treatment	1559.580	2.000	779.790	2.502	0.093
Error	14335.802	46.000	311.648		

4.3.3 Seagrass nutrients

Carbon, hydrogen and nitrogen

PERMANOVA+ results indicated significant differences between 'treatments' and within the various 'tissue' types for all % DW C, % DW H and % DW N analyses with the exception of % DW C; no 'treatment' effect was found. No significant 'treatment * tissue' interaction was found for % DW C, % DW H and % DW N (Tables 4.8, 4.9 and 4.10). Pairwise tests indicated that the significant differences were found between the ZM and ZZ treatments within the sheath 'tissue' for % DW C (p = 0.004) and % DW H (p = 0.007) and between the ZS and ZM treatment within the blade 'tissue' for % DW N (p = 0.012). PERMANOVA+ results for the combined C-H-N data revealed significant differences between 'treatments' and 'tissue' type, but no significant 'treatment * tissue' interaction was found (Table 4.11). Pairwise tests for the combined C-H-N data indicated the significant differences were found between the ZM and ZZ treatments within the sheath 'tissue' (p = 0.009).

Nitrogen content was variable across tissue types and treatments (0.60–2.71 % DW) with a median value of 1.84 % DW, but scaling the % DW N content against C:N ratios indicated most of the shoots were not nitrogen-limited as the majority of C:N ratios were lower than would be expected if nutrient limitation occurred within the tissue types (Fig. 4.9) (Duarte, 1990).

Phosphates and silicates

PERMANOVA+ results indicated no significant differences between 'treatments' and the various 'tissue' types and no significant 'treatment * tissue' interaction was revealed for the individual and combined Skalar PO_4 μg/L P and SiO_2 μg/L Si data (Fig. 4.10). Due to the extreme variability scaling the % DW phosphorus content against C:P ratios was not possible.

Combined nutrients

PERMANOVA+ results for the combined nutrients (% DW C, % DW H, % DW N, PO_4 μg/L P and SiO_2 μg/L Si) indicated significant differences between 'tissue' type. No significant differences between 'treatments' and no significant 'treatment * tissue' interaction were revealed (Table 4.12, Figs. 4.5, 4.6 and 4.7).

Table 4.8: PERMANOVA+ results for the mean % DW carbon within *Z. marina* tissues from the 2008 laboratory experiment. Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	2	18.177	9.089	2.208	0.125
Tissue	2	365.5	182.75	44.401	**< 0.001**
Treatment * Tissue	4	7.826	1.956	0.475	0.761
Res	63	259.3	4.116		
Total	71	912.89			

Table 4.9: PERMANOVA+ results for the mean % DW hydrogen within *Z. marina* tissues from the 2008 laboratory experiment. Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	2	0.723	0.362	3.378	**0.039**
Tissue	2	5.158	2.579	24.08	**< 0.001**
Treatment * Tissue	4	0.21553	5.3882E-2	0.503	0.743
Res	63	6.747	0.107		
Total	71	16.952			

Table 4.10: PERMANOVA+ results for the mean % DW nitrogen within *Z. marina* tissues from the 2008 laboratory experiment. Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	2	0.356	0.178	3.239	**0.033**
Tissue	2	14.626	7.313	133.24	**< 0.001**
Treatment * Tissue	4	0.362	9.0382E-2	1.647	0.166
Res	63	3.458	5.4889E-2		
Total	71	23.126			

Table 4.11: PERMANOVA+ results for the combined C-H-N data (% DW C, % DW H and % DW N) from the 2008 laboratory experiment. Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment for C, H, and N.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	2	5.535	2.768	2.953	**0.038**
Tissue	2	94.933	47.466	50.649	**< 0.001**
Treatment * Tissue	4	2.621	0.65532	0.699	0.636
Res	63	59.041	0.937		
Total	71	213			

Table 4.12: PERMANOVA+ results for the combined seagrass nutrient data (% DW C, % DW H, % DW N, PO_4 μg/L P and SiO_2 μg/L Si) from the 2008 laboratory experiment. Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment for C, H, and N and n = 26 for the ZS and ZM treatments, n = 21 for the ZZ treatment for both PO_4 μg/L P and SiO_2 μg/L Si.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	2	10.382	5.191	1.784	0.12
Tissue	2	102.74	51.369	17.649	**< 0.001**
Treatment * Tissue	4	6.024	1.506	0.517	0.868
Res	63	183.36	2.911		
Total	71	355			

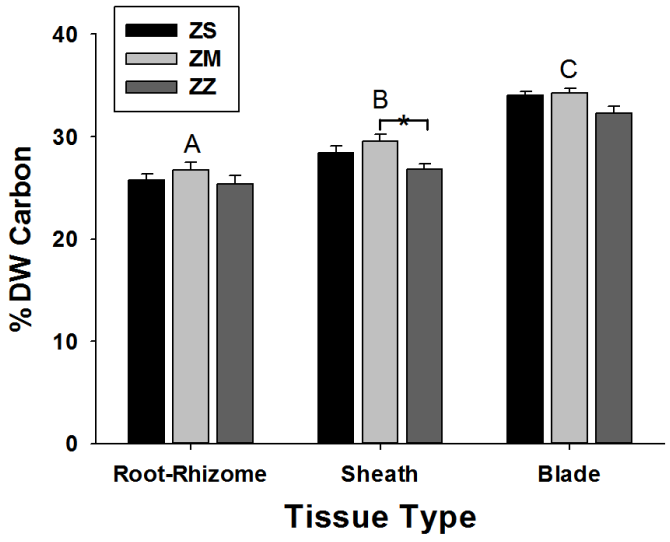


Figure 4.5: Mean (±SE) % DW carbon from the 2008 laboratory experiment for three treatments (ZS, ZM and ZZ) and three *Z. marina* tissue types (root-rhizome, sheath and blade). * indicates significant difference ($p < 0.05$); A, B, C indicates significant differences between tissue types ($p < 0.05$). Tank was used as the replicate; n = 10 for each treatment for root-rhizome and sheath tissue7, n = 7 for the ZS and ZM blades and n = 4 for the ZZ blades.

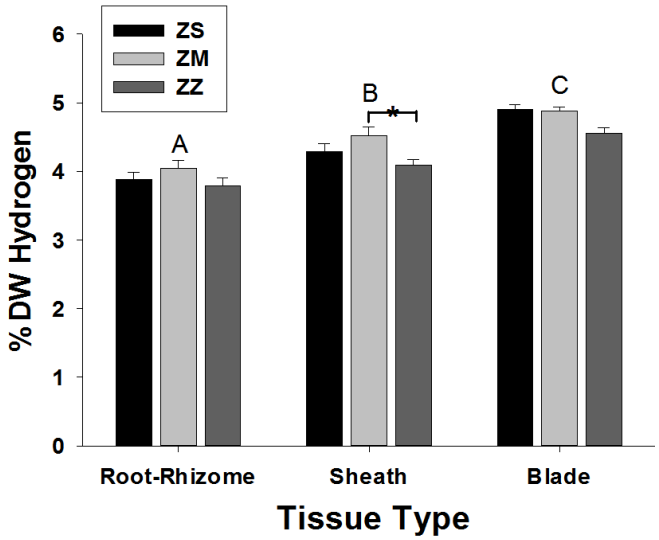


Figure 4.6: Mean (±SE) % DW hydrogen from 2008 laboratory experiment for three treatments (ZS, ZM and ZZ) and three *Z. marina* tissue types (root-rhizome, sheath and blade). * indicates significant difference ($p < 0.05$); A, B, C indicates significant differences between tissue types ($p < 0.05$). Tank was used as the replicate; n = 10 for each treatment for root-rhizome and sheath tissue7, n = 7 for the ZS and ZM blades and n = 4 for the ZZ blades.

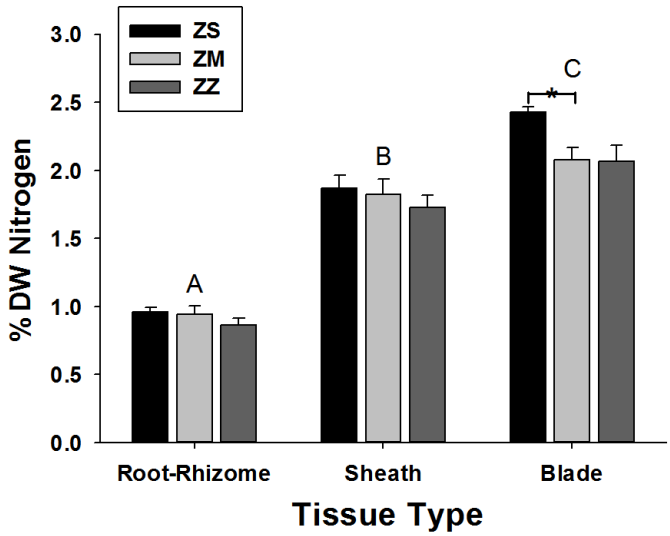
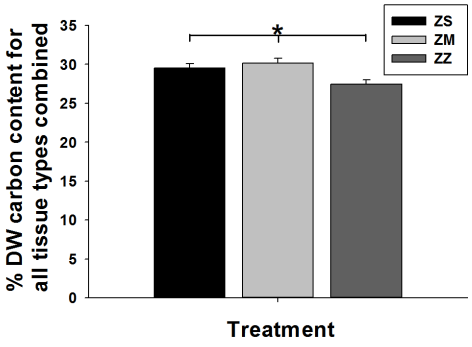
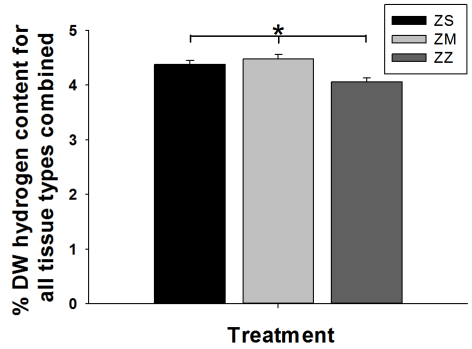


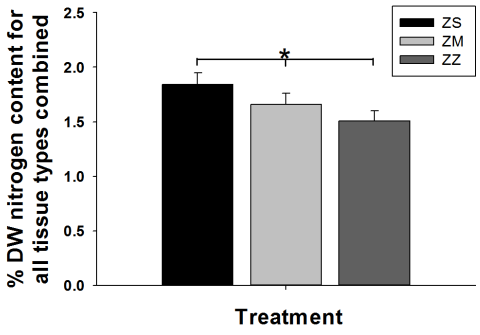
Figure 4.7: Mean (±SE) % DW nitrogen from 2008 laboratory experiment for three treatments (ZS, ZM and ZZ) and three *Z. marina* tissue types (root-rhizome, sheath and blade). * indicates significant difference ($p < 0.05$); A, B, C indicates significant differences between tissue types ($p < 0.05$). Tank was used as the replicate; n = 10 for each treatment for root-rhizome and sheath tissue7, n = 7 for the ZS and ZM blades and n = 4 for the ZZ blades.



(a) *Mean (±SE) % DW carbon*



(b) *Mean (±SE) % DW hydrogen*



(c) *Mean (±SE) % DW nitrogen*

Figure 4.8: Mean (±SE) % DW carbon (a), hydrogen (b), and nitrogen (c) averaged across all tissue types by treatment from the 2008 laboratory experiment. * indicates significant difference ($p < 0.05$). Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment for C, H, and N.

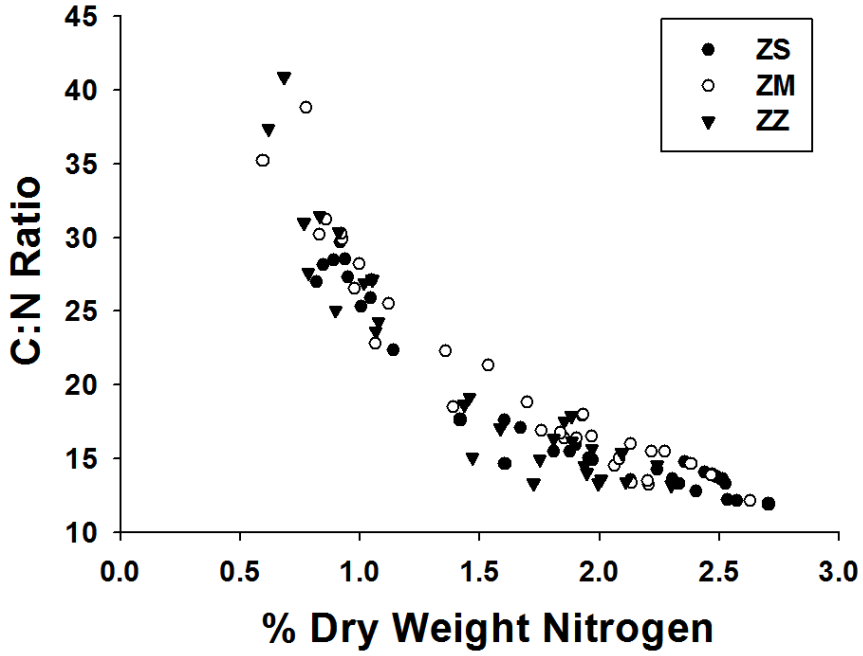


Figure 4.9: % DW nitrogen in three tissue types (root-rhizome, sheath and blade) for each treatment (ZS, ZM and ZZ) vs. % DW carbon-nitrogen ratios. Nitrogen content was variable across tissue types and treatments in all treatments. Tank was used as the replicate; n = 27 for the ZS and ZM treatments, n = 24 for the ZZ treatment.

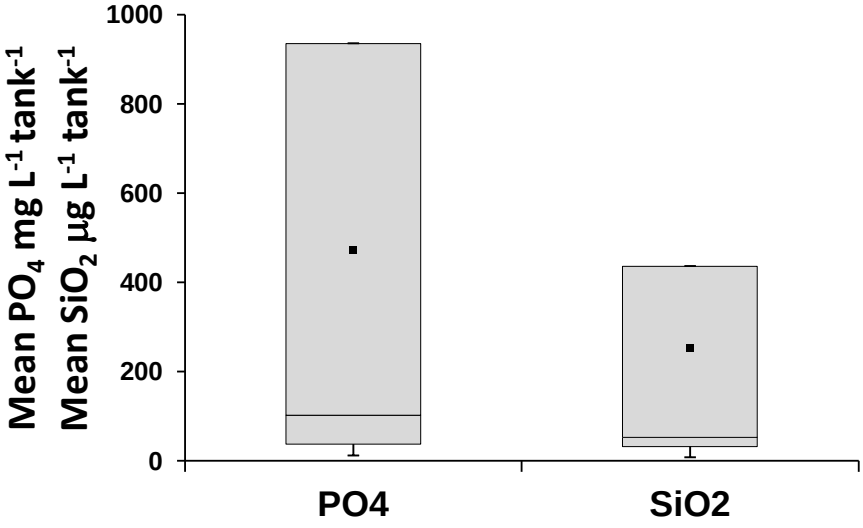


Figure 4.10: Skalar results for PO_4 μg L^{-1} P and SiO_2 μg L^{-1} Si. Data presented are the median, first and third quartiles. The black square indicates the mean value for each nutrient. Tank was used as the replicate; n = 26 for the ZS and ZM treatments, n = 21 for the ZZ treatment for both PO_4 μg L^{-1} P and SiO_2 μg L^{-1} Si.

4.4 Discussion

4.4.1 Chlorophyll fluorescence

Abiotic factors such as available light (Sand-Jensen, 1977; Short & Wyllie-Echeverria, 1996), water quality (Burkholder *et al.*, 1992; Short & Wyllie-Echeverria, 1996; Udy & Dennison, 1997a,b) and nutrient availability (Valiela *et al.*, 1997; Rabalais, 2002) are critical to all marine macrophytes and terrestrial plants (Hemminga & Duarte, 2000) and provide the necessary requirements for sustained growth and survival (Hemminga & Duarte, 2000). Dennison & Alberte (1985) found that the most important feature of the light environment controlling seagrass photosynthesis, growth and biomass accumulation is the length of the daily photoperiod in which the PPF exceeds or is equal to the species photosynthetic light saturation point (H_{sat}). As stated in the introduction to this chapter, the PPF required for *Z. marina* to reach its H_{sat} is highly variable and context dependent (Dennison & Alberte, 1982; Thom *et al.*, 2008).

Although PAM fluorometry has been suggested as the best method to determine chlorophyll fluorescence (Maxwell & Johnson, 2000; Beer *et al.*, 2002; Silva *et al.*, 2009), the maximum quantum yield of PSII (F_v/F_m) measurements for *Z. marina* were recorded using a PEA meter as a diving PAM was not available. F_v/F_m was used to determine chlorophyll fluorescence as it can indicate the onset and recovery from physiological stress. Therefore, any decrease in F_v/F_m may be associated with environmental stressors that directly affect PSII efficiency (Krause & Weis, 1991; Touchette & Burkholder, 2000a). Previous research at Salcombe revealed *in situ* F_v/F_m ranged from 0.80–0.84, but values as low as 0.72 ±0.04 SE were recorded in summer and values < 0.80 occurred in spring (Scarlett *et al.*, 1999). Results from the multiple laboratory experiments indicated the F_v/F_m ranged between ~0.77–0.83 at the start of each experiment, but declined over time to values < 0.70 in some years with chlorophyll fluorescence output varying over time within each treatment (Fig. 4.2).

Z. marina requires 5–6 hours of light-saturated photosynthesis per day (Alcoverro *et al.*, 1999) to maintain a positive carbon balance and vigorous growth, although this amount can vary greatly by location (Touchette & Burkholder, 2000a). Previous studies at similar latitudes to the UK reported that *Z. marina* required an average of at least 7 mol quanta m^{-2} d^{-1} during spring and summer, but only a minimum mean of 3 mol quanta m^{-2} d^{-1} for long-term survival of the species (Thom *et al.*, 2008). As the photoperiod for the seasonal differences was not stated, the PPF value equivalents range between 104–208 μmol m^{-2} s^{-1} for the winter and 121–162 μmol m^{-2} s^{-1} for the spring/summer based on the average day length for this region.

The photoperiod for the 2008 experiment was 16L:8D with an average PPF of 55–60 μmol m^{-2} s^{-1}. Although the 16 hour day length exceeded the field conditions for the time of year at which the experiment was conducted and the minimum 5–6 hours photoperiod necessary for light-saturated growth, the PPF in 2008 was at the low end of the reported range for the H_{sat} of *Z. marina* (Marsh Jr. *et al.*, 1986), and thus, perhaps too low for this species optimal requirements at this latitude. A repeated measures GLM indicated a significant interaction between date and treatment, suggesting a heterogeneous response within the three treatments over time, but the mean F_v/F_m values, especially in the ZM treatment, were below the *in situ* measurements reported by Scarlett *et al.* (1999). The low PPF in 2008 may have adversely affected the ability of *Z. marina* to achieve its full photosynthetic potential during the experiment and may have led to the results found (Fig. 4.1).

Analysis of the combined 2008–2009 data revealed no significant interaction between date and treatment, therefore suggesting a homogeneous response within the treatments over time across years. Although further analysis of the 2008–09 data indicated significant differences between treatments and years as well as a significant treatment * year interaction (Table 4.2, Fig. 4.1), pairwise comparisons revealed the PSII maximum quantum efficiency (F_v/F_m) was significantly different between the ZS and ZM treatments and the ZM and ZZ

treatments in 2008 only (Table 4.2). The differences in 2008 may indicate the upregulation of the photosynthetic apparatus within the shoots as a possible mechanism to cope with nutrient limitation and/or the presence of *S. muticum* and subsequent increased biomass within the ZS treatment and the increased biomass and seagrass shoot density within the ZZ treatment (see Chapter 3: Discussion).

Infrastructure improvements in 2009 allowed the PPF to be increased to 95–110 μmol m^{-2} s^{-1} and the day length was decreased to 12L:12D in 2009 and 2010, more in concordance with a wider range of previous studies (Dennison & Alberte, 1985; Dennison, 1987; Thom *et al.*, 2008; Jaschinski & Sommer, 2008). Although the PPF during the 2009–10 lab experiments was still low in comparison to some previous studies with PPF of 230 μmol m^{-2} s^{-1} or higher for *Z. marina* to achieve H_{sat} (Touchette & Burkholder, 2000a, and references therein), the length of the photoperiod still exceeded field conditions for the time of year the experiments were conducted. As there was no wave perturbation, sediment resuspension, self-shading or shading by *S. muticum*, the quantity of light available to the *Z. marina* shoots also exceeded normal field conditions (A. Foggo, unpublished data). If the PPF had been higher in the 2009 and 2010 experiments, increased radiant heat even from 'cool' brighter artificial daylight bulbs may have elevated seawater temperatures due to the close proximity of the lighting to the tanks, potentially leading to increased thermal stress which could have negatively affected seagrass photosynthesis, as in 2008.

Results for the combined 2008–2010 data indicated a significant interaction between date and treatment with the heterogeneous F_v/F_m response, making it difficult to interpret exactly how the presence of *S. muticum* affects the chlorophyll fluorescence of *Z. marina*. The ZM treatment had an overall higher F_v/F_m yield in 2010, whereas the ZS treatment had higher F_v/F_m values for both 2008 and 2009 as well as the final time point in 2010. These results perhaps indicate an environmental stress response, such as the downregulation of chlorophyll fluorescence within the *Z. marina* shoots in the ZM treatment, despite the lower biomass and potentially less competition for nutrient resources.

Seagrasses are in general relatively poor competitors for light (Hemminga & Duarte, 2000) as their light saturation point is higher than most of their competitors (Hemminga & Duarte, 2000), thus putting the larger, faster growing *S. muticum* at a distinct advantage in its ability to capture available light as its longer fronds can reach further towards the sea surface, reducing the light by nearly 95 % below them (Critchley *et al.*, 1990; Markager & Sand-Jensen, 1994; den Hartog, 1997; Strong *et al.*, 2006). This, coupled with comparatively inefficient light absorption by photosynthetic pigments in seagrasses compared to microalgae and most macroalgae (Hemminga & Duarte, 2000), means that *Z. marina* may notably suffer with continued unchecked proliferation of this invasive alga as found by den Hartog (1997) in Roscoff, France.

4.4.2 Growth

Z. marina achieves optimal growth when seawater temperatures range between 15–20 °C (see Lee *et al.*, 2007, and references therein) as the photon-saturated photosynthetic rate (P_{max}) and dark respiration are highly temperature sensitive (Dennison & Alberte, 1985). Optimal growth temperatures, however, can vary by season and location (Lee *et al.*, 2007). When *Z. marina* reaches or exceeds its light compensation point, the maximum net photosynthesis rate (i.e., O_2 production) of the leaves exceeds leaf respiration (O_2 consumption) by about five times (Hemminga, 1998). Thus, it is likely that plants grown under higher temperatures need increased light to maintain a positive carbon balance (Bulthuis, 1987; Lee *et al.*, 2007) than by those grown at lower temperatures. However, when grown under low light conditions (as would be found under the canopy of *S. muticum*) or inadequate PPF, seagrasses require lower seawater temperatures for optimal photosynthetic output than plants found growing in higher light environments (Bulthuis, 1987; Lee *et al.*, 2007). Therefore, the photosynthetic output of *Z. marina* in the 2008 experiment may have been reduced due to the higher seawater temperature and lower light conditions, as also found by Lee *et al.* (2007).

In 2009 and 2010, the temperature in the CT room was lowered to 10±1 °C to decrease the seawater temperature to reduce temperature stress. Repeated measures analysis of the combined laboratory experiments (2008–2010) revealed no significant treatment effect, indicating that the presence of *S. muticum* presence does not affect the total production shoot^{-1} tank^{-1} d^{-1} of *Z. marina* over time. The significant differences found between years (Table 4.7) appeared to be a result of the much higher mean total production shoot^{-1} tank^{-1} d^{-1} in 2010 compared to the means for 2008 and 2009 (78.5 mm versus 39.8 mm and 33.9 mm total production shoot^{-1} tank^{-1} d^{-1}, respectively) (Fig. 4.4). Differences found between 2010 and the other two years may have been a result of overall healthier wild-harvested plants as *L. zosterae* spread was not a concern in 2010 as it was in 2008–2009, but due to the large differences in the means between years, the experiment should be repeated for a more conclusive result.

F_v/F_m differences between treatments (Fig. 4.1) did not translate into differences in measurable growth during the duration of the experiment in 2008 (Fig. 4.3). No significant differences in growth (total production shoot^{-1} tank^{-1} d^{-1}) between treatments were found in 2009 or 2010 (Fig. 4.4). As light intensity may not have reached the light saturation point for *Z. marina*, this may have been a factor contributing to the low growth rates, which were below average for this species compared to previous findings (Hemminga & Duarte, 2000). Although no significant differences were found between treatments in 2008, there was an increase in the ZS treatment growth rate in the second half of the experiment, whereas the ZM treatment maintained steady growth throughout the experiment and the ZZ treatment declined significantly in the latter half of the experiment (Fig. 4.3), concomitant with an increase in the prevalence of *L. zosterae* infection within the ZZ treatment tanks (author's own observation). Growth remained steady in 2009 (Fig. 4.3) and 2010 (data not shown). Although PPF was perhaps too low for *Z. marina* to achieve maximum growth, nearly all shoots continued to produce new blades throughout each experiment, but to a lesser degree in the latter half of the 2008 experiment.

The root-rhizome below-ground biomass has been identified as an important factor regarding seagrass vulnerability to low light conditions (Hemminga, 1998; Evrard *et al.*, 2005). During periods of low photosynthetic output resulting from low light conditions, the roots and rhizomes can occasionally experience anaerobic conditions (Lee *et al.*, 2007). The extent to which seagrasses can respond to reduced irradiance via nutrient partitioning between above- and below-ground biomass is uncertain (Hemminga, 1998), but seagrass' ability to survive at suboptimal irradiance levels may be linked to their ability to store carbohydrates within their rhizomes (Alcoverro *et al.*, 1999; Peralta *et al.*, 2002). As the shoots in the 2008 experiment had a large proportion of below-ground biomass anchored within the sediment, respiration rates may have exceeded net photosynthesis, creating a carbon imbalance that may have been a factor in the limited shoot viability (Hemminga, 1998) for the duration of the experiment.

Seawater temperature: Effects on photosynthesis and growth

During the 2008 experiment, the seawater temperature ranged from 15–18.5 °C within the tanks, which was approximately 3–6 °C higher than early spring ambient *in situ* seawater temperatures measured along the southern Devon coast. This temperature range may have been too high for this population of seagrass and could have factored into the increased flowering of the shoots (Setchell, 1929) that was recorded during the second half of the experiment. The fact that many shoots developed inflorescences could have been a sign of temperature stress (de Cock, 1981; Short & Neckles, 1999).

It is interesting to note that when seagrasses are grown at higher than ambient temperatures, some of the phenotypic plasticity may be lost (Evans, 1983), and therefore they may lose their capacity to cope with environmental stressors. Results from experiments conducted on tropical seagrasses have indicated that seagrasses are susceptible to short-term changes in seawater temperatures, potentially irreversibly affecting their photosynthetic capacity and reducing their above-ground biomass (Campbell *et al.*, 2006). In 2008, shoots in all

treatments may have suffered from thermal stress and irradiance levels below the light compensation point for *Z. marina*, potentially adversely affecting growth rates. Although the seawater temperature in the 2008 experiment was well below the tropical temperatures tested by Campbell *et al.* (2006), it was abnormally high for early spring conditions for this temperate species found in SW England. For this temperate-adapted seagrass population, it may be possible that the higher seawater temperature induced a similar physiological response of the downregulation and closure of the PSII reaction centres due to low light levels as found by Campbell *et al.* (2006). With late summer sea surface temperatures within the range of the experimental temperature in 2008 (15–18.5 °C), any further increase in sea surface temperatures (Beardall *et al.*, 1998; Harley *et al.*, 2006) may irreversibly affect the Salcombe-Kingsbridge *Z. marina* population due to thermal stress.

4.4.3 Seagrass nutrients and nutrient limitation

Carbon, hydrogen and nitrogen

Nutrient limitation presents one of the largest challenges to marine organisms, often restricting their biomass accumulation (Rabalais, 2002). All photosynthetic organisms including vascular plants, macroalgae, and phytoplankton are affected by nitrogen and phosphorous limitation (Rabalais, 2002). Nitrogen is one of the most limited nutrients on earth with ~99.5 % unavailable for organic synthesis (White, 1993), but over the last 150 years there has been a substantial increase in the amount of reactive nitrogen (N_r) fixed by human activity (Vitousek *et al.*, 1997; Rabalais, 2002) and nitrogen and phosphorous may no longer be limited in coastal ecosystems due to direct and indirect anthropogenic nutrient enrichment (Short & Wyllie-Echeverria, 1996; Duarte, 2002).

Sources of nutrient-rich run-off consisting of inorganic dissolved nitrate and ammonium, dissolved organic amino acids, urea, and composite dissolved organic nitrogen (DIN) enter estuaries via river discharges, precipitation, drainage from wetlands, farmland, and in some cases, sewage systems (Gillanders & Kingsford, 2002). An increase in the number of

storms coupled with El Niño and La Niña Southern Oscillation events in the Pacific and the North Atlantic Oscillation and other boreal phenomena in the Atlantic can dramatically elevate river flow (Gillanders & Kingsford, 2002) and therefore increase their nutrient carrying capacity. As rivers terminate in estuaries and near-shore areas, nutrient enrichment alters ecological processes within these sensitive regions (Rabalais, 2002). Rainfall run-off alters flow rates and nutrient content of freshwater input into estuaries, ultimately affecting physical attributes including the rates of sedimentation, nutrient availability and concentration, temperature and salinity patterns, turbidity, and the amount of dissolved oxygen in estuarine ecosystems (Gillanders & Kingsford, 2002; Rabalais, 2002). When nutrient loading exceeds assimilation capacity, water degradation occurs with detrimental effects on ecosystem components and functioning (Rabalais, 2002).

Individual univariate GLMs for C-H-N components revealed significant differences between the % DW nutrient content of the three tissue types (Tables 4.8, 4.9 and 4.10). These results were expected due the inherent differences between plant components. Significant differences were also revealed between treatments, but pairwise comparisons using the multivariate dataset found significant differences only between ZZ, the biomass control treatment, and both ZS and ZM treatments. No significant differences were found between the ZS and ZM treatments. These differences may have been a result of the higher F_v/F_m measurements in the ZS and ZM treatments and/or the increased prevalence of *L. zosterae* within the ZZ treatment tanks. Research in the Isles of Scilly found a positive feedback loop within local populations; the higher the shoot density, the greater the prevalence of *L. zosterae* (Bull *et al.*, 2011).

The mean % DW ($\pm$ SE) C-H-N content within seagrass blades has been reported as ~33.6 $\pm$ 0.31 % DW C and 1.92 $\pm$ 0.05 % DW N (Duarte, 1990). Results from the 2008 experiment revealed the C-H-N partitioning within the blade tissues of *Z. marina* were consistent with the previously reported values (Figs. 4.5, 4.6, 4.7). The ZS and ZM treatments had the highest carbon content across all tissue types (Fig. 4.8), which may

have been a result of higher net photosynthesis within these treatments. Increased biomass within the ZS and ZZ treatment tanks may have limited nutrient resources within the tanks, decreasing the overall availability and uptake by a greater number of shoots and/or alga present. Results from the nutrient limitation experiment in 2011 (see Chapter 3: Nutrient limitation) indicated that although nutrients were consumed within all treatment tanks, no treatment effect occurred, indicating a homogenous response over time. In addition, it could be argued that nitrogen limitation was unlikely as the % DW N content was within the median range of *Z. marina* for all three treatments (Duarte, 1990; Hemminga & Duarte, 2000).

If nitrogen limitation was a factor in the 2008 experiment, excess carbon may have been diverted to phenolic production rather than used for growth (Bryant *et al.*, 1983; Tuomi *et al.*, 1984), leading to a reduction of shoot aerial biomass (Hemminga & Duarte, 2000). Although % DW N content was within the reported median range in 2008, blade loss within the ZZ treatment may have been due to a combination of nutrient limitation, wasting disease prevalence and increased phenolic production (Fig. 3.3). Nitrogen limitation may have occurred within the ZZ treatment as its % DW N:CA ratio was higher than the ZS and ZM treatments (Fig. 3.4). This result must be viewed with caution, however, due to the small sample size.

Phosphates and silicates

Results from phosphate and silicate analyses indicated a high degree of intra-sample variability within and between treatments (Fig. 4.10). Therefore, no evidence of an effect of the presence of *S. muticum* was deduced. The average phosphorus content in seagrass blades is 0.23 ± 0.011 (Duarte, 1990), but due to the high degree variability within these results, only an inaccurate mean phosphorus and silicate content could be calculated.

4.5 Conclusions

The annual laboratory experiments indicated complex interactions between *Z. marina* and *S. muticum*; presence of the invasive macroalga has been shown to alter seagrass tissue chemistry, photosynthesis and production of defensive compounds. It can be concluded from this work that *S. muticum* has the potential to affect *Z. marina* at a physiological level influencing its chlorophyll fluorescence output, both positively and negatively. The growth of *Z. marina* was not affected by the presence of *S. muticum*.

Nutrient partitioning revealed significant differences between paired treatments for various tissue types. The significantly lower % DW N content within the blade tissue in the ZM treatment may have been a result of reduced photosynthetic capacity perhaps due to nutrient limitation as the production of both % DW CA and % DW TA mg^{-1} equivalents was depressed within the ZM treatment (Fig. 3.3), despite no indication of such limitation occurring (Figs. 3.6, 4.9). The sheath tissue within the ZZ treatment had significantly lower % DW C and % DW H, perhaps related to the higher shoot density and prevalence of *L. zosterae* within the treatment tanks found in the latter half of the 2008 experiment. Physiological stress within the meristematic (i.e., sheath) region may have led to decreased growth, resulting in the lower % DW C and H contents. The possible positive feedback between high *Z. marina* shoot densities and increasing sea surface temperatures (Beardall *et al.*, 1998; Harley *et al.*, 2006), may challenge the long-term survival of *Z. marina*.

Chapter 5

Invasion Impacts: Densities & Physiognomy

Abstract

Seagrass meadows are dynamic coastal ecosystems that experience natural shifts in population densities and vegetative physiognomy due to seasonal influences, herbivory and outbreaks of disease, but the detrimental effects of invasive species and the consequences of sedimentation, direct mechanical damage and eutrophication may undermine seagrasses ability to survive. Within the Salcombe-Kingsbridge Estuary, boat anchoring and chain moorings have opened up 'pockets of opportunity' for the establishment of *S. muticum* stands. These 'forests' have the potential to influence the growth and density of *Z. marina* and its ability to recolonise these damaged areas both directly through competition for light and space and indirectly through weakened defences. As *S. muticum* encroaches upon the *Zostera* beds, the potential for competitive interactions between these two species arises. Previous research has found that blade lengths of *Posidonia oceanica*, an endemic seagrass in the Mediterranean Sea, decreased with increased interactions between the seagrass and *Caulerpa taxifolia*, an invasive green macrophytic alga. Numerous studies have also found that seagrass densities decline when overgrown by large macrophytic algal canopies, like those produced by *S. muticum*.

To determine if the presence of *S. muticum* had a similar affect on the density and vegetative physiognomy of *Z. marina* within the estuary, a four year field study was conducted using two treatments in permanent quadrats (ZS and ZM, as previously described in Chapter 2) and four permanent transects at increasing depths. Quadrat densities were sampled every six to eight weeks and seagrass morphometric data (blade length, width, area and number of blades per shoot) were recorded. *Z. marina* and *S. muticum* densities were sampled along each transect biannually in the spring and late summer/early autumn. *Z. marina* shoot densities in the permanent quadrats ranged from 136.5 ±9.1 to 246.6 ±16.1 shoots m^{-2} for the ZS treatment and 160 ±12.7 to 284.1 ±15.9 shoots m^{-2} for the ZM

treatment. Although results indicated significantly lower *Z. marina* densities within the ZS treatment permanent quadrats ($p < 0.001$) than the ZM treatment quadrats, densities within the quadrats increased annually, irrespective of treatment. A similar pattern emerged from the transect data: densities of both species increased over time. Morphometric data revealed no significant differences in mean length, width, area or number of blades per shoot between treatments, but mean blade lengths, and subsequently area, declined over time and the number of blades per shoot increased over time in both treatments. From these data, it appears that the presence of *S. muticum* significantly alters *Z. marina* densities in permanent quadrats where shoots are near the invasive alga, but natural densities along the transects appeared to be unaffected by its presence.

5.1 Introduction

Seagrass meadows, including their below-ground biomass, are highly productive coastal ecosystems, generating up to 1012 g DW C m^{-2} yr^{-1} (Duarte & Chiscano, 1999), but due to the anthropogenic effects of direct mechanical damage (Hastings *et al.*, 1995; Short & Wyllie-Echeverria, 1996; Reed & Hovel, 2006), invasive species (den Hartog, 1997; Ruesink *et al.*, 2006; Williams, 2007), eutrophication (Burkholder *et al.*, 1992; Short & Wyllie-Echeverria, 1996; Billen *et al.*, 1999; Hemminga & Duarte, 2000; Duarte, 2002; Howarth *et al.*, 2002; Wade *et al.*, 2005), sedimentation (Short & Wyllie-Echeverria, 1996; Erftemeijer & Lewis, 2006) and the naturally occurring wasting disease (Short *et al.*, 1988; Ralph & Short, 2002; Vergeer *et al.*, 1995), seagrasses, and the services they provide, face an uncertain future. An increasing number of studies have focussed on the direct consequences anchoring, moorings and dredging have on seagrass beds (Hastings *et al.*, 1995; Erftemeijer & Lewis, 2006; Collins *et al.*, 2010), and how they secondarily affect seagrass growth, density and ability to recolonise impacted areas (den Hartog, 1997; Kiparissis *et al.*, 2011). Damage to the shallow subtidal from mooring scars opens up ‘pockets of opportunity’ for invasive marine macrophytes (Walker & Kendrick, 1998; Kiparissis *et al.*, 2011), further facilitating their spread. The Salcombe-Kingsbridge

Estuary, with ~1000 foreshore and 250 deep-water moorings (Salcombe Harbour Authority, 2012), is a yacht and boating haven located on the southern coast of the UK. Due to inexperienced boat handlers coupled with the large number of boats that anchor within the estuary, environmental degradation has occurred. Such degradation is apparent by the mechanically generated anchoring and mooring scars visible in the substratum. As one of the most ubiquitous marine macrophyte invaders, *S. muticum* has directly benefited from this mechanical damage due to its ability to rapidly colonise any available space (den Hartog, 1997; Strong *et al.*, 2006), with such scars often acting as nurseries (Kraan, 2008).

Initially, it was believed that *S. muticum* posed an unlikely threat to seagrass meadows due to a mis-match of substrata preference: *S. muticum* prefers hard, rocky substratum whereas seagrasses are found in sandy and muddy sediments. Within the Salcombe-Kingsbridge Estuary, and in many other locations along the south coast of the UK, the substrata is a mixture of fine sandy sediment with small pebbles and shell fragments upon which *S. muticum* can attach with its small disc-like holdfast (den Hartog, 1997). These shell fragments and stones can become wedged within the root-rhizome matrix of *Z. marina*, enabling *S. muticum* to establish itself within the seagrass meadows aided by its ability to disperse peripatetically. As the density of *S. muticum* increases, alterations within ecosystems such as reduced ambient nutrient concentrations (Britton-Simmons, 2004), substrata domination (den Hartog, 1997; Strong *et al.*, 2006) and changes in macrophytic diversity (Stæhr *et al.*, 2000) and epifaunal assemblages occur (Gestoso *et al.*, 2010).

Previous studies have reported dramatic declines in the densities of *Z. marina* when shaded by large macrophytic algae, with total loss occurring in some instances (Hauxwell *et al.*, 2001). With its ability to exceed 5 m in length (Gorham & Lewey, 1984; den Hartog, 1997), *S. muticum* has negatively affected densities of understory kelps and other macrophytic algae (Farnham & Gareth Jones, 1974; den Hartog, 1997; Britton-Simmons, 2004), ultimately out-competing other species to become the dominant macrophyte within some coastal systems (Critchley *et al.*, 1986; den Hartog, 1997). To date, no studies

have investigated the direct consequences that *S. muticum* invasion has had on *Z. marina* densities.

Invasive species not only affect native species densities, they can also influence their growth. In previous research conducted in the Mediterranean Sea, Pergent *et al.* (2008) found that the blade lengths of *P. oceanica* decreased with increased interactions between the seagrass and *Caulerpa taxifolia*, an invasive macrophytic green alga. Interestingly, a negative correlation was also revealed; the frond length of *C. taxifolia* increased and the blade length of *P. oceanica* decreased with increasing interaction. Pergent *et al.* (2008) also found that leaf longevity was reduced, but that more leaves were produced annually, resulting in a faster leaf turnover rate with increased interaction between the seagrass and invasive alga.

With a high annual reproductive output, greater overall length and a plethora of available substrata, increasingly dense stands of *S. muticum* within the Salcombe-Kingsbridge Estuary pose a real threat to *Z. marina*. Therefore, the aim of this long-term study was to investigate how *Z. marina* densities changed over time, to determine any effects of this invader on seagrass vegetative physiognomy, and to track *S. muticum* spread and density changes within the estuary. I hypothesised that 1) *Z. marina* densities would decrease when near *S. muticum*, 2) *Z. marina* shoots would experience reduced growth (blade length and width), leading to smaller overall shoots in the presence of *S. muticum* and 3) *S. muticum* densities would increase within the estuary over time.

5.2 Methods

5.2.1 Permanent quadrat densities

Z. marina shoot densities were sampled within the same 20 1 m^2 permanent quadrats established in spring 2007, as described in Chapter 2: Methods, every six to eight weeks over a four year period from spring until autumn (spring: March–May, summer: June–

August and autumn: September–October). Mean seagrass densities m^{-2} were calculated from three random 0.0625 m^2 sub-samples from within each 1 m^2 permanent quadrat.

5.2.2 Transects

Four permanent transects, each approximately 70 m long and 12 m apart were established (Fig. 2.2) parallel to the tide line north of Woodville Rocks in the Salcombe-Kingsbridge Estuary. Transects were marked using geological markers (300 mm ground mark with raised head) painted with fluorescent anti-fouling paint to aid in the relocation of the end- and mid-points of each transect. Transects 1 and 4 were established at mean low water chart datum and at 1.2 m below mean low water chart datum, respectively. Transects 2 and 3 were established at intermediate depths between transects 1 and 4. Transects were sampled twice annually, once in early spring and again in late summer/early autumn to coincide with the vernal and autumnal equinoxes from autumn 2007 until spring 2011. *Z. marina* densities were determined by sampling 12 randomly located 1 m^2 quadrats along each transect. Within each 1 m^2 quadrat, four 0.0625 m^2 sub-samples were taken by counting the number of individual shoots per area. Data were averaged to produce the mean *Z. marina* density per 1 m^2 quadrat. After sampling *Z. marina* densities, the number of *S. muticum* thalli along each transect was counted based on individual holdfasts present in a 1 m wide strip centred on the transect. The mean number of *S. muticum* thalli within the field site was calculated and used as a proxy for overall *S. muticum* densities within the estuary.

To compare quadrat and transect densities (i.e., manipulated vs. unmanipulated), quadrat densities from the same months that transect sampling occurred were averaged together to produce the mean quadrat density for that sampled date. As the quadrats on the north side of Woodville Rocks were located at the same depth as transect 2 (see Fig. 2.2), only the mean density data collected from transect 2 were used in the comparison for all years sampled to eliminate any depth effects.

5.2.3 *Z. marina* vegetative physiognomy

Length (mm), width (mm) and total blade area (mm^2) moprhometric measurements were recorded for all blades from the three shoots collected at the field site for the research carried out in Chapters 3 (phenolics) and 6 (epibiota). The total number of blades per shoot was also recorded and used to calculate the total blade area per seagrass shoot sampled. These data were then averaged by quadrat to calculate the mean length (mm), width (mm) and area (mm^2) used in analyses. The total calculated area (mm^2) for each blade for each shoot sampled was summed; summed totals were then averaged across the three samples to calculate the mean total area per shoot. No data were available for early spring 2007 or autumn 2010. Values for these missing morphometric means for all vegetative characteristics measured were calculated by averaging the collected data for each season across all years. These averages then were used as the dummy data for the missing data points. These calculated mean data values were used only to validate overall trends in the data, removing any bias towards overall larger or smaller shoots, due to discrepancies in numbers of spring and autumn samples.

5.2.4 Data analysis

Permanent quadrats

Effects of experimental 'treatments', 'years' and 'seasons' upon the mean *Z. marina* densities in the permanent quadrats were analysed using a mixed model univariate GLM in SPSS 19 with the mean seagrass density per quadrat as the dependent variable. The GLM model had three factors, 'treatment' and 'year' were designated as fixed with two (ZS and ZM) and four (2007, 2008, 2009 and 2010) levels respectively and because of a lack of orthogonality 'season' was set as a random factor with three levels (spring, summer and autumn), nested within 'year'. Type III Sums of Squares were used and S-N-K *post hoc* tests were performed for 'years'. Non-conformity to assumptions of homogeneity of variances was indicated by results of Levene's Test, but examination of unstandardised

analytical residuals indicated normal distribution. Planned pairwise comparisons were used to identify significant differences between the ZS and ZM treatments within the interaction term 'year * treatment' ($p < 0.05$) with Tukey's LSD using estimated marginal means.

Sediment data and permanent quadrats

To determine sediment influence on the permanent quadrat densities of *Z. marina*, the complex relationships of particle size, % organic content (see Chapter 2) and seagrass densities from the permanent quadrats were explored using a GLM approach. A full factortial model using seagrass density (from the experimental quadrats) as a dependent variable and % organic content and the Method of Moments mean (logarithmic ϕ) particle size as covariates was explored first to eliminate the potential for heterogeneous slopes of factor * covariate combinations. All interactions between covariates and factors produced homogeneous slopes. The design specification was then amended to address the issue of correlated covariates and multiplicity of *p*-values by comparing models using the two different covariates and rejecting the least powerful predictor of the two by comparing Akaike Information Criterion (AIC) scores and AIC weights (Field, 2009). A final model used a type I Sums of Squares GLM to examine the effect of treatment on seagrass density after correcting for the influence of the particle size covariate. Non-conformity to assumptions of homogeneity of variances was indicated by results of Levene's Test; examination of unstandardised analytical residuals indicated need for a power transformation (X^2). Equality of variances was improved by transformation, but remained marginal, therefore further non-parametric analysis was carried out using PRIMER v6.1.13 with PERMANOVA+ v1.0.3 to confirm univariate GML results. Data were normalised and a Euclidean distance resemblance matrix was constructed. The same three-factor design described above was performed, with type III Sums of Squares, unrestricted permutation of raw data and 9999 permutations (Anderson *et al.*, 2008).

Transect densities

Effects of 'transects', 'seasons' and 'years' upon the mean transect densities for *Z. marina* were analysed using a mixed model univariate GLM in SPSS 19. The GLM model designated 'transect' with four (1, 2, 3 and 4) and 'year' with five (2007, 2008, 2009, 2010 and 2011) levels respectively as fixed factors and because of a lack of orthogonality, 'season' nested within 'year' was a random factor with two levels (spring and autumn). Type III Sums of Squares were used. Non-conformity to assumptions of homogeneity of variances were indicated by results of Levene's Test; examination of unstandardised analytical residuals indicated need for a square root transformation ($\sqrt{x}$). Equality of variances was improved by transformation. Planned pairwise comparisons were used to identify significant differences (Tukey's LSD: $p < 0.05$) between the transects using estimated marginal means.

Effects of 'seasons' and 'years' upon the mean number of *S. muticum* thalli per transect were analysed using a mixed model univariate GLM in SPSS 19 with two factors. 'Year' was set as fixed factor with five (2007, 2008, 2009, 2010 and 2011) levels and due to non-orthogonality, 'season' nested within 'year' with two (spring and autumn) levels was designated a random factor. A type III Sums of Squares model was employed and polynomial contrasts were conducted for 'year' to characterise any temporal changes; S-N-K *post hoc* tests were performed for 'year'. Conformity to assumptions of homogeneity of variances was indicated by Levene's Test.

Z.* marina *vegetative physiognomy

Effects of the experimental 'treatments', 'years' and 'seasons' upon the mean length, width, area and number of blades per shoot were analysed using separate mixed-model univariate GLMs in SPSS 19 for each dependent variable. The GLM models utilised a three-factor design where 'treatment' and 'year' were designated as fixed factors with two (ZS and ZM) and four (2007, 2008, 2009 and 2010) levels respectively and because

of a lack of orthogonality 'season' was set as a random factor with two levels (spring and autumn), nested within 'year'. Type III Sums of Squares were used and S-N-K *post hoc* tests were performed for 'year'. Non-conformity to assumptions of homogeneity of variances was indicated by results of Levene's Test for the mean width and area per blade only; examination of unstandardised analytical residuals indicated need for a power transformation (X^2) for mean width and a square root transformation ($\sqrt{x}$) for mean area per blade. Homogeneity of variances was improved by transformation for mean width, but visual inspection of the residuals for the transformed mean area indicated non-normality, therefore further non-parametric permutational analysis was performed using PRIMER v6.1.13 with PERMANOVA+ v1.0.3 to confirm univariate GML results. Planned pairwise comparisons were used to identify significant differences (Tukey's LSD: $p < 0.05$) between the transects using estimated marginal means. Analyses were confirmed in PRIMER-E v6.1.13 with PERMANOVA+ v1.0.3 using the same three-factor design as described above on normalised (when appropriate) data using Euclidean distance resemblance matrices. Type III Sums of Squares, unrestricted permutation of raw data and 9999 permutations were declared as design parameters. Individual PERMANOVA+s were calculated for each morphometric variable separately in addition to a PERMANOVA+ that analysed all dependent variables collectively.

5.3 Results

5.3.1 Permanent quadrat densities

Z. marina shoot densities for the permanent quadrats ranged from 136.5 ±9.1 (early spring) to 246.6 ±16.1 (summer) shoots m^{-2} for the ZS treatment and 160 ±12.7 (early spring) to 284.1 ±15.9 (summer) shoots m^{-2} for the ZM treatment (Fig. 5.1) over the duration of the long-term field study. GLM results indicated significant differences between 'treatments' and 'seasons' within 'years', but no significant differences were found between 'years' (Table 5.1). Planned pairwise comparisons for the factor 'treatment' within the interaction term 'treatment * season(year)' revealed the significant differences between 'treatment'

occurred in spring 2009 and summer 2010 (Table 5.2). Regardless of 'treatment', there was a general trend of increasing *Z. marina* densities within the estuary (Fig. 5.1).

Particle size, % organic content and the mean seasonal densities of Z. marina

Univariate GLM results of the mean seasonal densities of *Z. marina* within the permanent quadrats analysed with sediment data (Method of Moments mean (logarithmic ϕ) and loss on ignition % organic content) as covariates indicated a significant difference between 'treatments' (Table 5.3), as was found in the univariate GLM for *Z. marina* permanent quadrat densities (Table 5.1). A significant relationship between the average seasonal seagrass densities for *Z. marina* and particle size was revealed and after correcting for the influence of particle size; a highly significant difference was found between 'seasons' (Table 5.3). Results from PERMANOVA+ analysis confirmed the significant differences between 'treatments' and between 'seasons' (Table 5.4) found using the GLM covariate analysis. Fig. 5.2 illustrates an indicated weak positive correlation between increasing particle size and increasing *Z. marina* densities ($R^2 = 0.141$, $F = 22.647$, $df = 1$, $p < 0.001$).

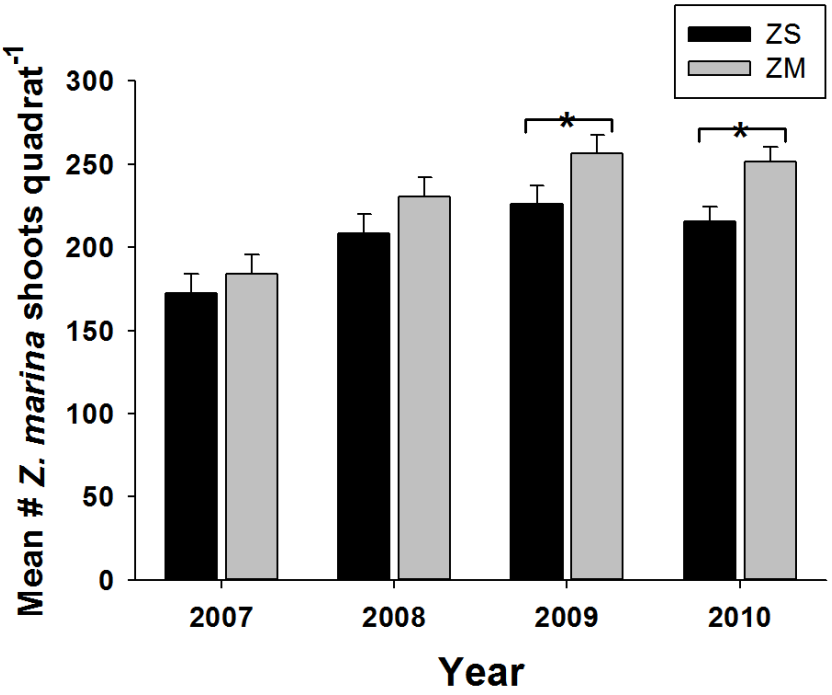


Figure 5.1: Mean (±SE) *Z. marina* shoots m^{-2} by treatment in permanent quadrats from the 2007–2010 long-term field study. Results represent the annual mean density for each treatment (calculated across all seasons). Quadrat was used as the replicate; n = 190 for each treatment over 4 years with 3 seasons per year.

Table 5.1: Univariate GLM results for *Z. marina* shoot densities in permanent quadrats from the 2007–2010 long-term field study. Se(Yr) = season nested within year. Quadrat was used as the replicate; n = 190 for each treatment over 4 years with 3 seasons per year.

Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypothesis	15151033.977	1	15151033.977	312.876	**< 0.001**
	Error	342026.203	7.063	48425.048		
Year	Hypothesis	182697.276	3	60899.092	1.160	0.391
	Error	364380.964	6.943	52480.086		
Treatment	Hypothesis	48409.433	1	48409.433	53.716	**< 0.001**
	Error	10906.257	12.102	901.215		
Season(Year)	Hypothesis	353245.168	7	50463.595	70.404	**< 0.001**
	Error	5017.435	7	716.776		
Year * Treatment	Hypothesis	7159.991	3	2386.664	4.467	0.104
	Error	1903.285	3.562	534.333		
Treatment * Se(Yr)	Hypothesis	5017.435	7	716.776	0.148	0.994
	Error	1734228.137	358	4844.213		

Table 5.2: Planned pairwise comparisons for the factor 'treatment' within the interaction term 'treatment * season(year)' for *Z. marina* shoot densities in permanent quadrats from the long-term field study from 2007–2010. No data are shown for autumn 2010 as samples were not collected for that season. Spring = March, April May; Summer = June, July, August; Autumn = September, October. Quadrat was used as the replicate; n = 190 for each treatment over 4 years with 3 seasons per year.

Year	Season	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Sig. (*p*)
2007	Spring	ZS	ZM	-23.467	0.451
	Summer	ZS	ZM	-8.800	0.690
	Autumn	ZS	ZM	-2.667	0.932
2008	Spring	ZS	ZM	-23.467	0.451
	Summer	ZS	ZM	-30.933	0.161
	Autumn	ZS	ZM	-11.733	0.706
2009	Spring	ZS	ZM	-40.178	**0.026**
	Summer	ZS	ZM	-36.267	0.245
	Autumn	ZS	ZM	-14.400	0.644
2010	Spring	ZS	ZM	-33.956	0.060
	Summer	ZS	ZM	-37.500	**0.038**
	Autumn	ZS	ZM	.	.

Table 5.3: Covariate GLM results for sediment data (Method of Moments (MoM) mean (logarithmic ϕ) (MoM; X^2 transformed)) and average *Z. marina* seasonal densities from the 2007–2010 long-term field study. MOM = Squared Method of Moments mean (logarithmic ϕ), Season(Yr) = season nested within year. Quadrat was used as the replicate; n = 70 for each treatment over 4 years with 2 seasons per year.

Source		Type I SS	df	MS	F	Sig. (*p*)
Intercept	Hypothesis	7065991.343	1	7065991.343	223.751	**0.001**
	Error	94397.419	2.989	31579.761		
MOM	Hypothesis	92096.336	1	92096.336	19.981	**0.001**
	Error	60289.937	13.081	4609.137		
Treatment	Hypothesis	14907.079	1	14907.079	39.782	**0.002**
	Error	1831.506	4.888	374.716		
Year	Hypothesis	111177.396	3	37059.132	1.176	0.449
	Error	94225.528	2.990	31509.224		
Season(Year)	Hypothesis	92922.195	3	30974.065	92.356	**< 0.001**
	Error	1346.289	4.014	335.377		
Treatment * Year	Hypothesis	3055.237	3	1018.412	3.540	0.171
	Error	816.817	2.839	287.664		
Treatment * Season(Yr)	Hypothesis	884.419	3	294.806	0.109	0.955
	Error	338075.455	125	2704.604		

Table 5.4: PERMANOVA+ results from long-term field study for sediment data (laser diffraction particle size (Methods of Moments mean (logarithmic ϕ) and % organic content)) and the mean seasonal densities of *Z. marina* for two treatments (ZS and ZM) and four years (2007–2010). Quadrat was used as the replicate; n = 70 for each treatment over 4 years with 2 seasons per year.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	5.396	5.396	7.229	**0.009**
Year	3	49.726	16.575	0.793	0.674
Season(Year)	3	62.716	20.905	8.962	**< 0.001**
Treatment * Year	3	2.182	0.728	0.975	0.522
Treatment * Season(Year)	3	2.239	0.746	0.320	0.961
Res	126	293.93	2.333		
Total	139	417			

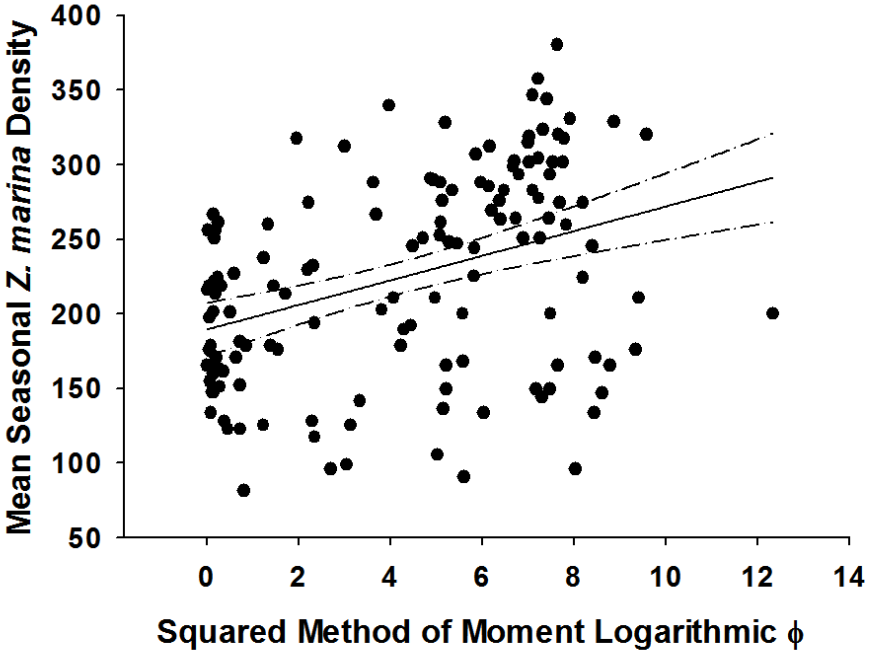


Figure 5.2: Method of Moments mean (logarithmic ϕ) particle size vs. the mean seasonal densities of *Z. marina* in permanent quadrats from the 2007–2010 long-term field study. Quadrat was used as the replicate; n = 70 for each treatment over 4 years with 2 seasons per year. $R^2 = 0.141$ (F = 22.647, df = 1; y = 0.0171x + 0.432), dashed lines indicate the 95% confidence intervals.

5.3.2 Transects densities

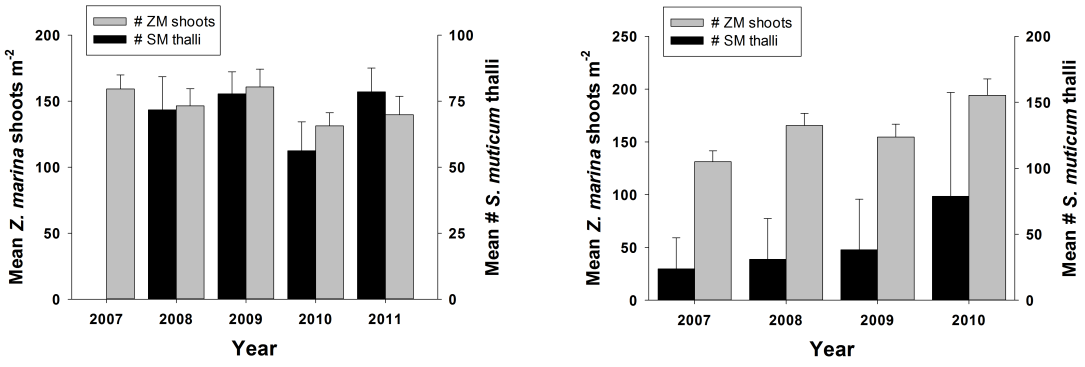
GLM results for *Z. marina* densities along the permanent transects indicated no significant differences between 'transects' or 'years', but significant differences were revealed between 'seasons' within 'years' (Table 5.5). Similar results were found for the mean number of *S. muticum* thalli, where no significant differences were found between 'years', but were found between 'seasons' (Table 5.6); polynomial contrasts for the number of *S. muticum* thalli indicated a significant linear response over 'years' (F = 10.687, df = 1, $p = 0.003$). Spring densities for both species were variable, but late summer/early autumn densities showed a steady annual increase (Fig. 5.3). The overall means for both *Z. marina* shoots m^{-2} and the total number of *S. muticum* thalli along each transect increased annually (using only the years where both seasons were sampled (2008–2010) (Fig. 5.4)). Visual inspection of the mean number of *S. muticum* thalli per transect graphed against the mean shoot densities m^{-2} along each transect for *Z. marina* revealed no conspicuous correlations between species densities (Fig. 5.5); *Z. marina* transect densities were not affected by the presence of *S. muticum*.

Table 5.5: Univariate GLM results for the mean densities of *Z. marina* for all transects from the 2007–2011 long-term field study. Quadrat was used as the replicate; n = 414 over 4 years with 2 seasons per year. Hypoth = hypothesis, Tran = transect, and Se(Yr) = season nested within year.

Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypoth	380560807554.028	1	380560807554.028	80.298	**0.001**
	Error	18887543916.160	3.985	4739353276.065		
Transect	Hypoth	5556662463.723	3	1852220821.241	2.290	0.131
	Error	9636825157.053	11.914	808860305.852		
Year	Hypoth	4883423842.164	4	1220855960.541	0.256	0.892
	Error	18953989220.151	3.979	4763202090.344		
Season(Year)	Hypoth	18728063973.113	4	4682015993.278	5.810	**0.007**
	Error	9844867380.764	12.217	805801040.973		
Tran * Year	Hypoth	19940832090.086	12	1661736007.507	2.049	0.116
	Error	9500490164.289	11.715	810940082.566		
Tran * Se(Yr)	Hypoth	9695759501.196	12	807979958.433	1.302	0.214
	Error	234503677067.985	378	620380098.063		

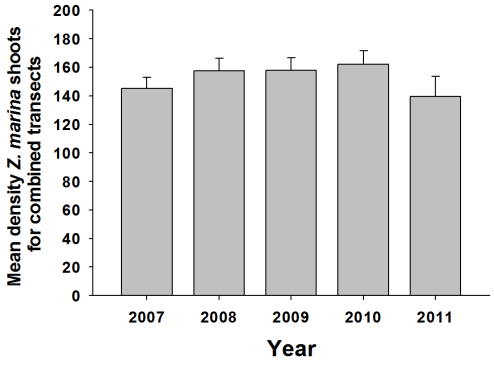
Table 5.6: Univariate GLM results for the mean number of *S. muticum* thalli for all transects from 2007–2011. Transect was used as the replicate; n = 32 over 5 years with 2 seasons per year in 2008–2010 and 1 season in 2007 and 2011.

Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypothesis	91395.200	1	91395.200	39.100	**0.008**
	Error	7124.027	3.048	2337.459		
Year	Hypothesis	6290.329	4	1572.582	0.666	0.657
	Error	7174.433	3.040	2360.022		
Season(Year)	Hypothesis	7454.125	3	2484.708	9.003	**< 0.001**
	Error	6347.417	23	275.975		

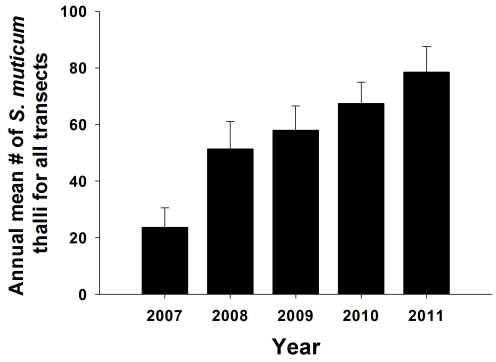


(a) *Spring transect densities* **(b)** *Autumn transect densities*

Figure 5.3: Transect densities for spring (a) and autumn (b) from 2007–2011 for *Z. marina* shoots and *S. muticum* thalli. *Z. marina* results are reported as the mean number of shoots m^{-2} averaged along all transects for each season and *S. muticum* results are reported as the mean number of thalli in a 1 m strip across all transects for each season. Quadrat was used as the replicate for *Z. marina* densities; n = 414. Transect was used as the replicate for *S. muticum* abundances; n = 32. Data was collected over 5 years with 2 seasons per year in 2007–2010 and 1 season in 2011 for *Z. marina* and over 5 years with 2 seasons per year in 2008–2010 and 1 season in 2007 and 2011 for *S. muticum*.



(a) *Annual mean* Z. marina *transect densities*



(b) *Annual mean* S. muticum *transect abundances*

Figure 5.4: Annual mean transect densities for *Z. marina* (a) and the annual mean number of thalli for *S. muticum* (b) from 2007–2011 field data. *Z. marina* results are reported as the mean number of shoots m^{-2} averaged across all transects for each year and *S. muticum* results are reported as the mean number of thalli in a 1 m wide strip centred on the transect and averaged across all transects for each year. Quadrat was used as the replicate for *Z. marina* densities; n = 414. Transect was used as the replicate for *S. muticum* abundances; n = 32. Data was collected over 5 years with 2 seasons per year in 2007–2010 and 1 season in 2011 for *Z. marina* and over 5 years with 2 seasons per year in 2008–2010 and 1 season in 2007 and 2011 for *S. muticum*.

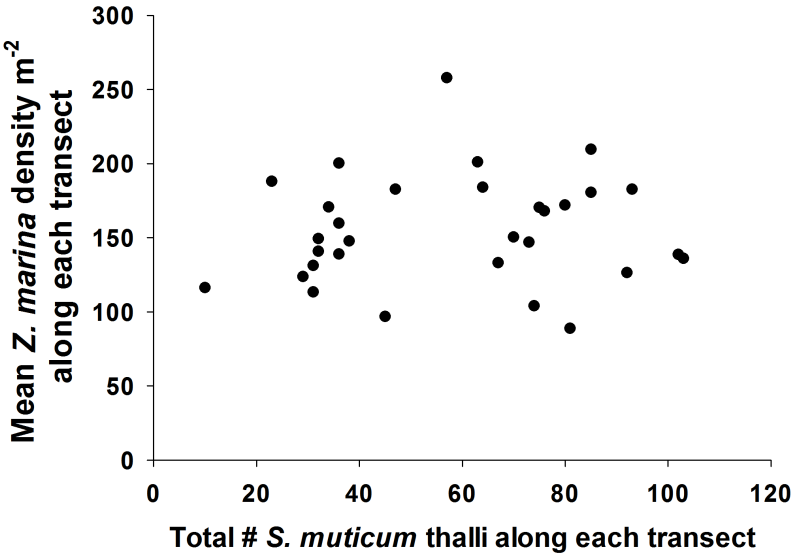


Figure 5.5: Mean number of *S. muticum* thalli per transect vs. mean *Z. marina* density per transect data from 2007–2011. Transect was used as the replicate; n = 32 for each species over 5 years with 2 seasons per year in 2008–2010 and 1 season in 2007 and 2011.

5.3.3 *Z. marina* vegetative physiognomy

Averaging across all 'years' and all 'seasons', the overall mean blade lengths of *Z. marina* shoots within the ZS treatment were 33.5 cm (±1 cm) and 33 cm (±1 cm) for the ZM treatment. The mean width was 4.8 mm (±0.04 mm) for both treatments. Although leaf widths remained constant throughout the duration of the long-term field study, there was an overall decrease in leaf lengths over time, which in turn led to a decrease in mean total leaf area (Fig. 5.6). Despite the decreasing leaf lengths, the number of leaves per shoot increased over time (Fig. 5.6). Morphometric data were used to calculate the mean total area (mm^2) per *Z. marina* shoot and the mean total leaf area (mm^2) for each 1 m^2 quadrat (Fig. 5.6).

GLM results for mean length, width, total blade area (mm^2) and number of blades per *Z. marina* shoot indicated no significant differences between 'treatments' or 'years', but significant differences were found between 'seasons' as expected (Tables 5.7, 5.8, 5.9, and 5.10). PERMANOVA+ results for the combined morphometric data (mean length, mean width, mean blade area, mean shoot area and mean number of blades per shoot) from the permanent quadrats indicated no significant differences between 'treatments' or 'years', but significant differences were revealed between 'seasons' within 'years' (Table 5.11).

Although no significant differences were found between 'treatments' for all vegetative characteristics measured, two trends emerged; blade lengths (and subsequently area) decreased over time whereas the number of blades per shoot increased over time. Comparisons of the averaged morphometric means to those years with missing data indicated decreases in blade length and subsequent area were not due to sampling bias (Figs 5.6 and 5.8).

Table 5.7: Univariate GLM results for the mean blade length of *Z. marina* from the 2007–2010 long-term field study. Quadrat was used as the replicate; n = 360 over 4 years with 3 seasons per year. Season(Yr) = season nested within year.

Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypothesis	40800999.022	1	40800999.022	99.201	**< 0.001**
	Error	2474154.691	6.016	411294.698		
Year	Hypothesis	1422769.749	3	474256.583	1.066	0.431
	Error	2667241.859	5.996	444872.280		
Treatment	Hypothesis	7.255	1	7.255	0.001	0.979
	Error	64739.956	6.720	9633.470		
Season(Year)	Hypothesis	2621247.530	6	436874.588	45.128	**< 0.001**
	Error	58085.355	6	9680.893		
Year * Treatment	Hypothesis	38037.202	3	12679.067	1.308	0.358
	Error	56231.167	5.800	9695.720		
Treatment * Season(Yr)	Hypothesis	58085.355	6	9680.893	1.089	0.368
	Error	3021730.251	340	8887.442		

Table 5.8: Univariate GLM results for the mean blade width of *Z. marina* from the 2007–2010 long-term field study. Quadrat was used as the replicate; n = 360 over 4 years with 3 seasons per year.

Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypothesis	171930.732	1	171930.732	417.206	**< 0.001**
	Error	2486.317	6.033	412.100		
Year	Hypothesis	402.764	3	134.255	0.302	0.823
	Error	2661.170	5.990	444.259		
Treatment	Hypothesis	18.098	1	18.098	1.431	0.269
	Error	91.609	7.244	12.645		
Season(Year)	Hypothesis	2618.611	6	436.435	35.714	**< 0.001**
	Error	73.322	6	12.220		
Year * Treatment	Hypothesis	41.906	3	13.969	1.156	0.404
	Error	68.302	5.652	12.084		
Treatment * Season(Year)	Hypothesis	73.322	6	12.220	0.627	0.709
	Error	6611.000	339	19.501		

Table 5.9: Univariate GLM results for the mean blade area (mm^2) of *Z. marina* from the 2007–2010 long-term field study. Quadrat was used as the replicate; n = 360 over 4 years with 3 seasons per year.

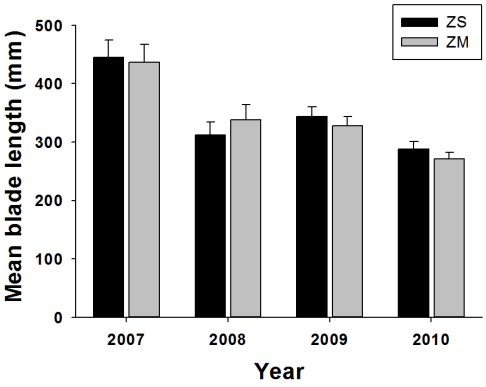
Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypothesis	499621.780	1	499621.780	235.749	**< 0.001**
	Error	12745.536	6.014	2119.298		
Year	Hypothesis	12769.066	3	4256.355	1.859	0.237
	Error	13726.997	5.996	2289.423		
Treatment	Hypothesis	1.115	1	1.115	0.024	0.881
	Error	308.581	6.700	46.054		
Season(Year)	Hypothesis	13488.197	6	2248.033	48.574	**< 0.001**
	Error	277.686	6	46.281		
Year * Trt	Hypothesis	185.481	3	61.827	1.334	0.351
	Error	268.814	5.799	46.354		
Treatment * Season(Year)	Hypothesis	277.686	6	46.281	1.092	0.367
	Error	14373.090	339	42.398		

Table 5.10: Univariate GLM results for the mean number of blades per shoot for *Z. marina* from the 2007–2010 long-term field study. Quadrat was used as the replicate; n = 360 over 4 years with 3 seasons per year.

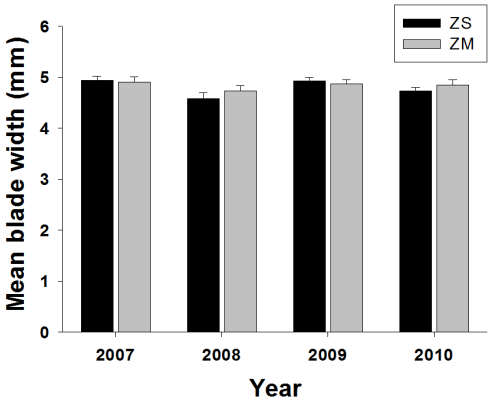
Source		Type III SS	df	MS	F	Sig. (p)
Intercept	Hypothesis	5421.246	1	5421.246	476.773	**< 0.001**
	Error	68.437	6.019	11.371		
Year	Hypothesis	60.504	3	20.168	1.640	0.277
	Error	73.702	5.995	12.295		
Treatment	Hypothesis	0.313	1	0.313	0.806	0.401
	Error	2.556	6.586	0.388		
Season(Year)	Hypothesis	72.448	6	12.075	30.650	**< 0.001**
	Error	2.364	6	0.394		
Year * Treatment	Hypothesis	0.687	3	0.229	0.579	0.651
	Error	2.310	5.836	0.396		
Treatment * Season(Year)	Hypothesis	2.364	6	0.394	1.331	0.242
	Error	100.601	340	0.296		

Table 5.11: PERMANOVA+ results for morphometric data from mean length, width and area for the permanent quadrats by treatment from the 2007–2010 long-term field study. Quadrat was used as the replicate; n = 360 for each morphometric character over 4 years with 3 seasons per year.

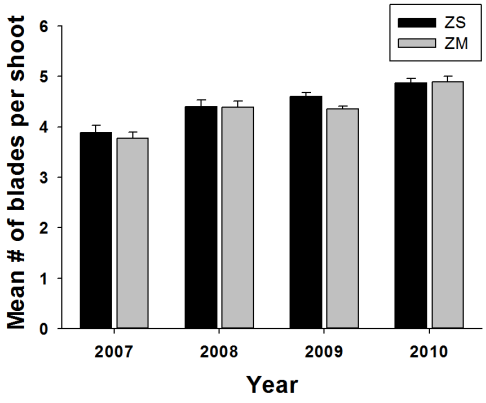
Source	df	Type III SS	MS	Pseudo-F	p (perm)
Treatment	1	1.034	1.034	0.664	0.472
Year	3	198.71	66.236	1.175	0.388
Season(Year)	6	332.32	55.387	32.448	**< 0.001**
Treatment * Year	3	5.517	1.839	1.185	0.392
Treatment * Season(Year)	6	9.299	1.55	0.908	0.504
Res	340	580.36	1.707		
Total	359	1077			



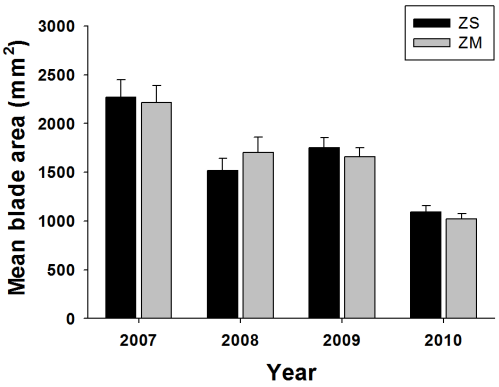
(a) *Mean leaf length (mm)*



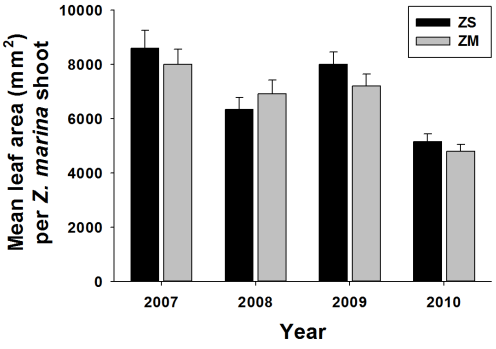
(b) *Mean leaf width (mm)*



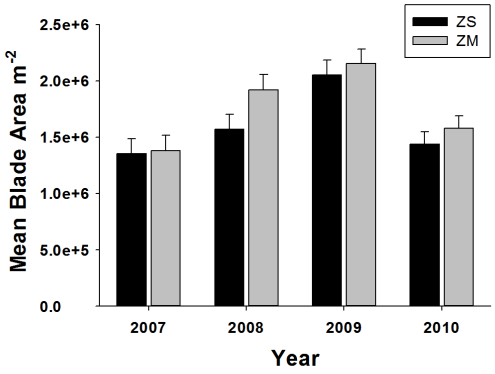
(c) *Mean number of leaves per Z. marina shoot*



(d) *Mean leaf area (mm^2)*



(e) *Total mean leaf area (mm^2) per Z. marina shoot*



(f) *Total mean leaf area (mm^2) per quadrat*

Figure 5.6: Mean length (mm) (a), width (mm) (b), number of leaves per *Z. marina* shoot (c), mean area per leaf (mm^2) (d), mean leaf area per *Z. marina* shoot (e) and mean *Z. marina* total leaf area m^{-2} quadrat (f) from *Z. marina* shoots collected from permanent quadrats between 2007–2010. Results represent the annual mean (±SE) (calculated across all seasons). Quadrat was used as the replicate; n = 360 for each morphometric character over 4 years with 3 seasons per year.

5.4 Discussion

5.4.1 Permanent quadrats and transect densities

Sediment disturbance and/or excavation is one of the most severe modifications that can occur within seagrass meadows (Erftemeijer & Lewis, 2006). This is due to alterations in the physical and chemical properties of the substrata, loss of seed banks and seagrass biomass, and changes in the local topography which then facilitates further sediment destabilisation, making regrowth of seagrasses into disturbed areas more difficult (DiCarlo & Kenworthy, 2008). Spatial heterogeneity (or 'patchiness') is a common and important feature in seagrass meadows (Reed & Hovel, 2006; Jackson *et al.*, 2006; Hirst & Attrill, 2008), but increasing frequency and intensity of unnatural perturbations (Vitousek *et al.*, 1997) may put the longevity of *Z. marina* at risk within the Salcombe-Kingsbridge Estuary due in part to diurnal tidal fluctuations of > 5 m and current velocities up to ~1.0 m s^{-1} at mid-flood/ebb during equinoctial spring tides (Kinetics Ltd., 1992). With such large volumes of water moving in and out of the estuary, continual erosion around the numerous mooring and anchoring scars within the seagrass beds is a substantive concern. As *Z. marina* meadows are vulnerable to anthropogenic disturbances (Campbell *et al.*, 2003; Jackson *et al.*, 2006), further fragmentation of the beds may increase the substrata available for *S. muticum* colonisation.

The Salcombe-Kingsbridge Estuary is one of the few locations within the UK where the foreshore is privately owned rather than owned as part of the Crown Estate. Therefore, enforced protection of the BAP species, *Z. marina* and its associated biota found within this area is more challenging despite the estuary's designation as a SSSI (Site of Special Scientific Interest), SAC (Special Area of Conservation) and ANOB (Area of Outstanding Natural Beauty). Considerable time and effort have been spent working with the landowners to reduce the number of swing/chain moorings on the foreshore to lessen the impact on the intertidal and shallow subtidal seagrass beds. Although there are restrictions on the number of moorings that can be installed on each private landowner's foreshore,

damage from existing moorings is already present. A moratorium on new moorings within the estuary may nonetheless help slow the spread of *S. muticum*. Previous research has indicated that recovery from large-scale disturbances within subtidal seagrass beds can take upwards of five years (Erftemeijer & Lewis, 2006). However, if *S. muticum* colonises anchoring and mooring scars, the re-establishment of *Z. marina* within these areas may not be possible (den Hartog, 1997; Kiparissis *et al.*, 2011).

Depth can also affect the influence invasive species have on seagrass densities. Within the Mediterranean Sea, Molenaar *et al.* (2005) found a significant decrease in *P. oceanica* densities at 6 m in the presence of the invasive alga, *C. taxifolia*, despite the invasive being much smaller in size. At 20 m, however, no significant differences were found in *P. oceanica* densities (Molenaar *et al.*, 2006). These results indicated that at shallower depths, *C. taxifolia* increased sedimentation and anoxia within the seagrass beds (Molenaar *et al.*, 2005). Results from this long-term study revealed that the transect densities of *Z. marina* did not differ significantly with increasing depth from the intertidal to shallow subtidal despite unmanipulated *S. muticum* presence along each transect. The effect of *S. muticum* presence on *Z. marina* densities within the permanent quadrats, however, was significant as the ZS treatment had overall lower densities than the ZM treatment. Although no significant differences were found in the sediment samples for either particle sizes or % organic content between the ZS and ZM treatments (see Chapter 2), the covariate GLM indicated significant differences between 'treatments' and 'seasons' within 'years' and a significant effect of mean particle size, suggesting that particle size exerts a significant influence upon determining *Z. marina* densities within the estuary (Fig. 5.2).

Interestingly, despite significant differences between the ZS and ZM treatments, *Z. marina* densities increased annually in both permanent quadrats and transects. The increased vegetative output could have been a result of biotic stress to the increased prevalence of the invasive alga within the estuary (see Fig. 5.3) as found by Pergent *et al.* (2008) in the Mediterranean Sea. Comparing the mean densities of *Z. marina* within the quadrats to the

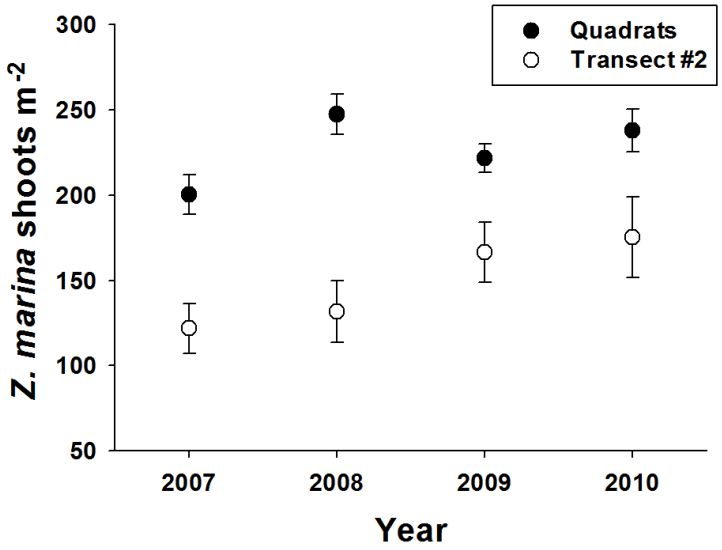


Figure 5.7: Comparison of manipulated vs. unmanipulated (quadrat vs. transect) *Z. marina* densities: mean *Z. marina* shoots m^{-2} per quadrat (n = 160 over 4 years and 2 seasons), irrespective of treatment, and mean *Z. marina* shoots m^{-2} for transect #2 (n = 82 over 4 years and 2 seasons) from the 2007–2010 long-term field study.

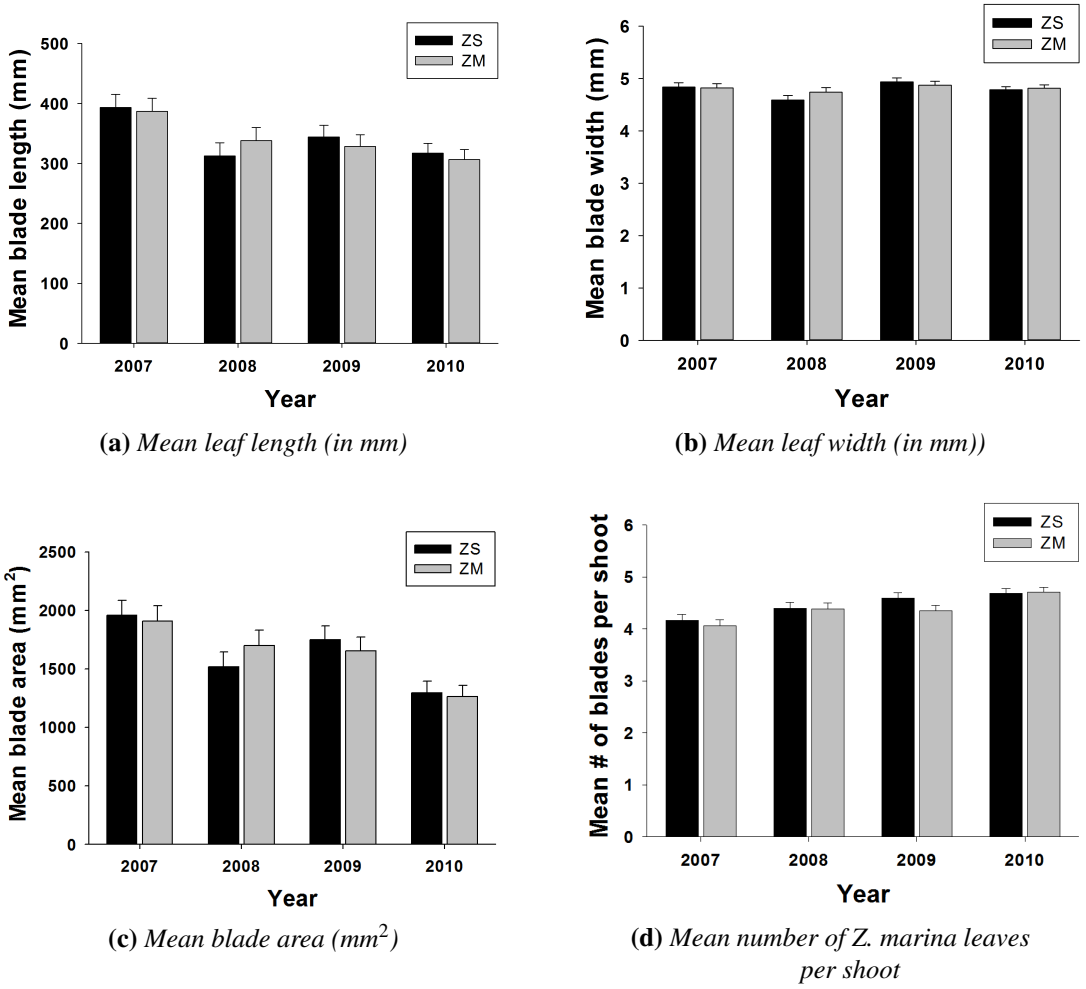
mean densities of *Z. marina* for transect #2 revealed that the unmanipulated transects had overall lower densities than the permanent quadrats (Fig. 5.7). The transects may have had overall lower *Z. marina* densities due to the the presence of mooring scars found within the field site. Few, if any, *Z. marina* shoots were counted within the scars, reaffirming the detrimental effects of anthropogenic disturbances on seagrass survival. Particle size differences due to anthropogenic disturbances may have also been a factor that contributed to the overall lower *Z. marina* densities within the transects.

5.4.2 *Z. marina* vegetative physiognomy

Estuaries may be hostile environments for photoautotrophs, especially when highly turbid. With minimal light requirements, algae only need ~1–3 % of surface irradiance for growth (Lüning & Dring, 1975; Markager & Sand-Jensen, 1992; Palacios & Zimmerman, 2007) whereas seagrasses require from 11 % (Hemminga & Duarte, 2000; Duarte, 2002) to 37 % of surface irradiation (Kenworthy & Fonseca, 1996), with light requirements tightly coupled to their morphology (Middelboe & Markager, 1997; Lee *et al.*, 2007). Although seagrasses can persist for a limited time in low light conditions, photo-adaptive

responses to continuously low irradiance levels are manifested as decreased plant size, shoot density, biomass, rates of leaf production and chlorophyll composition, including *a:b* ratios (Wiginton & McMillan, 1979; Dennison & Alberte, 1982; Peralta *et al.*, 2002; Lee *et al.*, 2007).

S. muticum can grow to five times the average length of the native seagrass, reducing the available light to organisms found under its canopy by 95 % (Critchley *et al.*, 1990; Markager & Sand-Jensen, 1994; den Hartog, 1997; Strong *et al.*, 2006), ultimately leading



(a) *Mean leaf length (in mm)*

(b) *Mean leaf width (in mm))*

(c) *Mean blade area (mm^2)*

(d) *Mean number of Z. marina leaves per shoot*

Figure 5.8: Mean length (mm) (a), width (mm) (b), mean blade area (c) of *Z. marina* leaves and total number of blades per shoot (d) from shoots collected from permanent quadrats between 2007–2010. Quadrat was used as the replicate; n = 400 for each morphometric character over 4 years with 3 seasons per year. Results include spring 2007 and autumn 2010 missing data points, determined by averaging the results from spring 2008-2010 and autumn 2007–2009, respectively and are reported as the annual mean (±SE) (calculated across all seasons).

to their decline by 50–75 % (Britton-Simmons, 2004). However, one possible 'advantage' that *Z. marina* has over *S. muticum* can be attributed to the annual senescence of the lateral fronds of *S. muticum* in late summer/early autumn, leaving only a perennial holdfast to over-winter (Norton, 1977b; Gorham & Lewey, 1984), enabling perennial seagrass beds to capture all available light throughout the autumn and winter months, building up carbohydrate reserves stored within its roots and rhizomes.

Vascular plants can respond and adapt to abiotic and biotic stress by altering their physiology and morphology (Lambers *et al.*, 1998; Hopkins & Hüner, 2004). Although significant seasonal differences were found and not unexpected, results from the long-term field study did not reveal any significant differences between the ZS and ZM treatments for any of the morphometric characters (Tables 5.7, 5.8, 5.9, 5.10, 5.11 and Fig. 5.6). Despite the lack of significant differences, two trends emerged: i) *Z. marina* mean blade length (and subsequent area) decreased over time and ii) the number of blades per shoot increased (Fig. 5.6), even after correcting for the missing data points (Fig. 5.8).

In all years except 2008, *Z. marina* shoots within the ZS treatment had longer blades than those within the ZM treatment, despite the decreasing lengths and mean total blade area over time. This may have been a result of acclimation to shading by *S. muticum* to try and capture more available light. Decreased blade lengths and mean blade area were perhaps a stress response (Peralta *et al.*, 2002) to the increased presence of the invader within the estuary (see Fig. 5.3) as was found by Pergent *et al.* (2008). Their research revealed that the intermediate blade lengths of *P. oceanica* decreased with increased *C. taxifolia* interaction, perhaps indicating acclimation of individual shoots or even adaptation of the population as a whole to the presence of *C. taxifolia*.

Biochemical changes within enzymes and other internal constituents, such as was found in the phenolic production discussed in Chapter 3, can cause a cascade of changes in specific processes such as photosynthesis, the growth rate of entire shoots and the morphology of organs or entire plants (Lambers *et al.*, 1998). Stress acclimation, whether to abiotic or

biotic stress, occurs usually within days to weeks within the individual plant. Although water stress is not a concern for marine macrophytes, water stress may induce a decrease in leaf area within terrestrial plants (Hopkins & Hüner, 2004). This may indicate that other abiotic factors, and potentially biotic factors, can affect the performance and production of biomass within seagrasses.

In the Mediterranean Sea, Pergent *et al.* (2008) found the leaf turnover rates of *P. oceanica* to be faster (i.e., shorter-lived leaves, but with an increased number of leaves produced annually) with increasing *C. taxifolia* interaction. There was no indication of a similar effect on *Z. marina* when in the presence of *S. muticum* as both ZS and ZM treatments had a similar number of blades per shoot across all seasons and all years, although the number of blades per shoot increased over time (Fig. 5.6), irrespective of treatment. Increases in vegetative output can also be a biotic stress response perhaps due to increasing invasive macroalga interaction as found in the Mediterranean (Pergent *et al.*, 2008). To elucidate if these results were a consequence of the presence of *S. muticum* only, further research is required.

In addition to the putative effects of *S. muticum* presence on *Z. marina* densities, further alterations in seagrass morphology and physiology may also result from decreased light availability by epiphytic shading (Phillips *et al.*, 1978; Fourqurean *et al.*, 1995; Moore & Wetzel, 2000) and rapid light attenuation due to increased phytoplankton blooms in eutrophic waters (Phillips *et al.*, 1978; Beer & Koch, 1996; Short & Wyllie-Echeverria, 1996; Rabalais, 2002). As epiphytic algae become more abundant on seagrass blades in eutrophic conditions, seagrasses experience reduced gas and nutrient exchange (Sand-Jensen, 1977; Hemminga & Duarte, 2000; McGlathery, 2001). However, as nutrients are trapped in slowly degrading seagrass tissues (Duarte & Cebrian, 1996), which in turn slows their return to the water column, the potential for rapid growth and turnover of phytoplankton that rely on these nutrients is reduced (Short & Wyllie-Echeverria, 1996; Hemminga & Duarte, 2000). Thus, the presence of seagrass may lessen the occurrence

of potentially harmful phytoplankton blooms that are often initiated in nutrient-enriched waters from either natural processes or anthropogenic events (Short & Wyllie-Echeverria, 1996; Hemminga & Duarte, 2000) due to their capacity for long-term nutrient storage (Risgaard-Petersen *et al.*, 1998; Hemminga & Duarte, 2000; Duarte, 2002). In addition, seagrasses also decrease turbidity through canopy friction and sediment accretion, resulting in increased light availability (de Boer, 2007). This positive feedback mechanism within seagrass meadows is one of the most important ecosystem services that seagrasses provide (de Boer, 2007) and is common wherever seagrasses are found (Koch, 2001).

5.4.3 Eradication and monitoring

In the early 1970s and 1980s, eradication of *S. muticum* was trialled in the Solent Estuary near Southampton (Farnham & Gareth Jones, 1974; Critchley *et al.*, 1986). All attempts at permanent removal of this nuisance species failed. Since that time, *S. muticum* has spread prolifically around European shores. The implications of the invasion of *S. muticum* give cause for concern for the long-term survival of *Z. marina* within the estuary considering the invader's rate of spread, persistence within coastal ecosystems, and negative effects on *Z. marina* in conjunction with increasing anthropogenic disturbances. Although eradication of *S. muticum* may no longer be an option, improved awareness and public education on how the invasive proliferates may help to slow its spread.

An annual monitoring programme such as SeagrassNet (Short *et al.*, 2006; SeagrassNet, 2008) should be implemented within the estuary to track the inter-annual variability of *Z. marina* from naturally occurring expansions and contractions (Reed & Hovel, 2006; Hirst & Attrill, 2008; Jackson *et al.*, 2006), long-term density changes, and the overall meadow health. A monitoring programme such as this may raise local awareness, enhancing the protection of *Z. marina* and the benefits the seagrass provides. In addition, laws prioritising conservation of BAP species and SSSI, SAC and AONB sites (i.e., habitats) over the planning and development system currently in place, which seeks voluntary conservation

agreements with landowners through financial incentives, would enhance and improve the current outlook for species and habitat conservation within the UK.

5.5 Conclusions

This chapter investigated the effects of *S. muticum* presence on the naturally occurring *Z. marina* densities along permanent transects over time and changes in *Z. marina* densities within manipulated permanent quadrats. Results from the long-term field study indicated that *S. muticum* negatively affected *Z. marina* densities within the quadrats, but there was no effect of its presence along the permanent transects. *Z. marina* densities within quadrats and along transects increased annually, as did the number of *S. muticum* thalli. Although no significant differences were found in the vegetative physiognomy of *Z. marina*, blade lengths and subsequently blade area decreased over time for both treatments and the number of blades per shoot increased over time. From these results it appears likely that the presence of *S. muticum* significantly affects *Z. marina* densities, but does not appear to directly influence the vegetative physiognomy of *Z. marina*.

Chapter 6

Invasion Impacts: Epibiotic Assemblage

Abstract

Invasive species can alter coastal ecosystems directly, for example through shading and competition for substrata and nutrients. Indirect effects of invasion may include the creation of dissimilar habitats (i.e., more or less complex in structure than those created/provided by native species), changes in food preferences and availability, alterations in associated epibiota, and potentially, thermal regime shifts. *Z. marina* supports a diverse, yet seasonally-driven epibiotic assemblage. However, with increasing encroachment of *S. muticum* into seagrass meadows, there exists the potential for changes in the epibionts found living on the blades of *Z. marina* where the seagrass and invasive alga are found intermixed as *S. muticum* can also host a diverse assemblage of epibionts. Previous research has shown that ecological problems arise when invasive algae establish themselves in existing habitats, modifying the native habitat architecture, disrupting intricately linked food webs and altering epifaunal communities.

To determine if, and how, the presence of *S. muticum* alters the epibiota of *Z. marina*, seagrass blades were sampled over a four year period every six to eight weeks from early spring to early autumn from 20 permanent 1 m^2 quadrats, with or without *S. muticum* present (ZS and ZM 'treatments', respectively). Epibiota were identified to the most detailed taxonomic level possible and/or functional group, where appropriate. Epiphytes and colonial epifauna were classed as either present or absent whereas all other individual epifauna were counted. PERMANOVA+ analyses revealed significant differences in species/functional groups between treatments ($p = 0.019$) across all years. Changes in epibiota species occurrence and assemblage composition were not reflected as significant differences in total biomass per sample in each treatment, but significant differences were found between years ($p = 0.045$). SIMPER analysis on log(X+1) transformed data revealed a 55.66 % dissimilarity between the two treatments, supporting the hypothesis that

with continued and increased *S. muticum* invasion into *Z. marina* meadows, the epibiota assemblages found on the blades of *Z. marina* are significantly altered. This conclusion is consistent with previous research affirming that invasive macroalgae may have an effect on epibiota assemblages and, potentially, processes within coastal ecosystems.

6.1 Introduction

Invasive species and their effects on 'natural' terrestrial environments have been widely studied with results indicating that when a habitat-forming or modifying species invades, it can have dramatic effects on the native biota (Braithwaite *et al.*, 1989). Our understanding of impacts of invasive plants and algae within coastal ecosystems pales in comparison, with few studies concerning how and to what extent these invaders modify the biodiversity and ecosystem services provided by native marine communities (Wikström & Kautsky, 2004). As the number of reported marine invasions increases due to the continued rise in global transportation (Bax *et al.*, 2003; Lodge *et al.*, 2006; Keller *et al.*, 2011) both in terms of human travel and cargo shipping, it is unlikely that any coastal ecosystem will escape the effects of invasive species.

Seagrass meadows provide a heterogeneous, complex habitat matrix and play host to an extensive and diverse range of biota found on and amongst their leaves including cyanobacteria (Novak, 1984), diatoms (Novak, 1984; Thursby & Davis, 1984), epiphytic algae (Bologna & Heck Jr., 1999), and sessile as well as mobile epifauna (Orth *et al.*, 1984; Hall & Bell, 1988; Vázquez-Luis *et al.*, 2008). They also provide shelter for larval fish and egg deposition substrata for many different organisms such as molluscs and fish (Bell & Pollard, 1989; Hemminga & Duarte, 2000; Jackson *et al.*, 2006). Research has shown a positive correlation between seagrass biomass / available surface area and macroinvertebrate abundance / species richness. The greater the complexity, the higher the species abundance (Parker *et al.*, 2001), but epibiota species richness and abundance can vary markedly between locations and from season to season (Lee *et al.*, 2001; Jones &

Thornber, 2010). Marine macrophytes, such as *S. muticum* and other phaeophytes can also host a diverse assemblage of epibionts due to their heterogeneous morphology (Wikström & Kautsky, 2004; Jones & Thornber, 2010). Ecological problems arise when non-native algal species establish themselves in 'new' habitats, altering the native habitat architecture and possibly disrupting intricately linked food webs (Valentine *et al.*, 2002). Previous research has shown that invasive macroalgae have the potential to change epifaunal communities (Wikström & Kautsky, 2004; Vázquez-Luis *et al.*, 2008; Lutz *et al.*, 2010; Gestoso *et al.*, 2011a), and thereby alter entire ecosystems (Vázquez-Luis *et al.*, 2008; Lutz *et al.*, 2010). With its high fecundity and rapid annual growth, *S. muticum* has the potential to adversely affect *Z. marina* and its associated epibiotic assemblages wherever it is found (Critchley *et al.*, 1986).

Although a range of research has been conducted regarding the effects of invasive species on epibiota within coastal ecosystems, no reported studies have examined how the presence of *S. muticum* influences the epibiotic community found on the blades of *Z. marina*. Therefore, the aim of this four year field study was to investigate how *S. muticum* invasion affects the associated epibiota of *Z. marina* within the Salcombe-Kingsbridge Estuary through a comparison of species assemblages found on *Z. marina* blades in the presence and absence of the invasive macroalga. The following questions were addressed: i) do species abundances of both flora and fauna decrease in the presence of *S. muticum*? ii) does the biomass of the epibiota decrease when *S. muticum* is present? and iii) if species assemblages are different, how do they differ? With the introduction and establishment of a dominant macrophytic alga, the magnitude of the effects from such an invasion will depend on the ability of the encrusting and mobile epibiota of *Z. marina* to withstand i) the increased shading provided by the increased architectural complexity of *S. muticum* (Hauxwell *et al.*, 2001; McGlathery, 2001; Britton-Simmons, 2004; Strong *et al.*, 2006) and ii) its chemical deterrents (Amsler & Fairhead, 2006; Plouguerne *et al.*, 2006) providing colonisation of the invasive alga is possible.

6.2 Methods

6.2.1 Epibiota

Epibiota samples were collected every six to eight weeks in the Salcombe-Kingsbridge Estuary from March until September over four years (2007–2010) from 20 permanent 1 m^2 quadrats, ten for each treatment (ZS and ZM), as described in Chapter 2. Samples were grouped by season; March–May samples were 'spring', June–August samples were 'summer' and September–October samples were 'autumn'. Shoots were only collected during these months due to the timing of *S. muticum* presence and its active growth within the estuary. *Z. marina* blades were cut directly above the meristematic tissue from three shoots within each experimental quadrat. The blades from each sample were subdivided, with one half preserved in 80 % IMS (Industrial Methylated Spirit) for epibiota analysis and the other half used for phenolic assays (see Chapter 3). Morphometric data (length, width, area and the number of blades per shoot) were recorded from all blades in each sample (see Chapter 5).

Epibiota sample containers were swirled gently before opening, the blades removed and the remaining IMS filtered through a pre-weighed 25 mm, 45 μm paper filter (Fisher Scientific, Loughborough). Epibiota collected on filters were viewed under a dissecting microscope (Meiji Techno UK, Axbridge) at 40x magnification and identified to functional group (algae) or functional type and if possible, species and counted. After identification, each filter paper was placed in a Gallenkamp IH-150 drying oven at 50 °C for 24 hours. Blades were measured (length and width), sectioned and analysed individually in 8 cm sections (Jacobs *et al.*, 1983), up to a total of 40 cm per blade. Blades were analysed according to location along the length: base (basal 8 cm), tip (top 8 cm, if present) and middle region (up to three 8 cm sections (24 cm maximum)). All blade sections from each sample were viewed on the same pre-weighed, labelled 9 cm paper filter (Fisher Scientific) in a 9 cm Petri dish to collect total sample biomass data. Using a combination of a compound Olympus BHB and Meiji Techno dissecting microscopes, organisms from

one side of each blade section were identified as described above, to functional group (algae) or functional taxonomic unit (FTU) (small invertebrates) and if possible, species.

All epiphytic algae were classed as either present or absent. If present, they were classed into six functional groups: filamentous, corticated filamentous, foliose, corticated foliose, saccate and coralline, after Steneck & Dethier (1994) and Saunders *et al.* (2003). If possible, epiphytes were classified to genus level or other unique identifying features, to allow for more detailed analyses. Epifauna were classed as present or absent if colonial and counted if non-colonial.

After organisms were identified, both sides of the blade were gently scraped onto the large filter to remove epibiota from the blade surface using a razor blade (Parker *et al.*, 2001; Tomas *et al.*, 2005; Jaschinski & Sommer, 2008). Once the entire sample was processed, the large filter within the Petri dish was dried at 50 °C for 24 hours. Once dried, the small and large filters for each sample were weighed, summed together, and used to calculate the biomass per total area of sample. The mean shoot area (mm^2) was multiplied by the mean number of shoots within each quadrat (see Chapter 5: Methods) to calculate the mean area per quadrat. This formed a proxy for total available colonisation space on *Z. marina* blades per 1 m^2 quadrat (see Fig. 6.1).

Data were summed to sample level as each sample contained one or more blades cut into one or more sections and total epibiota species abundances were calculated as the number of individuals per total surface area (both sides of blade) per sample. Confounding resulting from non-standardised sample areas in different shoots was a possibility, but unlikely given the lack of significant differences between 'treatments' in mean blade area revealed in Chapter 5 (Table 5.9, Fig. 5.6). Moreover, a GLM revealed no differences between the mean blade areas of the material analysed for epibiota ($F_{1,6011} = 1.72$, $p = 0.19$).

6.2.2 Data analysis

Epibiota species

Data were log(X+1) transformed to reduce the influence of abundant taxa (Clarke & Warwick, 2001) prior to analysis in PRIMER v6.1.13 with PERMANOVA+ v1.0.3. The severe transformation eliminated problems due to recording the abundance data on different scales (presence/absence vs. actual counts) (K. R. Clarke, personal communication, 2011). A Bray-Curtis similarity resemblance matrix was constructed and data were analysed using a four-factor PERMANOVA+ design. 'Treatment' and 'year' were defined as fixed factors with two (ZS and ZM) and four (2007, 2008, 2009 and 2010) levels respectively. Due to the lack of orthogonality, both 'season' with two levels (spring and autumn) nested within 'year' and 'quadrat' with 20 levels (quadrats 1–20) nested within 'treatment', were set as random factors. Resolving non-orthogonality in this manner made it possible for 'year' to be designated as a fixed, rather than random, factor in the PERMANOVA+ design. Type III Sums of Squares, unrestricted permutations of raw data and 9999 permutations were set as design parameters (Anderson *et al.*, 2008). Pairwise tests of the factor 'treatment' within the 'treatment * year' interaction term were also calculated. The proportional contributions of different epibiota species to the dissimilarity between treatments were investigated using SIMPER (Clarke & Warwick, 2001), with an 80 % cut-off.

Epibiota biomass

Data were analysed using SPSS 19, but non-conformity to assumptions of homogeneity of variances were indicated by results of Levene's Test; examination of unstandardised analytical residuals indicated need for transformation. Equality of variances could not be improved by transformation, therefore further analysis employed a non-parametric approach using PRIMER v6.1.13 with PERMANOVA+ v1.0.3. Total biomass data were summed to sample level and a Euclidean distance resemblance matrix was constructed to test the same PERMANOVA+ four-factor design described above. Type III Sums of Squares, unrestricted permutations of raw data and 9999 permutations were set as design

parameters. Due to the resulting non-significant *p*-value for 'quadrat' (> 0.7), 'quadrat' and all terms including this factor (Quadrat(Treatment), Year * Quadrat(Treatment) and Season(Year) * Quadrat(Treatment)), were pooled to simplify the model and to increase the power to detect any effects for this nested factor. Pairwise tests of the factor 'treatment' within the 'treatment * year' interaction term were also calculated.

6.3 Results

6.3.1 Epibiota species

A total of 226,798 individuals or occurrences, belonging to 87 taxa or FTUs, were identified on the blades of *Z. marina* from 1135 samples collected over four years (2007–2010). Copepods, nematodes and foraminifera were consistently the most abundant groups, regardless of treatment (see Appendix Table A.1). PERMANOVA+ analysis of the summed to sample level for species taxa / functional group data revealed significant differences between 'treatments', 'seasons' within 'years' and 'quadrats' within 'treatments', but no significant differences were found between 'years' (Table 6.1). Pairwise comparisons for the factor 'treatment' within the interaction term 'treatment * year' indicated the significant differences found between 'treatments' were more prevalent in 2008 (Table 6.2). SIMPER analysis revealed the average similarities between the ZS (43.27 %) and ZM (45.57 %) treatments (Table 6.3); the average dissimilarity within the ZS and ZM treatments was 55.66 % (Table 6.4). The results indicated a large number of species (20 out of 87 total taxa) each contributed a small amount to the dissimilarity between the two treatments, which may have been due in part to the severe log(X+1) transformation. Of the 20 different taxa or FTUs contributing to the dissimilarities, 10 taxa/FTUs had higher densities in the ZM treament, seven were more abundant in the ZS treatment and three had approximately equal abundances (Table 6.4). Results from the calculation of the mean area per quadrat (mm^2) indicated a seasonal increase as expected (Fig. 6.1).

Table 6.1: PERMANOVA+ results for epibiota species data from 2007–2010. Sample pot was the replicate; n = 564 for the ZS treatment and n = 571 for the ZM treatment over 4 years with 3 seasons per year.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	4110.6	4110.6	2.826	**0.019**
Year	3	1.4975E5	49918	0.986	0.501
Season(Year)	7	3.404E5	48629	39.62	**< 0.001**
Quadrat(Treatment)	21	42095	2004.5	1.629	**< 0.001**
Treatment * Year	3	5111	1703.7	1.094	0.310
Treatment * Season(Year)	7	10487	1498.1	1.221	0.168
Year * Quadrat(Treatment)	54	62846	1163.8	0.952	0.677
Season(Year) * Quadrat(Treatment)	125	1.5341E5	1227.2	0.971	0.717
Res	913	1.1542E6	1264.1		
Total	1134	1.8727E6			

Table 6.2: PERMANOVA+ pairwise comparisons for epibiota species data for the factor 'treatment' within the interaction term 'treatment * year' from 2007–2010. Sample pot was the replicate; n = 564 for the ZS treatment and n = 571 for the ZM treatment over 4 years with 3 seasons per year.

Year	Groups	t	P (perm)
2007	ZM, ZS	1.243	0.126
2008	ZM, ZS	1.439	**0.012**
2009	ZM, ZS	0.710	0.946
2010	ZM, ZS	1.517	0.067

Table 6.3: PRIMER SIMPER results for the epibiota species data from the 2007–2010 long-term field study. Sample pot was the replicate; n = 564 for the ZS treatment and n = 571 for the ZM treatment over 4 years with 3 seasons per year.

ZM Avg. Similarity = 45.57 %					
Species	Avg. Abund	Avg. Sim	Sim/SD	Contribution %	Cumulative %
Diatom unident.	1.79	7.96	2.34	17.47	17.47
Copepods	2.65	5.67	1.08	12.45	29.92
Nematode	2.08	4.42	0.93	9.70	39.62
Non-corticated filament	1.28	3.98	1.21	8.73	48.35
Sponge	1.12	3.61	0.86	7.92	56.27
Aora gracilis	1.36	3.54	0.86	7.77	64.04
Porcellidium viridis	1.02	2.73	0.86	6.00	70.03
'Scalloped shell' foram	1.47	2.38	0.73	5.23	75.27
Corticated filament	0.98	2.31	0.90	5.07	80.34
ZS Avg. Similarity = 43.27 %					
Species	Avg. Abund	Avg. Sim	Sim/SD	Contribution %	Cumulative %
Diatom unident.	1.78	8.19	2.11	18.92	18.92
Copepods	2.39	4.42	0.86	10.21	29.14
Non-corticated filament	1.26	4.04	1.17	9.33	38.47
Nematodes	1.96	4.01	0.90	9.27	47.74
Sponge	1.12	3.84	0.83	8.87	56.61
Aora gracilis	1.27	3.09	0.79	7.14	63.74
Porcellidium viridis	1.03	2.79	0.77	6.45	70.19
Corticated filament	0.94	2.04	0.80	4.71	74.91
Saccate algae	0.87	2.00	0.75	4.63	79.54
'Scalloped shell' foram	1.29	1.77	0.61	4.08	83.62

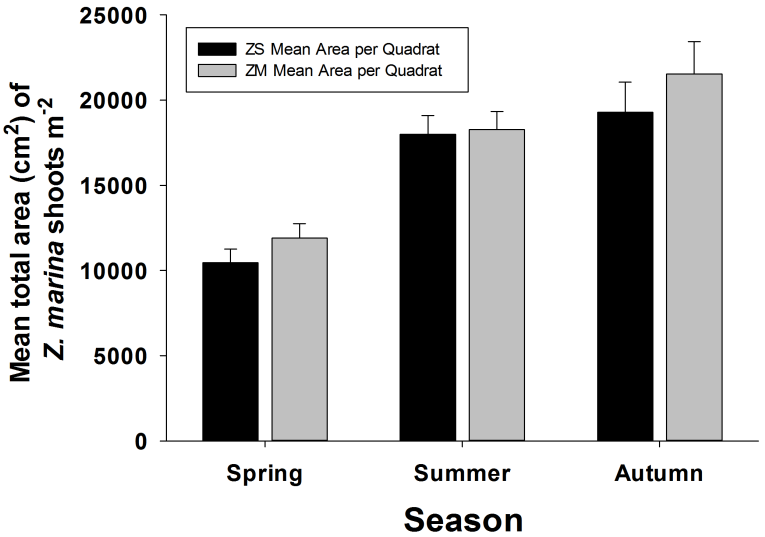


Figure 6.1: The mean total blade area (cm^2) m^{-2} quadrat for *Z. marina* by treatment and season calculated across all years (2007–2010). Quadrat was used as the replicate; n = 360 for each treatment over 4 years with 3 seasons per year.

Table 6.4: PRIMER SIMPER results for average dissimilarity between ZS and ZM treatments for the epibiota species data from the 2007–2010 long-term field study. Average dissimilarity between the two groups = 55.66 %. Sample pot was the replicate; n = 564 for the ZS treatment and n = 571 for the ZM treatment over 4 years with 3 seasons per year.

Species/FTU	ZM Avg. Abund	ZS Avg. Abund	Avg. Diss	Diss/SD	Contribution %	Cumulative %
Copepods	2.65	2.39	5.87	1.33	10.54	10.54
Nematodes	2.08	1.96	4.68	1.27	8.41	18.95
'Scalloped shell' foram	1.47	1.29	3.68	1.22	6.62	25.57
Aora gracilis	1.36	1.27	3.31	1.03	5.95	31.52
Ostracods	0.87	0.79	2.56	0.98	4.59	36.11
Porcellidium viridis	1.02	1.03	2.46	1.07	4.42	40.53
Sponge	1.12	1.12	2.24	0.98	4.02	44.55
Non-corticated filament	1.28	1.26	2.21	1.01	3.98	48.53
Corticated filament	0.98	0.94	2.18	1.15	3.92	52.45
Saccate algae	0.85	0.87	2.12	1.10	3.81	56.26
Flocculate bacteria	0.58	0.59	1.93	0.93	3.47	59.73
Copepod nauplii	0.62	0.62	1.77	1.05	3.19	62.92
Polysiphonia sp.	0.55	0.55	1.62	1.02	2.90	65.82
Bryozoan colony	0.39	0.44	1.36	0.88	2.44	68.26
Diatom unident.	1.79	1.78	1.27	0.75	2.27	70.53
Stolon tube-like alga	0.36	0.33	1.25	0.80	2.24	72.77
Ceramium sp.	0.37	0.38	1.20	0.89	2.16	74.93
Single, multi-cellular blade (foliose)	0.36	0.38	1.17	0.91	2.11	77.04
Nereid worm	0.27	0.31	1.00	0.67	1.79	78.83
Licmophora sp. (pennate diatom)	0.27	0.22	0.96	0.59	1.72	80.55

Table 6.5: PERMANOVA+ analysis of total sample biomass data with pooled 'quadrat' terms from the 2007–2010 long-term field study. Pooled = quadrat nested within year (Quadrat(Treatment), Year * Quadrat(Treatment) and Season(Year) * Quadrat(Treatment)). Sample pot was the replicate; n = 564 for the ZS treatment and n = 571 for the ZM treatment over 4 years with 3 seasons per year.

Source	df	Type III SS	MS	Pseudo-F	*p* (perm)
Treatment	1	557.94	557.94	1.165	0.363
Year	3	4345.4	1448.5	1.457	**0.045**
Season(Year)	7	7045.3	1006.5	0.949	0.567
Treatment * Year	3	2079.3	693.1	1.602	0.068
Treatment * Season(Year)	7	3245.2	463.6	0.437	0.998
Pooled	198	1.9091E5	964.21	0.519	0.870
Res	913	1.6977E6	1859.5		
Total	1132	1.9065E6			

6.3.2 Epibiota biomass

PERMNAOVA+ results using the pooled factor 'quadrat' indicated no significant differences between 'treatments' or 'seasons' within 'years', but significant differences were revealed between 'years' for the total biomass of each sample (Table 6.5).

6.4 Discussion

6.4.1 Epibiota species/FTU abundances

The main purpose of this study was to consider potential shifts in the epibiotic assemblage associated with *Z. marina* as a direct consequence of *S. muticum* invasion. Seagrasses, as well as large macrophytic algae, can provide organisms food and shelter from stressful conditions associated with intertidal life (Orth *et al.*, 1984; Jackson *et al.*, 2006; Gestoso *et al.*, 2011a). For example, seagrass meadows and long-lived kelp species can baffle currents, resulting in increased concentrations of particulate food (Orth *et al.*, 1984). They also create refuges for prey organisms, decreasing predation thanks to the greater morphological complexity of these marine macrophytes compared to bare sand (Orth *et al.*, 1984; Taylor & Cole, 1994). As shown in Chapter 5, however, *Z. marina* densities

have declined in the presence of *S. muticum*. This change may result in alterations in species or taxa dominance within the epibiota assemblage (Wikström & Kautsky, 2004; Chapman *et al.*, 2005; Vázquez-Luis *et al.*, 2008; Jones & Thornber, 2010) found within the Salcombe-Kingsbridge Estuary and elsewhere where the two species are found intermixed. Large habitat-forming invasive seaweeds may harbour entirely different epibionts (Schmidt & Scheibling, 2006), potentially resulting in modifications to the structure and function of epibiotic assemblages found within coastal ecosystems (Lutz *et al.*, 2010; Gestoso *et al.*, 2011b).

The habitat diversity hypothesis suggests that the species-area relationship changes with the sampling area size (Shen *et al.*, 2009); species diversity then increases with increasing spatial area and is controlled by the availability of differing habitats for colonisation by different species (Hirst & Attrill, 2008; Shen *et al.*, 2009). Previous research has reported epibiotic species richness and composition change when habitat complexity increases (Parker *et al.*, 2001; Gestoso *et al.*, 2010; Jones & Thornber, 2010). Although the study area (i.e., the permanent quadrats) did not increase in size, *per se*, the potential colonisation space available within the permanent quadrats was increased in the ZS treatment due to the presence of *S. muticum*. Buschbaum *et al.* (2006) and Gestoso *et al.* (2010) found that *S. muticum* increased habitat heterogeneity and substratum availability, and thus supported greater epibiota species richness than native algae. Schmidt & Scheibling (2006) also found higher epibiont diversity on the invasive *Codium fragile* subsp. *tomentosoides* (van Goor) P. C. Silva compared to the native kelps, *Laminaria sp.* in Canadian waters, whereas Lutz *et al.* (2010) found that species richness did not differ between the native and invasive species of macroalgae studied in Australia. This suite of results confounds the issue of how epibiotic assemblages respond to the presence of invasive macrophytes.

Results from this long-term study indicated that the epibiota assemblage composition differed between treatments in terms of abundance rather than species/FTU composition. *Z. marina* shoots within the ZS treatment had overall lower species abundances in the presence

of *S. muticum* than those in quadrats without the invasive alga (see Appendix Table A.1) and had marginally lower similarities between samples than the ZM treatment (Table 6.3). Very few species/FTUs were exclusively found on blades from one treatment or the other; nine species/FTUs were found only within the ZS treatment and five species/FTUs were found only in the ZM treatment. Significant seasonal differences were also revealed for the epibiota assemblages (Table 6.1), but this result was not unexpected as epibiotic organisms can vary across time and space due to differences in life histories (Gestoso *et al.*, 2010) and area available for colonisation (Fig. 6.1). SIMPER analysis identified the epibiotic species responsible for the similarities between treatments and those accounting for the differences. These results indicated that a small number of epibiota species/FTUs (9 total: see Table 6.4) accounted for over 50 % of the differences between the two treatments. Interestingly, despite the differences within the taxa present, no significant differences were revealed in biomass between treatments or seasons. The lack of differences in biomass may indicate that colonisation space was at a premium with different, yet similar-sized epibiotic species occupying any available space.

6.4.2 Habitat architecture

Habitat architecture drives faunal composition at the micro-scale whereas habitat size drives faunal abundances (Christie *et al.*, 2009). Despite the morphological differences between *Z. marina* and *S. muticum*, mobile invertebrates are generally non-specific in choosing a macrophyte host as distribution of these organisms appears to be largely determined by either food or habitat preferences (Hacker & Steneck, 1990; Taylor & Cole, 1994; Wikström & Kautsky, 2004). Seagrass beds with high shoot and blade densities as well as dense root-rhizome matrices provide rich habitat with high diversity (Attrill *et al.*, 2000; Saunders *et al.*, 2003). Comparisons made between seagrass species of similar biomass, but with significantly different surface areas, indicated amphipod abundances were higher on the plants with the greatest surface area (Orth *et al.*, 1984).

Results from this field study found at least 87 different epibiota species/FTUs, indicating that *Z. marina* plays a critical role in providing habitat or substrata for many different organisms within the estuary. The results from Chapter 5 indicated that the mean total surface area shoot^{-1} m^{-2} for *Z. marina* declined over the duration of the long-term field study as a result of a decrease in blade length (Fig. 5.6). With decreasing area available for epibiota to colonise, changes within the epibiotic assemblage of *Z. marina* may already be under way. Organisms that rely upon the seagrass may be affected due to loss of substrata for egg deposition (Wikström & Kautsky, 2004) and loss of preferred food and habitat (Buschbaum *et al.*, 2006; Gestoso *et al.*, 2011a) due to declining *Z. marina* densities (see Chapter 5) and decreasing blade lengths coupled with increasing numbers of *S. muticum* thalli within the estuary. Although *S. muticum* with its larger size and numerous branchlets can provide an increase in habitat availability and structure, any habitat enhancements are ephemeral due to its annual senescence and 'disappearance' from the UK coastline.

6.4.3 Benefits of *S. muticum* invasion?

Norton & Benson (1983) found only ~43 vagile species living on the fronds of *S. muticum* in research carried out in the Northeast Pacific, but previous work by Withers *et al.* (1975) found 74 species that consisted mostly of sedentary and encrusting macroscopic organisms from samples collected in the English Channel. Norton & Benson (1983) found similarities between the fauna that inhabited *Z. marina* beds and the fauna found on *S. muticum* and proposed that the seagrass beds 'restocked' the stands of *S. muticum* around Friday Harbor, WA. Although epibiota species/FTUs were not counted for *S. muticum* during the long-term study, an increasing number of studies have been conducted regarding the effect of invasive species on native flora and fauna, a large proportion of which focused on how invasive species alter epiphytic assemblages either through habitat modification or feeding preferences (Wikström & Kautsky, 2004; Gestoso *et al.*, 2010; Jones & Thornber, 2010; Lutz *et al.*, 2010). It may be worth considering that not all marine invasions are detrimental.

In general, invasion biology research has focused on the negative aspects of the arrival of non-native species into existing communities. However, invasive species have the potential to offer new or additional resources within their new environs, facilitating distribution and/or increased abundances of native and other species (Simberloff & Von Holle, 1999; Williams, 2007; Jones & Thornber, 2010). Emerging evidence has shown that some non-indigenous species can facilitate native species through habitat modification, trophic subsidy, pollination, competitive release and predatory release (see Rodriguez, 2006, and references therein). Although not tested in this study, previous research has shown that *S. muticum* offers suitable habitat for many invertebrates (Gestoso *et al.*, 2011b) and fish. For example, Polte & Buschbaum (2008) found that native pipefish in the Wadden Sea are promoted by the presence of *S. muticum*. Benthic organisms may also reap the benefits of invasive species presence as suggested by Vázquez-Luis *et al.* (2008) who found that detritus from the invasive *C. racemosa* facilitated changes in species assemblages due to its ability to persist year round in the Mediterranean Sea. This benefit may not hold true in the case of *S. muticum*, however, as its biomass appears to simply disappear off-shore due to its annual senescence in late summer/early autumn or breaks down completely as it never is found washed up onshore (author's own observation). Despite increases in epibiota species richness, Casas *et al.* (2004) found that *Undaria pinnatifida's* (Harvey) Suringar presence was associated with a dramatic decline in species richness and diversity of native seaweeds in Nuevo Gulf, Argentina. Unfortunately, species richness, diversity and evenness could not be calculated for this long-term study as the organisms were not enumerated on the same scales (presence/absence vs. actual counts).

6.4.4 Phenolic effects

Phlorotannins are considered to be anti-herbivory agents due to their unpalatability (Harrison, 1982; Amsler & Fairhead, 2006; Vergés *et al.*, 2007). For example, phenolic content within *S. muticum* ranges from 25 mg g^{-1} DW in the winter to > 50 mg g^{-1} DW in the summer (Gorham & Lewey, 1984) or ~10–30 % of its dry weight (Lobban & Harrison,

1994; Targett *et al.*, 1995; Amsler & Fairhead, 2006). Feeding experiments carried out in Portugal indicated that macro- and mesoherbivores preferred native seaweeds over the introduced *S. muticum* (Monteiro *et al.*, 2009). *Aplysia punctata* (Cuvier, 1803) grazed on *S. muticum*, but preferred the native red alga, *Osmundea osmunda* (S. G. Gmelin) K. W. Nam & Maggs, 1994 (Monteiro *et al.*, 2009). Results described in Chapter 3 established that phenolic content for both caffeic and tannic acids within the leaves of *Z. marina* decreased in the presence of *S. muticum*. Although useful against herbivory, previous research has reported that phenols found in *Z. marina* failed to deter settlement of epiphytic microalgae and invertebrates, as both groups formed dense assemblages on the blades by mid- to late summer (Harrison & Durance, 1989). As there were significant differences in the phenolics between the ZS and ZM treatments, it could be suggested that the lower phenolic content of the *Z. marina* shoots in the ZS treatment, for both the permanent quadrats and laboratory treatments, was not the driving factor that led to the differences found between treatments as *Z. marina* shoots within the ZM treatment had higher epibiota abundances.

6.4.5 Potential effects of *Z. marina* loss

Differences between the life cycles of *Z. marina* and *S. muticum* (perennial vs. annual) may also adversely affect epibiotic assemblages. Loss of *Z. marina*, whether through *S. muticum* competition or anthropogenic disturbances, may be detrimental to the native epibiotic assemblages due to species dispersal limitations as postulated in the unified neutral theory of biodiversity and biogeography (Hubbell, 2001). If *S. muticum* replaces *Z. marina* and becomes the dominant macrophyte within the estuary, maintenance of the epibiota assemblage may not be possible even with the provision of the increased complexity and heterogeneity of *S. muticum* due to the annual senescence of its vegetative thallus (Norton, 1977b; Gorham & Lewey, 1984; Buschbaum *et al.*, 2006). Epiphytes can play a critical role in the mean annual net production within seagrass meadows, with some epiphyte productivity as high as 20 % (i.e., 200 g C m^{-2} yr^{-1}) as reported in Florida and about 25

% of the annual production in *Thalassia testitudinum* Banks ex König, 1805 meadows off the North Carolina coast (Lewis III, 1982). Therefore, loss of critical epiphytic sustrata such as *Z. marina* can greatly influence the overall mean net producation within seagrass ecosystems and the herbivores and foragers that rely on the presence of *Z. marina*.

6.5 Conclusions

This chapter developed a framework to detect changes in the epibiotic assemblage of *Z. marina* in the presence or absence of *S. muticum*. Results from this long-term field study have shown that the overall biomass present on the blades is not significantly affected by the presence of *S. muticum* despite significant differences in epibiota assemblages between the ZS and ZM treatments. These findings therefore emphasise the need for further investigation into the changes within the epibiota species to determine if species richness and diversity are enhanced or lowered in the presence of *S. muticum*. Although the presence of *S. muticum* may increase the habitat and heterogeneity within the estuary, these benefits are restricted to six or seven months a year due to the ephemeral nature of the invasive alga.

Chapter 7

General Discussion

The overall aim of this study was to determine to what extent the presence of *S. muticum* affects the biochemistry, physiology and vegetative physiognomy of *Z. marina* and how these effects, in addition to the invader's presence, potentially influence the associated epibiota of *Z. marina*. Here I summarise the main findings of each chapter, highlight some of the issues faced while undertaking this project, set my findings within a wider context and discuss avenues of future research.

7.1 Anthropogenic disturbances

Seagrasses are wide-ranging and found bordering all continents except Antarctica, but are in decline worldwide. Great changes are occurring in the marine environment due to rapid globalisation and coastal development (Short & Burdick, 1996; Walker & Kendrick, 1998), increasingly marginalising seagrasses and the services they supply (Short & Wyllie-Echeverria, 1996). Seagrasses are vulnerable to the deleterious effects of climate change, fisheries, pollution, coastal development and invasive species. These combined factors have led to a 29 % loss of the known extent of seagrasses since the late 1800s, with seagrass meadows ultimately losing ~7 % of total global area annually.

It is widely accepted that humans are at the root of many species introductions (Carlton, 1989; Mack, 1996; Boudouresque & Verlaque, 2002; Bax *et al.*, 2003; Williams, 2007) and that these introductions have affected ecosystems and landscapes globally (Vitousek *et al.*, 1997). Whether initial introductions were accidental or deliberate, biological invasions have been facilitated by mechanisms such as shipping, fisheries (including aquaculture) and the escape of ornamentals. In the United States, for example, nearly 89 % of all coastal invasions have been attributed to the combined effects of shipping and fisheries (Ruiz *et al.*, 2000), further increasing the pressure on coastal ecosystems as humans come

to depend more heavily on marine resources. Although seagrass meadows are naturally disturbed and fragmented via wave action, currents and bioturbation (Reed & Hovel, 2006), intensified fragmentation may occur with continued unchecked mechanical damage caused by increased boating (anchoring, mooring, propeller scars) (Hastings *et al.*, 1995; Short & Wyllie-Echeverria, 1996) and fishery activities (dredging) (Erftemeijer & Lewis, 2006; Reed & Hovel, 2006). Once seagrass is removed and scars appear within the beds, scars can become colonised by *S. muticum*, making it harder for seagrass to regrow (den Hartog, 1997).

Although not tested during the long-term field study, it was apparent through visual observations that the mooring and anchoring scars within the *Z. marina* meadow were detrimental to the cohesiveness of the seagrass bed through continued erosion of the scar edges. In addition, rapid colonisation of the scars by *S. muticum* was also seen, as dense 'forests' of the invasive macroalga formed annually within the scars, allowing very little light penetration below their canopy. Eradication of *S. muticum* was trialled in the 1980s (Critchley *et al.*, 1986), but was unsuccessful. However, as most of *S. muticum* thalli within the scars are attached to small stones or shells, *S. muticum* removal from the *Z. marina* beds may be possible with concentrated effort. This, coupled with stricter policies governing placement and establishment of moorings and anchorage under UK and EU laws, may enhance the capacity of *Z. marina* within the Salcombe-Kingsbridge Estuary to recover after perturbation.

7.2 Abiotic factors

Worldwide loss of seagrass meadows mediated through direct and indirect anthropogenic changes in nutrient-loading was witnessed throughout the 20^{th} century. These losses have continued into, and are on the rise in the 21^{st} century (Short & Wyllie-Echeverria, 1996; Duarte, 2002). Management of eutrophication will determine whether or not seagrasses will continue to inhabit (or re-inhabit) future CO_2-rich waters. The aim of Chapter 2,

therefore, was to investigate the abiotic factors within the Salcombe-Kingsbridge Estuary and report the ambient conditions in which the long-term field study was conducted. Nutrient concentrations for NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} revealed inter- and intra-annually variability, possibly linked to the seasonal autotrophic drawn-down, but there was no indication that eutrophic conditions occurred for any measurable duration throughout the long-term study.

Results of sediment analyses indicated sediment samples were mainly composed of fine sand (> 93 %), making it ideal for *Z. marina* colonisation. The presence of *S. muticum* had no effect on particle size found within the experimental quadrats, but seasons differed significantly for both particle size and % organic content. The seasonal effect may have been due to decreased sediment stability related to lower *Z. marina* densities and the loss of *S. muticum* biomass throughout the winter months. Winter storms in conjunction with a decrease in standing biomass may alter the sediment composition and structure (i.e., particle size) due to increased wave action.

7.3 Phenolic compound production

In the early 1980s, the idea of "talking trees" (i.e., the concept of defence induction in plants via volatile transmissible signals) emerged (Baldwin & Schultz, 1983). Baldwin & Schultz (1983) described the elicitation of responses to herbivory via soluble signals produced in damaged trees and then 'communicated' to nearby or adjacent trees to prevent further herbivory. These results, in addition to results from several other studies, were heavily criticised by other authors (see Fowler & Lawton, 1985). Despite the criticism, there is evidence of communication between seaweeds and their associated fauna exemplified by tritrophic linkages between plants, herbivores and their predators (Coleman *et al.*, 2007). This research builds on past results, carrying forward the idea that the presence of the invasive macroalga, *S. muticum*, can elicit a biochemical response within *Z. marina*.

The aim of Chapter 3 was therefore to analyse how the invasion of *S. muticum* into *Z.*

marina meadows might change the seagrasses biochemistry, using phenolic compounds of caffeic and tannic acids as indicators. Results from the long-term field study and multiple laboratory experiments indicated that *S. muticum* invasion is not benign as the production of phenolic content is suppressed in the presence of *S. muticum* in both the field and laboratory experiments. Nutrient limitation may potentially have influenced macrophyte biology and biochemistry, but no effects of nutrient limitation were found in the 2011 study suggesting that phenolic production within the *Z. marina* shoots, or lack thereof, was not a direct result of a Redfield ratio imbalance.

Signalling through the production of inceptive chemicals such as phenolics, may be just one mode in which plants communicate. Release of water-soluble phenolic compounds into the water column from seagrass tissue may not deter or limit an invading alga (Zapata & McMillan, 1979; McMillan *et al.*, 1980), as phenolics can quickly dissipate within the water column. A more effective delivery method would be to release phenolic compounds into the sediment (Zapata & McMillan, 1979) via roots and rhizomes, but as *S. muticum* is a non-rhizomatous alga, any allelopathic defences produced by *Z. marina* may have little influence in directly deterring the continued spread of *S. muticum*. Given the apparent conservation of pathways producing phenols in phaeophytes and land plants, and evidence for common transduction pathways associated with the octadecanoid signalling pathway common to both (e.g. Coleman *et al.*, 2007), it is perhaps unsurprising that evidence for allelopathic consequences of close juxtaposition of the alga and the angiosperm. For this reason, further research into the exact pathways or signal transduction mechanisms underpinning this 'communication' in the marine environment are needed.

Unlike the findings for *P. oceanica*, which showed an increase in phenolic production with increasing invasive macroalgal interactions, the results of this study showed that phenolic production was suppressed in *Z. marina* in both the long-term field study and the short-term laboratory experiments. Results from the short-term nutrient limitation experiment suggested that this suppression was not likely to be a result of nutrient limitation within

the treatment tanks. These results, in conjunction with those from the Mediterranean, indicate that macroalgal invasions into seagrass beds may pose serious consequences for the seagrass' physiology and may indirectly lead to consequences such as weakening the defensive barrier of *Z. marina* to wasting disease (Harrison, 1982; Vergeer *et al.*, 1995). Seagrass die-off due to disease may then potentially aid the facilitation and spread of invasive species as new 'patches' become available for additional colonisation (den Hartog, 1997).

7.4 Physiology

The aim of Chapter 4 was to investigate the effects of the presence of *S. muticum* on the chlorophyll fluorescence output, growth and nutrient partitioning within the various tissue types (root-rhizome, sheath and blade) of *Z. marina*. Complex interactions between *Z. marina* and *S. muticum* were revealed, indicating that *S. muticum* may indeed have the potential to influence *Z. marina* at a physiological level, affecting the seagrass' chlorophyll fluorescence output. To determine if the F_v/F_m results were simply a photo-adaptive response to low PPF, measuring chlorophyll content, specifically the chl *a*:b ratio within the seagrass blades (Wiginton & McMillan, 1979; Dennison & Alberte, 1985; Abal *et al.*, 1994; Lee *et al.*, 2007) could have been used as a proxy for the response of *Z. marina* to reduced irradiance levels. An increase in chlorophyll content in conjunction with a decrease in the chl *a*:b ratio would indicate a photo-adaptive response to low light (Lee *et al.*, 2007). Despite previous studies investigating the light physiology of *Z. marina* conducted at similar latitudes under conditions similar to the 2009–2010 experiments, the PPF in 2009–10 still may have been too low for this population of *Z. marina*. Further research needs to be conducted to better understand the effects of *S. muticum* presence on the chlorophyll fluorescence of *Z. marina* under light-saturating conditions, preferably using a diving PAM fluorometer as *in situ* measurements could decrease any effects of environmental stress due to emergence.

Nutrient analyses for all years could have been conducted to validate the 2008 C-H-N, PO_4 and SiO_2 results, but due to the high costs associated with sample analysis, this was not possible. Despite the suboptimal laboratory conditions in 2008 in terms of seawater temperature and PPF, nutrient analyses indicated C-H-N % DW concentrations to be within the range of previously reported findings. This may be an indication of the resilience and ability of *Z. marina* to persist over short periods in suboptimal conditions. Nutrient partitioning analyses revealed that although there were differences between treatments for the % DW C-H-N components, these differences were mainly driven by significant differences found between sheaths in the ZZ biomass control treatment and the ZM treatment and between the blade tissues in the ZS and ZM treatments. This supports the proposition that observed changes were not merely consequnces of biomass effects within the ZS tanks or products of nutrient limitation. This leaves the possibility that 2008 results were an anomaly resulting from effects of *L. zosterae* within the tanks before the infection becoming apparent and the experiment terminating. The prevalence and spread of *L. zosterae* within *Z. marina* meadows may act in a density dependent fashion (Bull *et al.*, 2011); if *Z. marina* shoot densities become too high, the spread of *L. zosterae* increases due to increased physical contact between blades (Muehlstein *et al.*, 1991; Vergeer *et al.*, 1995; Ralph & Short, 2002). This possible positive feedback, coupled with increasing sea surface temperatures (Beardall *et al.*, 1998; Harley *et al.*, 2006), may negatively affect *Z. marina* populations as it is believed that *L. zosterae* flourishes in higher sea surface temperatures (Short *et al.*, 1988).

Each annual experiment was to have been both definitive and heuristic, ultimately producing a fully orthogonal, multi-year dataset. This would have enabled the use of parametric statistical testing rather than non-parametric tests, but due to the varying length of the laboratory experiments (three weeks in 2009 versus four weeks in 2008 and 2010), and the need to target resources at the most interesting questions, emerging differences in the number of treatments (ZS, ZM and ZZ in 2008–09, but only ZS and ZM in 2010) and the number of replicates for each treatment (ten for each treatment in 2008–09, but only six

in 2010) resulted, and analysis itself became the heuristic process. These problems were overcome through the use of PERMANOVA+ to test for significant differences between treatments. Additionally, since *Z. marina* is listed as a BAP species, Natural England, the body governing the UK, only granted permission to selectively harvest a limited number of shoots annually and therefore additional experiments could not be conducted. Experiments could not be carried out longer due to the decline in shoot health and an outbreak of wasting disease within the ZZ treatment tanks in 2008 and its emergence in 2009.

7.5 Densities and vegetative physiognomy

The aim of Chapter 5 was to investigate the effects of *S. muticum* presence on the densities of *Z. marina* within manipulated permanent quadrats and changes in the naturally occurring *Z. marina* densities along permanent transects over time. Results of the intra- and inter-annual transect analysis indicated that the presence of *S. muticum* appeared to have little influence on the naturally occurring densities of *Z. marina* within the field site. However, *Z. marina* densities within the experimental quadrats showed a significant decrease, perhaps indicating that shoot densities decline when near the invader, potentially driven by reduced irradiance levels. With decreasing *Z. marina* densities, infaunal communities may shift to greater numbers of hard-bodied taxa, as hard-bodied taxa are prevented from burrowing within the seagrass root-rhizome matrix more than soft-bodied taxa (Orth *et al.*, 1984).

Although *Z. marina* densities within the ZS treatment were significantly lower, the overall result for both the permanent quadrats and transects indicated that *Z. marina* densities increased over time within the estuary. This result was surprising given the number of mooring scars, pleasure-boating activities and increased *S. muticum* density. Abiotic conditions within the estuary, including water quality, irradiance levels and nutrient concentrations may therefore be ideal for *Z. marina* to flourish against all odds.

Significant differences were not found in the vegetative physiognomy of *Z. marina*, but blade lengths and subsequently blade area decreased over time in both treatments; it is

unlikely that this is due to the presence of *S. muticum*. One possible explanation for the decrease may have been a stress response due to declining water quality, but results from the ambient seawater nutrient concentrations did not indicate eutrophic conditions (see Chapter 2) arising over the duration of the field study. Interestingly, as blade lengths decreased, the number of blades per shoot increased over time. Increases in vegetative output can also be a stress response perhaps due to increasing invasive macroalga interaction as found in the Mediterranean (Pergent *et al.*, 2008).

7.6 Epibiota assemblages

The aim of Chapter 6 was to test for changes in the epibiotic assemblage of *Z. marina* in the presence or absence of *S. muticum*. The central questions were driven by previous studies that found differences between the epibiota found living on *S. muticum* and native kelps (Buschbaum *et al.*, 2006). As many epiphytes, planktonic organisms and small invertebrates are ephemeral and only occur seasonally, sampling every six to eight weeks across the three seasons when *S. muticum* presence was strongly evident throughout the estuary enabled a broad cross-section of the epibiotic organisms of *Z. marina* to be identified. Although seagrass density may be an important predictor for the variability in suspension feeders and grazers, variability in epifaunal taxa assemblages is predicted mainly by sediment chemistry, substratum coverage and geographical positioning (Gullström *et al.*, 2012).

Results from this long-term field study indicated significant differences between the ZS and ZM treatments with marginally greater between-sample similarities found within the epibiota from the ZM treatment. Results also showed that the overall biomass present on the blades was not significantly affected by the presence of *S. muticum* despite the significant differences in epibiota assemblage abundances between the ZS and ZM treatments.

These findings indicate the need for further specific investigation into the changes within the epibiota to determine how species richness and diversity in specific taxonomic and functional groups are affected by the presence of *S. muticum*. *S. muticum* is a suitable

habitat for invertebrates (Gestoso *et al.*, 2011b) as Buschbaum *et al.* (2006) found that *S. muticum* enhanced the epibiota diversity within the sediment potentially due to an increase in habitat heterogeneity and substrata availability. Any benefits of increased habitat architecture gained by the presence of *S. muticum*, however, are restricted to six or seven months a year due to the ephemeral nature of the invasive alga. What effects this annual senescence might have on epibiota that colonise or seek refuge within the heterogeneous architecture of *S. muticum* have yet to be revealed.

Although no experiments were conducted, initial field observations indicated thermal differences between the water located within the large summer growth of *S. muticum* and the surrounding water column, with the seaweed potentially providing warm water refuges for cold intolerant species. This was also observed in *S. muticum* populations in Ireland (Kraan, 2008) and is worth further consideration within the Salcombe-Kingsbridge Estuary. Due to this phenomenon, *S. muticum* fits within the "invasional meltdown" theory that well established invasive species facilitate other invasive species to settle and establish themselves, further altering the ecosystem (Simberloff & Von Holle, 1999; Grosholz, 2005), such as has been found in Willapa Bay, WA, USA (Ruesink *et al.*, 2006; Williams, 2007). These 'thermal islands' may therefore potentially increase invasion pressure on *Z. marina*. Thus, further research needs to be conducted studying *S. muticum* as a substratum.

7.7 Are we fighting a losing battle?

7.7.1 Global climate change

Over the last few decades, evidence supporting global climate change has come to the forefront of science. With a broad array of models, there have been wide-ranging predictions made regarding climate change (Caldeira & Wickett, 2003; Orr *et al.*, 2005; Borges *et al.*, 2006), including atmospheric CO_2 accumulation and rising global temperatures (Intergovernmental Panel on Climate Change, 2007). From these models,

it has been projected that by 2100, if no reduction in industrial outputs of CO_2 occurs, atmospheric CO_2 will increase three-fold (Beardall *et al.*, 1998) from its pre-industrial level of ~270 ppm (Wigley, 1983) reaching 650 ppm (Raven, 1991; Trenberth, 1996; Orr *et al.*, 2005) or higher (over 1000 ppm) (The Royal Society, 2005).

Predictions aside, there are now a number of observable trends with respect to climate change to which all species worldwide must adapt (Thomas *et al.*, 2004). These include increasing oceanic temperatures (~1–3 °C increase within the surface layers by 2050) (Trenberth, 1996; Harley *et al.*, 2006; Kaste *et al.*, 2006), melting polar ice caps resulting in rising sea levels currently at a rate of 3.1 mm (2.4 to 3.8) yr^{-1} (Intergovernmental Panel on Climate Change, 2007) (up from 2 mm yr^{-1} prior to 2003 (Bruun, 1962; Intergovernmental Panel on Climate Change, 2001; Harley *et al.*, 2006)), ocean acidification due to the burning of fossil fuels (Caldeira & Wickett, 2003), elevated UV-B exposure (Hader *et al.*, 1998; Smith *et al.*, 1992) and an increase in extreme weather (i.e., a greater number of storms with higher intensities and long-lasting droughts) (Burt *et al.*, 1998; Kaste *et al.*, 2006). Global climate change may represent the largest challenge that extant organisms have ever faced. How seagrasses, and the ecosystems they support, cope with changing conditions such as rising temperatures and sea level, ocean acidification and increased nutrient-loading among many other factors in combination with invasive species is beginning to emerge (Short & Wyllie-Echeverria, 1996; Duarte, 2002; Harley *et al.*, 2006).

7.7.2 Ocean acidification

During the Cretaceous period when seagrasses first appear in the fossil record (Beardall *et al.*, 1998; Hemminga & Duarte, 2000), oceanic pH was much lower (estimated to have been pH 7.6) (Caldeira & Wickett, 2003) due to nearly triple the approximate 380 ppm atmospheric CO_2 concentrations of today (Orr *et al.*, 2005), and was potentially more favourable for seagrasses (Beardall *et al.*, 1998; Hemminga & Duarte, 2000) than the current oceanic pH 8.2. As atmospheric CO_2 concentrations increase, oceanic pH decreases (Intergovernmental Panel on Climate Change, 2007). This decrease is detrimental

to calcifying organisms whose main source of C_i is bicarbonate (HCO_3^-) (Orr *et al.*, 2005; Harley *et al.*, 2006) by affecting their ability to maintain shell structure (Hall-Spencer *et al.*, 2008). Decreasing oceanic pH is also expected to have an effect on non-calcifying organisms such as seagrasses (Zimmerman *et al.*, 1997; Beardall *et al.*, 1998; Short & Neckles, 1999; Palacios & Zimmerman, 2007).

Although decreasing pH is the product of increasing dissolved CO_2 concentrations (Caldeira & Wickett, 2003), this results in only a small proportional change in HCO_3^- concentration, conferring no advantage to organisms relying on HCO_3^- as a C_i source (Beer & Koch, 1996; Beardall *et al.*, 1998). Seagrasses, however, are only half-saturated with respect to C_i at today's air-seawater CO_2 equilibrium (Beer & Koch, 1996), and may therefore express higher photosynthetic yields and growth rates as a result of decreasing oceanic pH and increasing near-shore CO_2 concentrations (Hall-Spencer *et al.*, 2008) as CO_2 can readily permeate their cells via passive diffusion. A rise in the rate of CO_2 passive diffusion to marine macrophytes would lower the overall metabolic costs associated with growth in terms of other resources (light, N, Fe, Zn) (Beardall *et al.*, 1998). Thus, fewer photons of light need to be absorbed per unit C assimilated, less Zn is needed for carbonic anhydrase used to convert HCO_3^- to CO_2 at the cell membranes, and less ATP and NADPH are needed for diffusive CO_2 entry from a high CO_2 concentration resulting in lower Fe requirements (Raven, 1991; Beardall *et al.*, 1998). Therefore, seagrasses may potentially benefit from ocean acidification (Zimmerman *et al.*, 1997; Palacios & Zimmerman, 2007; Hall-Spencer *et al.*, 2008; Martin *et al.*, 2008).

There is, nonetheless, an opposing opinion put forward by some of the same authors; seagrasses may not benefit from increasing oceanic CO_2 concentrations caused by an increase of atmospheric carbon absorption as previously thought as they have the ability to uptake HCO_3^- directly (Beer *et al.*, 2002). Increasing seawater CO_2 concentrations may therefore negate any potential gains in primary productivity of coastal macrophytes (Beer & Koch, 1996) as C_i acquisition deficiency may be driving the decline of seagrass

meadows worldwide (Short & Wyllie-Echeverria, 1996; Hemminga, 1998; Duarte, 2002). These differing opinions indicate that further research into how seagrasses respond to ocean acidification need to be conducted. Areas with active CO_2 vents, such as those found near Ischia and Vulcano, Italy and those near Papua New Guinea, provide ideal field sites to conduct such research as the seawater is naturally acidified along gradients (Hall-Spencer *et al.*, 2008).

7.7.3 Rising sea surface temperatures (SSTs)

Seagrass meadows are particularly vulnerable to rapid changes occurring in estuarine systems as seen by the 1930s wasting disease epidemic (Steele *et al.*, 2005), which has been linked to abnormally high sea surface temperatures (Short *et al.*, 1988). Rising sea temperatures due to climate change (Beardall *et al.*, 1998; Harley *et al.*, 2006), may further facilitate species invasions globally for the foreseeable future. Hales & Fletcher (1989) showed that an *S. muticum* germlings grow faster as sea temperature increases up to a maximum of 25 °C, after which survivability decreased markedly. With an average summer SST of ~16.6 °C along the British southern coast (Physical Oceanography DAAC, 2002), there is tremendous potential for even greater proliferation of *S. muticum* with increasing SSTs.

7.8 Marine policy, protection and long-term monitoring

Identifying and implementing optimal management plans to prevent, detect, and control invasive species should be of elevated importance due to the increasing economic and environmental losses caused by invasive species (Mehta *et al.*, 2007). A suite of transparent laws and regulations regarding the transport and trade of non-native species must be implemented, taking into consideration relevant and recent science on the ecological effects of invaders (Parker *et al.*, 1999). Coordinated efforts between regional, national and international governance over invasive marine species must also be achieved for successful

management to occur (Bax *et al.*, 2003).

According to Mack *et al.* (2000):

> *Failure to address the issue of biotic invasions could effectively result in severe global consequences, including wholesale loss of agricultural, forestry, and fishery resources in some regions, disruption of the ecological processes that supply natural services on which human enterprise depends, and the creation of homogeneous, impoverished ecosystems composed of cosmopolitan species. Given their current scale, biotic invasions have taken their place alongside human-driven atmospheric and oceanic alterations as major agents of global change. Left unchecked, they will influence these other forces in profound but still unpredictable ways.*

With approximately 60 % (von Bodungen & Turner, 2001) of the world's population living within 100 km of the coastal zone by the end of the 21^{st} century (Brambati, 2004), the need for increased public awareness of biological invasions as a component of global change is critical to help reduce the continued spread of present invaders and potentially halt future invasions. With improved public awareness and an increase in society's interest in biodiversity conservation, more sophisticated science-based information on the ecological consequences of invasive species may play a larger role in practical decision-making (Parker *et al.*, 1999). Unfortunately, the practicalities of incorporating good science into decision-making with respect to the protection and conservation of seagrasses within the UK may still be unrealistic at this stage as no long-term seagrass monitoring programmes currently exist within the UK (Foden & Brazier, 2007). With the implementation of EU legislation to protect seagrass beds from anthropogenic disturbance, the establishment of long-term monitoring programmes such as SeagrassNet (Short *et al.*, 2006) or Seagrass-Watch (McKenzie *et al.*, 2006-2008) would enable detection of responses to change in seagrass meadows (Duarte, 2002).

7.9 Summary

This thesis has examined a range of factors that may influence *Z. marina* as a species and the ecosystem it supports, specifically focussing on how the presence of *S. muticum* affects *Z. marina* and its associated epibiota. The fundamental question to this thesis therefore was: does the presence of *S. muticum* affect *Z. marina* and its associated epibiota? The answer is a guarded 'yes'.

The data accumulated are akin to circumstantial evidence in a murder trial, not quite a 'smoking gun' but the villain of the piece has certainly been placed squarely in the frame. There are weak forces in ecology that when coupled with unnatural forces, such as anthropogenic disturbances, can combine to have profound effects within ecosystems. The individual results have been mixed, each on its own may not unequivocally communicate the negative effects of *S. muticum* invasion on *Z. marina* and its associated epibiotic assemblage, but when considered collectively, they do.

Although more than 4000 plant species have been introduced to the US and Canada over the past 400 years, there is no evidence that even one 'native' species has been driven to extinction (Davis *et al.*, 2003). This, however, should not negate concern over the continued proliferation and spread of *S. muticum*. It is clear from the present study that there is still much to learn regarding the effects of *S. muticum* invasion into *Z. marina* meadows. As with most scientific investigations, the present study has raised as many questions as it has answered. Nevertheless, I believe that it has made a significant contribution to invasion biology by identifying previously unknown consequences of a well known invasive species.

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Appendix A

Species Taxa / Functional Taxonomic Unit (FTU) by treatment

Table A.1: Number of individuals per species taxa or FTU by treatment (ZS and ZM) for all epibiota data.

Species taxa / FTU	**ZS**	**ZM**
Diatom unident.	3048	3077
Non-corticated filament	1896	1957
Corticated filament	1317	1381
Polysiphonia sp.	681	679
Ceramium sp.	421	418
Stolon tube-like alga	375	427
Single, multi-cellular blade (foliose)	406	381
Saccate algae	1184	1135
Branching / bushy stolon-like filament	218	237
See-through tubular (branching) alga	58	81
Licmophora sp. (pennate diatom)	297	365
Coralline algae	1	3
Lomentaria orcadensis	4	0
Lomentaria articulata	0	3
Foliose, dichotomously branching alga	14	13
Antithamnionella-like alga	8	9
Wooden snake alga	5	1
Chylocaldia verticillata	10	25
Bottle brush red filament	43	35
Corticated foliose	1	0
Flocculate bacteria	788	768
Sponge	1625	1615
Scalloped shell foram	8590	9737
Encrusting foram	438	373
Donut-shaped foram	128	103
Brioche foram	341	617
Ice cream foram (*Brizalina spathulata*)	2	9
Rosette foram	1	5
Spiral eye foram	0	3
'Spaced' scalloped foram	20	25
Copepods	61852	64378
Copepod nauplii	876	950

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Species taxa / FTU	**ZS**	**ZM**
Porcellidium viridis	1723	1620
Bryozoan (encrusting)	465	478
Bryozoan (erect)	31	26
Nematode	14367	15518
Aora gracilis	3908	4916
Snail (all species)	136	380
Clam (all species)	101	76
Mussel	296	301
Limpet (all species)	12	20
Ostracods (all species)	2000	2180
Fish eggs	106	76
Non-polychaete worm	18	31
Polychaete worm (not Nereid)	31	33
Nereid worm	545	420
Ribbon worm/marine leech?	18	1
Sipunculid	4	0
Spirorbid worm	1	3
Nemertean	10	2
Terebellid worm	3	2
Arenicola defodiens	0	1
Encrusting bifurcated worm	3	4
Worm? Stuck to blade by threads, banding	8	6
Athecate hydroid	46	48
Thecate Hydroid	66	98
Erect Hydroid	1	3
Calycella hydroid	1	0
Egg cases (brown domes flat on blade)	294	333
Hennia reticulata egg cases	112	77
Eggs (various mollusc)	109	126
Egg mass (stringy white strands)	23	22
Jelly Bean eggs	9	6
Red-ringed brown egg sac	21	17
Chironomidae larvae	43	32
Caprellidae	155	157
Crab larva (nauplii/crab)	7	13
Hermit crab	0	2
Decapods	0	2
Prawns	3	0
Amphipods	522	436
Evansula inserta	8	4

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Species taxa / FTU	**ZS**	**ZM**
Isopods	9	14
Prostigmata	93	75
Stalked hydrozoan	27	24
Ascidian (colonial)	16	22
Ascidian (solitary)	10	10
Dorid	1	0
Aplysia punctata	12	15
Puckered brown bag	39	55
Diptera pupae (chironomidae)	4	0
Brittlestar	1	0
Iridescent bag with hole	2	0
Encrusting white blob (possible fungus)	36	36
White spores	26	32
Black volcanoes	44	57
Brown ‘beans’ attached to blade	3	1
TOTAL	**110177**	**116621**