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SPECIAL ISSUE: MACROALGAE IN A CHANGING WORLD

Invasive macroalgae in native seagrass beds: vectors of spread and impacts

Louise B. Firth, Andy Foggo, Thomas Watts, Antony M. Knights and Stacey deAmicis*

School of Biological and Marine Sciences, University of Plymouth, Plymouth, UK
*For correspondence. E-mail: stacey.deamicis@plymouth.ac.uk

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- **Background and Aims** Worldwide, invasive species are spreading through marine systems at an unprecedented rate with both positive and negative consequences for ecosystems and the biological functioning of organisms. Human activities from shipping to habitat damage and modification are known vectors of spread, although biological interactions including epibiosis are increasingly recognized as potentially important to introduction into susceptible habitats.
- **Methods** We assessed a novel mechanism of spread – limpets as transporters of an invasive alga, *Sargassum muticum*, into beds of the seagrass *Zostera marina* – and the physiological impact of its invasion. The association of *S. muticum* with three limpet species and other habitats was assessed using intertidal surveys on rocky shores and snorkelling at two seagrass sites in the UK. A 4-year field study tested the effect of *S. muticum* on *Z. marina* shoot density, dry weight and phenolic compounds (caffeic and tannic acid) content, and a laboratory experiment tested the impact of *S. muticum* on nutrient partitioning (C/H/N/P/Si), photosynthetic efficiency (F_v/F_m) and growth of *Z. marina*.
- **Results** On rocky shores 15 % of *S. muticum* occurrences were attached to the shells of live limpets. In seagrass beds 5 % of *S. muticum* occurrences were attached to the shells of dead limpets. The remainder were attached to rock, to cobblestones, to the seagrass matrix or embedded within the sand. *Z. marina* density and phenolics content was lower when *S. muticum* co-occurred with it. Over 3 years, photosynthetic responses of *Z. marina* to *S. muticum* were idiosyncratic, and *S. muticum* had no effect on nutrient partitioning in *Z. marina*.
- **Conclusions** Our results show limpets support *S. muticum* as an epibiont and may act as a previously unreported transport mechanism introducing invaders into sensitive habitats. *S. muticum* reduced production of phenolics in *Z. marina*, which may weaken its defensive capabilities and facilitate proliferation of *S. muticum*. The effect of *S. muticum* on *Z. marina* photosynthesis requires further work but having no effect on the capacity of *Z. marina* to sequester nutrients suggests a degree of resilience to this invader.

Key words: Biochemistry, ecosystem engineer, invasion, limpet, *Sargassum muticum*, seagrass, vector, *Zostera marina*, allelopathy.

INTRODUCTION

Ecosystem engineers have profound effects on ecosystem structure and functioning in terrestrial and aquatic ecosystems globally (Jones *et al.*, 1994; Crooks, 2002; Emery-Butcher *et al.*, 2020). When an invasive species is classed as both a habitat-forming and habitat-modifying ecosystem engineer, not only does it compete with native species (Mooney and Cleland, 2001; Morriën *et al.*, 2010), but it may also create novel habitat (Rodriguez 2006; Byers *et al.*, 2012; Firth *et al.*, 2021), and modify abiotic conditions such as light, temperature or sediment deposition (Levin *et al.*, 2006; McKinney and Goodell, 2010) that can have cascading environmental, economic and social impacts (Thomsen, 2010; Gribben *et al.*, 2019; Wood *et al.*, 2022; for a review see Guy-Haim *et al.*, 2018). The presence or activity of an ecosystem engineer can also modify access to resources or biochemical conditions present within an environment (Jones *et al.*, 1994; Lim *et al.*, 2020).

Whilst human activities such as shipping, habitat loss, fragmentation and proliferation of artificial structures are known to enhance invasive spread in aquatic environments (With, 2002; Bishop *et al.*, 2017; O'Shaughnessy *et al.*, 2020; Adomako *et al.*, 2021), some invasive species employ novel methods of spread between habitats across landscape scales. For instance, some species 'hitch-hike' as epibionts on the bills and feet of migratory birds (Green, 2016) and the carapaces of sea turtles (Harding *et al.*, 2011). Similarly, the increasing amounts of plastic flotsam in marine environments are providing durable novel substrata facilitating the spread of invasives (Kiessling *et al.*, 2015; Treneman *et al.*, 2018). Following the 2011 Japanese earthquake and tsunami, Carlton *et al.* (2017) documented the transport of 289 living Japanese coastal marine species from 16 phyla on floating objects that travelled thousands of kilometres across the Pacific Ocean to the shores of North America. With ever-increasing landscape connectivity and corresponding proliferation of invasive species (e.g. Clubley *et al.*, 2023), we are witnessing the homogenization of biota, changes in ecosystem functioning and the increased emergence of novel ecosystems (Hobbs *et al.*, 2006, 2009; Bulleri *et al.*, 2020).

Seagrass beds globally are particularly susceptible to invasion by macroalgae (Williams, 2007; Gallucci *et al.*, 2012; Thomsen *et al.*, 2012), and much work has been done on the impacts of invasive *Caulerpa* species. Whilst many authors have asserted that *Caulerpa taxifolia* can cause the regression of seagrasses (e.g. Boudouresque *et al.*, 1995; Stafford and Bell, 2006; Glardon *et al.*, 2008; Peirano *et al.*, 2011), and negative impacts on seagrass-associated biota (Wright *et al.*, 2007; Gribben *et al.*, 2009; Byers *et al.*, 2010), others have found no effect (for a review see Glasby *et al.*, 2013). Where competition does occur, *Caulerpa* overgrows seagrass rhizomes; interacting with both the below- and above-sediment surface tissues, affecting nutrient acquisition and light availability (Ceccherelli *et al.*, 2000). Previous research in the Mediterranean found that the native seagrass *Posidonia oceanica* increases its production of secondary metabolites (phenolic compounds such as caffeic acid) in response to the invasion of *C. taxifolia* 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.

In the UK, the invasive Japanese wireweed *Sargassum muticum* has successfully invaded seagrass (*Zostera marina*) beds (Fig. 1A; Druehl, 1973; Tweedley *et al.*, 2008). Whilst it was previously thought that *S. muticum* required a hard substrate for attachment, thus limiting its ability to invade seagrass beds (North, 1973), it is now known that it can spread from rocky habitats into seagrass beds through the drifting of detached fertile branches with air-filled gas bladders (Engelen *et al.*, 2015), or through 'stone-walking', whereby individuals attached to small stones may become buoyant and dispersed by local currents (Critchley, 1983). Firth *et al.* (2023) suggested that limpets may also be an important vector of spread of *S. muticum* from rocky shores. They reported that 24 % of 143 *S. muticum* individuals were attached to limpet shells (Fig. 1B; 83 % attached to the China limpet *Patella ulyssiponensis*, 17 % attached to the common limpet *P. vulgata*) on rocky shores. Like stone-walking, if a limpet that supports *S. muticum* becomes detached from the rock (Fig. 1C), the canopy provides buoyancy, thus enabling the shell to be transported to seagrass beds where it may become anchored though entanglement in the seagrass rhizome matrix or burial in sand or mud. To our knowledge, no research has focused on the importance of limpets as vectors of spread for *S. muticum* into *Z. marina* beds. Despite repeated concerns about the impacts of *S. muticum* on *Z. marina* beds since 1973 (Druehl, 1973; den Hartog, 1997), to our knowledge only a single paper has experimentally examined any impacts. DeAmicis and Foggo (2015) found that the epibiotic assemblages on the blades of *Z. marina* plants in plots that were invaded by *S. muticum* were significantly different from control uninvaded plots. Quantification of the impacts of *S. muticum* on the structure and functioning of seagrass beds remains a major knowledge gap.

The overarching aim of this study was to assess the role of limpets as vectors of spread of *S. muticum* into seagrass (*Z. marina*) beds and to quantify the impacts on a range of seagrass traits. First, we conducted field surveys to examine the association between *S. muticum* and attachment substrata (limpet shells, rock, cobbles, other substrata) both on rocky shores and in seagrass beds. Whilst both surveys were largely exploratory, we hypothesized that at least some *S. muticum* would be attached to limpet shells in both habitats and that shells of *P. ulyssiponensis* would be more important as an attachment substrate than *P. vulgata*. Second, a 4-year manipulative field experiment examined the impact of *S. muticum* on *Z. marina* phenolic compound production and density. We hypothesized that over time the relative phenolic content of *Z. marina* would be higher, and that density would be lower in the presence of *S. muticum* than without it. Finally, multiple laboratory experiments were conducted in a controlled environment to determine how *S. muticum* affects *Z. marina* photosynthesis [chlorophyll fluorescence output (F_v/F_m)], growth rates, and nutrient (C, H, N, P and Si) partitioning and allocation within its various tissue types (root-rhizome, leaf sheath

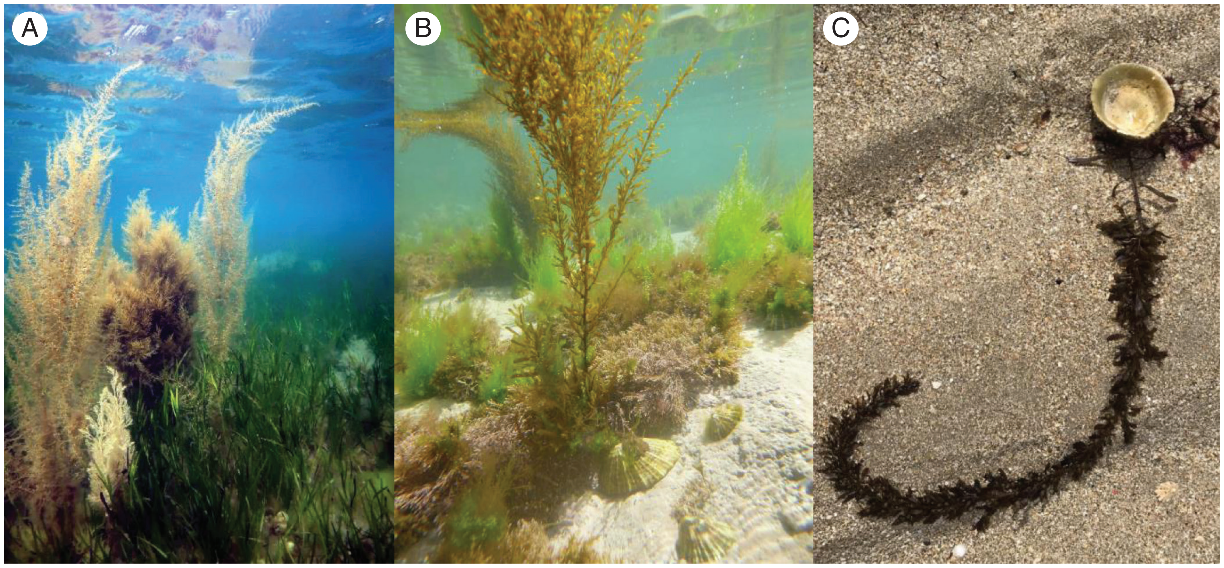

Fig. 1. Images of *Sargassum muticum* (A) colonizing a *Zostera marina* bed (photo credit Georgie Bull); (B) attached to the limpet *Patella ulyssiponensis* on a rocky shore (photo credit Louise Firth); (C) attached to a detached limpet shell washed up on the beach (photo credit Tony Legg).

and blade). We hypothesized that over time the presence of *S. muticum* would reduce *Z. marina* photosynthesis and growth rates and that it would alter nutrient partitioning and allocation within tissue types.

MATERIALS AND METHODS

Study system

All surveys and experiments in this study were conducted in Devon, SW England. The region is home to several seagrass beds located in shallow water (Green *et al.*, 2018). Cellars Cove (50.31018, −4.06676) is known to support ~0.14 ha of *Z. marina* with densities reaching 6.7 ± 7.01 plants per 50 cm^2 (Green *et al.*, 2018). These beds have also supported *S. muticum* since 1976 (Boalch and Potts, 1977). The total seagrass extent within the Salcombe–Kingsbridge ria (50.23129, −3.77330) meanwhile was estimated at 6.3 ha (in 2008) with known shoot density averages of 240 shoots m^{-2} (Tweedley *et al.*, 2008). The seagrass beds at Cawsand (50.33184, −4.19860) are the largest in the area covering 28.67 ha (Jenkin *et al.*, 2021). The surveys of limpets as vectors for spread of *S. muticum* were conducted at Cellar Beach and Cawsand in summer 2022. Due to difficulties obtaining permission for sampling, Salcombe–Kingsbridge was not sampled. All experimental work on the impacts of *S. muticum* on *Z. marina* was conducted in the Salcombe–Kingsbridge ria between 2007 and 2011.

Are limpets potential vectors of spread of S. muticum *in* Z. marina *beds?*

To assess the potential for limpets to act as vectors of spread of *S. muticum* into *Z. marina* beds, surveys were conducted in Cawsand and Cellars Cove which had both rocky shores and seagrass beds adjacent to one another. To test the potential association between *S. muticum* and patellid limpets on rocky shores, three 45-min searches were conducted across 25-m transect lines ($n = 3$, ~1 m on either side of the transect line was observed as a sample area) in each of the lower, middle and upper regions of both rocky shore study sites during low-water spring tides. For every *S. muticum* thallus that was located, the ‘attachment’ substratum was noted [i.e. limpet (*P. vulgata*, *P. ulyssiponensis*, *P. depressa*), bedrock platform or loose cobblestones].

To test the association between *S. muticum* and limpet shells in seagrass beds, subtidal snorkelling surveys were conducted across shore-perpendicular weighted 25-m transect lines ($n = 32$) during low water slack of spring tides. Water depths at Cellars Cove and Cawsand survey sites ranged from ~<1–3.0 and ~3.3–3.6 m, respectively. Transect lines were systematically snorkelled along and ~1 m on either side of the transect line was observed as a sample area. For every *S. muticum* thallus that was located, the ‘attachment’ substratum was noted (as above, but also including ‘seagrass matrix’ which classifies a habitat created by intertwined blades and rhizomes of *Z. marina* forming a matt-like area that can be colonized by various settling species; Tanner, 2006; Tweedley *et al.*, 2008).

Field study: what are the impacts of S. muticum *upon* Z. marina *density and phenolic compound production?*

Long-term field experimental set up. To investigate how *S. muticum* potentially affects *Z. marina*, a 4-year field study (March 2007 to March 2011) was conducted in the Salcombe–Kingsbridge ria. Twenty permanent 1 × 1-m quadrats were established at a depth of 0.5 m below chart datum: ten each for two experimental treatments: with and without *S. muticum* (hereafter ‘Z+S’ and ‘Z’, respectively). Two similarly sized *S. muticum* individuals (~75–90 cm long) were harvested intact from nearby locations and attached to 25 × 25-cm plastic grids using cable ties; and two grids were secured within ten randomly selected permanent quadrats using reinforcing bar ‘hooks’, driven deep into the sediment. Control (Z) treatments were established by affixing two ‘empty’ grids within the remaining ten quadrats. Seagrass blades were carefully pulled through all grids to remain upright within the water column and not trapped underneath. Any additional *S. muticum* individuals that colonized the control Z treatments were removed during each sampling session.

Density measurements. Four permanent 70-m-long shore-parallel transects were established between mean low water low (MLWL) and 1.2 m below MLWL. *Z. marina* densities were determined by sampling 12 randomly located 1-m^2 quadrats along each transect. Within each quadrat, four 0.25 × 0.25-m 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* plants 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* plants within the field site was calculated and used as a proxy for overall *S. muticum* densities within the estuary. To compare quadrat and transect *Z. marina* densities (i.e. manipulated vs. unmanipulated), quadrat densities from the same months that transect sampling occurred were averaged to produce the mean quadrat density for that sampled date.

Phenolic compound measurement. Seagrass samples were collected within the established permanent quadrats every 6–8 weeks from three seasons (spring: March–May, summer: June–August and autumn: September–October, the active growing period for *S. muticum*) over a 4-year period (2007–2010). 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 laboratory, 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 and frozen at −20 °C.

To quantify the percentage dry weight (% DW) content of caffeic (CA) and tannic acid (TA) equivalents within blade tissues, samples were dried at 65 °C for 24 h, ground and ~150 mg of weighed sample was extracted in 50 % MeOH for 24 h in a dark refrigerator at 4 °C. Phenols in blade tissue were assayed using an adapted Folin-Ciocalteu colorimetric assay (Harrison and Durance, 1989; Hargrave *et al.*, 2017), processed in triplicate and read against CA and TA standard dilution series at 725 and 765 nm respectively using a Unicam Helios Epsilon spectrophotometer (Unicam Ltd, Cambridge, UK).

Statistical analyses. To test the effect of *S. muticum* on *Z. marina* density, data from the permanent quadrats were analysed using a mixed model univariate generalized linear model (GLM) in SPSS 19 with the mean seagrass density per quadrat as the dependent variable. The GLM had three factors, 'treatment' and 'year' were designated as fixed with two (Z+S and Z) and four (2007, 2008, 2009 and 2010) levels respectively, but we set 'season' as a random factor with two levels (spring and autumn), nested within 'year', because we wanted to capture the overall growth season mean density of shoots, and thus 'season' here is akin to a temporal 'block'. We were also unable to access the site in both seasons in all years due to tidal variations, meaning the final samples were not orthogonally distributed. We experimented with including a first-order autocorrelation term in the model to account for the repeated disturbance of the permanent quadrats, but this addition did nothing to the model fit or its interpretation. Type III sums of squares were used and Student-Newman-Keuls (SNK) *post hoc* tests were performed for 'years'. Conformity to assumptions of normality and homogeneity of variances were confirmed by plots of fits and residuals. Pairwise comparisons with Tukey's honest significant difference (HSD) tests using estimated marginal means were used to identify significant differences between the Z+S and Z treatments within the interaction term 'year × treatment' ($P < 0.05$).

To test the effect of *S. muticum* on overall seagrass phenolics, we used a three-factor PERMANOVA in PRIMER ver. 6.1. based on standardized CA and TA equivalents, using Euclidian distances and factors: Treatment (two levels: Z+S, Z; fixed), Year (four levels: 2007–2010, fixed) and Season (three levels: spring, summer, autumn, random due to lack of orthogonality as described above). Unrestricted permutations of raw data, type III sums of squares and 9999 permutations were set as design parameters (Anderson *et al.*, 2008).

Laboratory study: what are the impacts of S. muticum *on* Z. marina *photosynthetic performance, growth and nutrient partitioning?*

Laboratory experimental conditions. To investigate the impacts of *S. muticum* on *Z. marina* nutrient partitioning and physiological responses, four, 3- to 4-week laboratory experiments using wild-harvested *Z. marina* were undertaken annually from 2008 to 2011 in a constant temperature (CT) room. Seagrass shoots were hand-harvested locally in early spring and acclimated to laboratory conditions for 2 weeks in aerated tanks at *in situ* densities (~160 plants m^{-2}). Ten glass tanks (30 × 23 × 39 cm; 27-L capacity) of seawater were partitioned into two, unequally sized compartments (60:40) by 1-cm grid plastic fencing to allow for water exchange while keeping algae or control seagrass shoots from physically interacting with the focal *Z. marina* plants. Experimental samples were all collected from the seagrass in the large tank compartment. Three treatments were established in 2008 and 2009: *Z. marina* + *S. muticum* (Z+S), *Z. marina* only (Z) and a biomass control, *Z. marina* + *Z. marina* (Z+Z) (Fig. 2). After 2009, only the Z+S and Z treatments were tested, following preliminary analysis indicating a lack of biomass (Z+Z vs. Z) effect. After epiphytes were gently removed by lightly scraping with a razor blade, five *Z. marina* shoots [each ~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. One *S. muticum* individual (~60 g WW) attached to a small stone was added to the smaller compartment of each tank for the Z+S treatment. For the Z+Z biomass control treatment, five to seven additional *Z. marina* shoots (each ~12 g WW total biomass) were added to the smaller compartment. The Z treatment consisted of five *Z. marina* shoots anchored within the larger tank compartment only.

Chlorophyll fluorescence measurements. Chlorophyll fluorescence was used to examine effects of *S. muticum* proximity upon photosynthetic efficiency of the seagrass. In 2008, shoots were held at 15 ± 2 °C in a CT room with ~55–60 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) on a 16-h/8-h light/dark (16L:8D) cycle (equivalent to 3.17–3.46 quanta mol m^{-2} d^{-1}). In 2009, we were able to increase the photosynthetic photon flux density (PPFD) 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 ± 2 °C to match the ambient conditions as the experiments occurred earlier in the spring than in 2008. To determine the maximum photochemical efficiency

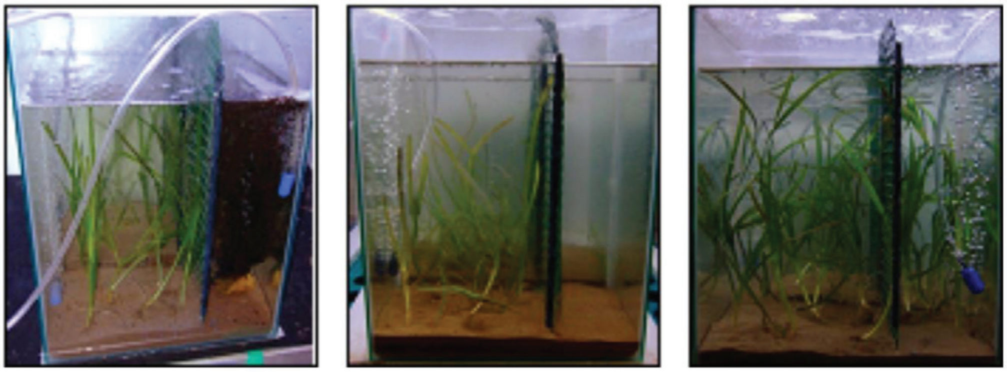

FIG. 2. Laboratory experimental design using three treatments (left to right): *Zostera marina* + *Sargassum muticum* (Z+S), *Z. marina* only (Z) and *Z. marina* + *Z. marina* (Z+Z) (photo credits: Stacey DeAmicis).

of the photosystem II (PSII) apparatus in dark-adapted seagrass blades, (F_v/F_m) measurements were recorded over a 5-s period at 100 % light intensity on four dates (T = 0, and weekly thereafter) throughout the experiment using an MK2 Plant Efficiency Analyser (PEA meter) (Hansatech Instruments Ltd, King's Lynn, UK). Once 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. The mean F_v/F_m for each tank at each date was used for statistical analysis to avoid pseudo-replication.

Growth measurements. All blades in five individual shoots were punctured with a fine needle at the blade–sheath interface at the start of the experiment and again after ~14 d. Growth was measured as the distance the hole had grown away from the interface (Westera and Lavery, 2006). Length measurements between the top of the sheath and the puncture holes were taken on two dates, once at ~2 weeks after the start of the experiment and again at the end of the experiment. 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 per shoot per tank. The mean total production per shoot per tank d^{-1} was calculated by dividing the total production per shoot per tank by the number of days from the initial hole punch.

Tissue nutrient measurements. Three *Z. marina* tissue types (root-rhizome, sheath-meristem region and blades) were harvested at the end of the 2008 laboratory experiment and analysed separately for carbon and nitrogen content. These analyses were carried out to determine nutrient partitioning and allocation within each tissue type. Details of analytical chemistry are given in Supplementary Data S1.

Statistical analyses. The effect of *S. muticum* on *Z. marina* chlorophyll fluorescence (F_v/F_m) was analysed in SPSS 19 using repeated-measures type III sums of squares linear models. The combined 2008 and 2009 analysis utilized a two-way design where both 'year' with two (2008 and 2009) and 'treatment' with three (Z+S, Z and Z+Z) levels respectively were fixed factors; 'date' was designated the within-subject factor with four levels (T = 0, 1, 2 and 3). Box's test of equality of covariance matrices was used 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 visual inspection of fits and residuals. Pairwise comparisons were used to identify significant differences between the Z+S, Z and Z+Z treatments ($P < 0.05$) using Tukey's HSD based upon estimated marginal means.

The effect of *S. muticum* on *Z. marina* growth [total production per shoot per tank d^{-1} (in mm)] was analysed in SPSS 19 using a repeated-measures lm with type III sums of squares and tests for conformity to analytical assumptions (as described above). To avoid pseudo-replication, multiple data per tank were amalgamated and a single datum per tank was used as the replicate. The combined 2008 and 2009 analysis used a two-way design where both 'year' with two (2008 and 2009) and 'treatment' with three (Z+S, Z and Z+Z) levels respectively were fixed factors. 'Date' was set as the within-subject factor with two levels (mid- and end experiment measurements). SNK *post hoc* tests were performed for 'year' and pairwise comparisons were used to identify significant differences between 'treatments' ($P < 0.05$) using Tukey's HSD based upon estimated marginal means.

The effect of *S. muticum* on *Z. marina* carbon and nitrogen partitioning was analysed using type III sums of squares univariate lms in SPSS 19; data were tested using a two-factor design, with 'treatment' with three (Z+S, Z and Z+Z) 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.

RESULTS

Are limpets potential vectors of spread of S. muticum *into* Z. marina *beds?*

S. muticum was attached to limpet shells at both rocky shores sampled. These were primarily restricted to rockpools in the mid- to low shore. Of the 654 *S. muticum* individuals that were observed across both locations, 95 (15 %) were attached to limpet shells, whilst 517 (79 %) and 42 (6 %) were attached to the rock platform and loose cobblestones, respectively. A total of 384 were found at Cellars Cove (299 on rock, 43 on limpet shells, 42 on cobblestones) and 270 at Cawsand (218 on rock, 52 on limpet shells, none on cobblestones). *P. ulyssiponensis* was the only limpet species found to support *S. muticum* epibionts.

S. muticum was only observed in the seagrass bed at Cellars Cove. No individuals were found at Cawsand. Of the 168 individuals observed at Cellars Cove, eight (5 %) were attached to limpet shells (five on *P. ulyssiponensis*, three on *P. vulgata*), with the remaining 160 attached to a range of substrata (78 on cobbles, 69 in sand, nine in seagrass matrix, four on submerged rock). One limpet shell was observed to still have the soft body of the limpet still in the shell. This suggests that *S. muticum* settled on the limpet while it was alive and attached to a rocky substrate. This contrasts with the alternative scenario with the shell being deposited in the seagrass matrix first and colonized by *S. muticum* subsequently.

What are the impacts of S. muticum *on* Z. marina *density and phenolic compounds?*

Z. marina shoot densities in the permanent quadrats ranged from 162.9 ± 9 to 273.8 ± 16 shoots m^{-2} for the Z+S treatment and 136.1 ± 11 to 306.1 ± 22 shoots m^{-2} for the Z treatment (summarized in Fig. 3A). Results indicated significantly lower *Z. marina* density within the Z+S treatment permanent quadrats ($P < 0.001$) than in the control Z treatment quadrats, with significant treatment effects evident particularly in the final 2 years of the study. Densities within the quadrats broadly increased over time and a similar pattern emerged for *S. muticum* in the transects (linear model $F_{1,30} = 13.840$, $P < 0.001$; Fig. 3B) whilst *Z. marina* densities across the transects remained relatively stable (Fig. 3A).

Phenolic contents in *Z. marina* varied throughout the study with average CA equivalents ranging from 1.39 to 1.48 % dry weight (DW) and TA equivalent contents ranging from 1.76 to 1.89 % DW. There was a significant main effect of treatment for both analyses (Fig. 4A and B), with *Z. marina* shoots in the Z+S treatment exhibiting significantly lower % DW phenolic content, for both CA ($P < 0.05$) and TA equivalents, ($P < 0.01$) than shoots in the Z treatment across all years.

What are the impacts of S. muticum *on* Z. marina *photosynthetic efficiency, growth and nutrient partitioning?*

Chlorophyll fluorescence analyses revealed significant differences between treatments with and without *S. muticum* (Fig. 5; $P < 0.01$), but pairwise comparisons indicated these differences only occurred in 2008 ($P < 0.001$) and were less prominent in the comparison of the *S. muticum* treatment with the biomass control treatment.

Repeated-measures GLM indicated that there were no differences between responses of growth in the different treatments or years to the passage of time in the laboratory (Supplementary Data Fig. S1). Neither was there a main effect of treatment. Across the time periods, mean growth was 35.46 (Z+S treatment), 35.18 (Z treatment) and 32.10 (Z+Z treatment) mm per shoot per tank d^{-1}. There was a significant interaction between Treatment and Year, but this was attributable to the biomass control treatment differing from the control; there was no indication of any effect involving the Z+S treatment.

Significant differences were also found in nutrient (carbon and nitrogen, Supplementary Data Fig. S2) partitioning amongst functional regions of the shoots ($P < 0.05$); again, pairwise comparisons indicated that these differences lay between the biomass control treatment (Z+Z) and the *Sargassum* treatment (Z+S) and between Z+Z and the unmanipulated seagrass (Z) treatments ($P < 0.05$) but not between Z+S and Z treatments. Plots of tissue N against C:N ratio indicated a lack of nutrient limitation.

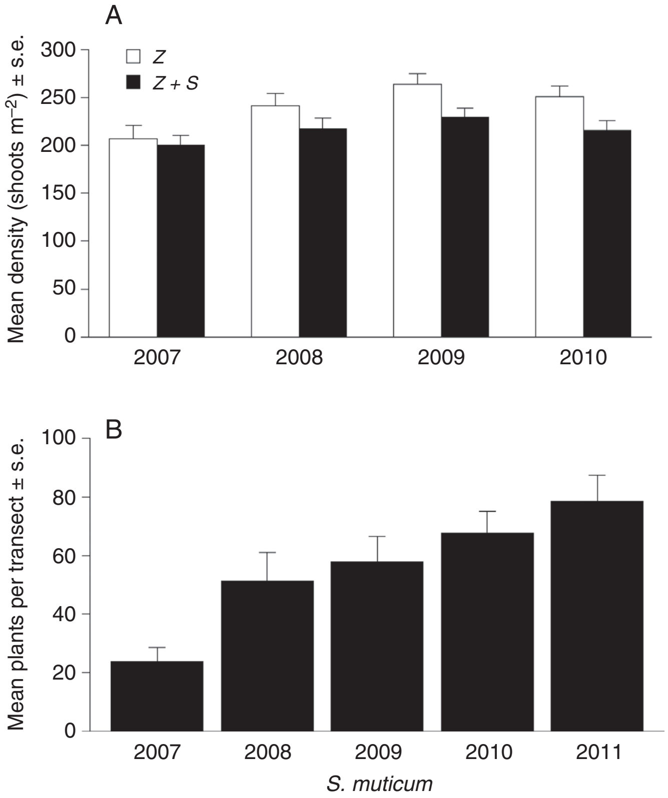

FIG. 3. Mean (±s.e.) *Zostera marina* shoot densities in (A) two permanent quadrat treatments from 2007 to 2010: *Z. marina* + *S. muticum* (Z+S) and *Z. marina* only (Z). Results represent the annual mean density for each treatment (calculated across all seasons). Annual mean transect densities for (A) *S. muticum* and (B) *Z. marina* 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 plants in a 1-m-wide strip centred on the transect and averaged across all transects for each year. Sampling occurred only in autumn and spring in 2007 and 2011, respectively; all $n = 18$ per quadrat per year.

DISCUSSION

S. muticum was found living attached to limpets both on rocky shores and in seagrass (*Z. marina*) beds suggesting that limpets may represent a vector of spread for *S. muticum* across landscapes from rocky shores into seagrass beds. Of all the *Z. marina* traits that were assessed, *S. muticum* was only found to have a negative effect on *Z. marina* density and phenolic compounds (both CA and TA equivalents), but there was little evidence of any effect on *Z. marina* photosynthesis [chlorophyll fluorescence output (F_v/F_m)], growth rates, and nutrient (C, H, N, P and Si) partitioning and allocation within its various tissue types (root-rhizome, leaf sheath and blade).

Despite limpets only accounting for attachment substrata for *S. muticum* in 5 % of cases in seagrass beds, they appear to be more important as attachment substrate on natural rocky shores (15 % of individuals were attached to limpets; see Firth *et al.*, 2023 who reported 24 %). We found that *S. muticum* was attached to both *P. ulyssiponensis* and *P. vulgata* in seagrass beds, but it was only observed on *P. ulyssiponensis* on rocky shores. An emerging body of evidence is revealing that *P. ulyssiponensis* represents important habitat for algal epibionts (Pereira *et al.*, 2022; Firth *et al.*, 2023; see also Martins *et al.*, 2014 for closely related *P. aspera*), particularly in relatively exposed conditions where densities of *P. ulyssiponensis* are high, and consequently grazing of the primary rock substrata is high. Firth *et al.* (2023) suggested that this is due to *P. ulyssiponensis* exhibiting aggressive behaviour towards limpet competitors, preventing mutual grazing on their shells, thereby indirectly providing an associational refuge for algae on their shells. More research is required to ascertain the exact mechanism underpinning this emergent pattern.

No *S. muticum* was found in the *Z. marina* beds at Cawsand. This could possibly be due to recent introduction of advanced mooring systems (Solandt, 2022), which aim to prevent the disturbance of seagrass by lifting mooring chains off the seabed. The introduction of these moorings has seen a

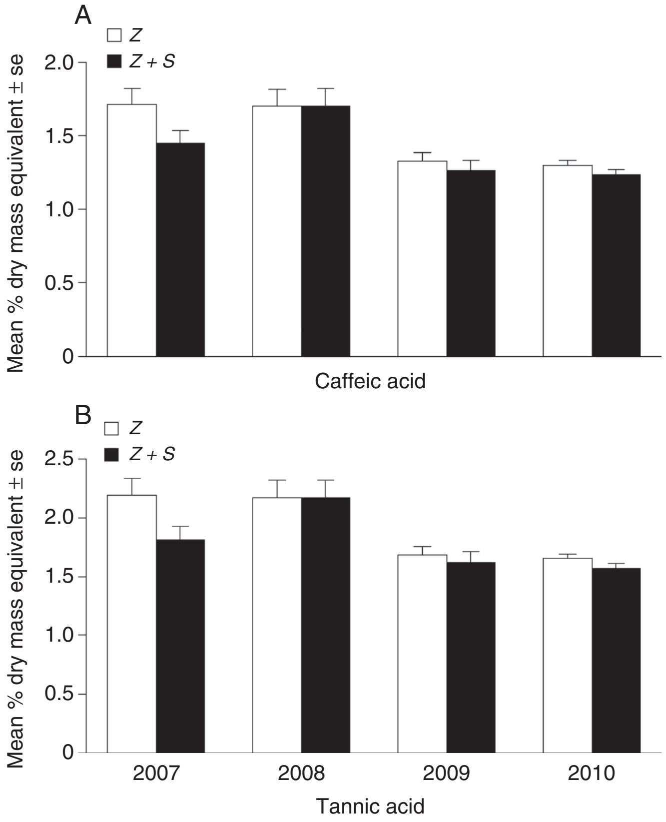

FIG. 4. Mean (±s.e.) percentage dry weight for (A) caffeic and (B) tannic acid content of *Zostera marina* from long-term field study for two treatments: *Z. marina* + *S. muticum* (Z+S: $n = 39, 40, 50, 60$) and *Z. marina* only (Z: $n = 40, 40, 50, 60$) from 2007 to 2010.

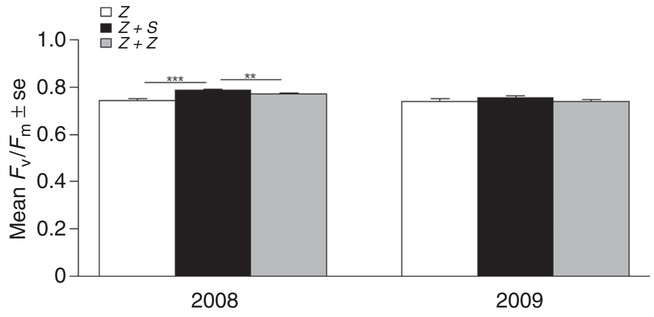

FIG. 5. Mean F_v/F_m (±s.e.) from combined 2008–2009 data for three treatments (Z, Z+S and Z+Z), all $n = 40$. *Significant difference ($P < 0.05$).

reduction in anchor disturbance and scarring in the Cawsand Bay seagrass beds and an increase in seagrass density around mooring points (Solandt, 2022). In contrast, Cellars Cove experiences high levels of recreational boating traffic particularly during the summer months (peak growing seasons for both *Z. marina* and *S. muticum*) and is not home to any fixed moorings, so boats must anchor disturbing and uprooting nearby seagrass and leaving the area exposed and susceptible to invasion. Survey observations from Cellars Cove saw patchy distributions of seagrass with scar-like marks from anchoring with many of these cleared patches colonized by small sprouts of carpet-like *S. muticum* (T. Watts, pers. observ.). Whilst we did not quantify percentage cover of *Z. marina* in our transects in the 2022 survey, it is likely that this may have been due to high *Z. marina* densities at Cawsand preventing *S. muticum* from successfully invading the beds there (T. Watts, pers. observ.). Large numbers of individuals were observed on the adjacent rocky shore ($n = 270$), suggesting that lack of supply was not a limiting factor in this instance. In the Mediterranean, resistance to the invasion of *Caulerpa cylindracea* has been attributed to native seagrass *Posidonia oceanica* shoot density, suggesting that some factors correlated with the canopy structure must be involved in the reduced capacity of *C. cylindracea* to penetrate the meadows, such as space limitation, water motion, nutrient supply or canopy shading (Ceccherelli *et al.*, 2000). Future work should quantify densities of both invader and recipient habitat.

Results from the long-term transect analysis indicated that *S. muticum* had little influence on naturally occurring *Z. marina* densities. Densities of *Z. marina* within the permanent experimental quadrats, however, showed a significant decrease, perhaps indicating shoot density declines in proximity to 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). Such an increase in hard-bodied taxa into native seagrass beds may exacerbate further invasion of *S. muticum* (Strong *et al.*, 2006) and other non-native taxa (e.g. *Codium fragile*; Thomsen and McGlathery, 2006; Drouin *et al.*, 2016).

Our results revealed that *Z. marina* phenolic content is suppressed in the presence of *S. muticum*. Nutrient limitation may potentially have influenced macrophyte biology and biochemistry, but no effects of nutrient limitation were found, suggesting that phenolic production within the *Z. marina* shoots, or lack thereof, was not a direct result of a Redfield ratio imbalance. These results contrast with findings for *P. oceanica*, which showed an increase in phenolic production with increasing invasive macroalgal interactions (Dumay *et al.*, 2004; Pergent *et al.*, 2008). Collectively, our findings indicate that macroalgal invasions into seagrass beds may have subtle, yet synergistic influences upon the physiology of seagrass, potentially leading indirectly to insidious consequences such as changing *Z. marina*'s defensive barrier 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 colonization (den Hartog, 1997).

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 and 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 and 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 exists 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.

The data accumulated in this study 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*'s invasion on *Z. marina*, but when considered collectively, they do. Although more than 4000 plant species have been introduced to the USA 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 invasion of *S. muticum* into *Z. marina* meadows. As with most scientific investigations, the present study has raised as many questions as it has answered. More research is required to examine the multitude of possible impacts of *S. muticum* on vulnerable seagrass beds.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Figure S1: Mean growth (mm per tank d^{-1}, ±s.e.) from three laboratory study with three treatments: *Zostera marina* only (Z), *Zostera marina* + *Sargassum muticum* (Z+S) and *Z. marina* + *Z. marina* (Z+Z) repeated across two years (2008, 2009); *n* = 10 tanks per treatment per year.

Figure S2. Mean tissue contents (a = carbon, b = nitrogen) in three regions of the seagrass (blades, roots, sheaths) in each of three laboratory treatments: *Zostera marina* only (Z), *Z. marina* + *Sargassum muticum* (Z+S), and *Z. marina* + *Z. marina* (Z+Z); *n* = 10 per treatment other than for blades, where reproductive activity resulted in seven, seven and four replicates respectively in the three treatments.

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