Vol. 130: 51–63, 2018
https://doi.org/10.3354/dao03263

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 28

Environment, dosage, and pathogen isolate moderate virulence in eelgrass wasting disease

P. D. Dawkins[1], M. E. Eisenlord[1,*], R. M. Yoshioka[2], E. Fiorenza[3], S. Fruchter[1], F. Giammona[1], M. Winningham[1], C. D. Harvell[1]

[1]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA
[2]Oregon Institute of Marine Biology, University of Oregon, Charleston, OR 97420, USA
[3]School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195, USA

ABSTRACT: Eelgrass wasting disease, caused by the marine pathogen *Labyrinthula zosterae*, has the potential to devastate important eelgrass habitats worldwide. Although this host–pathogen interaction may increase under certain environmental conditions, little is known about how disease severity is impacted by multiple components of a changing environment. In this study, we investigated the effects of variation in 3 different *L. zosterae* isolates, pathogen dosage, temperature, and light on severity of infections. Severity of lesions on eelgrass varied among the 3 different isolates inoculated in laboratory trials. Our methods to control dosage of inoculum showed that disease severity increased with pathogen dosage from 10^4 to 10^6 cells ml^{-1}. In a dosage-controlled light and temperature 2-way factorial experiment consisting of 2 light regimes (diel light cycle and complete darkness) and 2 temperatures (11 and 18°C), *L. zosterae* cell growth rate *in vitro* was higher at the warmer temperature. In a companion experiment that tested the effects of light and temperature in *in vivo* inoculations, disease severity was higher in dark treatments and temperature was marginally significant. We suggest that the much greater impact of light in the *in vivo* inoculation experiment indicates an important role for plant physiology and the need for photosynthesis in slowing severity of infections. Our work with controlled inoculation of distinct *L. zosterae* isolates shows that pathogen isolate, increasing dosage of inoculum, increasing temperature, and diminishing light increase disease severity, suggesting *L. zosterae* will cause increased damage to eelgrass beds with changing environmental conditions.

KEY WORDS: Virulence · Environmental stress · *Labyrinthula zosterae* · Eelgrass wasting disease · EGWD · *Zostera marina* · Seagrass

INTRODUCTION

Eelgrass beds are among the most productive marine ecosystems in our oceans, providing a multitude of ecosystem services that include critical nursery habitats for economically important species, stabilization of sediments, carbon sequestration, and water filtration and purification (Lamb et al. 2017, Nordlund et al. 2017), as well as valuable cultural benefits (Wyllie-Echeverria & Cox 2000, de la Torre-Castro & Rönnbäck 2004). However, this valuable ecosystem is currently experiencing serious declines worldwide (Orth et al. 2006, Waycott et al. 2009). Although declines have most recently been associated with anthropogenic disturbances such as eutrophication, overharvesting, and sediment runoff (Barbier et al. 2011), the most remarkable historic declines in eelgrass beds have been attributed to outbreaks of eelgrass wasting disease (EGWD) (Renn 1934, 1935, Short et al. 1987, Muehlstein et al. 1991, Godet et al. 2008).

The disease symptoms associated with EGWD are blackened streaks and lesions with defined borders, often with a pale necrotic center (Muehlstein et al. 1991). During the 1930s, seagrass populations along

*Corresponding author: me367@cornell.edu

the Atlantic coasts of Europe and North America suffered catastrophic declines, reaching 90% mortality (Renn 1934, 1935, Short et al. 1987, Godet et al. 2008). In the late 1980s, seagrasses in Cape Ann, Massachusetts, Great Bay, New Hampshire, and Niantic River, Connecticut (USA), suffered declines also attributed to EGWD (Short & Wyllie-Echeverria 1996). EGWD outbreaks have had detrimental environmental and ecological consequences, including alteration of sediment distribution, disruption of coastal food chains and fisheries, and losses of major populations of migratory waterfowl (Short et al. 1986). It was not until the acute declines along the western Atlantic coast in the 1980s that a *Labyrinthula* sp. was conclusively identified as the causative agent of EGWD (Short et al. 1987). In 1991, *L. zosterae* was named and confirmed to cause EGWD in the eelgrass species *Zostera marina* (Muehlstein et al. 1991).

L. zosterae has been considered an opportunistic pathogen, meaning it is present in the environment but only becomes pathogenic under specific conditions of host susceptibility and/or change in the environment (Burge et al. 2013, 2014, Groner et al. 2014). Examples of this type of pathogen include the herpesviruses associated with high mortality of larval and juvenile stages of a number of molluscan species in commercial hatcheries and nurseries where pathogenesis is promoted by rearing conditions such as higher temperature and high density (Farley et al. 1972, Le Deuff et al. 1996, Arzul et al. 2001). Another pathogen considered an opportunist is Quahog Parasite Unknown, a thraustochytrid closely related to related to *L. zosterae*, which causes molluscan disease and mortalities regulated by temperature and salinity (Dahl 2015). Other examples include the temperature-dependent disease in corals caused by *Vibrio shiloi* AK1 and *V. coralliilyticus* (Frydenborg et al. 2014). Further study is needed to establish *L. zosterae* as opportunistic and understand the factors affecting its virulence in eelgrass.

In this study, we used controlled experiments to investigate the impact of light and temperature, 2 major environmental stressors to eelgrass in the Salish Sea, on the virulence of *L. zosterae.* We hypothesized that the interaction between these 2 stressors may lead to a new physiological regime for the *Z. marina* host and its corresponding *L. zosterae* pathogen isolate that facilitates increased EGWD. Under this new regime, the compromised immune response of the host and the increased virulence and/or increased growth rate of the pathogen may increase risk of a disease outbreak (Burge et al. 2014).

Recent work investigating environmental factors affecting EGWD has shown that multiple different strains of *L. zosterae* are present in the environment (M. E. Eisenlord unpubl. data). The characterization of a pathogen as opportunistic, when this pathogen may exist as a consortium of diverse strains, must take into account that virulence may vary among strains. Virulence, used here as a metric of disease damage, is not an independent characteristic of the microbe; rather, it is contingent upon characteristics of the pathogen and the susceptibility of the host, as well as the environmental context (Casadevall & Pirofski 2001). Thus, an increase in virulence driven by changes in the environment could be caused by either a community-level change of non-pathogenic strains switching to pathogenic strains, or by a change in respective strain pathogenicity. Recent work by Martin et al. (2016) has shown that both pathogenic and non-pathogenic strains of *L. zosterae* are widespread globally, but further studies are needed to elucidate the environmental factors that affect the virulence of these pathogenic strains and identify where they occur in nature.

Research investigating environmental factors that affect marine infectious disease is a priority in our changing oceans (Burge et al. 2013, 2016, Groner et al. 2016, Lafferty & Hofmann 2016). While direct impacts of a changing climate have been documented on diseases of corals, shellfish, and finfish (Harvell et al. 2002, Burge et al. 2013), these drivers, as well as the other environmental factors, have yet to be clearly linked to EGWD. In this study we tested how pathogen dosage, isolate, light, and temperature affect EGWD virulence. We investigated the combined influence of light and temperature on virulence and *L. zosterae* growth *in vitro*. We hypothesized that (1) *L. zosterae* isolates vary in virulence, (2) *L. zosterae* virulence increases with increased dosage, (3) *L. zosterae* virulence increases with warmer temperatures, and (4) *L. zosterae* virulence increases with reduced light. The goal of this study was to elucidate effects of environmental factors on the EGWD system.

MATERIALS AND METHODS

Culturing and identification of the etiologic agent

Labyrinthula zosterae cells were isolated from *Zostera marina* leaves with characteristic sharp-edged black or dark brown lesions symptomatic of EGWD (Muehlstein et al. 1991). Small sections of lesioned tissue were surface-rinsed with sterilized seawater, blotted

dry, and placed onto serum seawater agar (SSA) plates, subsequently wrapped in Parafilm and grown at 20–24°C. Cultures were then re-plated to create axenic cultures and transferred monthly. SSA was modified from Porter (1990) as described by Groner et al. (2014).

Identification of *L. zosterae* cells includes observation of characteristic cell growth on SSA media coupled with observation of characteristic cell morphology via light microscopy. *L. zosterae* has an identifiable growth pattern on SSA; irregular masses of aggregated cells form on the agar surface as well as within the agar matrix, exhibiting intricate patterns of branching along the culture's spreading margin (Muehlstein et al. 1991). The most obvious feature of the fusiform cells is their prominent central nuclei as well as gliding movement within their ectoplasmic networks (Muehlstein et al. 1991). *L. zosterae* identification is further confirmed by observation of fusiform or spindle-shaped vegetative cells linked with mucous strands via light microscopy.

Expt 1: Impact of dosage and temperature on *L. zosterae* virulence

We ran a full-factorial experiment comparing virulence of 3 *L. zosterae* dosage treatments (10^4, 10^5, and 10^6 cells ml^{-1}) at 2 temperatures (14 and 19°C) in *Z. marina* leaf tissue (*in vivo*).

Forty *Z. marina* plants were collected on 26 April 2015 from Collin's Cove, Friday Harbor, Washington (WA) (48° 32′ 60″ N, 123° 0′ 36″ W), and kept at ambient temperature in sterile seawater for 2 h to acclimate. We selected the third longest blade and recorded its length, using only blades with no visible signs of disease or physical damage. The third longest is less likely to have been infected than older blades (Groner et al. 2014), while still providing the length needed for this experiment. Each blade was scraped clean of epiphytes and fouling with a glass cover slide, gently to avoid mechanical damage to the leaf surface, and subsequently cut into 5 cm sections. The base and tip of each blade were discarded. The blades were then quickly rinsed in reverse osmosis water to reduce potential *L. zosterae* contamination from seawater or the leaf exterior, as fresh water inhibits *Labyrinthula* (Muehlstein et al. 1988). Blades were then placed into a sterile seawater bath for 60 s and blotted dry.

The *L. zosterae* isolate (8.16.D) used for this experiment was isolated from non-flowering adult *Z. marina* shoots in 2011 that were collected from Picnic Cove, Shaw Island, WA (48° 34′ 12″ N, 122° 55′ 12″ W), in 2006 and subsequently grown in a continuous flow mesocosm at Friday Harbor Laboratories (Groner et al. 2014). Previous experiments have confirmed that this is a virulent isolate (Groner et al. 2014). *L. zosterae* cells were gently scraped from their SSA plates and suspended in filtered seawater, and a stock inoculum of 8.3×10^6 cells ml^{-1} was prepared using a hemocytometer (Groner et al. 2014). Inoculations of 3 cell concentrations were prepared by diluting the stock inoculum: 1:8 for a concentration of 10^6 cells ml^{-1} (high dosage), and then serially diluted 1:10 for concentration of 10^5 cells ml^{-1} (medium dosage) and 1:100 for a concentration of 10^4 cells ml^{-1} (low dosage).

Each dosage treatment consisted of 10 replicate 5 cm leaf sections. A sterile razor was used to score an 'X' through the center of each leaf section to serve as a controlled point of entry for the pathogen to reduce variability of infectivity. Ten µl of the dosage-controlled *L. zosterae* inoculum was pipetted directly onto the scored area of each eelgrass leaf section. While a number of transmission modes have proven to be successful, including infected leaf-drift, infected close-neighbor plant, and attached-infected leaf piece, we developed this dosage-controlled inoculation protocol specifically to test virulence (Martin et al. 2016). Experimental leaf sections were laid onto petri dishes containing a thin layer of sterile seawater and agar. This moist surface prevents the clips from desiccating during the inoculation.

In addition to the 10 replicates for each treatment, 5 replicates of negative controls, consisting of a 10 µl sham inoculum (0 cells ml^{-1} in sterile seawater) were inoculated. Five positive controls (a dosage of 10^4 *L. zosterae* cells ml^{-1}) placed directly onto the seawater serum agar were added to each temperature-controlled chamber to confirm inoculum viability. All plates were sealed with Parafilm and placed into climate-controlled chambers set to 12 consecutive hours of light per day. After 24 h, 2 ml of sterile seawater were added to all plates (except positive controls) to prevent blade desiccation. Pendant loggers (HOBO) recorded temperature in each of the climate-controlled chambers every 30 min throughout the experiment. The average ± SD temperatures of the incubators were 19.1 ± 0.09°C and 14.38 ± 2.31°C.

The experiment was terminated after 10 d. Each leaf section was photographed on the final day of the experiment. Lesion area was measured using ImageJ (Schneider et al. 2012). When examining leaf sections for the presence of *L. zosterae*, only distinct, dark brown or black-bordered lesions were measured

(Muehlstein et al. 1991). We calculated disease prevalence (number of diseased leaf sections/total number of leaf sections × 100%) and severity (total lesion area/leaf section area) as per Groner et al. (2014).

Expt 2: Virulence of *L. zosterae* isolates

We compared the virulence of 3 *L. zosterae* isolates in *Z. marina* leaf tissue (*in vivo*) under controlled conditions (12:12 h light:dark cycle at 20°C).

Twenty *Z. marina* plants were collected on 19 June 2016 from Indian Cove, Shaw Island, WA (48° 33′ 36″ N, 122° 56′ 24″ W), and kept at ambient temperature in filtered seawater for 3 h to acclimate. Only second-rank leaves with no signs of *L. zosterae* infection were used. Epiphytes and fouling were gently scraped off blade surfaces, and 4 cm leaf sections (n = 8) were taken from each blade. The base and tip of each blade were discarded. Four leaf sections from each plant were distributed evenly across our 3 isolate treatments to spread potential genetic variation. The remaining leaf sections from each plant were used as negative controls.

The 3 isolates used for inoculations were cultured from the San Juan Islands (WA) on 8 June 2015: 1 isolate (Isolate A) from North Cove, San Juan Island (48° 43′ 12″ N, 123° 1′ 48″ W), and 2 isolates (Isolates B and C), from Shoal Bay, Lopez Island (48° 32′ 60″ N, 122° 52′ 48″ W). These isolates were chosen based on their variable growth rates in culture. Mean ± SD inoculums of (0.87 ± 0.22) × 10^5 cells ml^{-1} (Isolate A), (1.33 ± 0.39) × 10^5 cells ml^{-1} (Isolate B), and (0.77 ± 0.04) × 10^5 cells ml^{-1} (Isolate C) were prepared using a hemocytometer. Prior to cell quantification, each inoculum was centrifuged with 1 µm zirconia/silica beads for 25 s to break up aggregations of clumped cells to improve confidence in our cell concentrations. Unlike in Expt 1, leaf tissue was not scored prior to inoculation since we were testing both infectivity and virulence of the isolates in this experiment. Leaf sections were laid onto petri dishes containing a thin layer of sterile seawater with agar and placed in temperature-controlled chambers set to a 12:12 h light:dark cycle at 20°C. Pendant loggers that recorded light and temperature were placed on the center of each of the 3 shelves in 2 climate-controlled chambers, set to record every 30 min. Each treatment consisted of 20 experimental samples, 20 negative controls (sterile seawater sham inoculum), and 10 positive controls (inoculum on sterile seawater agar media).

Twenty µl of the prepared inoculum were carefully pipetted onto the center of each experimental section. Petri dishes were wrapped with Parafilm to keep samples sealed. After 4 h, the samples were unwrapped and flooded with 3 ml of sterile seawater to keep the leaf sections hydrated. The experiment was terminated after 12 d. Photo analysis using ImageJ was used as in Expt 1. Recording only characteristic lesions, we calculated disease prevalence and severity.

Expt 3: Impact of light and temperature on *L. zosterae* virulence

We ran a full-factorial experiment comparing the impact of 2 light conditions (12:12 h light:dark cycle and full dark) and 2 temperatures (12 and 18°C) on *L. zosterae* virulence in *Z. marina* leaf tissue (*in vivo*) and on the cell growth rates of the *L. zosterae* in liquid culture (*in vitro*).

Z. marina shoots were collected on 31 March 2016 from False Bay, Friday Harbor, WA (48° 28′ 48″ N, 123° 4′ 12″ W), and kept at ambient temperature in sterile seawater for 2 h. The second-rank leaf was chosen and its length recorded. The blade was then gently scraped clean of epiphytes and fouling with a glass coverslip, and its central section was cut into 4 cm sections. The base and the tip of the blade were discarded. The 4 cm sections were placed in 0.37 µm filtered seawater with an aerator under LED grow lights set on a 12 h diel light cycle to acclimate.

The *L. zosterae* isolate used for our experiments was cultured on 21 March 2016 from the eelgrass tank on the Friday Harbor Laboratories premises where the culture used in Expt 1 was isolated (see Expt 1 methods). An inoculum of (1.20 ± 0.27) × 10^5 cells ml^{-1} (mean ± SD) was centrifuged with 1 µm zirconia/silica beads for 25 s to break up cell clumps and prepared using a hemocytometer (see Expt 2 methods).

Pendant loggers that recorded light and temperature were placed on the center of each of the 3 shelves in 2 climate-controlled chambers, set to record every 30 min. The average temperatures of the climate-controlled chambers were 12.02 ± 1.02°C and 18.74 ± 0.58°C, and respective average light intensities were 1100.39 ± 243.16 and 1066.27 ± 322.70 lux. The temperatures were chosen to be consistent with temperature ranges observed in intertidal eelgrass beds in the San Juan Islands during summer months.

In vivo experiment

A single 2 mm cut was made across the center of each leaf section using a sterile razor. Each treatment consisted of 25 experimental samples, 15 negative controls (sterile seawater sham inoculum), and 10 positive controls (inoculum on sterile seawater agar media). Twenty µl of the prepared inoculum were carefully pipetted onto the center of the cut of each experimental section. Petri dishes were wrapped with Parafilm to keep samples sealed. After 4 h, the samples were unwrapped and flooded with 3 ml of sterile seawater to keep the leaf sections hydrated. The dark treatments were wrapped in 2 layers of tinfoil to ensure no light penetration after flooding was completed. The experiment was terminated 7 d after inoculations. Photo analysis using ImageJ was done as in Expts 1 and 2.

In vitro experiment

A cell growth experiment with the same full-factorial treatments was run in tandem to the *in vivo* experiment within the same climate-controlled chambers. *L. zosterae* cells were diluted to 10^4 cells ml^{-1} in serum seawater broth within a 1.5 ml microcentrifuge tube (500 µl of 10^5 cells ml^{-1} *L. zosterae* inoculum combined with 1000 µl of serum seawater broth). The ingredients of serum seawater broth are identical to previously described SSA (Groner et al. 2014), omitting agar. Fifteen tubes were prepared for each treatment. Daily over 5 d, 3 replicates for each treatment were removed and counted 3 times each. Prior to cell quantification using a hemocytometer, each microcentrifuge tube was centrifuged with 1 µm zirconia/silica beads for 25 s to break up aggregations of clumped cells and improve precision of counts.

Statistical analyses

All analyses were done in R (v. 3.0.1) and were performed at a significance level of $\alpha = 0.05$, except in post hoc tests where Bonferroni-corrected p-values are given.

Disease prevalence was analyzed using generalized linear models (GLMs) with binomial error distributions and logit links (function glm in the 'lme4' package, Bates et al. 2015). Disease severities were analyzed using GLMs with beta error distributions and log links (function 'betareg' in the 'betareg' package, Cribari-Neto & Zeileis 2010). Beta models account for the proportion of clearly lesioned area for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Cell growth was analyzed using GLMs with negative binomial error distribution and log link (glmer.nb, MASS, Venables & Ripley 2002). Where appropriate, post hoc tests were performed using least square means analysis with Bonferroni correction of p-values ('lsmeans,' Lenth 2016). For each analysis, full models and all reduced models were compared using Akaike's information criterion (AIC). R code (Supplement 1) and datasets (Supplement 2) for these analyses are available at www.int-res.com/articles/suppl/d130p051_supp/.

Expt 1: Impact of dosage and temperature on *L. zosterae* virulence

Differences in levels of disease prevalence were analyzed using a GLM with a binomial error distribution and logit link (function 'glm' of the 'lme4' package). Dose and temperature were treated as continuous fixed factors, with dose characterized by the order of magnitude of the pathogen concentration. Model selection was based on AIC (Table 1).

Differences in levels of disease severity were analyzed using a generalized linear model with a beta error distribution and log link (function betareg of the betareg package). Dose and temperature were treated as continuous fixed factors with dose characterized by the order of magnitude of the pathogen concentration. The models accounted for the proportion of clearly lesioned area for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Model selection was based on AIC (Table 2).

Expt 2: Virulence of *L. zosterae* isolates

Differences in levels of disease prevalence across different *L. zosterae* isolates were analyzed using a GLM with a binomial error distribution and logistic link (function 'glm' of the 'lme4' [v1.1-13] package). The different isolates were treated as categorical fixed factors. Model selection was based on a likelihood ratio test between the model including isolate and the null model (function 'anova' with 'test=lrt' of the package 'stats' [v3.4.1]). To test for differences between the different isolates, a least-squares mean analysis was performed on the full model (function 'lsmeans' with Bonferroni p-value adjustment of the package 'lsmeans' [v2.26-3]) (Table 3).

Table 1. Expt 1. Generalized linear model selection used to assess the effects of pathogen dose and temperature on eelgrass wasting disease prevalence. **Bolded** column represents the best model. LogLik: log-likelihood; AICc: Akaike's information criterian corrected for small sample size; NA: not applicable. ***p < 0.001

	[1] Full	[2] Additive	**[3] Dose**	[4] Temperature	[5] Null
Intercept estimate	−12.183	−9.926***	**−10.960*** **	0.910	0.268
Intercept SE	13.181	2.994	**2.678**	1.263	0.261
Dose estimate	2.781	2.313***	**2.2882*** **	NA	NA
Dose SE	2.723	0.555	**0.5485**	NA	NA
Temperature estimate	0.0661	−0.0699	**NA**	−0.0389	NA
Temperature SE	0.774	0.101	**NA**	0.0746	NA
Interaction estimate	−0.0281	NA	**NA**	NA	NA
Interaction SE	0.159	NA	**NA**	NA	NA
df	4	3	**2**	2	1
logLik	−25.661	−25.676	**−25.920**	−40.918	−41.053
AICc	60.049	57.782	**56.051**	86.046	84.176
Delta	3.998	1.731	**0**	29.995	28.125
Weight	0.0870	0.270	**0.642**	1.97×10^{-7}	5.02×10^{-7}

Model equations are:

[1] logit(Disease Presence) ~ $\beta_1 + \beta_2$Dose + β_3Temperature + β_4Dose×Temperature
[2] logit(Disease Presence) ~ $\beta_1 + \beta_2$Dose + β_3Temperature
[3] logit(Disease Presence) ~ $\beta_1 + \beta_2$Dose
[4] logit(Disease Presence) ~ $\beta_1 + \beta_2$Temperature
[5] logit(Disease Presence) ~ β_1

Table 2. Expt 1. Generalized linear model selection used to assess the effects of pathogen dose and temperature on eelgrass wasting disease severity. **Bolded** column represents the best model. Abbreviations as in Table 1. *** p < 0.001

	[1] Full	[2] Additive	[3] Dose	[4] Temperature	[5] Null
Intercept estimate	**−11.202*** **	−5.500***	−4.669***	−1.328***	−0.563***
Intercept SE	**0.753**	0.163	0.143	0.0748	0.0153
Dose estimate	**1.763*** **	0.746***	0.743***	NA	NA
Dose SE	**0.133**	0.0254	0.0254	NA	NA
Temperature estimate	**0.381*** **	0.0492***	NA	0.0462***	NA
Temperature SE	**0.0424**	0.00450	NA	0.00439	NA
Interaction estimate	**−0.0592*** **	NA	NA	NA	NA
Interaction SE	**0.00751**	NA	NA	NA	NA
df	**4**	3	2	2	1
logLik	**−7324.208**	−7355.825	−7415.761	−7841.884	−7897.363
AICc	**14657.796**	14718.450	14835.910	15688.156	15796.851
Delta	**0**	60.653	178.113	1030.359	1139.055
Weight	**1**	6.75×10^{-14}	2.10×10^{-39}	1.82×10^{-224}	4.54×10^{-248}

Model equations are:

[1] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Dose + β_3Temperature + β_4Dose×Temperature
[2] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Dose + β_3Temperature
[3] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Dose
[4] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Temperature
[5] logit(Lesioned Area, Healthy Area) ~ β_1

Differences in levels of disease severity across different *L. zosterae* isolates were analyzed using a GLM with a beta error distribution and log link. The different isolates were treated as fixed factors and modeled against the proportion of lesioned tissue for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Model selection was based on AIC. To compare the differences between the different isolates, a least-squares mean analysis was performed on the full model (function 'lsmeans' with Bonferroni p-value adjustment of the package 'lsmeans' [v2.26-3]) (Table 5).

Expt 3: Impact of light and temperature on *L. zosterae* virulence

In vivo experiment. Differences in levels of EGWD severity were analyzed using a GLM with a beta error distribution and log link. The full model

Table 3. Expt 2. Least-squares means table comparing eelgrass wasting disease prevalence across different pathogen isolates (A: North Cove; B: Shoal 1; C: Shoal 2). Significant p-values ($p < 0.05$) are shown in **bold**

Comparison	Estimate	SE	*Z*-ratio	p
Control–A	−0.211	0.712	−0.297	1.000
Control–B	−0.847	0.617	−1.373	1.000
Control–C	−2.565	0.578	−4.438	**0.0001**
A–B	−0.636	0.812	−0.784	1.000
A–C	−2.354	0.782	−3.009	**0.0157**
B–C	−1.718	0.697	−2.463	0.0827

Table 4. Expt 2. Least-squares means table comparing eelgrass wasting disease severity across different pathogen isolates (A: North Cove; B: Shoal 1; C: Shoal 2). Significant p-values ($p < 0.05$) are shown in **bold**

Comparison	Estimate	SE	*Z*-ratio	p
Control–A	−0.032	0.024	−1.324	0.548
Control–B	−0.034	0.025	−1.392	0.505
Control–C	−0.152	0.040	−3.787	**0.001**
A–B	−0.002	0.033	−0.066	0.999
A–C	−0.120	0.045	−2.636	**0.041**
B–C	−0.117	0.045	−2.580	**0.049**

Table 5. Expt 3. Statistical summary for differences in eelgrass wasting disease severity across treatments. Pairwise comparisons were completed using Wilcoxon rank sum. Reported p-values are Bonferroni-corrected. L: light; D: dark; number indicates temperature (°C). *significant at $p < 0.05$

Comparison	*W*	r	p
L12–L18	218.5	0.672307692	0.06965
D12–D18	288	0.886153846	3.8484
L12–D12*	485.5	1.493846154	0.0048876
L12–D18*	124	0.381538462	0.0009294
L18–D12	428.5	1.318461538	0.14994
L18–D18*	457	1.406153846	0.02718

included the interaction of both temperature and light treatments, and all subsequent models were reduced from this model. Both light and temperature were treated as fixed factors, with light treatment as categorical and temperature treated as continuous. Severity was modeled as the proportion of diseased area for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Model selection was based on AIC (Table 5).

In vitro experiment. Differences in cell counts of *L. zosterae* were analyzed using a generalized linear mixed-effects model with a negative binomial error distribution and log link function 'glmer.nb' of the MASS package. The full model included the interaction of temperature, light, and time, with all subsequent models being reduced forms of this model. Light, temperature, and time were treated as fixed factors, with light treatment as categorical and temperature and time treated as continuous factors. The vial in which each sample was held was treated as a random factor to maintain consistency with the study design. Model selection was based on AIC (Table 6).

RESULTS

Expt 1: Impact of dosage and temperature on *Labyrinthula zosterae* virulence

From comparison of models using AIC, we found that the model best supported by the data only includes dosage and not temperature when analyzing prevalence. From this model and the averaged model, we found that disease prevalence increases with the log of pathogen dose ($Z = 2.360$, $p = 0.0183$, relative importance [RI] = 1.0) and is only weakly influenced by temperature ($Z = 0.091$, $p = 0.9276$, RI = 0.36; Fig. 1). The analysis of severity found that the best model was the full model including the interaction of dosage and temperature. From this model and the averaged model, we found that that dosage ($Z = 2.251$, $p = 0.02$, RI = 0.99) was significant, while both temperature ($Z = 0.127$, $p = 0.89$, RI = 0.30) and the interaction of temperature and dosage were not ($Z = 0.259$, $p = 0.79$, RI = 0.07; Fig. 1). *L. zosterae* cell growth in the positive controls showed that the inoculum was viable.

Expt 2: Virulence of *L. zosterae* isolates

Analysis of the 3 potentially unique isolates of *L. zosterae* and controls showed that the models allowing for differences in severity and prevalence between the different isolates fit the data better than

Table 6. Expt 3. Statistical summary for differences in cell counts across treatments. *significant at $p < 0.05$. Treatments are defined in Table 5

Comparison	p
L12–L18*	0.005756
D12–D18*	0.0102862
L12–D12	0.7747686
L12–D18*	0.0031316
L18–D12*	0.0200634
L18–D18	0.9560524

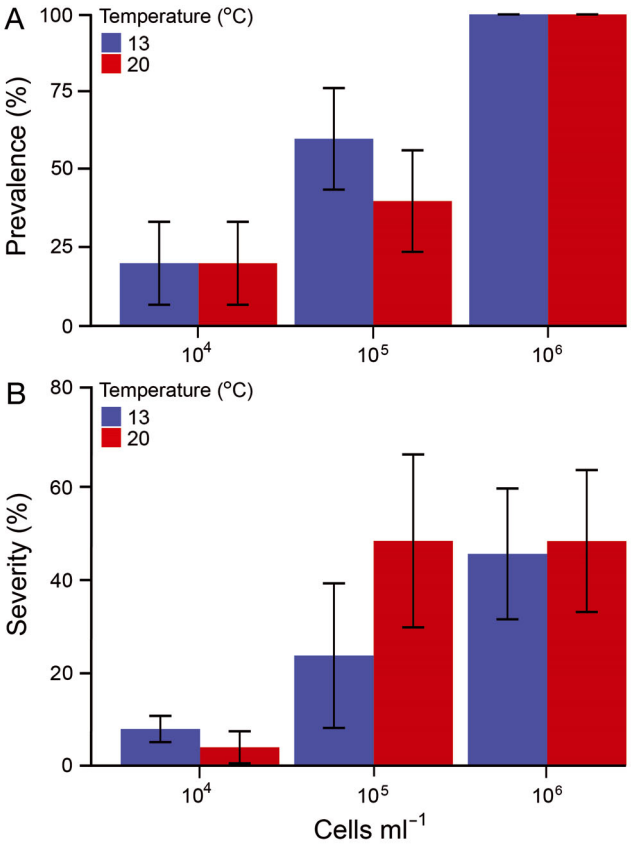

Fig. 1. Increasing pathogen dosage increases both prevalence and severity of eelgrass wasting disease. Bars represent the mean ±1 SE for (A) disease prevalence and (B) disease severity across 3 dosage treatments and 2 temperatures. Significant effects of temperature were only found in panel (B)

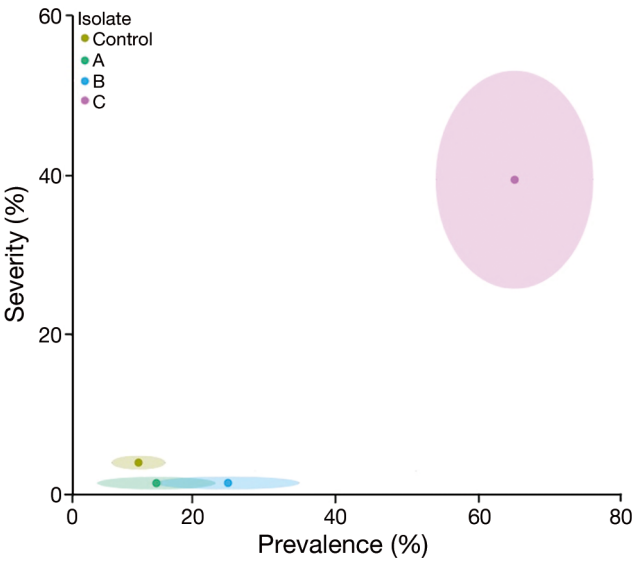

Fig. 2. Different isolates of *Labyrinthula zosterae* vary in disease prevalence and severity. Points represent mean levels of prevalence and severity for the different isolates with the ellipse boundaries representing ±1 SE. Differences in prevalence and severity between isolates are significant ($p < 0.05$)

the models that did not allow for the variation (prevalence likelihood ratio test: chi-squared = 22.456, $p < 0.001$; Fig. 2). From subsequent post hoc least-squares means analysis with a Bonferroni p-value correction, we found 3 distinct groups for prevalence, with Isolate A from North Cove and Isolate B from Shoal Bay. For severity, Isolate C from Shoal Bay was significantly different from the rest. *L. zosterae* cell growth in the positive controls showed that the inoculum was viable.

Expt 3: Impact of light and temperature on *L. zosterae* virulence

In vivo experiment

Photo-analysis of our negative controls and treatments confirmed that our inoculation methods were effective. A total of 3 out of 60 of our negative controls showed disease, with lesion area confined to small specks of blackened tissue. The ubiquitous nature of the pathogen makes picking completely uninfected plants difficult, with the possibility of *L. zosterae* existing commensally in the plants before the experiment and shifting to pathogenic once the plants became stressed. Conversely, 99 out of the 100 inoculated leaf sections developed lesions, further supporting the effectiveness of our inoculation methods.

Comparison of the models using AIC showed that the best model includes only the light regime. The averaged model supports this and we can conclude that reductions in light increase severity of the disease ($Z = 2.207$, $p = 0.02$), while increasing temperature does not impact severity ($Z = 0.177$, $p = 0.86$), and there are no interactive effects ($Z = 0.255$, $p = 0.82$) (Fig. 3). *L. zosterae* cell growth in the positive controls showed that the inoculum was viable.

In vitro experiment

The cell growth experiment was run for a total of 5 d. However, due to a lack of resources — either space or nutrients — cell growth in the 18°C treatment plateaued by Day 3. For this reason, Days 4 and 5 of the cell growth experiment were not included in our analysis.

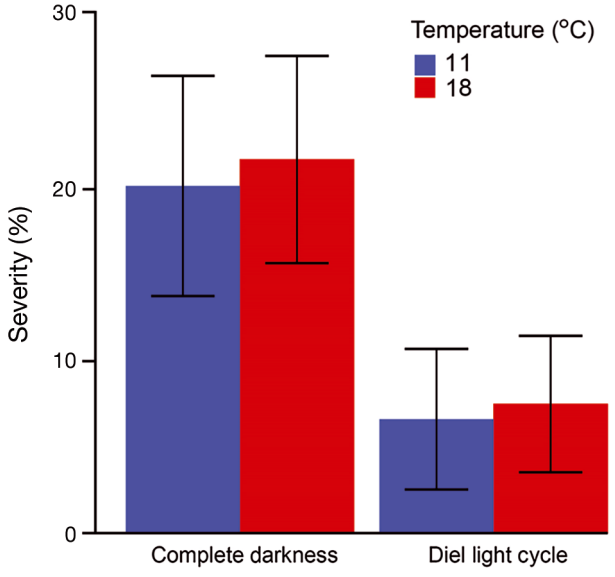

Fig. 3. *In vivo* experiment. Light and temperature influence eelgrass wasting disease severity. Mean ± SE disease severity showing that reductions in light and increasing temperatures both increase severity

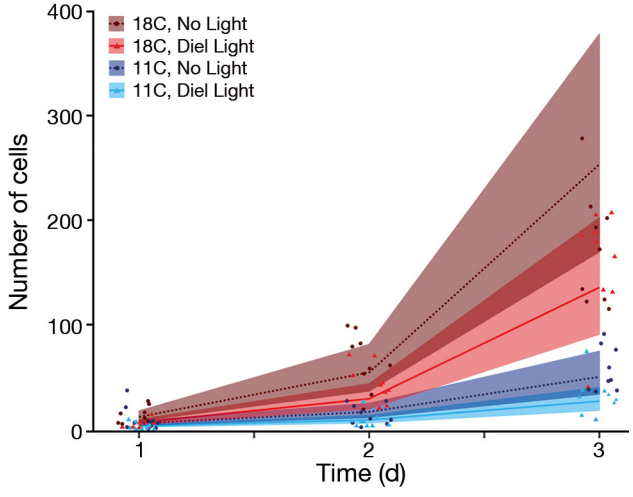

Fig. 4. *In vitro* experiment. Light and temperature influence the population growth rates of *Labyrinthula zosterae*. Predicted growth curves ±1 SE from the best model that has increasing temperature increasing the growth rate and a constant difference between the 2 light treatments. The points represent individual counts at various time points for each of the treatments

From the comparison of the models using AIC, the best model includes a changing impact of temperature over time with a constant light effect. From this model, growing *L. zosterae* in complete darkness does not alter the growth rate, although there are consistently more cells in the culture at all time points (Z = 3.828, $p < 0.001$). The 2 different temperature treatments showed no difference at the start of the trial (Z = 0.715, $p = 0.475$), but the cells grown at the higher temperature showed an increased growth rate (Z = 2.145, $p = 0.0320$) (Fig. 4).

DISCUSSION

Opportunistic pathogens are those that coexist commensally with their hosts, leading to pathogenesis only under specific conditions such as host immunosuppression or environmental change (Burge et al. 2013, 2014). Understanding the changeable biology of opportunistic pathogens is a large knowledge gap, gaining increased attention within disease ecology (Burge et al. 2013) as opportunistic outbreaks increase with ocean change (Burge et al. 2014). Since at least some strains of *Labyrinthula zosterae* are ubiquitous in the marine environment, the factors that facilitate an outbreak of EGWD are likely to rely on either a change in environment or emergence of new strains. *L. zosterae* strains are readily cultureable and so the EGWD pathosystem is unusually tractable to experimentally investigate key drivers of variation in virulence of infections.

We developed an improved method to control dosage in experimental inoculations of eelgrass to quantitatively test differences in *L. zosterae* performance across environments and for different isolates. Our dosing method provides increased control of cell concentrations to study the host–pathogen interaction between *Zostera marina* and *L. zosterae*. *L. zosterae* cells secrete an ectoplasmic filamentous net through bothrosome organelles by which their spindle-shaped cells can move and absorb nutrients (Porter 1972). This ectoplasmic net results in irregular, sticky aggregations of *L. zosterae* cells in culture which are difficult to count and impossible to quantitatively dose. In previous studies, inoculations of *L. zosterae* on healthy seagrass plants were performed by several methods: direct contact with active lesions (Vergeer et al. 1995), direct contact with gauze bandages containing the pathogenic *L. zosterae* cells (Brakel et al. 2014), or by inoculation in a liquid culture homogenized with a vortex and estimated with a hemocytometer (Groner et al. 2014). To ensure consistency in pathogenic cell concentrations across inoculations in this study, *L. zosterae* cells were vortexed for 25 s with 1 µm zirconia/silica beads and diluted to appropriate concentrations with sterile seawater before being pipetted directly onto plant tissue. Vortexing the cells with glass beads broke up irregular aggregates of *L. zosterae* cells and allowed consistent cell counts between treatments.

Results from the *L. zosterae* dosage experiment show that higher concentration of pathogenic cells leads to higher disease severity at both temperatures. Inoculation of 10^6 pathogenic cells ml^{-1} caused 17.8% (1.67 times) higher severity compared to 10^5 cells ml^{-1}, and 30.4% (3.11 times) higher severity compared to 10^4 cells ml^{-1} (when temperature was held at 16.5°C). Further studies are needed to determine environmental levels of *L. zosterae*, and subsequently test if patterns of higher disease severity at higher pathogenic cell concentrations hold up under naturally occurring conditions.

In the isolate inoculation experiment, we tested for differences in virulence of 3 pathogen isolates cultured from the San Juan Islands. The activity of our isolates fell into 2 significantly different categories of severity—1 isolate stood out as being much more virulent, with lesions developing approximately 25% faster, while the other 2 isolates grouped together at a lower severity with the control treatment. Over the course of our 12 d trial, our most virulent isolate fell into a higher prevalence category than the control treatment and the other 2 isolates, with at least 25% more leaf sections developing lesions. The high-virulence isolate and 1 of the low-virulence isolates came from the same field site (Shoal Bay), showing that mixes of different virulence isolates exist in nature.

This study has implications for future conservation and management of critical marine habitats. To offset the effects of eelgrass bed decline, conservation efforts have turned to transplanting eelgrass to accelerate recolonization and expansion at sites with suitable ecological conditions (for example Leschen et al. 2010, Goehring et al. 2015). Given our results that different isolates of *L. zosterae* can vary from relatively non-pathogenic to highly virulent, it is important to use care in moving transplants, as they might harbor unusually virulent isolates of *L. zosterae*. The introduction of novel isolates to different populations is risky, and is well documented as an epidemiological phenomenon causing spread of infectious disease. Our study suggests that more work needs to be done on strain diversity of this pathogen, since virulent strains coupled with transplantation stress will undermine restoration efforts. Use of transplants to accelerate growth and bed coalescence is a proactive way to restore declining eelgrass beds, but these transplants need to be informed by knowledge of local disease metrics to avoid increasing the range of more virulent isolates of *L. zosterae.*

The ability to culture *L. zosterae* allowed us to distinguish between the separate response of the pathogen in isolation (*in vitro*) and inside its eelgrass host (*in vivo*). Our *in vitro* study with 2 light levels and 2 temperatures at a constant dosage showed that *L. zosterae* cell growth is marginally affected by light levels, with slightly higher cell counts in dark treatments compared to light treatments. However, isolated pathogen cells grew much faster at the warmer temperature; cells of *L. zosterae* grew 2.13 times faster at 18°C than at 11°C. Thus the small effect of light was overwhelmed by the huge effect of temperature on *in vitro* growth of *L. zosterae* cells. The *in vivo* light/temperature experiment showed the opposite result. Disease severity was much higher in the dark, but only marginally affected by temperature. Since light level had an almost negligible affect on *in vitro* growth, we conclude that this significant role of light in the *in vivo* experiment is driven by the capability of *Z. marina* to mount a defense response to the infection. Therefore, plants grown in the dark were unable to mount an effective defense and consequently infections spread more rapidly.

Light, directly associated with photosynthetic capability, controls a plant's resources that could be allocated to produce secondary metabolites used as defense mechanisms. Secondary metabolites play an important role in plant disease resistance, but the biochemical basis of seagrass defense responses is understudied (Ross et al. 2008). Seagrasses produce a range of secondary metabolites, including phenolic-based compounds (Trevathan-Tackett et al. 2015). Phenolic-based compounds have diverse ecological roles in marine angiosperms and are capable of mediating biotic interactions on both broad and localized scales (Sieg & Kubanek 2013). One proposed mechanism for increased disease resistance in eelgrasses is the production of these phenols. Vergeer & Develi (1997) found that eelgrass plants kept under higher light intensities produced a higher concentration of phenolic compounds in response to infection than plants kept under low light intensities. They also found lower infection rates at higher light intensities. Induction of phenols is a significant energy investment (Vergeer & Develi 1997), so it is no surprise that our data suggest that decreased light and thus photosynthetic capability is directly correlated with the ability of *Z. marina* to mount an effective response to infection. This finding may help explain variable levels of EGWD in nature between beds and across seasons that have variable canopy light conditions.

Shifts in the environmental context of EGWD caused by climate change and physical intervention by overwater structures are imminent, and will have impacts on plant–pathosystem dynamics. Our results and previous studies (Kaldy 2014, Sullivan et al.

2017) suggest that increased temperature associated with recent trends of climate change may increase the risk of disease of *L. zosterae* in the marine environment. These results are consistent with earlier studies investigating the temperature range of *Labyrinthula* spp. (Young 1943, Pokorny 1967), but our study is the first to pair *in vitro* with *in vivo* experiments at a controlled dosage to quantify how increased temperatures directly increase reproductive rates of the pathogen. In the coming century, average annual temperatures in Washington are projected to rise at a rate of 0.1 to 0.6°C (0.2 to 1.0°F) decade^{-1} (Lawler & Mathias 2007). Global average temperatures have increased by 0.7°C (1.3°F) over the last century and are projected to rise between 1.1 and 6.4°C (2.0–11.5°F) by 2100 (Alley et al. 2007). Light attenuation events that may be caused by algal blooms or turbid plumes occur with increased frequency in coastal waters and during high water temperature periods in the summer (Kim et al. 2015). Furthermore, light quality and quantity are often altered by human actions in coastal areas (Zimmerman 2006), such as construction and expansion of structures such as piers, docks, and bridges (Short & Wyllie-Echeverria 1996, Shafer 1999), channel dredging (Moore et al. 1997, Longstaff & Dennison 1999), and runoff from watersheds (Longstaff & Dennison 1999, Cabello-Pasini et al. 2002). While the effects of light and temperature on eelgrass growth and survival as well as on EGWD dynamics have been investigated previously (Backman & Barilotti 1976, Vergeer et al. 1995, Hauxwell et al. 2001, Thom et al. 2008, Kim et al. 2015), our studies test hypotheses about the role of these environmental factors to facilitate EGWD with quantitative methods. These studies further elucidate the affects of shifting environmental parameters on both global and localized scales, and contribute to the body of literature that informs the building of coastal structures over seagrass beds that may exacerbate EGWD.

Understanding emergence of EGWD requires knowledge of its host–pathogen biology, which will involve the use of models parameterized for many environmental, ecological, and biological factors. Characterization of the status of *L. zosterae* as an opportunistic pathogen will help define the mechanisms of EGWD and may lead to further research on defining potential approaches for managing and projecting seagrass diseases. To improve our ability to predict and respond to eelgrass epidemics in wild populations, it will be necessary to take into consideration the interplay of abiotic factors in plant–pathosystem dynamics (Sullivan et al. 2013).

Acknowledgements. Our deepest thank-you to Susan Lynch for her generous support of the Cornell Ocean Research Apprenticeship for Lynch Scholars (www.eeb.cornell.edu/harvell/corals/), which provided support for the student authors and brought us together to collaborate on this valuable research. We thank Sandy Wyllie-Echeverria and Ann Jerell for the invaluable assistance in learning about the study system and their methods development suggestions. We thank Billie Swalla, director of the University of Washington Friday Harbor Marine Labs, for providing access to facilities and equipment. We are especially grateful for field and lab assistance provided by M. Fisher, J. McDowell, J. Morreale, and K. Kingsbury. Field and experimental work was supported by the Cornell Ocean Research Apprenticeship for Lynch Scholars Program (generously funded by Susan Lynch) to C.D.H. and the Atkinson Center for a Sustainable Future's Sustainable Biodiversity Fund grant to M.E.E. Partial support for M.E.E was provided by the National Science Foundation Graduate Research Fellowship Program. We declare we have no competing interests.

LITERATURE CITED

Alley R, Berntsen T, Bindoff NL, Chen Z and others (2007) Climate change 2007: the physical science basis. Summary for policymakers. Working Group I contribution to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva

Arzul I, Renault T, Lipart C, Davison AJ (2001) Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. J Gen Virol 82:865–870

Backman TW, Barilotti DC (1976) Irradiance reduction: effects on standing crops of the eelgrass *Zostera marina* in a coastal lagoon. Mar Biol 34:33–40

Barbier EB, Hacker SD, Kennedy C, Koch EW, Stier AC, Silliman BR (2011) The value of estuarine and coastal ecosystem services. Ecol Monogr 81:169–193

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67: 1–48

Brakel J, Werner FJ, Tams V, Reusch TBH, Bockelmann AC (2014) Current European *Labyrinthula zosterae* are not virulent and modulate seagrass (*Zostera marina*) defense gene expression. PLOS ONE 9:e92448

Burge CA, Kim CJS, Lyles JM, Harvell CD (2013) Special issue oceans and humans health: the ecology of marine opportunists. Microb Ecol 65:869–879

Burge CA, Eakin CM, Friedmn CS, Froelich B and others (2014) Climate change influences on marine infectious diseases: implications for management and society. Annu Rev Mar Sci 6:249–277

Burge CA, Closek CJ, Friedman CS, Groner ML, Jenkins CM, Shore-Maggio A, Welsh JE (2016) The use of filter-feeders to manage disease in a changing world. Integr Comp Biol 56:573–587

Cabello-Pasini A, Lara-Turrent C, Zimmerman RC (2002) Effect of storms on photosynthesis, carbohydrate content and survival of eelgrass populations from a coastal lagoon and the adjacent open ocean. Aquat Bot 74:149–164

Casadevall A, Pirofski L (2001) Host-pathogen interactions: the attributes of virulence. J Infect Dis 184:337–344

Cribari-Neto F, Zeileis A (2010) Beta regression in R. J Stat Softw 34:1–24

Dahl S (2015) Ecology of QPX disease in the hard clam *Mercenaria mercenaria*. PhD dissertation, Stony Brook University, New York, NY

de la Torre-Castro M, Rönnbäck P (2004) Links between humans and seagrasses—an example from tropical East Africa. Ocean Coast Manag 47:361–387

Farley CA, Banfield WG, Kasnic G Jr, Foster WS (1972) Oyster herpes-type virus. Science 178:759–760

Frydenborg BR, Krediet CJ, Teplitski M, Ritchie KB (2014) Temperature-dependent inhibition of opportunistic vibrio pathogens by native coral commensal bacteria. Microb Ecol 67:392–401

Goehring M, Gaeckle J, Hass T, Brandt S (2015) Puget Sound eelgrass (*Zostera marina*) recovery strategy. Washington State Dept of Natural Resources, www.dnr.wa.gov/publications/agr_nrsh_eelgrass_strategy_final.pdf

Godet L, Fournier J, van Katwijk MM, Olivier F, Le Mao P, Retière C (2008) Before and after wasting disease in common eelgrass *Zostera marina* along the French Atlantic coasts: a general overview and first accurate mapping. Dis Aquat Org 79:249–255

Groner ML, Burge CA, Couch CS, Kim CJS and others (2014) Host demography influences the prevalence and severity of eelgrass wasting disease. Dis Aquat Org 108: 165–175

Groner ML, Maynard J, Breyta R, Carnegie RB and others (2016) Managing marine disease emergencies in an era of rapid ocean change. Philos Trans R Soc B 317:20150364

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP (2002) Climate warming and disease risk for terrestrial and marine biota. Science 296:2158–2162

Hauxwell J, Cebrian J, Furlong C, Valiela I (2001) Macroalgal canopies contribute to eelgrass (*Zostera marina*) decline in temperate estuarine ecosystems. Ecology 82: 1007–1022

Kaldy JE (2014) Effect of temperature and nutrient manipulations on eelgrass *Zostera marina* L. from the Pacific Northwest, USA. J Exp Mar Biol Ecol 453:108–115

Kim YK, Kim SH, Lee KS (2015) Seasonal growth responses of the seagrass *Zostera marina* under severely diminished light conditions. Estuaries Coasts 38:558–568

Lafferty KD, Hofmann EE (2016) Marine disease impacts, diagnosis, forecasting, management and policy. Philos Trans R Soc B 371:20150200

Lamb JB, van de Water JAJM, Bourne DG, Altier C and others (2017) Seagrass ecosystems reduce exposure to bacterial pathogens of humans, fishes, and invertebrates. Science 355:731–733

Lawler JL, Mathias M (2007) Climate change and the future of biodiversity in Washington. Report prepared for the Washington Biodiversity Council. https://www.rco.wa.gov/documents/biodiversity/WA-Climate-Biodiversity Report.pdf

Le Deuff RM, Renault T, Gerard A (1996) Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. Dis Aquat Org 24:149–157

Lenth RV (2016) Least-squares means: the R package lsmeans. J Stat Softw 69:1–33

Leschen AS, Ford KH, Evans NT (2010) Successful eelgrass (*Zostera marina*) restoration in a formerly eutrophic estuary (Boston Harbor) supports the use of a multifaceted watershed approach to mitigating eelgrass loss. Estuaries Coasts 33:1340–1354

Longstaff BJ, Dennison WC (1999) Seagrass survival during pulsed turbidity events: the effects of light deprivation on the seagrasses *Halodule pinifolia* and *Halophila ovalis*. Aquat Bot 65:105–121

Martin DL, Chiar Y, Boone E, Sherman TD and others (2016) Functional, phylogenetic and host-geographic signatures of *Labyrinthula* spp. provide for putative species delimitation and a global-scale view of seagrass wasting disease. Estuaries Coasts 39:1403–1421

Moore KA, Wetzel RL, Orth RJ (1997) Seasonal pulses of turbidity and their relations to eelgrass (*Zostera marina* L.) survival in an estuary. J Exp Mar Biol Ecol 215:115–134

Muehlstein LK, Porter D, Short FT (1988) *Labyrinthula* sp., a marine slime mold producing the symptoms of wasting disease in eelgrass, *Zostera marina*. Mar Biol 99:465–472

Muehlstein LK, Porter D, Short FT (1991) *Labyrinthula zosterae* sp. nov., the causative agent of wasting disease of eelgrass, *Zostera marina*. Mycologia 83:180–191

Nordlund LM, Jackson EL, Nakaoka M, Samper-Villarreal J, Beca-Carretero P, Creed JC (2017) Seagrass ecosystem services—What's next? Mar Pollut Bull, doi: 10.1016/j.marpolbul.2017.09.014

Orth RJ, Carruthers TJB, Dennison WC, Duarte CM and others (2006) A global crisis for seagrass ecosystems. Bioscience 56:987–996

Pokorny KS (1967) *Labyrinthula*. J Protozool 14:697–708

Porter D (1972) Cell division in the marine slime mold, *Labyrinthula* sp., and the role of the bothrosome in extracellular membrane production. Protoplasma 74:427–448

Porter D (1990) Phylum Labyrinthulomycota. In: Margulis L, Corliss JO, Melkonian M, Chapman DJ (eds) Handbook of the Protoctista: the structure, cultivation, habits and life histories of the eukaryotic microorganisms and their descendants exclusive of animals, plants and fungi. Jones & Bartlett Publishers, Boston, MA, p 388–397

Renn CR (1934) Wasting disease of *Zostera* in American waters. Nature 134:416

Renn CR (1935) A mycetozoan parasite of *Zostera marina*. Nature 135:544–545

Ross C, Puglisi MP, Paul VJ (2008) Antifungal defenses of seagrasses from the Indian River Lagoon, Florida. Aquat Bot 88:134–141

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671–675

Shafer DJ (1999) The effects of dock shading on the seagrass *Halodule wrightii* in Perdido Bay, Alabama. Estuaries 22: 936–943

Short FT, Wyllie-Echeverria S (1996) Natural and human-induced disturbance of seagrasses. Environ Conserv 23: 17–27

Short FT, Mathieson AC, Nelson JI (1986) Recurrence of the eelgrass wasting disease at the border of New Hampshire and Maine, US. Mar Ecol Prog Ser 29:89–92

Short FT, Muehlstein LK, Porter D (1987) Eelgrass wasting disease: cause and recurrence of a marine epidemic. Biol Bull (Woods Hole) 173:557–562

Sieg RD, Kubanek J (2013) Chemical ecology of marine angiosperms: opportunities at the interface of marine and terrestrial systems. J Chem Ecol 39:687–711

Smithson M, Verkuilen J (2006) A better lemon squeezer? Maximum-likelihood regression with beta-distributed dependent variables. Psychol Methods 11:54–71

Sullivan BK, Sherman TD, Damare VS, Lilie O, Gleason FH (2013) Potential roles of *Labyrinthula* spp. in global seagrass population declines. Fungal Ecol 6:328–338

Sullivan BK, Trevathan-Tackett SM, Neuhauser S, Govers LL (2017) Review: Host-pathogen dynamics of seagrass diseases under future global change. Mar Pollut Bull, doi:10.1016/j.marpolbul.2017.09.030

Thom RM, Southard SL, Borde AB, Stoltz P (2008) Light requirements for growth and survival of eelgrass (*Zostera marina* L.) in Pacific Northwest (USA) estuaries. Estuaries Coasts 31:969–980

Trevathan-Tackett SM, Lane AL, Bishop N, Ross C (2015) Metabolites derived from the tropical seagrass *Thalassia testudinum* are bioactive against pathogenic *Labyrinthula* sp. Aquat Bot 122:1–8

Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th edn. Springer, New York, NY

Vergeer LHT, Develi A (1997) Phenolic acids in healthy and infected leaves of *Zostera marina* and their growth-limiting properties towards *Labyrinthula zosterae*. Aquat Bot 58:65–72

Vergeer LHT, Aarts TL, de Groot JD (1995) The 'wasting disease' and the effect of abiotic factors (light intensity, temperature, salinity) and infection with *Labyrinthula zosterae* on the phenolic content of *Zostera marina* shoots. Aquat Bot 52:35–44

Waycott M, Duarte CM, Carruthers TJB, Orth RJ and others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proc Natl Acad Sci USA 106:12377–12381

Wyllie-Echeverria S, Cox PA (2000) Cultural saliency as a tool for seagrass conservation. Biol Mar Mediterr 7:421–424

Young EL III (1943) Studies on *Labyrinthula*. The etiologic agent of the wasting disease of eel-grass. Am J Bot 30: 586–593

Zimmerman RC (2006) Light and photosynthesis in seagrass meadows. In: Larkum WD, Orth RJ, Duarte CM (eds) Seagrasses: biology, ecology and conservation. Springer, Dordrecht, p 303–321

Editorial responsibility: James Jancovich, San Marcos, California, USA

Submitted: January 15, 2018; Accepted: June 18, 2018
Proofs received from author(s): August 7, 2018