High mortality of *Zostera marina* under high temperature regimes but minor effects of the invasive macroalgae *Gracilaria vermiculophylla*

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**A B S T R A C T**

The present study tested for density-dependent effects of the invasive drift macroalgae *Gracilaria vermiculophylla* (Ohmi) Papenfuss on growth and survival of the native eelgrass, *Zostera marina* L., under different temperature levels. Three weeks laboratory experiments were conducted in Odense, Denmark, combining three algae densities (control, low 1.9 kg WW m⁻², high 4.5 kg WW m⁻²) with typical Danisch summer temperatures (18 °C) and elevated temperatures (21 °C and 27 °C). There was a significant effect of temperature on shoot survival with an average 68% mortality in the high temperature treatment but almost no mortality at the two lower temperatures. The higher mortality was probably caused by high sulphide levels in the sediment pore water (0.6 mmol l⁻¹ at 18 °C compared to 3.7 mmol l⁻¹ at 27 °C). Above-ground growth of the surviving shoots was also significantly affected by temperature, with leaf elongation rates being negatively affected, while the leaf plastochrone interval increased. Relative growth rate was significantly higher at 21 °C than at 18 °C or 27 °C, whereas rhizome elongation was significantly lowest at 27 °C. Elemental sulphur content in the plant tissues increased significantly with temperature and was up to 34 times higher (S⁰ in rhizomes) at 27 °C compared to the lower temperatures. In contrast to the temperature effects, cover by *G. vermiculophylla* did not cause significant effects on any seagrass responses. However, there was a (non-significant) negative effect of algal cover at the highest temperature, where the seagrass is already stressed. The latter results suggest that more studies should test for interaction effects between temperature and other anthropogenic stressors given that temperature is predicted to increase in the near future.

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1. Introduction

Seagrasses are coastal foundation species that protect shorelines by wave attenuation, provide habitat and nursery grounds for fish and invertebrates and filter land-derived nutrients (Heck et al., 2003; Orth et al., 2006). However, seagrasses are under threat worldwide from a growing number of anthropogenic stressors, in particular invasive species, global warming, nutrient pollution and drift algal accumulations (Short and Wyllie-Echeverria, 1996; Orth et al., 2006; Williams, 2007). In recent years much research effort has targeted specific impacts on seagrass performance from each of these stressors; for example accumulations of drift algae, high temperatures and certain non-native species have all, in separate studies, been shown to have negative effects on seagrass growth and survival (Nelson and Lee, 2001; Greve et al., 2003; Williams, 2007). However, in nature, these stress-factors often act together, with possible synergistic adverse effects, that cannot be detected in single-factor experiments. In addition, the stress-factors are generally density-dependent processes, with specific thresholds that, if exceeded, may cause population decline. Thus, to better understand impacts of multiple co-occurring anthropogenic stressors on seagrass performance, multi-factorial and density-dependent stress-experiments are needed.

Global warming can have direct and indirect effects on seagrasses (Short and Neckles, 1999). Direct effects of elevated temperatures include increasing rates of photosynthesis and respiration and possibly also changes in the P:R ratio (e.g., *Zostera marina* respiration exceeds photosynthesis above 30 °C (Greve et al., 2003)). Indirect effects comprise plant community changes, changes in frequency and intensity of extreme weather events and rising sea levels (Short and Neckles, 1999). Elevated temperature also increases the bacterial metabolism in the sediment, usually by
a factor of 2–3 for each 10 °C increment (Thamdrup et al., 1998; Sand-Jensen et al., 2007). In addition, to global warming, anthropogenic eutrophication can also stress seagrasses, e.g., via provision of organic matter and associated high sulphide levels in sediments; sulphide is a phytotoxin that can inhibit metaloenzymes e.g. cytochrome oxidase which is essential for ATP synthesis (Raven and Scrimgeour, 1997; Armstrong and Armstrong, 2005). Accordingly, increased sulphide levels can reduce seagrass growth and potentially ultimately lead to increased mortality.

Zostera marina is one of the most important meadow-forming seagrasses in temperate ecosystems (Short et al., 2007) and it is therefore of paramount importance to know if this species can tolerate the combined effects of increased temperatures and competition from drift algae. By contrast, eutrophication and elevated temperatures may give a competitive advantage to macroalgal species that are tolerant to high temperatures and that can convert nutrients into rapid growth (Pedersen and Borum, 1996). The spatial structure of seagrass beds may also enhance accumulation of drifting algae (Tweedley et al., 2008), particularly in shallow lagoons and estuaries which are important habitats for populations of drifting algae (Tweedley et al., 2008), particularly in temperate seagrass beds as seen for California seagrass beds (Huntington and Boyer, 2008). More specifically, we hypothesize that negative effects on Z. marina performance are highest when both temperature and drift algae levels are highest. In this study, seagrass performance measures include shoot mortality and several above- and belowground growth indices. Levels of elemental sulphur in the seagrass tissue and of sulphide in the sediment were also quantified. Sulphide has been suggested to be a contributing factor of seagrass mortality during stress (Holmer et al., 2005a, 2005b; Holmer and Nielsen, 2007).

2. Materials and methods

2.1. Field sampling

Sediments, eelgrass, and algae were collected around the island of Fyn in August, October, and November 2007. Sediment, characterized by low iron (<5 μmol cm⁻³) and organic matter content (~0.2%) was collected at Svenstrup Strand (55° 28’ N/9° 45’ E, Fig. 1, Table 1) between 0.5 and 1.5 m depth. The sediment was transported to the laboratory, sieved through a 1000 μm sieve and homogenised. Undamaged Zostera marina shoots were collected at ca. 1.5 m depth at Gals Klint (55° 30’ N/9° 40’ E), where the seagrass is found in extensive shallow water meadows. Average temperatures in the waters around Fyn at the sampling times were 15.7 °C in August, 12.8 °C in October and 8.4 °C in November (ICES Oceanographic Database, 2009). Shoots were cleaned free of sediments and transported to the laboratory in buckets with seawater (ca. 20 psu) and left aerated for 3–5 days until the start of the experiment. Gracilaria vermiculophylla was collected at Fyns Hoved (55° 36’ N/10° 36’ E, Fig. 1) by picking up clumps of algae at about 30 cm water depth. Transport, laboratory processing and acclimatisation were otherwise similar to the procedures described for seagrasses.

2.2. Experimental design

The experiment was initially setup as a two-factorial design in three replicates (Table 1), i.e. three buckets combining either of three levels of temperature (means of 18 °C, 21 °C and 27 °C) and 3 levels of Gracilaria vermiculophylla biomass (0, ca. 2000, ca. 4500 g m⁻²). The chosen temperatures were based on observations of average temperatures during July and August, measured in Odense Fjord. Kertinge Nor and Holckenhavn Fjord from 2000 to 2006 (MADS Database, 2009) and regional climate models, based on the IPCC-DCC emission models SRES A2 and B2 (Cubasch et al., 2007).
which predict an increase of mean sea surface temperature in the Baltic sea area of 2–4 °C until the end of the 21st century (HELCOM, 2007). The high temperature corresponds to a ‘worst case scenario’ and was chosen deliberately to be close to but below an upper lethal temperature tolerance of ca. 30 °C for Zostera Marina (Greve et al., 2003).

Due to logistic constraints we had to divide the experiment into three sub-experiments. The first two sub-experiments were run at 18 and 21 °C and differed in the number of buckets. In the first sub-experiment it was 1 per treatment combination, for a total of six. In the second experiment there were 2 per treatment, for a total of twelve. Both were conducted at Marine Biological Research Station, Kerteminde, where two 600 l tanks were equipped with a 500 W heater and two circulation pumps each (Fig. 2A) and half of the water was exchanged each week. Due to logistic constraints the replicates for the first setup were divided into two sub-experiments (Experiments 1 and 2).

### Experimental setup. Densities (g WW m⁻²) are given as average for each treatment (±1 SE n = 2–3). Due to logistic constraints the replicates for the first setup were divided into two sub-experiments (Experiments 1 and 2).

<table>
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<th>Exp. Start</th>
<th>End</th>
<th>Temperature (°C)</th>
<th>Treatment</th>
<th>T_start</th>
<th>T_end</th>
<th>No. replicates</th>
<th>Wet density (g ml⁻¹)</th>
<th>H₂O content (%)</th>
<th>Medium grain size (μm)</th>
<th>Org. matter (μmol cm⁻³)</th>
<th>Fe(III) (μmol cm⁻³)</th>
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<td>1808 ± 0</td>
<td>1879 ± 0</td>
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<td>4.8 ± 2.6</td>
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</table>

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### Weekly sampling

Each week, sulphide content in the pore water was measured. Three sub-replicates per bucket were taken at 3 cm sediment depth, with needles attached to syringes, for a total of 81 subsamples each week. The needles were closed at the end and had the first root bundle was measured. To standardize the planting units, rhizomes were trimmed to three rooted nodes, one end-node bearing leaves, the other two only roots. For the underground parts the time interval between measurements was 28 days (1 week acclimatisation, 3 weeks with treatment) so that uprooting the plants for measurement before the algae were added could be avoided. After planting the shoots, the buckets were put into the aquaria tanks freshly filled with water from the sea (19.6 ± 0.4 psu). The heaters were used to warm the water to the desired temperatures over the course of one week, depending on the initial temperature of the seawater at daily rates of 0.3 °C in August, 0.7 °C in October and 2.7 °C in November. Leaves were then marked with a syringe needle through the leaf sheath (Figure 8-2A; Short and Duarte, 2001) before adding drift algae biomass and then left for 3 weeks of growth. This was well above the plastochrone intervals for leaves as well as rhizomes which both are ca. 15 days (Short and Duarte, 2001). Epiphytes, sediments and mesograzers were gently removed from healthy tissue of Gracilaria vermiculophylla (i.e. without any visible indications of rot). Blotted wet weight of the algae was measured before and after the experiment. Approximately 60 g (2000 g m⁻²) and 150 g (4500 g m⁻²) were added to buckets, corresponding to low (L) and high (H) density treatments, respectively. Control buckets (C) did not have any algae. The two algal addition treatments were chosen to represent algal patches in seagrass beds as they are found in nature where dense aggregations occur, with large free spaces in between. As 10 cm of sediment filled only half of the bucket, the remaining 10 cm of the side walls created a protected microcosm that prevented the advection of low oxygenated water out of the bucket. Temperature and light under the algal mats were measured with HOBO data loggers. Temperature averages were 18.3 ± 1.0 °C, 20.8 ± 1.0 °C and 27.0 ± 2.6 °C and light was 13.5 ± 1.9 μmol photons m⁻² s⁻¹. In the low algae density treatments and 5.0 ± 0.6 μmol photons m⁻² s⁻¹ in the high density treatments. In the control buckets, light at the sediment surface was constantly above 130 μmol photons m⁻² s⁻¹.
four 0.4 mm holes in the sides (Berg and McGlathery, 2001). The samples were preserved in a solution of 0.5 M ZnAc until distillation. Sulphide concentrations were quantified photometrically at 670 nm as described by Cline (1969). AVS and CRS samples were distilled as described by Fossing and Jorgensen (1989) and analyzed photometrically at 670 nm following Cline (1969).

Water column oxygen levels were measured weekly with a YSI 55 field electrode mounted on a lab-stand at 10, 5 and 2 cm above the Gracilaria-mat and subsequently for every cm through the mat all the way down to the sediment surface. Measurements were taken in the last 2 h of the light and the dark period.

2.4. Growth measurements and elemental sulphur

After the 3 weeks of each sub-experiment all seagrass shoots were harvested, counted and examined for survival by examining structural integrity in the meristem region (dead shoots dis-integrated between fingers). To determine borderline cases of structural integrity, a 30 g weight was attached to the rhizome, and if the shoot broke at the meristem region it was classified as ‘dead’. The mortality rate was calculated as a percentage of dead shoots per day, for 21 days in total. For measuring growth ten shoots were randomly selected from each bucket. The number of leaves and side shoots was counted on them and each leave with a punch mark was measured from the meristem to the mark and from the meristem to the tip. New, unmarked, leaves were measured only from the meristem to the tip. The sum of the first measurement gave the total leaf growth, the sum of the second gave the total aboveground shoot length. Measurements from meristem to tip of new, unmarked, leaves went into both sums. The number of leaves and side shoots was counted too. Plastochrone Intervals (PL) for leaves and rhizomes (PR), relative growth rate (RGR), and leaf and rhizome elongation were calculated according to Short and Duarte (2001). All shoots from each individual bucket were separated into leaves, rhizomes and roots and the pooled dry weight for each compartment was measured. The ten shoots chosen for further measurements were freeze dried while the remaining shoots were dried at 60 °C for 24 h. The content of elemental sulphur was measured in the roots and rhizomes in the first two experiments and in addition the leaves in the third experiment were also analyzed after extraction in methanol following Zopfi et al. (2001) in a Dionex UV 170 U detector with HPLC grade methanol as the eluent.

2.5. Sediment characterization

The initial sediment characteristics were determined by quantifying wet density, water content organic matter, iron content and grain size. Wet density was determined by measuring the weight of 5 ml of sediment. Water content was measured by drying wet sediment at 105 °C for 24 h and reweighing. Organic content was measured by loss on ignition (560 °C for 6 h). Iron (Fe_total) was extracted by suspending wet sediment in 0.5 M HCl and shaking for 30 min. The supernatant was then measured by photometric absorption at 562 nm using the ferrozine method (Stookey, 1970; Sørensen, 1982). Finally, grain size was determined after Wentworth (1922) by sieving the wet sediment through a succession of sieves (1000, 500, 250, 125 and 63 μm) defining the last
fraction that passed through the 63 μm sieve as ‘fine’. Percentage of each fraction was determined after drying at 105 °C for 24 h.

2.6. Statistical procedures

Effects of temperature and drift algal levels were tested with a 2-way ANOVA (Statistica, 2001, version 6, Tulsa, OK, Statsoft Inc.). Data was tested for normality with the Shapiro–Wilks test and if necessary transformed by either log (+0.1), root or arcsine (for percentages) transformation. Regrettably the samples of AVS and CRS from the first sub-experiment were lost. To achieve the necessary number of replicates for the ANOVA test on AVS and CRS we pooled the two lower temperatures into a low temperature group and compared it to the high temperature, thus reducing the number of temperature treatments to two for this test. Test for homogeneity of variance was done with Levene’s test and post-hoc pair wise comparison was done with Student–Newman–Keul test.

Results were accepted as significant if \( p < 0.05 \) and termed near-significant if \( 0.05 < p < 0.1 \). Linear regression was conducted to examine potential relationships between sediment pore water sulphide as well as AVS vs. plant tissue S0 content and seagrass mortality. A possible relation between oxygen concentration at the sediment surface and mortality rate was also examined by linear regression.

3. Results

3.1. Sediment sulphur and water column oxygen levels

Temperature significantly affected AVS, CRS and pore water H2S (Table 2a). In general, the 18 °C and 21 °C treatments had similar values, which clearly differed from 27 °C treatment. AVS (Fig. 3A) and CRS (Fig. 3B) were both significantly affected by temperature, when the lower temperatures were pooled (Table 2a). Values ranged from 0.9 ± 0.1 mm cm⁻³ to 2.4 ± 0.2 mm cm⁻³ for AVS (Fig. 3A) and 16.0 ± 1.5 mm cm⁻³ to 29.4 ± 2.2 mm cm⁻³ for CRS (Fig. 3B). The average H2S concentrations were 787 ± 122 μmol l⁻¹ for the lower temperatures (18 and 21 °C) and 3186 ± 467 μmol l⁻¹ for the high temperature at 27 °C (Fig. 3C). The development of H2S over time did not follow a consistent pattern and only at the high temperature was the concentration rising constantly, although with a decreasing rate in the last measurement (Tables 3 and 4).

The effect of temperature on the average oxygen concentrations was much stronger than the effect of Gracilaria density treatments (Fig. 4). The high Gracilaria density treatment differed from the other two treatments at 18 °C both during the day (6.4 mg l⁻¹ compared to >8 mg l⁻¹) and the night (3.2 mg l⁻¹ compared to >5.5 mg l⁻¹) and at 27 °C (7.7 mg l⁻¹ compared to >11 mg l⁻¹) during the day. The lowest values were measured at 27 °C during the night ranging from 3.1 to 4.3 mg l⁻¹. In the high temperature treatment the difference between day and night was more pronounced with the daylight values being between 2.5 and 3.5 times higher than those at night, while at the lower temperatures it was only 1.2–1.9 times higher.

3.2. Mortality and biomass

Only temperature alone had significant effects on the mortality of eelgrass (Table 2c). While in the two lower temperatures only 3 out of 450 shoots died, the mortality at 27 °C was much higher with one sub-replicate having zero survivors. The highest average mortality rate was 3.1% shoots d⁻¹ for the high Gracilaria density treatment at 27 °C (Fig. 5A).

Leaf biomass per bucket at the two lower temperatures was 3.36 ± 0.4 g DW on average. At 27 °C it was reduced with only 1.4 ± 0.4 g DW on average (Fig. 5B). Root biomass was significantly affected by temperature, while rhizome biomass (\( p = 0.06 \)) was not significant at the alpha = 0.05 level. Rhizome and root biomass per bucket were 0.9 ± 0.1 g and 0.6 ± 0.1 g at the lower temperatures and, at 0.2 ± 0.1 g and 0.1 ± 0.02 g respectively were lowest at 27 °C (Table 2c, Fig. 5C and D).

Although there were no single or interactive significant effects of Gracilaria, a graphical analysis indicated a consistent negative effect of drift algal additions at the highest temperature only (Fig. 5). Thus, at 27 °C mortality was highest at the high Gracilaria levels and lowest in the controls. The effect on biomass was

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**Table 2**

Results of 2-way ANOVA on fourteen response variables. Sub-table a presents the results for sulphur compounds in the sediments and in the pore water. Sub-table b shows the results for sulphur compounds in the sediments and in the pore water. Sub-table c presents the results for sulphur compounds in the sediments and in the pore water.

| Source of variation | DF | MS | F | p | MS | F | p | MS | F | p | MS | F | p |
|---------------------|----|----|---|---|----|---|---|----|---|---|----|---|---|---|
| a) AVS               |    |    |   |   |    |   |   |    |   |   |    |   |   |   |
|                      |    |    |   |   |    |   |   |    |   |   |    |   |   |   |
| Temperature         | 2  (1) | 2.09 | 7.51 | 0.015* | 500.41 | 40.87 < 0.001* | 2457.00 | 21.88 < 0.001* | 2.36 | 5.30 0.016* | 3206.19 | 3.30 0.050 |
| Gracilaria level     | 2   | 0.14 | 0.52 | 0.607 | 1.51 | 0.12 | 0.885 | 154.80 | 1.50 | 0.250 | 0.98 | 2.21 | 0.139 |
| Temp × Grac         | 4   (2) | 0.98 | 3.53 | 0.056 | 13.11 | 1.07 | 0.368 | 56.10 | 0.54 | 0.706 | 0.45 | 1.00 | 0.432 |
| Error               | 18  (15) | 0.28 | 12.25 | 0.45 | 103.30 | 0.45 | 9727.5 |
| b) Relative growth rate |    |    |   |   |    |   |   |    |   |   |    |   |   |   |
|                      |    |    |   |   |    |   |   |    |   |   |    |   |   |   |
| Temperature         | 2   | 0.00005 | 3.76 | 0.005* | 172.73 | 10.21 | 0.001* | 33.98 | 1.53 | 0.002* | 19.98 | 0.886 | 0.429 |
| Gracilaria level     | 2   | 0.00002 | 1.62 | 0.226 | 1.19 | 0.07 | 0.932 | 17.88 | 0.56 | 0.580 | 1.28 | 0.057 | 0.345 |
| Temp × Grac         | 4   | 0.00001 | 0.84 | 0.515 | 14.29 | 0.84 | 0.515 | 29.68 | 0.93 | 0.467 | 60.50 | 2.684 | 0.065 |
| Error               | 18  | 0.00001 | 16.91 | 31.84 | 22.54 | 0.02 |
| c) Mortality rate (arc sine transformed) |    |    |   |   |    |   |   |    |   |   |    |   |   |   |
|                      |    |    |   |   |    |   |   |    |   |   |    |   |   |   |
| Temperature         | 2   | 0.00116 | 86.78 < 0.001* | 0.55 | 3.76 | 0.004* | 0.53 | 3.24 | 0.063 | 0.73 | 9.46 0.002* |
| Gracilaria level     | 2   | 0.00005 | 2.33 | 0.123 | 0.16 | 1.11 | 0.351 | 0.06 | 0.35 | 0.708 | 0.18 | 2.39 | 0.129 |
| Temp × Grac         | 4   | 0.00004 | 1.90 | 0.154 | 0.12 | 0.82 | 0.530 | 0.03 | 0.20 | 0.935 | 0.03 | 0.35 | 0.839 |
| Error               | 18  | 0.00002 | 0.15 | 0.16 | 0.08 |

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reversed with highest values for controls and lowest values at the high *Gracilaria* levels.

### 3.3. Aboveground and belowground growth

The algae mats were left untouched for the duration of the experiment, to provide minimal experimental disturbances to sediments and *Z. marina* growth, resulting in some algal net growth, particularly in the high temperature treatment (Table 1). For the eelgrass there were significant effects of temperature for all aboveground test-variables (Table 2b). In contrast, all *Gracilaria* treatments, and temperature × *Gracilaria* interactions were not significant (p > 0.14). Mean relative growth rate was higher at the lower two temperatures at 1.5 ± 0.1% d⁻¹ compared to a mean of 1.0 ± 0.2% d⁻¹ at 27 °C across all 3 levels of *Gracilaria* additions (Fig. 6A). The mean PL ranged from 9.0 ± 1.9 d at 27 °C to 16.3 ± 0.6 d at lower temperatures (Fig. 6B). Leaf elongation rate ranged between 9.4 ± 2.1 mm d⁻¹ at 27 °C and 18.6 ± 1.3 mm d⁻¹ at the lower temperatures (Fig. 6C). The general trend in the effect of temperature on leaf growth was that higher temperatures stimulated the production of new leaves but caused slower growth of existing leaves.

The belowground data were slightly less consistent compared to the aboveground data (Table 2b, Fig. 6). In general, as for the aboveground data, there were no effects of *Gracilaria* additions, except for a near-significant *Gracilaria* × temperature interaction on rhizome plastochrone interval, indicating a complex interaction, with positive effects of *Gracilaria* addition at the two lowest temperatures, but a reversed pattern for the highest temperature...
Again all the measured response variables (except rhizome plastochrone intervals, see above) were significant or near-significant for the temperature treatments. Rhizome elongation was lowest at the highest temperature (Fig. 6E, Table 2b). It ranged from $0.4 \pm 0.03$ mm d$^{-1}$ at the lower temperatures to $0.1 \pm 0.03$ mm d$^{-1}$ at $27^\circ C$.

A graphical analysis revealed consistent negative effects of drift algal additions at the highest temperature. As for the biomass the pattern was reversed compared to mortality rate (Figs. 5 and 6). Thus, at $27^\circ C$ all response variables showed highest values without drift algae, intermediate values at low *Gracilaria* levels and lowest values at high *Gracilaria* levels.

### 3.4. Elemental sulphur in the plant tissue

$S^0$ in the leaves was only measured at $27^\circ C$ because concentrations in the underground parts were low in samples of the two lower temperatures. At $27^\circ C$ the highest concentration was $0.003 \pm 0.002$ μmol gDW$^{-1}$ which was measured in the control treatment without drift algae. Similarly, plant sulphur levels in the belowground tissue were affected by temperature only, with highest $S^0$ levels at $27^\circ C$ (although only near-significant for sulphur in the roots, Table 2a, Fig. 7A). For rhizomes and roots, sulphur levels ranged from $5.9 \pm 0.8$ and $31.1 \pm 4.9$ μmol gDW$^{-1}$ at the lower temperatures to $288.1 \pm 40.2$ and $197.7 \pm 126.1$ μmol gDW$^{-1}$ at the high temperature, respectively. Within the same *Gracilaria* treatment, $S^0$ levels were much higher at $27^\circ C$, up to 34 times for rhizomes but only up to 6 times in roots.

### 4. Discussion

This study showed that a three-week cover by the invasive drift macroalgae *Gracilaria vermiculophylla* did not significantly affect growth or survival of *Zostera marina*, whereas the increase in temperature following predicted climate scenarios significantly affected both parameters.

#### 4.1. Mortality, sulphur compounds and oxygen

The most obvious effect on *Zostera marina* was the much higher mortality at $27^\circ C$. The temperature significantly affected the concentrations of pore water sulphide, which in turn were strongly correlated with the mortality of the eelgrass. The difference between leaf biomasses of low and high temperature treatments
may also be attributed to the different mortalities, as in 5 out of 9 replicates at the high temperature only a few leaves survived. Several other studies have shown detrimental effects of pore water sulphide at lower concentrations than those measured. Goodman et al. (1995) found reduced photosynthesis vs. irradiance ratios at concentrations of 400–800 $\mu$mol l$^{-1}$ and Holmer and Bondgaard (2001) used concentrations of 100–1000 $\mu$mol l$^{-1}$ in the water column and observed negative effects on growth, meristems and aboveground biomass. In the present experiment meristem damage occurred mostly at high concentrations at 27 °C, similar to Martínez-Lüscher and Holmer (2010). Their correlation between pore water sulphide and mortality rate was, however, rather weak, but they had indications of an anoxic and sulphidic layer in the lower water column at night, thus concluding that the high mortality was caused by sulphide poisoning during the night.

The mortality rates associated with sulphide exposure appear to differ between seagrass species. *Thalassia testudinum* was able to tolerate 2–10 mmol l$^{-1}$ (Koch and Erskine, 2001), while *Halophila engelmannii* showed tissue necrosis and eventual mortality at concentrations as low as 200 $\mu$mol l$^{-1}$ (Pulich, 1983). For Zostera marina Pedersen et al. (2004) found that as long as the internal oxygen concentration was above 35% air saturation sulphide intrusion into the plant was prevented even at sulphide concentrations >1000 $\mu$mol l$^{-1}$. *Zostera marina* usually grows in highly reduced sediments and accordingly has high sulphide intrusion (Frederiksen et al., 2006, 2008; Holmer and Frederiksen, 2007), with significantly detrimental effects only occurring in the interaction with other stressors such as water column anoxia (Holmer and Bondgaard, 2001; Holmer and Nielsen, 2007). The internal oxygen balance during the day is maintained by oxygen produced in photosynthesis while the uptake of oxygen from the water column maintains the balance in the dark (Greve et al., 2003; Pedersen et al., 2004). The oxygen produced during the day is not stored, at least not in large amounts for use during night time (Sand-Jensen et al., 2005). At night oxygen from the water column is diffusing into the plant leaves and down to the belowground system via the lacunae. Therefore, low oxygen concentrations during the night may be detrimental to the plants. Frederiksen and Glud (2006) examined the oxygenation of the rhizosphere at 12 °C and found that leakage of O$_2$ from roots stops at water column concentrations between 70 and 150 $\mu$mol l$^{-1}$ (1–2.5 mg l$^{-1}$). Holmer and Nielsen (2007) suggested that the critical question is whether the meristems are in a zone with low oxygen concentration and potentially high sulphide invasion, when the seagrass is covered with drift algae. As the saturation at the sediment surface was low (e.g. average 41.6 ± 7.9% in 27H) the high mortality, up to 77% of all shoots in the high density treatment died, in the present study appears to support their conclusion. The importance of elevated temperatures on the survival of *Z. marina* under anoxic conditions was recently demonstrated by Pulido and Borum (2010) where shoot mortality started after 24 h in anoxic water at 20 °C but at 30 °C no shoot survived longer than 8 h.
Given the results of this study the effects of elevated temperatures under hypoxic conditions seem to be similar but on a longer timescale.

Mortality in this study was, in addition to correlation with pore water H$_2$S, also correlated with elemental sulphur accumulation in the rhizomes. Plant S$^{0}$ concentrations at 27 °C were up to 34 times higher than in the two lower temperatures and are comparable to the values measured by Holmer et al. (2005b) for Zostera marina exposed to high sulphide concentrations. The large difference in S$^{0}$ between the high and the two lower temperatures indicates a potential threshold for S$^{0}$ accumulation and that seagrasses at high temperature had to cope with high pore water H$_2$S concentrations and thus more sulphide intrusion. At 27 °C the S$^{0}$ concentration in rhizomes and roots showed a declining trend with increasing density of Gracilaria vermiculophylla. This may be counterintuitive but is consistent with observations by Koch et al. (2007) where the capacity of the plants to reoxidize sulphides was reduced in the presence of multiple stressors (temperature, hypoxia and salinity) at high levels. They suggested that a lack of reoxidation capacity increases the exposure of the sensitive parts of the plants to sulphide and increases mortality. The similar effect on the S$^{0}$ in rhizomes at 18 °C may be due to the rot on the underside of the algal mat in the treatment with high Gracilaria density.

The significant effects on AVS and CRS resemble the effects shown by Holmer and Nielsen (2007). In the present experiment the cause was elevated temperatures while in their experiment, which was done at constant temperature (15 °C), it was coverage with filamentous algae.
Apart from indirect effects by sulphide, elevated temperature alone causes direct physiological stress for seagrasses, which, when sustained over a longer time can cause a higher mortality rate. Greve et al. (2003) found anoxic conditions in the meristems of Zostera marina due to oxygen depletion by enhanced respiratory demands, when exposed to temperatures higher than 30 °C. Nejrup and Pedersen (2008) found a 12-fold increased shoot mortality compared to the optimal range, when exposing Z. marina to 25–30 °C. These authors emphasized that their setup was well aerated and therefore explained the detrimental effects with physiological stress alone, as accumulation of sulphide was unlikely.

### 4.2. Aboveground and belowground growth

Growth of the aboveground parts of Zostera marina was significantly affected by the temperature, documenting that temperature is a stronger stress factor than drift algae which had no significant effect on the aboveground growth. A similar negative effect of high temperature on growth was documented by Nejrup and Pedersen (2008) who observed a reduction in leaf elongation rate by 75% which is comparable up to 77% reduction in the present study (27H vs. 21H). Holmer and Bondgaard (2001) observed initially high leaf elongation rates under high sulphide levels and anoxia, however, leaf elongation stopped after 14 days and meristem damage occurred. In their experiment no drift algae were added and the temperature was lower (15 °C), this may account for the different results, as the low temperature may have delayed the effect of sulphide in their experiment. Although the effects of drift algal cover were not significant, noticeable trends of decreasing growth, leaf elongation, and plastochrone interval in leaves were observed at 27 °C. These decreases are consistent with Holmer and Nielsen (2007), where reduced growth in Z. marina exposed to filamentous drift alga was noticed. Similarly Lamote and Dunton (2006) observed a reduction in effective quantum yield (a proxy for photosynthesis) in treatments with 50% macroalgal cover.

For the belowground compartments of the plants there was the same significant effect of temperature but also a trend for negative effects of Gracilaria vermiculophylla on plastochrone interval in rhizomes in the lower temperatures and a negative trend of G. vermiculophylla on rhizome elongation rate at 27 °C. Morphological adaptations to environmental stress have been observed for various seagrass species (Irlandi et al., 2002; Miller et al., 2005; Peralta et al., 2005). For example, Halophila ovalis grows shorter internodes under high sulphide pressure (Kilminster et al., 2008) and stunted root growth has been noticed in several studies (Holmer and Bondgaard, 2001; Holmer and Nielsen, 2007; Mascaro et al., 2009; Holmer et al., 2010). The lower rhizome elongation rate may not only be due to exposure to high sulphide levels, but also due to a decreasing capacity of the seagrass to cope with similar levels of sulphide concentration (Koch et al., 2007). Most studies about temperature effects on seagrasses have been done on sub- and tropical species. Campbell et al. (2006) exposed seven tropical species to temperature peaks of 35–45 °C for 1–4 h for several days. For four species Cymodocea rotundata, Cymodocea serrulata, Halophila universis, and Thalassia hemprichii, all restricted to (sub)tropical waters, they found no lasting damage after treatments of up to 40 °C. The other three species H. ovalis, Syringodium isoetifolium and Zostera capricornis, which can also be found in temperate regions already exhibited thermal stress symptoms at 40 °C. Treatments with 45 °C on the other hand were detrimental to all species. Effects were a decrease in photosynthetic yield and a shift toward non-photochemical quenching. At 45 °C irreversible damage to photosynthetic enzymes and the photosystem II occurred rapidly. Long term temperature tolerances are lower. When comparing the two tropical species Halodule wrightii and Thalassia testudinum, Koch et al. (2007) found reduced growth and quantum efficiency for T. testudinum starting at 33 °C while H. wrightii maintained a high photosynthetic rate up to 34–35 °C. In this study the authors also experimented with added organic material to enhance sediment sulphide. In this case T. testudinum exhibited higher tolerances under high temperatures. The authors concluded that temperature and sulphide concentration have synergistic effects with different effects on different species. Generally species from warmer climate zones seem more capable to increase photosynthesis and respiration over a wider range than temperate species which have their optima at temperatures below seasonal maxima (Short and Neckles, 1999).

### 4.3. Ecological aspects of drift algae in a warmer climate

Although there was a noticeable trend of negative effects of drift algal covers on Zostera marina, only temperature had significant effects on eelgrass response variables. Other studies have found more pronounced and significant effects of drift algal cover on seagrass, in field as well as laboratory experiments. Macià (2000) documented a reduction in shoot density in a Thalassia testudinum bed through cover by drift algae (Laurencia spp. and Dictyota
spp.), an effect that was enhanced under increased grazing pressure by sea urchins. Irlandi et al. (2004) maintained dense cover by drift algae (Laurencia spp. and Polysiphonia spp.) on small plots (0.25 m²) of T. testudinum for 2–3 months and observed a reduction in aboveground biomass by 25% but no further loss after additional 3 months of cover. They also observed no short term (10 days) effects on the growth of T. testudinum and attributed this to resource sharing with adjacent shoots. The loss of biomass was attributed to lowered light availability due to the algal mats. Nelson and Lee (2001) excluded Ulvaia obscura over 5 weeks from plots within a Z. marina bed and found a large shoot loss in the presence of drift algae (54% vs. 12% in non-stressed control plots). Huntington and Boyer (2008) conducted enclosure/exclosure experiments of 3 months duration with Gracilaria andersonii in Z. marina beds. They reported significant reduction of Z. marina shoot density with high densities of G. andersonii (1700 g m⁻²) when compared with low densities (0–325 g m⁻²). The authors attributed this loss of shoot density mainly to the loss of newly recruited shoots buried under the mat.

Most of these studies were of longer duration (several months) and used algae species of different morphological types (sheet-like or filamentous) compared to this study. The shading effect of sheet-like algae or dense mats of filamentous algae may be greater than that of an openly structured mat of Gracilaria vermiculophylla, and, given the relatively short duration of our experiment, this may partially explain the lack of significant effects. As G. vermiculophylla can be found year-round in temperate estuaries (Thomsen et al., 2006), with rapid growth in spring, this will cause longer and probably stronger competition compared to our experiment. The open spatial structure of the G. vermiculophylla mats may allow more water movement, compared to a tighter packing of sheet-like or filamentous mats and therefore also higher oxygen concentrations. Holmer and Nielsen (2007) used very similar incubation time, but they observed larger negative effects probably through the rotation of the filamentous algae resulting in oxygen depletions and large pools of sulphides within a very short time (<3 weeks). Significantly higher sulphide concentrations in the sediments at just 3 °C higher than here (30 °C) were also found by Martínez-Lüscher and Holmer (2010).

5. Conclusions

As shown by other studies, elevated temperatures had large-scale negative effects on Zostera marina which illustrates its overriding importance compared to other environmental stressors. When comparing 18 °C, 21 °C and 27 °C the high mortality in the highest temperature is notable, indicating that a change to a warmer climate regime will likely cause higher eelgrass mortality, either independently or in combination with other environmental stressors. In contrast, the relatively short-term cover by Gracilaria vermiculophylla had no major effect on eelgrass survival, although consistent, but non-significant, trends suggested a negative influence on Z. marina performance at the highest temperature. This latter finding supports the suggestion that synergistic stressors (here drift algae and elevated temperatures) over longer time scales, could be causative agents resulting in significant loss of seagrass beds and local extinctions in areas affected by invasive algae.

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