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# Seagrass wasting disease varies with salinity and depth in natural *Zostera marina* populations

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ABSTRACT: In the 1930s, the wasting disease pathogen *Labyrinthula zosterae* is believed to have killed 90% of the temperate seagrass *Zostera marina* in the Atlantic Ocean. Despite the devastating impact of this disease, the host–pathogen interaction is still poorly understood, and few field studies have investigated factors correlating with the prevalence and abundance of *L. zosterae*. The present study measured wasting disease in natural populations of *Z. marina* on the Swedish west coast, and showed a strong correlation between the disease and both salinity and water depth. No infection was detected in *Z. marina* shoots from low-salinity (13–25 PSU) meadows, whereas most shoots carried the disease in high-salinity (25–29 PSU) meadows. Shallow (1 m) living *Z. marina* shoots were also more infected compared to shoots in deeper (5 m) meadows. In addition, infection and transplantation experiments showed that *Z. marina* shoots from low-salinity meadows with low pathogen pressure were more susceptible to *L. zosterae* infection. The higher susceptibility could not be explained by lower content of inhibitory defense compounds in the shoots. Instead, extracts from all *Z. marina* shoots significantly reduced pathogen growth, suggesting that *Z. marina* contains inhibitory compounds that function as a constitutive defense. Overall, the results show that seagrass wasting disease is common in natural *Z. marina* populations in the study area and that it increases with salinity and decreases with depth. Our findings also suggest that low-salinity areas can act as a refuge against seagrass wasting disease.

KEY WORDS: *Labyrinthula zosterae* · Eelgrass · Chemical defense · Host–pathogen interaction · Infection · Transplantation

## INTRODUCTION

Seagrass meadows are one of the most valuable ecosystems in the world (Costanza et al. 2014). They have important roles in carbon sequestration and sediment stabilization (Duarte 2000, Mateo et al. 2006), and constitute nursery grounds for commercially important species of fish and shellfish (Beck et al. 2001, Heck et al. 2003). A large decline of seagrass populations has been documented worldwide, threatening the health of these coastal systems (Waycott et al. 2009). The most dominant seagrass species in the northern hemisphere is the eelgrass *Zostera marina* L. In the 1930s, large parts of the *Z. marina* populations along the Atlantic coasts were killed in an epidemic believed to have been caused by the marine pathogen *Labyrinthula zosterae* D. Porter & Muehlstein, 1991 (Petersen 1934, Renn 1935). This endophytic protist causes black necrotic lesions on *Z. marina* leaves and is commonly referred to as seagrass wasting disease (Muehlstein et al. 1991). More recently, only a few outbreaks have been reported (Short et al. 1987), even though *L. zosterae* and other pathogenic *Labyrinthula* spp. have been identified in seagrasses in many parts of the world (Sullivan et al. 2013). *Z. marina* and *L. zosterae* presently co-exist and the pa-

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thogen does not appear to be virulent in northern European populations (Brakel et al. 2014, S. Jakobsson-Thor pers. obs.).

It is still not known what caused *L. zosterae* to become pandemic in the 1930s (Short et al. 1987), but many of the surviving or less affected *Z. marina* populations were found in areas with low salinity (e.g. Cottam 1935, Young 1943, Stevens et al. 1950, Rasmussen 1977, den Hartog 1987), leading to the hypothesis that low salinity mitigates the infection (Young 1938, 1943). Several studies have therefore investigated whether variation in salinity affects the virulence of the pathogen (Young 1943, Short et al. 1986, Giesen et al. 1990, Burdick et al. 1993), and it has been reported that *Labyrinthula* spp. becomes inactive below 10 PSU (Young 1943, Muehlstein et al. 1988, Martin et al. 2009). Additionally, laboratory experiments have found a correlation between salinity and lesion coverage (Burdick et al. 1993), and a positive linear relationship between salinity and *L. zosterae* lesion area (McKone & Tanner 2009), further supporting the hypothesis that low salinity is a limiting factor for *L. zosterae* infection. A yearlong field survey showed that wasting disease increased during increasing salinity events in a *Z. marina* meadow in Great Bay, New Hampshire, USA, and that prolonged disease was sustained during salinities above 20 PSU (Burdick et al. 1993). The impact of *Labyrinthula* sp. disease on the subtropical seagrass *Thalassia testudinum* K. D. Koenig, 1805 has also been found to be low in areas below 15 PSU salinity (Blakesley et al. 2002), but to our knowledge, quantitative measurements of how *L. zosterae* disease and pathogen abundance varies in different salinity environments among natural *Z. marina* populations are still missing.

Despite the devastating effects *L. zosterae* can have on *Z. marina* populations (Sullivan et al. 2013), few published studies have specifically aimed at investigating how *L. zosterae* prevalence and abundance varies in the field. So far, the degree of infection has been found to vary spatially within and between meadows, and temporally within and between years (Hily et al. 2002, Bockelmann et al. 2013). The infection rate has also been shown to correlate with several seagrass parameters, including shoot length (Groner et al. 2014), biomass (Trevathan-Tackett et al. 2013), epiphyte load (Groner et al. 2016), and seagrass density (Bull et al. 2012, Groner et al. 2016). Furthermore, pathogen prevalence has been connected to water depth in intertidal *Z. marina* meadows, suggesting that the disease is more prevalent on shoots at shallower depths (Groner et al. 2014), but it is presently unknown whether this pattern also applies to non-tidal systems such as the ones found on the Swedish west coast.

One way for *Z. marina* to withstand infection by *L. zosterae* is to be chemically defended through the production of secondary metabolites. Extracts and isolated compounds from several seagrass species show inhibitory effects against associated microorganisms and pathogens (Zidorn 2016). For example, extracts from 2 species (*Halodule beaudettei* den Hartog, 1964 and *Syringodium filiforme* Kützing, 1860) both deter a stramenopile pathogen (*Schizochytrium aggregatum* Goldstein & Belsky, 1964) related to *Labyrinthula* (Engel et al. 2006). Chemical defenses against *L. zosterae* are, however, still poorly understood, and no specific defense compound against the pathogen has been identified. Phenolic acids have been shown to correlate with lesion occurrence (Vergeer & Develi 1997), and to increase in response to *L. zosterae* infection (McKone & Tanner 2009). Trevathan-Tackett et al. (2015) also found that phenolic acids can inhibit growth of pathogenic *Labyrinthula* sp. isolates from turtlegrass *T. testudinum*. However, the inhibitory concentrations were much lower than the levels measured within the plant, indicating that phenolic acids may have other roles in the seagrass. Furthermore, additional unidentified compounds with inhibitory effects towards *Labyrinthula* sp. were isolated from *T. testudinum* (Trevathan-Tackett et al. 2015).

The aim of the present study was to investigate the occurrence of wasting disease in natural populations of *Z. marina* shoots growing in different salinities and at different depths on the Swedish west coast. For this purpose, *Z. marina* shoots were collected and analyzed for *L. zosterae* lesion coverage and cell abundance. Furthermore, we investigated if *Z. marina* is chemically defended against *L. zosterae* and whether low infection rates can be correlated to increased resistance against the pathogen. Since *L. zosterae* has been shown to be sensitive to low salinity, we specifically hypothesized that *Z. marina* shoots from high-salinity meadows would be more heavily infected by *L. zosterae* compared to shoots from low-salinity meadows. Furthermore, we hypothesized that *Z. marina* shoots which grow at shallow depths are more infected than those which grow at greater depths. If these hypotheses are correct, shoots in high-salinity, shallow environments would be exposed to a higher selection pressure by the pathogen. We therefore hypothesized that these shoots would contain higher levels of chemical defenses and be more resistant to infection when exposed to *L. zosterae*, compared to shoots from low salinity and greater growing depths.

## MATERIALS AND METHODS

### Field survey

A total of 288 *Zostera marina* shoots were collected by SCUBA on the Swedish west coast in August 2013. Shoots were taken from 6 different meadows, 3 of which were located in a high-salinity area in the archipelago outside the Tjärnö marine laboratory, and 3 in a low-salinity area at the mouth of the Ide Fjord (Table 1, Fig. 1). Within each meadow, shoots were collected from both shallow (1 m) and deep (5 or 2 m) sites. The collection depths at the deep sites correspond to the lower depth distribution of *Z. marina* for high- and low-salinity meadows, respectively. Within each depth, 4 shoots were collected from each of 6 haphazardly chosen 25 × 25 cm squares spaced approximately 10 m apart. Salinity and irradiance measurements were carried out in the high- and low-salinity areas, with single measurements taken once a week over the course of a month prior to the time of collection (range values in Table 1). All sampled shoots were photographed and stored at –80°C, pending analyses of lesion coverage, *Labyrinthula zosterae* cell abundance, and chemical defense.

Table 1. Ranges of salinity and irradiation measurements from shallow (1 m) and deep (2 or 5 m) sites in the 6 sampled seagrass meadows (see also Fig. 1). Ranges marked * refer to the 5 m sites

| Meadow | GPS coordinates | Salinity (PSU) | | Irradiation (µmol $s^{-1}$ $m^{-2}$) | |
|---|---|---|---|---|---|
| | | 1 m | 2 or 5 m | 1 m | 2 or 5 m |
| 1 | 58° 53.134′ N, 11° 08.526′ E | 25–29 | 25–29* | 218–333 | 85–146* |
| 2 | 58° 52.208′ N, 11° 08.944′ E | 25–29 | 25–29* | 218–333 | 85–146* |
| 3 | 58° 51.392′ N, 11° 08.420′ E | 25–29 | 25–29* | 218–333 | 85–146* |
| 4 | 59° 04.898′ N, 11° 12.953′ E | 13–22 | 14–25 | 120–200 | 47–85 |
| 5 | 59° 04.830′ N, 11° 13.023′ E | 13–22 | 14–25 | 120–200 | 47–85 |
| 6 | 59° 05.718′ N, 11° 13.351′ E | 13–22 | 14–25 | 120–200 | 47–85 |


Fig. 1. Study area showing the 6 sampled *Zostera marina* meadows growing in high salinity (Meadows 1–3) and low salinity (Meadows 4–6). See also Table 1

### Lesion coverage and cell abundance

Pathogen load and signs of disease connected to *L. zosterae* infection were quantified using 2 different measurements: lesion coverage (% of total shoot area) and cell abundance (no. of *L. zosterae* cells $mg^{-1}$ *Z. marina* dry weight). Lesion coverage measures the extent of tissue necrosis caused by *L. zosterae* infection (Burdick et al. 1993), while cell abundance is a direct measurement of the pathogen quantity within a shoot (Bergmann et al. 2011). Both measures of infection (lesion coverage and cell abundance) were also used to calculate the infection prevalence in each high-salinity meadow by dividing the number of infected shoots by the total number of collected shoots.

Photographs from the field survey were analyzed using ImageJ software to determine the wasting disease lesions and total leaf area of the shoots. *L. zosterae* cell abundance in leaf tissue was quantified according to the protocol described in Bockelmann et al. (2013). The third leaf of each shoot was freeze-dried and homogenized using a ball mill for 3 × 2 min at 30 Hz. DNA was extracted from a 2–3 mg sample using the Invisorb Spin DNA Extraction Kit (Stratec Molecular) according to the manufacturer's protocol, with the exception that 1 µl salmon sperm was added to saturate the silica columns with unspecified DNA. DNA was eluted in 100 µl elution buffer, and the target DNA was subsequently purified using a one-step

PCR inhibitor removal kit (Zymo Research). Real-time quantitative PCR (qPCR) was performed on a StepOne Plus qPCR machine (Applied Biosystems) as described in Bockelmann et al. (2013). Each sample ran in technical triplicates, and cycle threshold ($C_T$) was calculated with a fixed threshold of 0.05. A standard curve with known *L. zosterae* cell concentrations of 0.5 cells $\mu l^{-1}$ ($C_T$: 33.51 ± 0.79 SD), 15 cells $\mu l^{-1}$ ($C_T$: 27.75 ± 0.76 SD), and 150 cells $\mu l^{-1}$ ($C_T$: 23.49 ± 0.21 SD) ran alongside the samples on each qPCR plate. $C_T$ values above 39 were not considered, and samples where the triplicates were exceeding an SD of 0.5 were excluded from further analysis.

### Chemical defense assays

In order to investigate a possible chemical defense, and whether this differs between infected and uninfected *Z. marina* shoots, leaves of each *Z. marina* sample were freeze-dried, homogenized, and extracted in dichloromethane and methanol (1:1) for 1 h. The extraction was limited to the 3 youngest leaves of each shoot to standardize the extraction between shoots with different numbers of leaves. The plant material was removed and the solvents evaporated using a SpeedVac. The *L. zosterae* strain used to test inhibitory capacity of *Z. marina* extracts was isolated from *Z. marina* leaves haphazardly collected by snorkeling in the Tjärnö archipelago in July 2013. Leaves with apparent *L. zosterae* lesions were cut into 3 cm pieces and surface-sterilized in 0.5% sodium hypochlorite for 20 s, rinsed in distilled water for 10 s, and submerged in filtered (0.2 µm) seawater for 1 min (Bockelmann et al. 2013). The pieces were placed on agar plates containing serum-seawater agar (SSA) consisting of filtered (0.2 µm) seawater, 12 g $l^{-1}$ agar-agar, 1 g $l^{-1}$ glucose, 0.1 g $l^{-1}$ peptone, 0.1 g $l^{-1}$ yeast extract, 3 mg $l^{-1}$ germanium dioxide, 25 ml $l^{-1}$ streptomycin/penicillin (10 000 units penicillin and 10 mg streptomycin $ml^{-1}$), and 10 ml $l^{-1}$ horse serum. The agar solution was autoclaved and cooled down to 50°C before antibiotics and horse serum were added. *L. zosterae* was maintained by transferring 7 mm diameter plugs from growing cultures and placing them face down in the center of new agar plates every third week. Cultures were incubated in the dark at 25°C.

The inhibitory effect of the extracted *Z. marina* metabolites on *L. zosterae* growth was tested using a modified version of the bioassay described by Martin et al. (2009). One ml of liquid media, SSA medium minus agar (see previous paragraph), containing 1% dimethyl sulfoxide and 1 ml volumetric aliquot of extract per individual shoot, were transferred into 6 well plates (∅ = 35 mm). *L. zosterae* was transferred to the wells by taking 7 mm diameter plugs from cultures growing on agar plates, prepared as in the previous paragraph, and placed face down in the center of each well. Media containing 1% dimethyl sulfoxide served as a negative control. The well plates were incubated in the dark for 18 h at 25°C, after which the media was removed. To determine *L. zosterae* growth, the outer edge of the *L. zosterae* colony growing attached to the bottom of the well plate was outlined with a marker and photographed and the total area of the *L. zosterae* colony was thereafter measured using ImageJ software. The inhibitory effect of the extracted *Z. marina* metabolites was calculated as percent *L. zosterae* growth in plates with *Z. marina* extracts compared to controls.

### Transplantation and infection experiments

Two experiments were carried out in order to test if *Z. marina* shoots from meadows with different salinity have different susceptibility to *L. zosterae* infection. Collection of lesion-free *Z. marina* shoots was conducted at 1–2 m depth by snorkeling in May 2015 from the same meadows as in the field study (Table 1, Fig. 1). At that time of year, *L. zosterae* infection is low, and lesions on *Z. marina* shoots are uncommon (S. Jakobsson-Thor pers. obs.). A field experiment was performed by transplanting a total of 15 shoots from both high- and low-salinity meadows into each of 3 meadows in the high-salinity area at the end of June 2015. A full, reciprocal transplantation design, including moving infected high-salinity shoots to the low-salinity meadows, was not feasible since *L. zosterae* was not present in the low-salinity area (see 'Results'). The transplanted *Z. marina* shoots were marked and haphazardly attached to 25 × 25 cm concrete steel squares that were placed 2 cm into the sediment at 1–1.5 m depth within the meadows. After 1 mo, shoots were photographed, and ImageJ software was used to calculate the percent coverage of the wasting disease lesions as described in 'Lesion coverage and cell abundance' above.

The susceptibility to *L. zosterae* infection by shoots of different salinity origin was further studied in a laboratory infection experiment in June 2015. After epibiont removal, 25 shoots from each of the 6 meadows (i.e. a total of 150 shoots) were planted in individual 6 l containers by submerging the shoot rhizomes in 600 ml sterilized sediment. The containers

were placed in a flowthrough system with filtered (25 µm) surface water (26 PSU, 20°C). Shoots were acclimatized for 1 wk, after which 15 shoots from each meadow (i.e. a total of 90 shoots) were infected with *L. zosterae* for 7 d, and the remaining untreated shoots served as controls (n = 10 from each meadow). Shoots were infected using a method modified from Brakel et al. (2014). *Z. marina* leaves were collected haphazardly in the Tjärnö archipelago by snorkeling in May 2015 and *L. zosterae* was isolated on agar as described in 'Chemical defense assays' above. *L. zosterae*-covered agar plugs (19.6 $mm^2$) taken from the edge of the colonies were attached to the middle part of the third youngest leaf of each shoot. Control shoots received agar plugs without *L. zosterae.* The infection was quantified in the same way as in the field infection experiment (see previous paragraph).

### Statistical analyses

*L. zosterae* lesion coverage (% of total shoot area) and cell abundance (no. of *L. zosterae* cells $mg^{-1}$ *Z. marina* dry weight) were statistically analyzed using partially nested ANOVA with meadow (3 levels) as a random factor, depth (2 levels) as a fixed factor, and square (6 levels) as a random factor nested within meadow (see Tables 2 & 3). Since no signs of infection were detected in the low-salinity meadows, lesion coverage and cell abundance were statistically compared only in the high-salinity meadows. Thus, the factor salinity was not included in the statistical analyses.

The inhibitory effect of the extracted *Z. marina* metabolites on *L. zosterae* growth in the bioassays was analyzed using a partially nested ANOVA with salinity (2 levels) and depth (2 levels) as fixed factors, meadow (3 levels) as a random factor nested within salinity, and square (6 levels) as a random factor nested within meadow (see Table 4).

Lesion coverage on *Z. marina* shoots in the transplantation experiment was analyzed using a 2-factor ANOVA, with salinity origin (2 levels) as a fixed factor, and meadow (3 levels) as a random factor. Lesion coverage on *Z. marina* shoots in the laboratory infection experiment was analyzed using nested ANOVA with salinity (2 levels) as a fixed factor, and meadow (3 levels) as a random factor nested within salinity. Control shoots did not show any signs of infection and were therefore not included in the analysis.

When the factors or interaction between factors in the ANOVAs had p-values > 0.25, a pooling procedure was applied where the mean-square for the factor was pooled with the residual mean-square to increase the power of the analyses (Underwood 1997). This was done with the depth × meadow interaction for data on lesion coverage in the field survey. The pooled data are shown in Table 2. The same was done for the salinity × meadow interaction for the field infection experiment. Prior to all statistical analyses, data were tested for homogeneity of variances with Cochran's test and transformed when required (Underwood 1997). Data on lesion coverage and *L. zosterae* cell abundance from the field survey, as well as data on lesion coverage from the infection and transplantation experiments, were square root-transformed. Multiple mean comparisons were made with the Student-Newman-Keuls (SNK) test (Underwood 1997).

## RESULTS

### Lesion coverage and cell abundance

No lesions or *Labyrinthula zosterae* cells were found on *Zostera marina* in the low-salinity meadows, clearly showing an absence of the pathogen and any signs of the disease in this area (Fig. 2). Lesion coverage on individual *Z. marina* shoots in high-salinity meadows ranged from 0 to 15.4% of the total shoot area (Fig. 2a). No statistically significant difference in mean lesion coverage on shoots growing in different high-salinity meadows was detected, but we found statistically significant small-scale variation in lesion coverage among sampling squares within the same meadow (Table 2). Furthermore, *Z. marina* growing at shallow depths had significantly higher lesion coverage ($\bar{x}$ = 3.3% ± 0.4 SE) compared to the shoots collected at deeper sites ($\bar{x}$ = 1.9% ± 0.2 SE) (Fig. 2a, Table 2).

*L. zosterae* cell abundance on infected *Z. marina* shoots ranged between 9.8 and 571.3 cells $mg^{-1}$ *Z. marina* dry weight (Fig. 2b). In accordance with the results for lesion coverage, there was no significant difference in mean *L. zosterae* cell abundance between shoots growing in different meadows, but cell abundance varied significantly among squares within meadows (Table 3). In contrast to the results on lesion coverage, no significant difference in mean *L. zosterae* cell abundance was detected between shoots at different depths (Fig. 2b, Table 3).

In the high-salinity meadows (Fig. 3), lesion prevalence on *Z. marina* shoots ranged from 96 to 100% for shallow sites, and from 87 to 96% for deep sites (Fig. 3b). The corresponding *L. zosterae* cell prevalence was 87–91%, and 61–82% respectively (Fig. 3a).

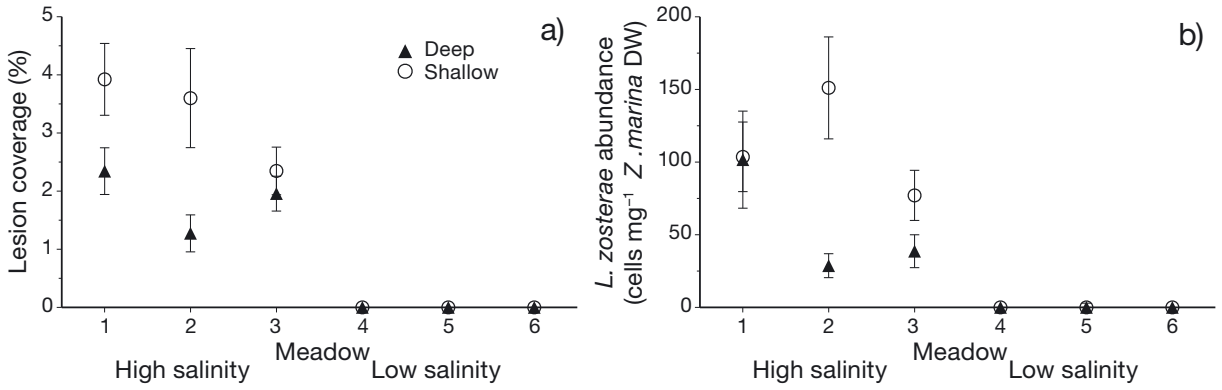


Fig. 2. Field survey. Average *Labyrinthula zosterae* infection on *Zostera marina*, measured by (a) lesion coverage and (b) *L. zosterae* cell abundance. *Z. marina* collected from high-salinity meadows 1–3, and low-salinity meadows 4–6 (see Fig. 1, Table 1). Non-transformed data is shown. Error bars show SEM, n = 24. DW = dry weight

Table 2. Field survey. Partially nested ANOVA of lesion coverage (%) on *Zostera marina* shoots from different meadows, depths, and collection squares in the high-salinity area. Data was square root-transformed to meet the assumptions of ANOVA. Bold indicates significance ($p < 0.05$)

| Source of variation | df | MS | *F* | p | Error term |
|---|---|---|---|---|---|
| Meadow | 2 | 1.42 | 1.80 | 0.18 | Square (Depth) |
| Depth | 1 | 7.28 | 15.83 | **<0.01** | Residual |
| Square (Depth) | 32 | 0.79 | 1.72 | **0.02** | Residual |
| Residual | 108 | 4.60 | | | |

### Transplantation and infection experiments

All shoots, independent of origin, showed signs of infection when exposed to *L. zosterae* both in the field and in the laboratory (Fig. 4). Additionally, shoots collected from low-salinity meadows became significantly more infected compared to shoots from high-salinity meadows, both in the transplantation (2-factor ANOVA, $F_{1,86} = 4.79$, $p = 0.03$, Fig. 4a) and the infection experiment (nested ANOVA, $F_{1,84} = 77.18$, $p < 0.01$, Fig. 4b). Low-salinity shoots had 44.3% ± 5.8 SE or 39.2% ± 0.02 SE higher lesion coverage compared to high-salinity shoots in the transplant and infection experiments, respectively. There was also a statistically significant difference in lesion coverage between shoots planted in different meadows in the transplantation experiment (2-factor ANOVA, $F_{1,86} = 12.63$, $p < 0.01$), whereas no difference in lesion coverage was detected between shoots from different meadows in the laboratory experiment (nested ANOVA, $F_{1,4} = 0.26$, $p = 0.91$).

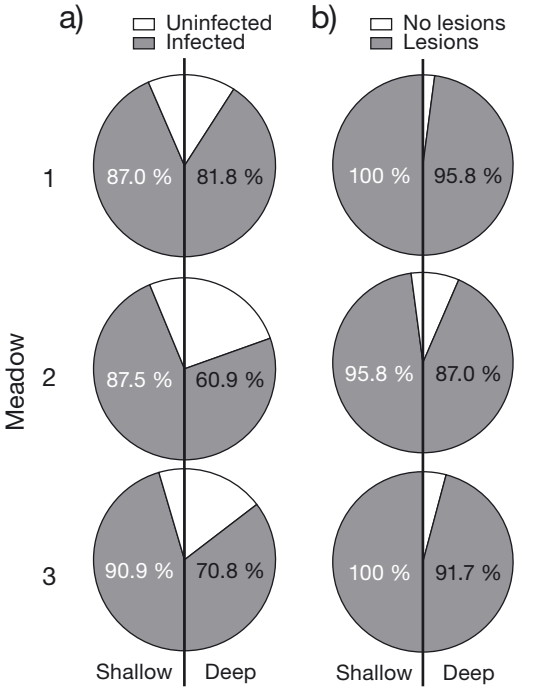


Fig. 3. Field survey. Prevalence of (a) *Labyrinthula zosterae* cells and (b) lesions on *Zostera marina* shoots from shallow and deep sites in high-salinity meadows 1–3 (see Fig. 1, Table 1). n = 24

Table 3. Field survey. Partially nested ANOVA of *Labyrinthyla zosterae* cell abundance (no. of *L. zosterae* cells $mg^{-1}$ *Zostera marina* dry weight) in *Z. marina* shoots from different meadows, depths, and collection squares in the high-salinity area. Data was square root-transformed to meet the assumptions of ANOVA. Bold indicates significance ($p < 0.05$)

| Source of variation | df | MS | *F* | p | Error term |
|---|---|---|---|---|---|
| Meadow | 2 | 97.46 | 0.83 | 0.44 | Square (D × M) |
| Depth | 1 | 422.54 | 4.71 | 0.16 | Depth × Meadow |
| Depth × Meadow | 2 | 179.62 | 1.54 | 0.23 | Square (D × M) |
| Square (Depth × Meadow) | 30 | 1755.45 | 2.39 | **<0.01** | Residual |
| Residual | 108 | 2643.86 | | | |

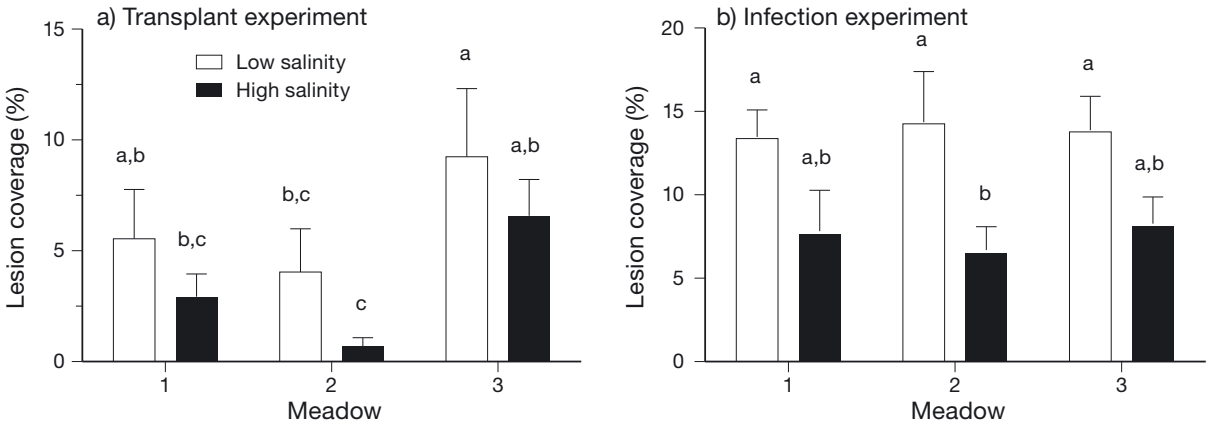


Fig. 4. Transplantation and infection experiments. Lesion coverage (%) following infection by *Labyrinthula zosterae* upon *Zostera marina* shoots originating from low and high salinity in the (a) field and (b) laboratory (see Fig. 1, Table 1 for locations of meadows 1–3). Letters above bars indicate significant differences between mean values (Student-Newman-Keuls test, $p < 0.05$). Non-transformed data is shown. Error bars show SEM, n = 15

### Chemical defense assays

Extracts from all *Z. marina* shoots, regardless of infection status, depth, or salinity origin, inhibited *L. zosterae* growth at natural concentrations (Fig. 5). Overall, pathogen growth in media treated with *Z. marina* extracts was strongly inhibited (growth was ≤31% relative to growth on control media). No statistically significant difference in inhibitory effect between extracts from shoots growing in different salinities was detected (Table 4). However, there was a statistically significant interaction between the factors meadow and depth (Table 4). When means were compared using the SNK test, we found that extracts from shoots growing in shallow water in 2 meadows inhibited *L. zosterae* growth more compared to extracts from shoots growing in deeper water, while there was no significant difference between shoots growing at different depths in the rest of the meadows (Fig. 5). There was also a significant difference in the inhibitory effect of the extracts from shoots sampled from different squares, illustrating a small-scale variation in possible defense levels between plants within meadows (Table 4).

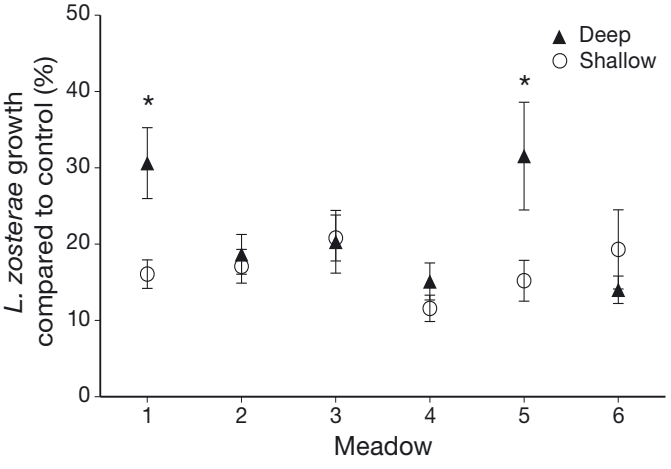


Fig. 5. Bioassays. Chemical defense of *Zostera marina* against *Labyrinthula zosterae* measured as average *L. zosterae* growth compared to control on *Z. marina* crude extracts (from deep sites and shallow sites) after 18 h incubation. Meadows 1–3 = high salinity, 4–6 = low salinity (see Fig. 1, Table 1). *Significant difference between shallow and deep sites (Student-Newman-Keuls test, $p < 0.05$). Error bars show SEM, n = 24

Table 4. Laboratory bioassays. Partially nested ANOVA of inhibition (%) of *Labyrinthula zosterae* growth by extracts of *Zostera marina* from different salinities, meadows, depths, and collection squares compared to controls. Bold indicates significance ($p < 0.05$)

| Source of variation | df | MS | *F* | p | Error term |
|---|---|---|---|---|---|
| Salinity | 1 | 450.99 | 0.53 | 0.51 | Meadow (Salinity) |
| Depth | 1 | 1843.69 | 1.58 | 0.28 | Square (Depth × Meadow (Salinity)) |
| Meadow (Salinity) | 4 | 845.47 | 2.09 | 0.09 | Depth × Meadow (Salinity) |
| Salinity × Depth | 1 | 2.29 | 0.002 | 0.97 | Depth × Meadow (Salinity) |
| Depth × Meadow (Salinity) | 4 | 1170.86 | 2.90 | **0.03** | Square (Depth × Meadow (Salinity)) |
| Square (Depth × Meadow (Salinity)) | 60 | 404.23 | 2.32 | **<0.01** | Residual |
| Residual | 216 | 174.54 | | | |

## DISCUSSION

Results from the present study show that the degree of *Labyrinthula zosterae* infection in the temperate seagrass *Zostera marina* varies significantly on different spatial scales. No infection was detected in low-salinity meadows, and shallow-growing *Z. marina* shoots had more disease symptoms than deeper-growing shoots. Although *Z. marina* from low-salinity habitat was more susceptible to *L. zosterae* infection, there was no difference in inhibitory effect against *L. zosterae* between *Z. marina* extracts from low- and high-salinity meadows. This is the first study to show that inhibitory compounds against *L. zosterae* are omnipresent in natural *Z. marina* populations, independent of the origin of the shoots and the ambient pathogen pressure.

We detected clear differences in *L. zosterae* infection in terms of disease signs (lesion coverage) and pathogen load (cell abundance), between high- and low-salinity meadows of *Z. marina* on the Swedish west coast. The pathogen was present in all high-salinity meadows studied, whereas *Z. marina* shoots growing in low-salinity environments were free from infection. No significant difference in *L. zosterae* infection was found between the 3 high-salinity meadows, but a significant small-scale variation among shoots within meadows was detected. Overall, our results show that high-salinity meadows of *Z. marina* from the study area have high prevalence of *L. zosterae* infection compared to what has been reported from other regions (Bockelmann et al. 2013, Groner et al. 2016). We found that 87 to 100% of the shoots had lesions and that 61 to 91% carried *L. zosterae* cells. To our knowledge, few other regions have shown equally high prevalence of lesions in *Z. marina* during recent years. Lesion prevalence has previously been found to vary between 0 and 95% ($\bar{x}$ = 23%) in *Z. marina* meadows in northern Europe (Bockelmann et al. 2013), and between 6 and 79% ($\bar{x}$ = 44%) in the San Juan archipelago, USA (Groner et al. 2014, 2016). The prevalence of *L. zosterae* cells in *Z. marina* is also higher in our study compared to the few areas where this has previously been measured. The only other study using a qPCR assay to quantify *L. zosterae* cells was performed in northern European meadows, where the pathogen was found in 0–89% ($\bar{x}$ = 21%) of the *Z. marina* shoots sampled, and cell abundance ranged between 1.26 and 50 400 cells $mg^{-1}$ *Z. marina* dry weight (Bockelmann et al. 2013). The highest mean abundance on infected shoots was detected in the western Baltic Sea, Germany, at 1640 cells $mg^{-1}$ dry weight (Bockelmann et al. 2013), compared with 172.7 cells $mg^{-1}$ dry weight measured in our study. Contrary to our findings, Bockelmann et al. (2013) also discovered *L. zosterae* in a Swedish meadow with a relatively low salinity (6–14 PSU). However, since the prevalence of *L. zosterae* cells was low (5%), this further supports the notion that low-salinity meadows are less infected compared to high-salinity meadows.

*L. zosterae* cells could be detected in 83.9% of the *Z. marina* shoots with apparent lesions. A previous survey of 5 European *Z. marina* meadows showed that *L. zosterae* cells are detected more often in shoots with lesions, but pointed out that shoots can have lesions without cells being detected by qPCR (Bockelmann et al. 2013). Necrotic tissue in seagrasses can be caused by factors other than disease, e.g. salinity stress (Biebl & McRoy 1971) and temperature (Collier & Waycott 2014). This highlights the importance of investigating actual pathogen load and not only signs of the disease, such as lesions, when studying seagrass wasting disease (Bergmann et al. 2011).

The observed difference in *L. zosterae* infection between *Z. marina* meadows growing in different salinities was most likely due to the absence of *L. zosterae* in the low-salinity environment, rather than the low-salinity shoots being inherently resistant to pathogen infection. To our knowledge, this is the first study to perform a transplantation of *Z. marina* shoots from low- to high-salinity meadows with the aim of investigating infection by *L. zosterae*. The results from the transplantation as well as from the infection experiments reveal that *Z. marina* shoots from low salinities can become extensively infected when transferred to high-salinity environments. These findings corroborate previous studies where low salinity has been found to reduce *Labyrinthula* spp. growth in laboratory experiments (Muehlstein et al. 1988, Martin et al. 2009). The results from the present study further show that although *Z. marina* shoots from high-salinity meadows are more infected *in situ*, they are actually more resistant to *L. zosterae* infection compared to shoots from low-salinity meadows. The higher resistance in *Z. marina* shoots from high-salinity meadows could reflect local adaptation (genetic differentiation) to a high pathogen pressure and/or environmentally induced resistance (phenotypical plasticity), e.g. through increased chemical defense production, in the transplanted seagrass shoots. To examine this further, multigenerational transplantation or common garden experiments using seagrass seeds from high- and low-salinity meadows would be required (cf. Ågren & Schemske 2012).

Apart from differences in *L. zosterae* abundance between meadows with different salinities, infection also correlated with depth: *Z. marina* shoots growing at 1 m depth had higher lesion coverage than at 5 m. This coincides with findings from the San Juan archipelago, where infection was more dominant on *Z. marina* higher up in the intertidal region than further down (Groner et al. 2014). Similar effects of depth were found with the presence of endophytic brown algal pathogens on the brown algae *Laminaria hyperborea* Foslie, 1884, *Laminaria digitata* J. V. Lamouroux, 1813, and *Saccharina latissima* C. E. Lane, C. Mayes, Druehl & G. W. Saunders, 2006, where symptoms were more severe on shallow-living individuals compared to deeper ones (Ellertsdottir & Peters 1997). Those authors hypothesized that higher irradiance increases the development of the pathogens, leading to higher pathogen pressure on shallow-living individuals. It is uncertain if light has the same positive effect on *L. zosterae* virulence, since the pathogen is a non-photosynthetic organism. However, in some terrestrial host–pathogen interactions, light can control infection, and shading has been found to either have a positive or a negative impact, depending on the studied system (Roberts & Paul 2006). The mechanisms of light connected to virulence are often hard to disentangle and it is not always clear if light itself is the direct cause of the observed effect, or if other indirect factors of light, e.g. temperature changes, play a role (Roberts & Paul 2006). Nevertheless, laboratory studies show that light composition directly influences spore release in the ascomycete fungus *Venturia inaequalis* G. Winter, 1875 (Gadoury et al. 1998), and that zoospore production is reduced at low light in a chytrid pathogen on the diatom *Asterionella formosa* Hassall, 1850 (Bruning 1991). At this stage, it is still unclear if *L. zosterae* infection at different depths can be linked to irradiance, due to the low number of experimental studies. So far, infection by *Labyrinthula* sp. in *Thalassia testudinum* has been negatively correlated with canopy light (Trevathan-Tackett et al. 2013), and laboratory studies by Vergeer et al. (1995) showed that an increase in irradiance decreased lesion coverage on *Z. marina*. However, the irradiance levels in Vergeer et al. (1995) were lower than the levels found in both the *T. testudinum* meadows (Trevathan-Tackett et al. 2013) and the *Z. marina* meadows in this study. An experimental study investigating the effect of ecologically relevant light levels on *L. zosterae* and *Z. marina* separately, and on the interaction between the two, is therefore needed to gain a better understanding of the effect of light on seagrass wasting disease in this system.

Even though there were significant differences in lesion size among the transplanted shoots from different origins, the *L. zosterae* growth assay showed that crude extracts from all *Z. marina* shoots inhibit *L. zosterae* growth, independent of origin or infection status of the shoots. This suggests that the production of inhibitory compounds is not induced by a high pathogen pressure, as previously suggested (Buchsbaum et al. 1990, Vergeer et al. 1995), but rather that *Z. marina* contains inhibitory compounds that function as a constitutive defense. In contrast, the most studied potential defense compounds against *L. zosterae* infection so far, the phenolic acids, have been shown to increase in infected seagrass shoots, and to correlate with lesion size in laboratory infection experiments (McKone & Tanner 2009). In addition, trans-cinnamate 4-monooxygenase, an enzyme for phenol synthesis, is upregulated in *L. zosterae*-inoculated shoots (Brakel et al. 2014). However, increased phenolic acid production in turtlegrass *T. testudinum* is only found in infected leaves above the lesions, not below (Steele et al. 2005). Those authors therefore suggest that the production of phenolic acids is not a response to *L. zosterae* infection, but is a pseudo-induction caused by a disruption in carbohydrate allocation within a leaf leading to higher abundance of carbon above the lesions and thereby an accumulation of phenolic acids. Furthermore, metabolites other than phenolic acids with inhibitory effects against *Labyrinthula* sp. have been isolated (but not elucidated) from *T. testudinum* using bioassay-guided fractionation (Trevathan-Tackett et al. 2015). We therefore suggest that more studies applying an open approach, rather than using *a priori* assumptions about defensive roles of specific groups of compounds, could lead to identification of novel defense compounds against *L. zosterae* in *Z. marina*. The isolation and identification of the compounds that are inhibitory in laboratory growth assays is necessary to further evaluate if these compounds also serve a significant role as a chemical defense in natural *Z. marina* populations.

## CONCLUSION

Although seagrass wasting disease can have a devastating impact on seagrass meadows, information on how and why infection varies in natural populations is still limited. Here we show that the degree of *Labyrinthula zosterae* infection differs between *Zostera marina* meadows from different salinity areas, and between shoots growing at different depths. Even

though *Z. marina* shoots from low-salinity environments seem to be more susceptible to *L. zosterae* than shoots from high-salinity areas, our findings suggest that low-salinity areas can still act as a refuge against seagrass wasting disease, due to the poor performance of the pathogen in low salinity. This study is also the first to show that all *Z. marina* shoots in natural populations contain compounds that inhibit *L. zosterae* growth, independent of origin or infection status of the shoots.

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