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Effects of multi-stress exposure on the infection dynamics of a *Labyrinthula* sp.–turtle grass pathosystem

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ABSTRACT: To assess the relationship among environmental stressors, seagrass susceptibility and *Labyrinthula* virulence, specimens of *Thalassia testudinum* were exposed to common abiotic stressors (hypersalinity, elevated temperature, nighttime hypoxia and elevated sulfide). Stressors were applied in isolation and in combination using an orthogonal design, incorporating either pulsed (Days 1–7) or sustained (Days 1–14) stress, with pathogen exposure occurring on Days 8–14. Seagrass infection responses were variable yet contingent upon environmental conditions affecting *Labyrinthula* viability. Following a 1 wk exposure period to any abiotic stressor (pre-infection), *T. testudinum* samples failed to show any significant drop in effective quantum yield. However, plant photochemistry declined significantly in response to successful infection, which was most prevalent under ambient conditions. Hypersalinity appeared to be the major factor which inhibited *in vitro Labyrinthula* sp. growth and *in planta* virulence. These data suggest that in the absence of selected abiotic stressors, *Labyrinthula* sp. has an enhanced capability of successful infection and can efficiently diminish host health (i.e. suppress photochemistry and lead to enhanced lesions). In summary, relatively short-term exposure to common environmental stressors has a generally negative influence on *Labyrinthula* sp. viability and virulence that can outweigh the effects of reduced *T. testudinum* photosynthetic health, essentially yielding an asymmetry favoring host defenses, but this asymmetry also reverses when stress is alleviated. When considering further the potential for complex interactions in this multi-stress system, positive antagonism may be more likely than synergistic or negatively antagonistic outcomes.

KEY WORDS: Seagrass · Stress · Disease · *Labyrinthula*

INTRODUCTION

Increasing evidence demonstrates that on a global level, emerging diseases are coupled with climate change and anthropogenic activity (Daszak & Cunningham 2000, Harvell et al. 2004). While the importance of pathogens in terrestrial ecosystems has long been documented (Grenfell & Dobson 1995, Anderson et al. 2004, Eviner & Likens 2008), the role of diseases in marine habitats has not been as well studied, as demonstrated by the paucity of information on the effects of climate change on marine host–pathogen interactions (Harvell et al. 2009, Burge et al. 2014). Marine diseases can have severe negative impacts on benthic community structure by causing host population declines, compromising ecosystem engineers and altering overall ecosystem functionality (Lessios 1988, Harvell et al. 2007, Hofmann & Ford 2012, Quere et al. 2015). Evidence suggests that many marine diseases are caused by opportunistic pathogens whose pathogenicity is not only contingent upon host demographics (Groner et al. 2014) but also upon the interacting effects of environmental conditions and host defense responses (Burge et al. 2013). Increasing our knowledge of host–pathogen relationships, in the context of a changing environment, is critical

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to understanding the future trajectory of coastal ecosystem health.

Opportunistic pathogens of the genus *Labyrinthula* have been identified as the cause of 'wasting disease' in seagrass beds in both temperate and subtropical biogeographical regions. *Labyrinthula* spp. move within a self-produced ectoplasmic network and degrade host cell wall tissue by use of extracellular enzymes (Muehlstein 1992). Infections are often characterized by notable brown-black lesions and are spread by blade-to-blade contact (Muehlstein et al. 1988, Muehlstein1992, Raghukumar 2002). Wasting disease outbreaks have had long-lasting ramifications on seagrass populations. In the 1930s, a widespread epidemic resulted in the destruction of up to 90% of *Zostera marina* beds on both sides of the temperate North Atlantic Ocean (Muehlstein et al. 1991). Since then, smaller-scale sporadic die-offs have occurred in both the northern and southern hemispheres (reviewed in Sullivan et al. 2013). As *Labyrinthula* spp. are widespread in the marine environment (Raghukumar 2002) and not always associated with seagrass lesions (Bockelmann et al. 2013), incidences of disease are suspected to be associated with poor environmental conditions which suppress or weaken the hosts' defenses. These same environmental factors, however, can simultaneously influence the growth, virulence and pathogenicity of the pathogen, thus effectively serving as the mediating factor that can shift the interaction in favor of host or pathogen (Blanford et al. 2003, Cróquer & Weil 2009).

Seagrass beds in subtropical environments, such as *Thalassia testudinum* in Florida Bay (a semi-enclosed estuary in South Florida, USA), are commonly exposed to environmental stressors including elevated temperature, hypersalinity, porewater sulfide toxicity and hypoxia (Koch et al. 2007a). While untested, it is plausible that exposure to varying combinations of these factors may promote wasting disease and lead to sudden mortality events as observed in 1987 and thereafter (Robblee et al. 1991, Carlson et al. 1994, Hall et al. 1999, Blakesley et al. 2002). Correlational data suggest that areas of elevated salinity, enhanced plant density and low light have a greater prevalence of wasting disease in *T. testudinum* (Blakesley et al. 2002, Trevathan-Tackett et al. 2013). Temperature has also been implicated as a trigger for disease outbreaks in temperate seagrass meadows (Rasmussen 1977, Bull et al. 2012). In Florida Bay, temperatures exceeding the optimal range for *T. testudinum* (>31°C) have been found to be associated with beds that experienced some of the most extreme die-offs (Blakesley et al. 2002). These warm sub-tropical temperatures, aside from promoting hypersalinity, can augment microbial sulfate reduction rates which in turn promote porewater sulfide production (Koch et al. 2007a). Although *T. testudinum* has demonstrated a high tolerance to sulfide toxicity due to its ability to utilize photosynthetically derived oxygen to oxidize its rhizosphere (Erskine & Koch 2000), the combination of sulfide and other stressors may contribute to increased susceptibility to *Labyrinthula* infection. As environmental stressors are often present concurrently (and non-additively) yet studies integrating stressor effects are relatively lacking (Crain et al. 2008, Darling & Côté 2008), this study was conducted to determine if common abiotic stressors (both alone and in combination) promote wasting disease susceptibility in *T. testudinum*.

MATERIALS AND METHODS

Collection and maintenance of *Thalassia testudinum* and *Labyrinthula* sp.

T. testudinum was collected off the Gulf coast of Florida (29° 20' N, 83° 23' W), cleaned of epiphytes and subsequently transplanted into terracotta pots filled with Arag-Alive!™ (CaribSea). Plants were maintained in aquaria under greenhouse conditions at the University of North Florida, Jacksonville, Florida, at a salinity of 30, representative of the initial collection site. Instant Ocean Sea Salt (Instant Ocean®) was used to create all seawater used in this study. Aquaria diel temperature values were maintained between 25 and 27°C, and photosynthetically active radiation levels were <300 µmol m^{-2} s^{-1}. Partial seawater changes were conducted on a weekly basis to maintain adequate levels of nutrients. Specimens were allowed to acclimate for no less than 1 wk prior to use in any experiment.

A known pathogenic isolate of *Labyrinthula* sp., termed isolate 8b (Martin et al. 2016), was maintained in culture and used for all experiments described herein. Serum-seawater agar (SSA) described in Trevathan et al. (2011) was used for *Labyrinthula* sp. culture and contained 500 ml of prepared seawater (salinity of 25) with 6 g agar, 0.5 g glucose, 0.05 g nutritional yeast, 0.05 g peptone, 1.5 mg germanium dioxide, 12.5 ml streptomycin/penicillin (stock: 1.25 g streptomycin + 1.25 g penicillin per 100 ml de-ionized H_2O), and 5 ml horse serum. All chemicals were purchased from Sigma-Aldrich.

General experimental design

Two full factorial design experiments were implemented in order to test the hypothesis that exposure to pulsed environmental stressors compromise the health of *T. testudinum* and promote *Labyrinthula* infection. In the first experiment, specimens of *T. testudinum* were exposed to combinations of environmental stressors for 1 wk and then simultaneously exposed to *Labyrinthula* sp. leaf-vectors and stressors for an additional week (sustained). In the second experiment, seagrasses were initially exposed to environmental stressors for 1 wk and then subsequently returned to ambient conditions (recovery) where they were exposed to *Labyrinthula* sp. leaf-vectors (which were not exposed to stressors) for an additional week. For both infection experiments, individual shoots were each kept in 3.8 l polyethylene terephthalate containers (Rubbermaid). Power-FLO T5 HO bulbs (Hagen) were used to obtain full spectrum lighting (200 µmol m^{-2} s^{-1}) and run on a 12:12 h light:dark photoperiod. In both experiments, seagrass shoots were randomly assigned a treatment group and incubated under their respective stressor(s) for 7 d, at which time their photochemical efficiency was assessed (described below). At this time, seagrass shoots were either permitted to stay under their respective treatment group or returned to ambient conditions, depending on the experiment to which each shoot belonged. Samples were then infected with *Labyrinthula* sp. according to established methods (Steele et al. 2005). Briefly, sterile (autoclaved) segments (2 cm) of *T. testudinum* were incubated for 1 wk on prepared plates of *Labyrinthula* sp. Vectors were then attached to the second rank leaf of each replicate using a clamp made from segments of Tygon tubing. The control group received sterile vectors (i.e. without *Labyrinthula* sp.). Plants were then left to incubate for 7 d until visible signs of infection were evident (i.e. necrotic lesions). At that time, photochemical efficiency and lesion size were quantified as described below. An overview of the experimental design scheme is shown in Fig. 1.

Salinity, temperature, sulfide and hypoxia treatments

Seagrass shoots were randomly assigned to a treatment in an experimental setup that utilized a full factorial design for the 4 stressors (salinity, temperature, hypoxia and sulfide; Table 1). An additional control group consisted of seagrass shoots exposed to the stressors and to mock (sterile) leaf-vectors after the

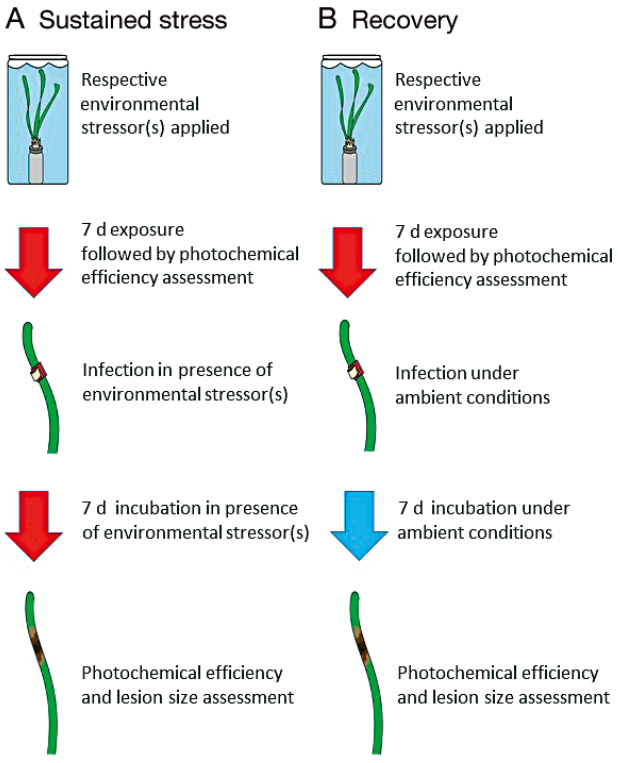

Fig. 1. Experimental design scheme for *Thalassia testudinum* infection studies

Table 1. Experimental conditions for sustained stress (n = 85) and recovery (n = 85) experiments, testing the effects of salinity, temperature, sulfide and hypoxia on the virulence of *Labyrinthula* sp. infection of *Thalassia testudinum*. Parameters: ambient salinity (31), high salinity (45); ambient temperature (23°C), high temperature (30°C); no sulfide (0 mM), sulfide (6 mM); normoxic (>8.00 mg l^{-1}), hypoxic (<2.00 mg l^{-1}). n = 5 for each treatment group

Group(s)	Experimental conditions			
	Salinity	Temperature	Sulfide	Oxygen
Treatment	Ambient	Ambient	No	Hypoxic
	Ambient	Ambient	No	Normoxic
	Ambient	High	No	Hypoxic
	Ambient	High	No	Normoxic
	Ambient	Ambient	Yes	Hypoxic
	Ambient	Ambient	Yes	Normoxic
	Ambient	High	Yes	Hypoxic
	Ambient	High	Yes	Normoxic
	High	Ambient	No	Hypoxic
	High	Ambient	No	Normoxic
	High	High	No	Hypoxic
	High	High	No	Normoxic
	High	Ambient	Yes	Hypoxic
	High	Ambient	Yes	Normoxic
	High	High	Yes	Hypoxic
	High	High	Yes	Normoxic
Control	High	High	Yes	Hypoxic

first week. Each treatment (including the control) contained 5 replicates for a total of 85 microcosms in each of the 2 experiments.

Over the course of the experiments, mean (±SE) salinity values were maintained at 45.6 ± 0.16 (high) and 31.2 ± 0.21 (ambient), respectively. Elevated salinity values were achieved with the addition of Instant Ocean Sea Salt, and de-ionized water was added as needed throughout any experiment to account for evaporation and maintain the respective salinities. Control temperatures (±SE) were maintained at 23.2 ± 0.15°C and elevated temperatures were kept at 30.2 ± 0.04°C using individual 25 W adjustable aquarium heaters (Commodity Axis). All microcosms were monitored on a daily basis to maintain their respective salinities and temperatures using a YSI model 85 multiprobe meter (YSI). Salinity and temperature values were chosen based upon preliminary experimentation (authors' unpubl. data) that would not induce senescence yet would represent ecologically relevant parameters experienced in Florida Bay (Stabenau & Kotun 2012). All microcosms were aerated throughout the experiment to prevent hypoxic conditions where applicable.

Seagrass shoots were exposed to sulfide (6 mM) and night-time hypoxia (<2.00 mg l^{-1}) in quantities known to elicit a stress response (Koch & Erskine 2001). To restrict sulfide exposure to below-ground tissue, root chambers were constructed as per Koch & Erskine (2001) with minor modification. Briefly, seagrass short shoots were threaded through 5 mm holes in rubber stoppers and were sealed on non-photosynthetic tissue using marine epoxy (West Marine®). Stoppers with shoots were then inserted into glass vials (300 ml) which served as root chambers and contained the appropriate sulfide concentration. Root chambers with shoots were then placed in individual microcosms. During the light cycle of the photoperiod, all microcosms were aerated. At the beginning of the dark cycle, N_2 gas was bubbled into the appropriate microcosms until hypoxic conditions were obtained. Normoxic treatments were continuously aerated.

Sulfide speciation is pH-dependent. At the ambient pH of seawater (~8.2), the dominant sulfide species is the hydrosulfide anion (HS^-). Although HS^- is toxic at high concentrations (Koch & Erskine 2001), hydrogen sulfide (H_2S) is a potent phytotoxin and is the dominant sulfide species in seagrass sediments in Florida. At a pH of 7.0, the ratio of HS^- to H_2S is 50:50. Therefore, sodium sulfide ($NaS \cdot 9H_2O$) was dissolved in deoxygenated seawater that had an adjusted pH of 7.0 to obtain a hydrogen sulfide concentration of 6 mM. Sulfide concentrations in the root chambers were monitored with a solid-state silver/sulfide probe (Model 27504-28, Cole-Parmer®). Additionally, the seawater of the microcosm was monitored to ensure sulfide was restricted to root chambers. To account for sulfide oxidation, root chambers received fresh seawater/sulfide mixture every 3 d and were maintained at a sulfide concentration of 6 mM. Shoots in treatments that were not exposed to sulfide were still sealed in root chambers whose below-ground tissues were exposed to deoxygenated seawater with a pH of 7.0. These chambers also received fresh seawater every 3 d to prevent nutrient limitation.

Pulse amplitude modulated fluorometry

To determine if the application of a given abiotic stressor or *Labyrinthula* sp. infection induced sublethal stress, plant photochemical efficiency was assessed using a diving pulse amplitude modulated (PAM) fluorometer (Heinz-Walz). Effective quantum yield (EQY) was measured utilizing the following parameters: measuring intensity = 5, gain = 6, damp = 2, saturation intensity = 2. To obtain consistent measurements, a leaf clip (DIVING-LC) was attached 1 cm above the site of infection and measurements were taken 4 mm away from the leaf surface (Durako & Kunzelman 2002). EQY measurements were taken after the first week and prior to infection (pre-infection), and at the end of any experiment (1 wk post-infection).

Lesion measurements

Post-treatment, surface area measurements of necrotic lesions were utilized as a proxy indicator of plant susceptibility to infection and as a measure of virulence. Photographs of each shoot were taken using a Canon Powershot SX260 HS digital camera and lesion size for each plant was measured and quantified using ImageJ software (Schneider et al. 2012). A post-hoc analysis of lesion size was also used to investigate potential differences in overall pathogenicity (i.e. symptomatic or not) and resulting virulence levels.

In vitro *Labyrinthula* sp. growth assay

To assess the effects of salinity and temperature on growth of *Labyrinthula* sp., 4 treatment groups (n = 6 per treatment) were prepared that mimicked some of

the conditions of the *T. testudinum* infection experiments: (1) ambient salinity (31) + ambient temperature (23°C); (2) ambient salinity + high temperature (30°C); (3) high salinity (45) + ambient temperature; (4) high salinity + high temperature. A 6 mm diameter cork borer was used to extract standard-sized SSA plugs of *Labyrinthula* sp. from the growing edge of cultures incubated under conditions mentioned above. Each plug was then placed surface-side down into a 12 well microplate (Costar®, Corning) and randomly assigned to one of the treatment groups. Two ml of liquid media were then carefully added to each well. The liquid media was prepared according to the SSA recipe, but was augmented with salinities corresponding to treatment groups and the agar component was omitted. To obtain elevated temperatures, the microplates were immersed in a water bath.

After a 72 h incubation period, the microplates were removed from their treatment groups and the liquid media and agar plug were carefully discarded. *Labyrinthula* sp. cells were stained using 1 ml of 0.1% Crystal Violet histological stain (Fisher Scientific). After 1 min, the stain was removed, rinsed with deionized water and dried at 37°C for 30 min. The microplates were then inverted and the colony edge was traced for each well. Photographs of each well were taken using a Canon Powershot SX260 HS digital camera and colony size was measured and quantified in square millimeters (mm^2) using ImageJ software.

Statistical analyses

The normality of all residuals was assessed using the Shapiro-Wilk test, and the equality of error variances was analyzed using Levene's test. All statistical analyses were conducted using IBM SPSS Statistics19 (IBM) or R Software version 3.3.1.

Due to space limitations, each seagrass infection experiment (sustained stress and recovery simulation) had to be conducted in 2 parts (i.e. temporally separated) where replicates within each treatment were randomly assigned to one of the 2 groups. A *t*-test was performed to determine if significant differences existed between the 2 groups. If differences existed, a randomized block design with time point as the block was incorporated into the analysis. Data for lesion size were square root transformed to meet the assumptions of ANOVA and a randomized block ANOVA (4-way) was conducted with time as a nuisance factor when applicable. When interactions were present, ANOVAs were used to determine individual effects.

Data for EQY could not be transformed to meet the assumptions of ANOVA, thus nonparametric tests were utilized. Differences in post-infection EQY values between the control and the elevated salinity + elevated temperature + sulfide + hypoxic group were analyzed using a Kruskal-Wallis test. Wilcoxon signed ranks tests were conducted to determine if statistically significant differences were present between pre-infection and post-infection EQY values for each main effect. Kruskal-Wallis tests were also performed to determine if there were statistically significant differences in post-infection EQY values among the main effects and to determine if there were differences in lesion sizes between the sustained stress experiment and recovery simulation experiment. A 2-way ANOVA was conducted to test the effects of salinity and temperature on the growth (measured as colony area) of *Labyrinthula* sp.

In studies similar to this, tiny lesions (<3 mm^2) were omitted in order to focus on lesions more clearly linked to infectious disease and thus provide a more conservative view of pathogenicity. When followed for more than 7 d in another experimental system that also utilized *Labyrinthula* sp. 8b, such small spots failed to show progressive growth and thus were not considered to be symptoms typifying wasting disease (D. Martin & A. Boettcher pers. obs.); similarly, spots <2.7 mm^2 in *Posidonia oceanica* control plants were omitted (Olsen et al. 2015). Segmented linear regression (using R) was used here in a post-hoc analysis of lesion size across all experiments to objectively state where a change in lesion size occurred and thus help infer what lesion range was likely due to pathogenesis (essentially eliminating any tiny spots as lesions). The segmented linear regression determines breakpoints in continuous data where 2 different slopes meet. Breakpoints were assessed using an iterative search procedure and choosing the model that had the lowest residual mean squared error. These 'adjusted' larger-lesions-only data (non-transformed) were then used to compare overall percent pathogenicity between recovery and sustained scenarios using a 2-tailed *t*-test and for deriving the recovery experiment means used in the following section.

The most common model underlying ANOVA is additive (Folt et al. 1999), whereby interaction effects are evaluated for relative to the sum of individual factor effects; it may also be considered the most parsimonious model for general use (Kendler & Gardner 2010). This additive null model is used when applying Piggott et al.'s (2015a,b) directional classification scheme for synergism versus antagonism (and the

null), but we did so only in a generic sense when re-evaluating virulence levels from the recovery experiment. Consequently, we re-analyzed the raw data (for the 'adjusted' lesion sizes data; see above) graphically using means-only in order to apply their method conceptually. In other words, any outcomes are not true statistical interactions from ANOVA; alternatively, this was applied post-hoc to see if these 11 factorials, in the aggregate, might suggest a particular overall direction or tendency — as opposed to the original individual factorial ANOVA outcomes where small sample sizes may preclude detecting significant interaction terms (see below for further rationale/discussion). Briefly, deviation from an additive model may yield either positive or negative synergism or antagonism, respectively, if higher or lower in absolute magnitude than is predicted by the null — depending on the factorial value relative to each delimiting value of consequence (e.g. the control, highest individual effect, lowest individual effect, and/or summed individual effects; for detailed methodology see Piggott et al. 2015a).

To further derive a more general perspective from the recovery scenario (again, using the adjusted lesion sizes data), means of each treatment type were combined to yield overall treatment means for 3 sets of factor level (1×, n = 4; 2×, n = 6; >2×, n = 5) for comparison using 1-way ANOVA. The effect of factor level on this conservative estimate of percent pathogenicity was evaluated for all recovery scenario data, using a Kruskall-Wallis test since ANOVA assumptions were not met.

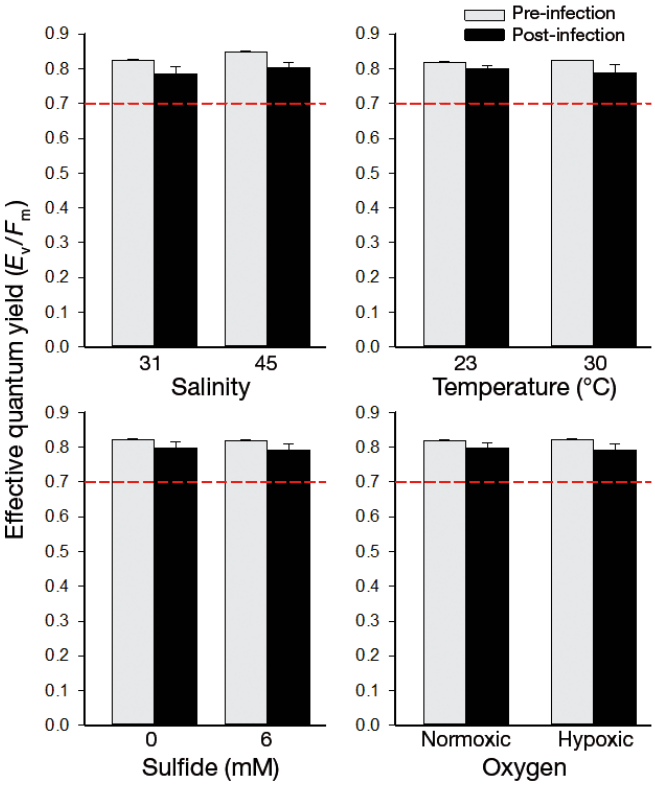

Fig. 2. Effective quantum yield (EQY) of *Thalassia testudinum* under sustained stress conditions. Specimens were exposed to abiotic stressors (salinity, temperature, sulfide and oxygen) for 1 wk (pre-infection) followed by a week of exposure to *Labyrinthula* sp. (post-infection) under sustained stress conditions. Red line indicates EQY threshold of 0.700. Bars represent mean + SE

RESULTS

Seagrass photochemistry

Following a 1 wk exposure period to any abiotic stressor (pre-infection), *Thalassia testudinum* samples failed to show any drop in EQY below 0.700 (threshold for compromised photochemical efficiency as per Koch et al. 2007b) (Figs. 2 & 3). Under the sustained stress experiments, pre-infection EQY values differed from post-infection values in a variable manner (Fig. 2 and Table 2). However, EQY values never dropped below the critical threshold of 0.700. Conversely, post-infection EQY values were markedly lower than pre-EQY values in all cases represented under the recovery scenario (Fig. 3), indicating ambient conditions represented under recovery are conducive to *Labyrinthula* infection and consequently cause a decline in host EQY. There was found to be a significant negative correlation between lesion presence and EQY ($p < 0.001$; $r = -0.81$). A summary of the statistical results pertaining to the pre- and post-infection EQY data is provided in Table 2.

Virulence (lesion size) and pathogenicity

Nearly all exposures to *Labyrinthula* sp. 8b caused lesions (overall pathogenicity was 99.3%, less the controls; but see the more conservative adjusted estimate below). However, virulence, and thus individual seagrass infection responses, was variable and highly contingent upon environmental conditions and the effects of those environmental conditions on the viability of the *Labyrinthula*. A summary of the statistical results associated with lesion size (virulence) is provided in Table 3. There was a significant interaction between salinity and sulfide (ANOVA, $F_{1,61} = 5.95$, $p = 0.018$, effect size = 0.46) under sustained stressors. Salinity was found to have a significant negative impact on lesion size (ANOVA, $F_{1,76} = 22.01$,

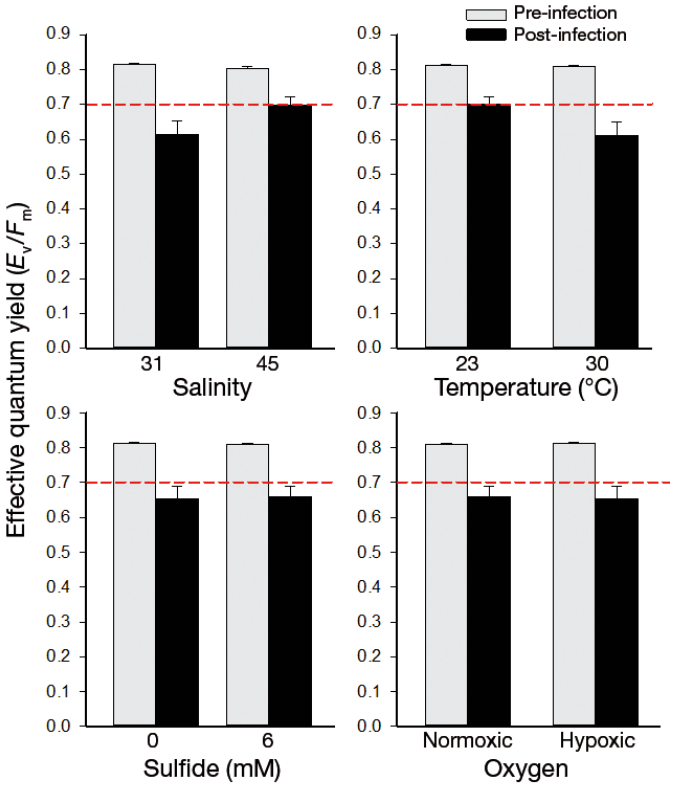

Fig. 3. Effective quantum yield (EQY) of *Thalassia testudinum* under recovery conditions. Specimens were exposed to abiotic stressors (salinity, temperature, sulfide and oxygen) for 1 wk (pre-infection) followed by a week of exposure to *Labyrinthula* sp. (post-infection) under ambient or recovery conditions (post-infection). Red line indicates EQY threshold of 0.700. Bars represent mean + SE

Table 2. Treatment effects on effective quantum yield (EQY) in *Thalassia testudinum* based on Wilcoxon signed ranks test (pre- vs. post-infection with *Labyrinthula* sp.), significance at $p < 0.05$ (in **bold**)

Pre- vs. post-infection EQY		Pre-infection (mean ± SE)	Post-infection (mean ± SE)	*Z*	p
Sustained					
Salinity	Ambient	0.825 ± 0.002	0.791 ± 0.019	−3.587	**<0.001**
	High	0.817 ± 0.004	0.802 ± 0.016	−0.323	0.747
Temperature	Ambient	0.818 ± 0.004	0.806 ± 0.005	−2.373	**0.018**
	High	0.823 ± 0.002	0.787 ± 0.023	−1.340	0.180
Sulfide	Ambient	0.822 ± 0.003	0.798 ± 0.016	−2.157	**0.031**
	High	0.819 ± 0.004	0.794 ± 0.018	−1.741	0.082
Oxygen	Ambient	0.820 ± 0.003	0.802 ± 0.015	−1.102	0.270
	Low	0.822 ± 0.004	0.790 ± 0.019	−2.882	**0.004**
Recovery					
Salinity	Ambient	0.817 ± 0.002	0.615 ± 0.038	−5.429	**<0.001**
	High	0.803 ± 0.004	0.870 ± 0.177	−4.668	**<0.001**
Temperature	Ambient	0.811 ± 0.004	0.700 ± 0.021	−5.394	**<0.001**
	High	0.809 ± 0.002	0.780 ± 0.177	−4.759	**<0.001**
Sulfide	Ambient	0.812 ± 0.003	0.819 ± 0.175	−4.846	**<0.001**
	High	0.808 ± 0.003	0.660 ± 0.030	−5.330	**<0.001**
Oxygen	Ambient	0.809 ± 0.003	0.834 ± 0.179	−4.720	**<0.001**
	Low	0.811 ± 0.003	0.650 ± 0.034	−5.444	**<0.001**

$p < 0.001$, effect size = 0.58), but sulfide did not (ANOVA, $F_{1,76} = 0.54$, $p = 0.465$, effect size = 0.27), indicating that the effects of salinity were not the same when sulfide was present. Under high salinity, regardless of sulfide, lesion sizes were smaller. However, with ambient salinity, high sulfide resulted in larger lesions.

The recovery experiment resulted in a significant interaction (ANOVA, $F_{1,64} = 4.43$, $p = 0.039$, effect size = 0.27) for the 4×-factorial among salinity, temperature, oxygen and sulfide. However, salinity was the only significant (salinity × temperature × sulfide ANOVA, $F_{1,72} = 8.71$, $p = 0.004$, effect size = 0.53; salinity × temperature × oxygen ANOVA, $F_{1,72} = 9.19$, $p = 0.003$, effect size = 0.54; salinity × sulfide × oxygen ANOVA, $F_{1,72} = 9.27$, $p = 0.003$, effect size = 0.54) main effect with ambient salinity groups resulting in larger lesions, regardless of the other stressors. Additionally, there was a significant difference in lesion size between the sustained stressors group (mean ± SE, 0.78 ± 1.54 mm) and the recovery simulation group (45.00 ± 4.95), with the latter resulting in larger lesions ($t_{155} = -7.28$, $p < 0.001$, effect size = 0.77; Fig. 4) when including all lesion sizes.

While nearly all exposures to the pathogen led to lesions of some size, a more conservative, post-hoc interpretation using segmented linear regression showed a cut-off or break-point value at very small lesion sizes (≤2.5 mm^2, $p < 0.001$; see inset Fig. 5), after 7 d of exposure. Overall pathogenicity, when re-calculated (adjusted) in this light, is then reduced to only 24%. However, recovery scenario treatments alone showed an 'adjusted' pathogenicity of 47%, thus accounting for nearly all such cases, while sustained scenario treatments averaged just over 1% (Fig. 5). This result is also reflected in the similar outcome based on area of all lesions/spots (Fig. 4).

For the adjusted (larger lesions only) recovery scenario data, sample size was small and variance high within any given treatment, as total recovery scenario N was reduced from 85 to 37 among the 11 treatments. Thus, we employed only a graphic (conceptual) application of Piggott et al.'s (2015a) directional synergism/antagonism classification scheme (see Fig. A1 in the Appendix for detailed graphic used to derive the classifications). Most outcomes were antagonistic (91%), with

Table 3. Treatment effects on lesion size in *Thalassia testudinum* after infection with *Labyrinthula* sp., based on 4-way ANOVA with elevated salinity, elevated temperature, high sulfide and low oxygen as fixed factors, significance at $p < 0.05$ (in **bold**)

Source of variation	df	*F*	p
Sustained stress			
Salinity	1	40.627	**<0.001**
Temperature	1	7.763	**0.007**
Sulfide	1	1.528	0.221
Oxygen	1	0.356	0.553
Salinity × temperature	1	2.180	0.145
Salinity × sulfide	1	5.951	**0.018**
Salinity × oxygen	1	0.702	0.406
Temperature × sulfide	1	0.179	0.673
Temperature × oxygen	1	1.464	0.231
Sulfide × oxygen	1	0.307	0.581
Salinity × temperature × sulfide	1	2.652	0.109
Salinity × temperature × oxygen	1	1.462	0.231
Salinity × sulfide × oxygen	1	0.798	0.375
Temperature × sulfide × oxygen	1	0.794	0.376
Salinity × temperature × sulfide × oxygen	1	1.027	0.315
Total	78		
Recovery simulation			
Salinity	1	9.869	**0.003**
Temperature	1	0.246	0.622
Sulfide	1	1.030	0.314
Oxygen	1	4.017	**0.049**
Salinity × temperature	1	0.134	0.715
Salinity × sulfide	1	0.696	0.407
Salinity × oxygen	1	2.973	0.089
Temperature × sulfide	1	0.666	0.418
Temperature × oxygen	1	1.091	0.300
Sulfide × oxygen	1	0.317	0.576
Salinity × temperature × sulfide	1	1.883	0.175
Salinity × temperature × oxygen	1	0.447	0.506
Salinity × sulfide × oxygen	1	0.557	0.458
Temperature × sulfide × oxygen	1	3.781	0.056
Salinity × temperature × sulfide × oxygen	1	4.425	**0.039**
Total	80		

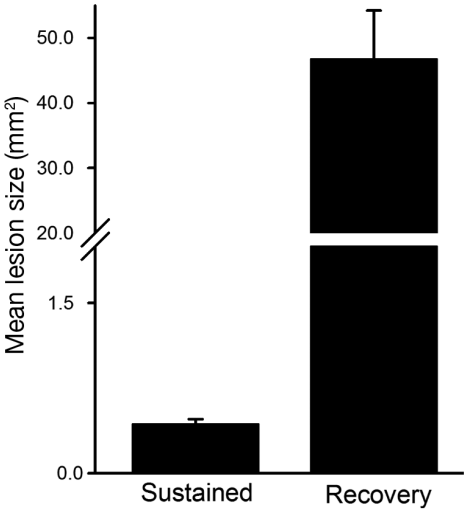

Fig. 4. Lesion area of *Thalassia testudinum* blades infected with *Labyrinthula* sp. following infection under sustained stress or recovery scenarios. Each group contains data pooled from all abiotic exposure treatments. Bars represent mean + SE

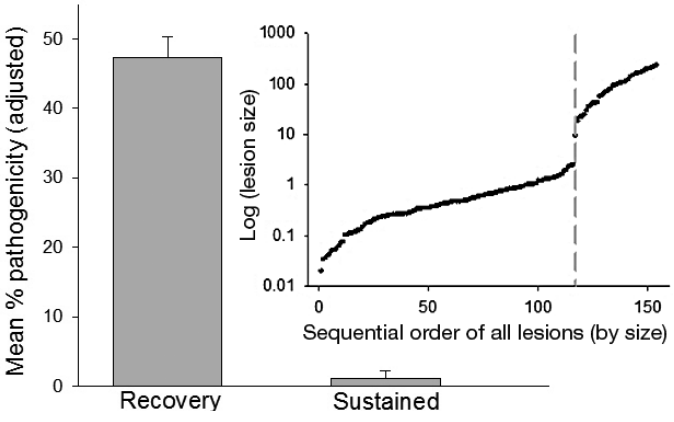

Fig. 5. Infection results (percent pathogenicity) adjusted for apparent non-symptomatic or non-significant spots/lesions. Bars represent mean + SE. Inset: all spots/lesions plotted by increasing size and evaluated for discontinuity in the regression (note vertical line; only those above this were used in the main plot)

the majority in the direction of positive antagonism (82%); only one of the eleven factorials (9%) presented as synergistic (Table 4). Meanwhile, the only factorial treatment from the main (all lesion sizes) recovery scenario data set to show a significant interaction term was the 4× (Table 3), but it was not distinguishable from the additive model based on overlapping 95% confidence intervals (and not shown here).

When asking whether adjusted mean lesion size (i.e. average virulence, and using the same recovery-only data) was generally influenced by factor level, we found no significant difference among 1×, 2× and >2× treatments (Fig. 6; 1-way ANOVA, $F = 0.137$, $p = 0.873$, effect size = 0.16). Similarly, percent pathogenicity (using the conservative, adjusted measure) examined by factor level for the overall recovery data set suggests cumulative stressor effects on pathogenicity were not different from single factor effects or each other (Fig. 7; Kruskall-Wallis $\chi^2 = 1.789$, $p = 0.409$, effect size = 0.31).

In vitro Labyrinthula sp. growth assay

Labyrinthula growth was impacted by the hypersalinity treatment (Fig. 8) as colony areas were signif-

Table 4. Treatments (by column) for the 'adjusted' lesion size (virulence) data from the recovery scenario were re-analyzed using the directional classification scheme presented by Piggott et al. (2015a), but based on means only, i.e. not true statistical interaction terms (see 'Materials and methods: Statistical analyses' and 'Discussion' for rationale). Results: '**–A**' negative antagonism, '**+A**' positive antagonism, and '**+S**' positive synergism. Specifically, these data represent the seagrass *Thalassia testudinum* plus pathogen *Labyrinthula* sp. 8b infection response (necrotic lesion size, our proxy for virulence) for the pulsed stressor(s) with recovery experiment. In this summary, factorial treatment means have been evaluated graphically relative to the magnitude and direction of the various individual mean factor effects, predicted (summed) factorial effects and the control (Piggott et al. 2015a,b). See Fig. A1 in the Appendix for the detailed graph used to derive the classifications

	Factorial (level)										
Stressors	**2×**						**3×**				**4×**
Salinity	x	x		x			x	x	x		x
Sulfide	x		x		x		x	x		x	x
Oxygen		x	x			x	x		x	x	x
Temperature				x	x	x		x	x	x	x
Result (classification)	**–A**	**+A**	**+A**	**+A**	**+A**	**+A**	**+S**	**+A**	**+A**	**+A**	**+A**

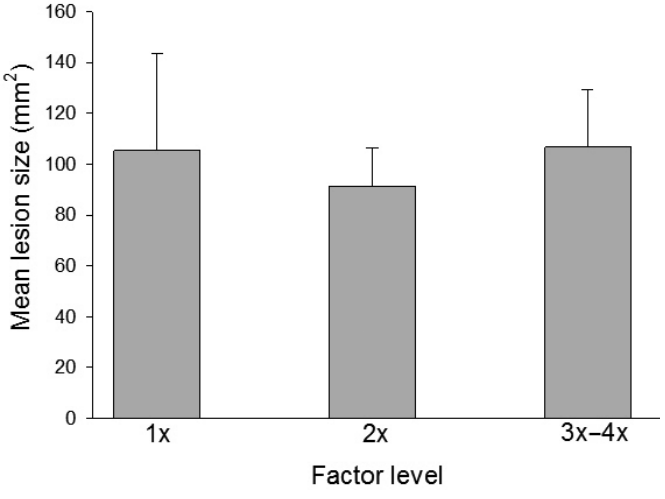

Fig. 6. Aggregate mean lesion size (virulence) of recovery-only treatments, by factor level. Bars represent mean + SE

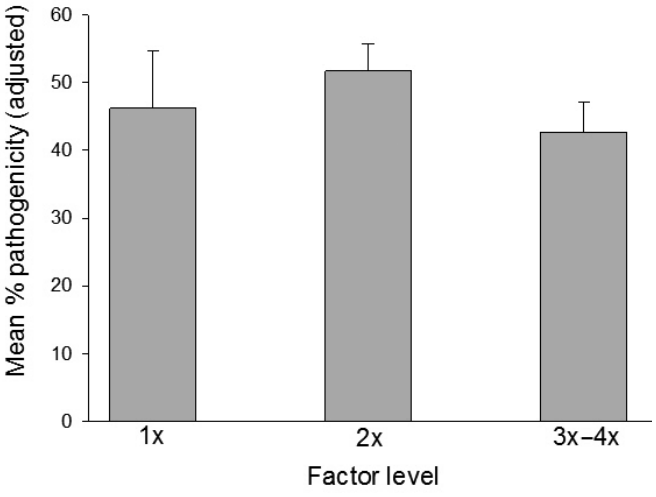

Fig. 7. Aggregate mean pathogenicity of recovery-only treatments, by factor level. Bars represent mean + SE

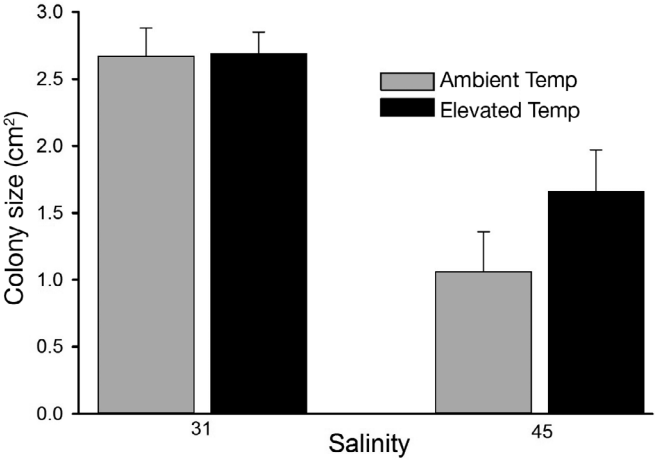

Fig. 8. Effects of salinity and temperature on *in vitro Labyrinthula* sp. growth. Bars represent mean + SE. Ambient temperature = 23°C; high temperature = 30°C

icantly reduced ($F_{1,25}$ = 24.81, p < 0.001). Temperature did not significantly affect growth ($F_{1,20}$ = 1.20, p = 0.286), and the interaction between salinity and temperature was not significant ($F_{1,20}$ = 1.37, p = 0.256).

DISCUSSION

Seagrass beds and their associated microbiota represent diverse plant pathosystems that are under the influence of numerous confounding abiotic and biotic factors that may facilitate or impede disease progression (reviewed in Sullivan et al. 2013). In this study, we assessed the relationship between host susceptibility and pathogen virulence in the presence/absence of select environmental stressors commonly found in subtropical *Thalassia testudinum* grass beds. It was initially hypothesized that the presence of multiple

abiotic stressors would cause an additive or possibly even synergistic decrease in photochemical efficiency coupled with a concomitant increase in disease susceptibility. However, the results of this study suggest that infection responses are variable and that short-term exposure to select environmental stressors can have a negative influence on *Labyrinthula* that can outweigh the effects of diminished *T. testudinum* health as assessed by photosynthetic efficiency. In general, abiotic stress (especially hypersalinity) negatively influenced *Labyrinthula* sp. 8b growth *in vitro*, virulence and pathogenicity in seagrass shoots, and was found to be a major limiter of lesion spread (but not necessarily lesion initiation). When lesion formation (and not short-term abiotic stress exposure) did occur, it had a significant negative impact on seagrass EQY.

While it is generally accepted that global-scale environmental stressors (e.g. climate warming) have the potential to promote disease transmission and host susceptibility in the marine environment (Harvell et al. 2002), some studies have shown that stressors, such as elevated temperature, can promote host fitness and possibly reduce pathogen pressure (Ward et al. 2007, Olsen et al. 2015). *T. testudinum* has a remarkable capacity to endure exposure to a combination of stressors without significantly compromising growth, shoot survival or tissue-oxidizing capacity (Koch et al. 2007a). Our results are in general agreement with others that have found that salinity values up to 45 do not negatively impact the quantum yield of *T. testudinum* under short-term (7 d) or even longer-term conditions (30–60 d) (Trevathan et al. 2011, Koch et al. 2007a,b). Even though select combinations of stressors (e.g. elevated temperature and sulfide exposure) have the potential to disrupt seagrass carbon metabolism (Koch et al. 2007b), we found no evidence that, under short-term conditions, *T. testudinum* susceptibility to infection is reproducibly enhanced, regardless of the stressor being applied.

Hypersalinity appears to be a major factor in the regulation of growth, virulence and pathogenicity of *Labyrinthula* isolate 8b as evidenced through *T. testudinum* infection and *in vitro* growth assays. Trevathan et al. (2011) noted a 70% reduction in lesion size when *T. testudinum* samples were infected with the same isolate at a salinity of 45 compared to controls maintained at 30. Similarly, in both of our sustained stress and recovery infection experiments, an ambient salinity of 31 resulted in larger seagrass lesions following infection. Hypersaline conditions may compromise the structural integrity of *Labyrinthula's* external ectoplasmic network and ultimately reduce its ability to adhere to and infect host tissue (Young 1943, Martin et al. 2009). When comparing pre- and post-infection seagrass EQY values, post-infection values were significantly lower in ambient salinity groups regardless of experiment. These data suggest that in the absence of stressors, *Labyrinthula* sp. has a relatively enhanced capability of successful infection (in these laboratory trials) and can efficiently diminish host health (i.e. suppress photochemistry and lead to enhanced lesion formation). Once lesions have spanned the width of the blade, a decrease in photosynthesis as well as a reduction in oxygen and carbohydrate transport can ensue (Durako & Kuss 1994, Ralph & Short 2002, Steele et al. 2005, Olsen & Duarte 2015).

The results of the *in vitro Labyrinthula* sp. growth assays support the *T. testudinum* infection response data in that colony size was negatively affected by hypersalinity. Similar findings have been reported by Martin et al. (2009), who demonstrated that *Labyrinthula* sp. isolated from *T. testudinum* have larger colony areas and higher cell counts when grown at an intermediate salinity of 30 and very reduced areas and counts at high salinity. Interestingly, wasting disease studies on the temperate *Zostera marina*–*Labyrinthula zosterae* pathosystem show evidence for a positive correlation between hypersalinity exposure and wasting disease symptoms (Burdick et al. 1993, McKone & Tanner 2009), with a pathogen salinity optima in culture from about 20–40 (Young 1943). Consequently, even though *T. testudinum* and *Z. marina* can both exist under euryhaline conditions (Zieman 1975, Nejrup & Pedersen 2008), it is evident that salinity may serve as one of the more important factors mediating this pathogen's success. Similar cases highlighting impacts of salinity on disease progression in estuarine organisms have been noted, including variable effects (Bushek et al. 2012), but also increasing (Ford & Haskin 1988, Chu et al. 1993, Perrigault et al. 2012) and decreasing (Reid et al. 2003) effects. However, patterns of *Labyrinthula* distribution in relation to seagrass hosts and salinity are limited mainly to symptoms (lesions), with the only quantified measures (via qPCR) existing for *L. zosterae* in European eelgrass. The latter show lower-salinity (≈15–25, or mesohaline-polyhaline waters) areas can still yield relatively high pathogen levels in terms of both prevalence and abundance (Bockelmann et al. 2013, Brakel et al. 2014). Therefore, while it appears that various seagrass-associated *Labyrinthula* are generally more stenohaline and/or mixoeuhaline than their hosts, and may be tolerant of

salinity changes in the short term, more euryhaline and/or mesohaline-polyhaline populations do exist — at least in European eelgrass beds.

We did not observe any significant effect of temperature on *Labyrinthula* growth when comparing 23 versus 30°C. These results are in contrast to the findings of Olsen & Duarte (2015) and Olsen et al. (2015), who showed that *Labyrinthula* obtained from *Cymodocea nodosa* and *Posidonia oceanica* undergo a reduction in areal growth (and cell division) in response to water temperatures ≥28°C, but these particular isolates were also not identical in their response. The variability among these findings might be attributed in part to differences in the particular species of *Labyrinthula* found in seagrass beds around the world (Martin et al. 2016). Such differences in tolerances may result in very different outcomes in relation to disease transmission and severity, further complicating basic predictions in the wild. Thus, various stressors (within limits) may act quite differently depending on the pathosystem in question.

As an example of differential outcomes within a regional system, warming in the Mediterranean was seen as reducing pathogen pressure in *P. oceanica*, yet this effect was less consequential to *C. nodosa*. This was based not only on summer temperature maxima differentially inhibiting pathogen growth, but on the capacity of the host to fend off infection (Olsen & Duarte 2015, Olsen et al. 2015). At this point, it is worth noting that nearly all 'primary' outbreaks of seagrass wasting disease, i.e. those without obvious or fairly extreme environmental perturbations considered a leading cause (as seen in the Florida Bay *T. testudinum* system; Robblee et al. 1991), are from temperate seagrass systems, and primarily that of eelgrass *Z. marina* (e.g. Bull et al. 2012). Tropical Australian populations of *Z. muelleri* may be the exception (Sullivan et al. 2013), though it is considered a temperate/tropical species and near the end of its range in that region (Collier et al. 2011, York et al. 2013). While this trend does reflect where much of the research is taking place (shorelines of developed countries in the northern hemisphere), it may also indicate an emerging pattern of a larger mismatch in host versus pathogen temperature optima, as noted early-on for eelgrass by Young (1943), and more recently for *P. oceanica* by Olsen & Duarte (2015). Similarly, at least some temperate species (e.g. *Z. marina*) are known to have temperature optimas below the seasonal maxima, owing to a photosynthesis-to-respiration ratio that declines with rising temperatures, while warmer water species tend to keep positive net production more in pace with rising seasonal temperatures (Short & Neckles 1999). Thus, temperate seagrasses may be more prone to wasting disease owing to a relatively large temperature mismatch, though susceptibility likely reflects an amalgam of many life-history traits.

To help summarize the various effects of abiotic stress on this seagrass–*Labyrinthula* pathosystem, we also produced a conceptual diagram (Fig. 9) to visualize how both symmetrical (affecting both host and pathogen) and asymmetrical (affecting one or the other) stress effects can yield overlapping results in terms of being symptomatic, depending on intensity of the stressor(s). For example, salinity ranging from 30 to 45 may act asymmetrically by representing none-to-high stress to the pathogen, but essentially none to the host, near the higher end of this treatment range (e.g. note lack of shading Fig. 9i,iii), possibly by inhibiting *Labyrinthula* transmission in the first place

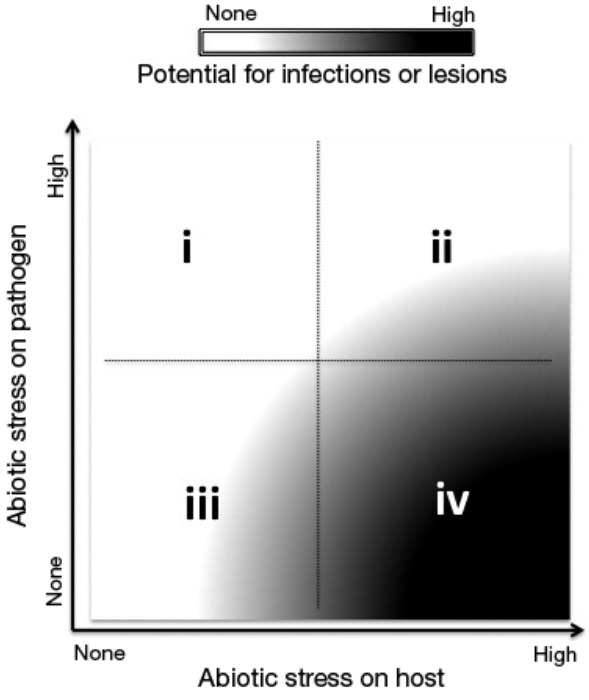

Fig. 9. Conceptualizing the symmetry of host–pathogen outcomes relative to abiotic stress levels in this seagrass–*Labyrinthula* pathosystem. Density (shading) reflects the probability or potential for host infection (or extent of lesion formation) when subject to the various combinations of abiotic stress conditions stated, with host exposure levels on the *x*-axis, and pathogen exposure levels on the *y*-axis. The quadrants (i–iv) summarize 4 general outcomes for the 2 basic stress scenarios (no or lower stress, or moderate to high stress) as applied to each of the 2 players (host and pathogen). For example, higher levels of infection are expected (darkest shading) only in cases of higher plant stress and low or no pathogen stress (region iv); alternatively, no host damage or infection is expected when the host is never stressed but the pathogen is (region i)

(as discussed above). In cases considering multiple abiotic stressors together, Fig. 9 may help demonstrate why there was little to no lesion development under sustained stress as most of the tested treatments seemed to affect both host and pathogen (represented in 9ii), but relatively intense lesion development in the recovery treatments (given recently stressed plants were subject to non-stressed pathogen populations, Fig. 9iv; although not tested here directly, this situation also suggests that any lag-time in recovery for seagrasses versus their microbial symbionts may prove pivotal). However, the normal or non-stressed baseline scenario is to the lower left of Fig. 9, in which biotic factors may otherwise determine outcomes (e.g. Brakel et al. 2014); of course, biotic factors can also alter abiotic interactions.

Of substantial importance for predicting future ecological change is whether the cumulative effects of multiple stressors is additive, making predictions simpler, or otherwise (e.g. Willsteed et al. 2017). The recovery scenario data based on lesion size, when tiny spots were excluded as a disease response, offers the most significant and arguably the most direct results for further investigating the consequences of disease intensity among the various treatments. We also believe the interaction concept is important enough to at least attempt a more generic approach with this smaller data set, by comparing treatment means only. Using the basic directional concept behind Piggott et al.'s (2015a) synergism/antagonism classification scheme, factorial means were evaluated relative to the positive control, the main effects of individual constituent factors, and their particular additive models (see Table 4 and Fig. A1). This is done in an attempt to yield useful interpretations (relative to true statistical interaction terms, given the small sample sizes of individual factorials) in the sense of suggesting any directionality when factorials are assessed in the aggregate (e.g. Are they all in the same direction?). By applying the compelling re-conceptualization presented by Piggott et al. (2015a,b) 82% of these factorial outcomes suggest positive antagonism (i.e. positive, but still less than expected relative to the additive model), though we have precluded use of the additive case given our crude method (except for an exact match, yet only one case came close to that; Fig. A1). Just one of 11 (9%) suggests synergism, and positively so. Relative to Piggott et al.'s (2015a) re-analysis of Crain et al.'s (2008) meta-analysis, our data may be in the opposite direction (yet similar in magnitude) of the norm for non-additive situations, as they found negative antagonism among the most common (28%) and positive antagonism least common (15%). However, our data agree with Côté et al.'s (2016) assessment in that synergism may be relatively rare.

We further asked whether multi-factorial (2×, or >2×) treatment means from these recovery data, in the aggregate (pooled), were significantly different from the aggregated single-factor (1×) means or each other (although we are dealing with 4 different factor variables, the same 4 were used repeatedly in the various factorial groupings). For the case of both virulence (mean lesion size) and percent pathogenicity, response mean values appear quite similar and independent of factor level, further suggesting a lack of additive and synergistic outcomes for cumulative effects in this system (Figs. 6 & 7).

As a changing climate alters marine ecosystem functionality, there is a pressing need to understand the mechanisms that underpin the etiology of seagrass wasting disease in order to predict and mitigate outbreaks. However, spatial and temporal effects of environmental stressors on host–pathogen interactions yield complex and dynamic responses (Lafferty & Holt 2003), and additive effects seen at the organismal level may lead to antagonism or synergism at the population or community level (Kroeker et al. 2017). While seagrass wasting disease is a widespread occurrence, host demographics, *Labyrinthula* diversity and environmental conditions (duration and intensity) all influence disease prevalence and severity (Groner et al. 2014, Martin et al. 2016). Some have proposed that environmental stressors may exacerbate the effects of wasting disease by decreasing seagrass' ability to resist infection from *Labyrinthula* spp. (Blakesley et al. 2002, Trevathan et al. 2011). Contrary to the hypothesis that host health would be compromised, *Labyrinthula* sp. isolate 8b health/virulence appears equally sensitive to short-term abiotic stressors under laboratory conditions, specifically elevated salinity. Thus, effects of common abiotic stressors in this system may be explained in part by how symmetrical the particular responses are between the host and pathogen, i.e. whether they favor one or the other. In general, relatively short-term exposure to common environmental stressors has a negative influence on *Labyrinthula* sp. 8b viability and virulence that can outweigh the effects of reduced *T. testudinum* photosynthetic health, essentially yielding an asymmetry favoring host defenses, but this asymmetry also reverses when stress is alleviated.

Furthermore, the suggested absence of additive and synergistic outcomes in factorial treatments relative to single factor treatments — and that antagonism may be positive — indicate an important aspect

for future studies to consider when addressing cumulative environmental effects in such pathosystems. Conservation efforts may use such information to prioritize which stressors to address, if any, when considering wasting disease. As is becoming more evident, it will depend on the host and region in question as much as the particular stressor(s). Some stressors like low light (e.g. turbidity due to point-source eutrophication) will necessarily be more tractable than warming (owing to more global issues; Côté et al. 2016). This particular stressor, considered among the most common and significant affecting seagrasses globally (Short & Wyllie-Echeverria 1996), should lead to an asymmetry affecting the host but not the pathogen (see Fig. 9iv). However, managers may still be challenged when interactions present differently depending on level of organization (Kroeker et al. 2017).

Recent sequencing of the *Z. marina* genome (Olsen et al. 2016) will assist with understanding the molecular basis of seagrass–microbe interactions (Davey et al. 2016). Future omic and profiling studies on both hosts and pathogens may also begin to unravel whether sub-lethal *Labyrinthula* spp. effects have the potential to alter seagrass population dynamics, especially when considering complex functions with multiple layers of regulation

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Appendix

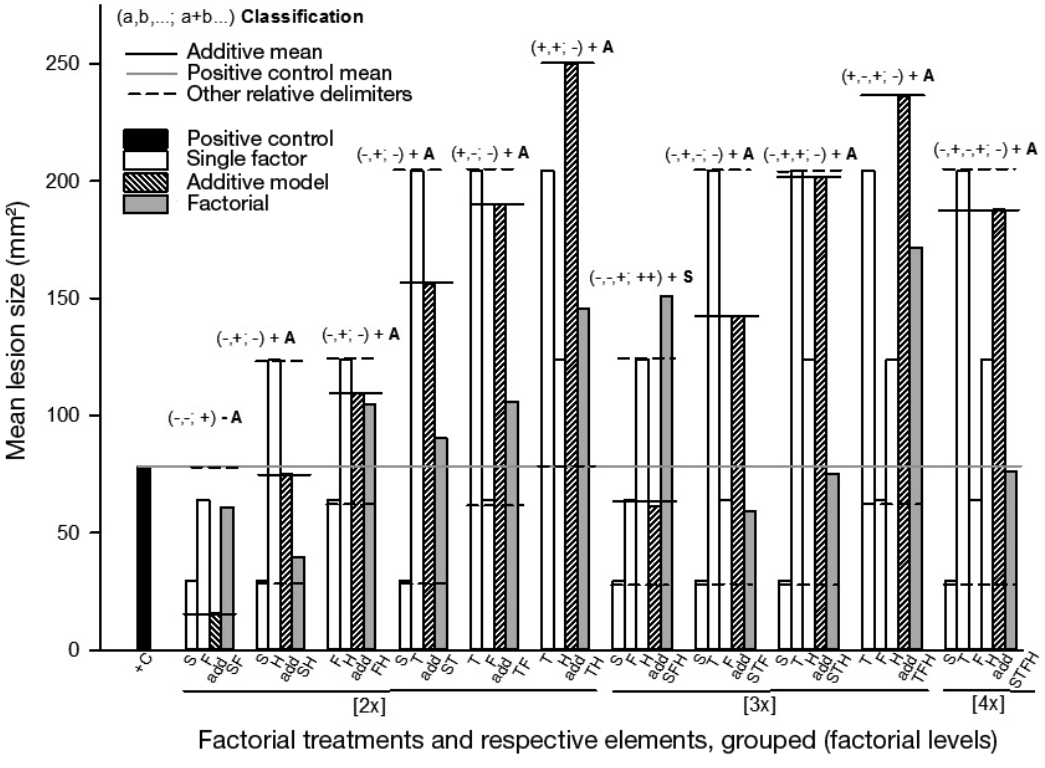

Fig. A1. Seagrass (*Thalassia testudinum*) × pathogen (*Labyrinthula* sp. 8b) infection response (necrotic lesion size) for 'pulsed stressor(s) with recovery' experiment. Factorial results are presented relative to (see horizontal lines) positive control, and to single-factor constituent test results and their respective additive model outcomes for a particular grouping. Additive model: C + (A – C) + (B – C)… where C = positive control mean, and A, B, … are individual factor test means. For *x*-axis treatment type or predicted model: +C = positive control, add = additive model, S = hypersalinity, F = high sulfide, H = hypoxia, and T = high temperature; combined treatment letters indicate factorials. For classification: within parentheses above each bar grouping, 'a,b,…' are individual factor results coded as +/0/− relative to control, followed by semicolon and then the interaction 'a+b…' (i.e. factorial) result coding relative to the additive, as described by Piggott et al. (2015a); the overall classification follows in **bold**, where **+S** = positive synergistic, **+A** = positive antagonistic, and **−A** = negative antagonistic (see Table 1 in Piggott et al. 2015a); however, results are simplistic and based on means only, not on significant statistical interaction effects (see 'Materials and methods: Statistical analyses' and 'Discussion')

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