Eelgrass disease dynamics:

An experimental analysis of the eelgrass - *Labyrinthula zosterae* interaction

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Für meine Eltern

und Philip

Contents

Summary

Seagrasses are ecosystem engineers and build up ecologic and economic highly valuable ecosystems in shallow marine waters, providing a wide range of ecosystem services, supporting human health, food security and protection of the coasts. However, seagrass ecosystems are threatened and decrease at alarming rates on global and local scale. Causes for the loss of seagrass meadows include, among mostly anthropogenic influences, infectious diseases. The most prominent disease in seagrass is the 'wasting disease'. In the 1930s 'wasting disease' hit trans-Atlantic *Zostera marina L.* (eelgrass) populations, provoking the biggest ever reported seagrass die-off. The proposed agent of this disease is the marine net-slime mold *Labyrinthula zosterae*. It has been suggested that wasting disease outbreak might have been favored by unfavorable conditions for eelgrass. However, these hypotheses were hardly targeted by experimental investigation. Some recent molecular studies detected locally high prevalence of the *L. zosterae* in northern European eelgrass meadows, raising the question whether these are a potential threat for eelgrass stands. In my thesis, I aimed to characterize the interaction of contemporary *L. zosterae* - eelgrass and to test the influence of diverse environmental factors. Therefore, I performed a series of experimental infections with naive eelgrass plants raised from seeds and *L. zosterae* isolates from the study area the south western Baltic and the North Sea.

In the first chapter, virulence and pathogenicity of *L. zosterae* isolates was assessed depending of its origin and the interaction of the origin of the eelgrass plant. *L. zosterae* infection caused higher leaf growth rates in the host and was not associated to mortality independent from host or protist origin.

In the second chapter I follow up on the increased growth rates in inoculated plants. I hypothesized that *L. zosterae* would facilitate eelgrass growth under nutrient limitation by enhanced internal recycling of nutrients. The alternative exclusive hypothesis was that nutrient limitation would enhance *L. zosterae* infection in eelgrass plants. In this study, inoculation with *L. zosterae* and nutrient limitation both reduced eelgrass growth additively. No interaction of nutrient level and *L. zosterae* infection could be detected. Similar to the first experiments plants were however able to clear high infection levels within 3 wk to ambient background levels of infection. Thus I conclude that eelgrass plants were well capable to hinder the spread of the infection.

Finally, in the third chapter I assessed the effect *L. zosterae* infection and its effect on host fitness under unfavorable conditions for the host. Therefore, I designed a fully-factorial experiment, exposing *Z. marina* plants to combinations of *L. zosterae* infection,

heat stress, light limitation and different salinity levels. I hypothesized a synergistic effect of eelgrass stress factors on eelgrass infection dynamics increasing negative effects of *L. zosterae* infection on host fitness. Contrary to my expectation, inoculation with *L. zosterae* did not reduce fitness associated traits under any condition. However, we detected a strong interaction between salinity and temperature on pathogenicity, namely *L. zosterae* was not able to infect eelgrass under high temperature and low salinity.

This work corroborate the idea that contemporary *L. zosterae* isolates do not represent an immediate risk for eelgrass beds in the south-western Baltic, however, they might represent a reservoir from where more virulent forms may evolve.

Deutsche Zusammenfassung

Seegräser sind sogenannte "Ökosystem-Ingenieure", die im küstennahen Flachwasserbereich der Ozeane großflächige Wiesen bilden. Diese Wiesen stellen sowohl ökologisch als auch ökonomisch sehr wertvolle Ökosysteme dar, denn sie beherbergen eine Vielzahl von Organismen und sichern z.B. den Schutz der Küsten. Trotz dieser Wertschöpfung sind Seegrasbestände weltweit besonders durch menschliche Einflüsse bedroht und verschwinden mit alarmierender Geschwindigkeit. Neben anthropogenen Einflüssen spielen aber auch Infektionskrankheiten für den Rückgang des Seegrases eine Rolle. Die bekannteste Seegraskrankheit ist die sogenannte Siecht-Krankheit des Seegrases, in der englischen Fachliteratur bekannt als "wasting disease". Diese verursachte in den 1930er Jahren das größte jemals dokumentierte Seegrassterben. Seegraspopulationen beidseits des Atlantiks wurden binnen weniger Jahre vernichtet. Man geht davon aus, dass die Krankheit durch den Netz-Schleimpilz *Labyrinthula zosterae* ausgelöst wurde. Desweiteren wurde gemutmaßt, dass der Krankheitsausbruch durch für das Seegras ungünstige Umweltbedingungen begünstigt wurde. Jedoch wurden diese Thesen bisher kaum experimentell untersucht. Kürzlich ergaben molekulare Studien, dass Seegrasblätter an einzelnen Standorten in Nordeuropa häufig mit *L. zosterae* infiziert sind. So stellt sich die Frage, ob heutige Infektion des Seegrases mit *L. zosterae* eine Bedrohung für die rezenten Seegrasbestände darstellt. Ziel meiner Dissertation war es deshalb, die Interaktion zwischen rezentem Seegras und *L. zosterae* unter unterschiedlichen Umweltbedingungen zu charakterisieren. Dafür führte ich eine Reihe von Infektionsexperimenten mit Seegräsern durch.

In dem ersten Kapitel meiner Dissertation wurde die Virulenz und Pathogenität dreier *Labyrinthula zosterae* Isolate abhängig von deren Herkunft und in Interaktion mit der Herkunft der Seegräser erfasst. Die Infektion mit *L. zosterae* führte zu einem erhöhten Blattwachstum des Seegrases und war nicht mit erhöhter Sterblichkeit verbunden.

In dem zweiten Kapitel verfolgte ich die Tatsache des erhöhten Blattwachstums weiter. Ich stellte die Hypothese auf, dass *L. zosterae* das Seegraswachstum fördert, indem es das interne Recyceln von Nährstoffen bei Nährstoffmangel erhöht. Die alternative Hypothese war, dass die Nährstofflimitation die *L. zosterae* Infektion im Seegras verstärkt. Tatsächlich verringerten in diesem Experiment sowohl die Nährstofflimitation als auch *L. zosterae* Infektion das Seegraswachstum. Eine Interaktion von Nährstofflimitation und Infektion war jedoch nicht nachzuweisen. Wie im ersten Experiment konnte 21 Tage nach der experimentellen Infektion kaum noch *L. zosterae*

Zellen in den neu gewachsenen Seegrasblättern nachgewiesen werden, d. h. die Pflanze war in der Lage die Infektion auf ein sehr geringes Maß einzudämmen.

Schließlich untersuchte ich im dritten Kapitel den Einfluss der *L. zosterae* Infektion unter ungünstigen Bedingungen für das Seegras. Meine Erwartung war, dass Seegras-Stressoren, wie erhöhte Temperatur und geringe Lichtintensität, die Infektion verstärken würden und sich damit auch die negativen Auswirkungen auf die Wirtsfitness erhöht. Entgegen meiner Hypothese, wurde das Seegras nicht wesentlich von der *L. zosterae* Infektion beeinflusst, weder unter nicht-stressvollen noch unter stressigen Bedingungen. Ich stellte jedoch eine synergistische Interaktion zwischen einem niedrigen Salzgehalt und erhöhter Temperatur fest, in der Weise, dass unter diesen Bedingungen keine Infektion erfolgte.

Diese Arbeit unterstützt die Annahme, dass die rezenten *L. zosterae* Stämme in unserem Untersuchungsgebiet der süd-westlichen Ostsee und Nordsee zurzeit keine akute Gefahr für den Seegrasbestand darstellen. Jedoch bieten diese ein Reservoir in dem sich virulentere Stämme entwickeln könnten.

Introduction

Symbiotic host – microbe interactions in the light of global change

While for decades symbiosis, i.e. the living together of unlike organisms (*sensu* de Bary 1879), has been believed to be something rather exceptional, the omnipresence and relevance of symbiotic microorganism for the earth ecosystems is by now without doubt (Mendes et al. 2013, McFall-Ngai et al. 2013, Alivisatos et al. 2015). Symbiosis is meant here in its broader sense including the range from mutualistic to parasitic interactions. It exists a huge diversity of strategies, how microbes interact with their hosts e.g. as parasites, pathogens, mutualists or commensals, classified by fitness costs and benefits of the interaction for the symbionts. However, host - microbe interactions are seldom stable over life time, but may shift within the continuum between parasitism and mutualism depending on environmental condition and life stage (Bronstein 1994, Newton et al. 2010). Terrestrial and marine systems are changing in unprecedented rates driven by anthropogenic activity e.g. climate change, increased deposit of anthropogenically fixed nitrogen, pollution or land use change (Halpern et al. 2008, Rockström et al. 2009, Doney et al. 2012), which might affect fine-tuned species interactions. To understand how anthropogenic induced changes affect the earth ecosystems is one of the big challenges for scientists these days (Lubchenco 1998).

Global environmental change can disrupt or weaken symbiotic interactions. For example, due to different ecologic tolerances the symbiont may not be able to endure the new environmental conditions. As a consequence, it dies off or is not able to interact in the same way as before with the host. As an example, it has been shown that beneficial gut microbiota of a stink bug (*Nezara viridula*) is sensible to elevated temperatures, which presumable limits the distribution of its host (Kikuchi et al. 2016). Furthermore, changes in metabolic rates of symbionts might be altered, which can imbalance the fine-tuned interaction as observed during coral bleaching, i.e. the loss of endosymbiotic zooxanthellae of corals (Wooldridge 2010). In addition, global environmental change can affect the timing of developmental stages and result in a temporal disruption of the interaction, which is called phenologic impairment (Yang & Rudolf 2010).

The above outlined examples concerned disrupting or weakening of symbiotic interactions. However, similarly interactions can switch, and turn e.g. from commensals to parasites or pathogens. For example Italian ryegrass (*Lolium multiflorum)* is frequently infected by the fungal endophyte *Neotyphodium occultans.* Whether this infection is beneficial or harmful for its host depends on environmental conditions like water supply (Miranda et al. 2011). Similarly, new interactions can form or existing interaction

strengthen, as with opportunistic pathogens, which are by definition microorganisms that turn pathogenic upon environmental change or the availability of susceptible hosts (Burge et al. 2013).

Infectious diseases in the marine realm

Infectious diseases in the marine realm may have severe ecologic and socio-economic implications, especially if key stone or foundation species are affected such as reef building corals, sea stars (as top predators) or seagrasses (Kershaw 2009, Groner, Maynard, et al. 2016). While disease is ubiquitous and belong to a healthy ecosystem (Hudson et al. 2006), there is nevertheless the concern, that infectious diseases are becoming more frequent due to anthropogenic change and represent a greater threat to conservation, ecosystem services (Harvell et al. 1999, 2002). In some taxonomic groups as corals, turtles and mollusks, indirect evidence indicates an increase of infectious diseases over the time span from 1970 to 2010 (Ward & Lafferty 2004). However, the link between environmental change and disease outbreak is not well understood for many marine host - pathogen systems.

The outcome of the mutual interaction depends on host ability to fend of the pathogen (host defense status), complemented by the pathogens ability to infect and harm the host (pathogenicity and virulence). Environmental stressors may decrease host defense status by resource allocation towards mitigation of stressor due to e.g. in the case of warming increased metabolic activity (Roth et al. 2010). If the immune response is compromised, the host will exhibit a higher susceptibility. However, resource allocation will affected only non-permanent defense mechanisms, already build up defenses might not be altered. In parallel, the pathogen will react to the host specific environmental stressor either by an increased or decreased fitness, depending on its optimum towards the respective environmental factor (Lafferty 1997). Thus reproductive output might increase or decrease. As well the capacity to infect the host or to damage the host might vary with environmental influence, e.g. some virulence genes are expressed only upon a certain temperature (Maurelli et al. 1984). Therefore, while for some microorganisms a certain environmental condition can favor its spread, others might be hindered, resulting in the relief of the host from its parasite. This illustrates, the careful consideration of various factors to predict how environmental factors affect a certain host - pathogen system (Lafferty et al. 2004, Rohr et al. 2011).

Compared to terrestrial ecosystem, marine infectious diseases are less studied and understood (McCallum et al. 2004), although they differ substantially to terrestrial systems e.g. they contain a greater taxonomic diversity of phyla in hosts and pathogens

and different modes of disease transmission. In this thesis, I aim to contribute to a not well understood marine host – pathogen system, which has been hypothesized to have tremendous ecological impact upon disease outbreak. I investigate the seagrass species *Zostera marina* L. and the frequently associated foliar endophyte *Labyrinthula zosterae*. For this potential pathosystem the influence of environmental factors for disease outbreak is only poorly understood. In this thesis I use the term endophyte in its literal sense, i.e. organism living inside the plant, without inferring a mutualistic relationship between plant and microorganism (see e.g. Schulz & Boyle 2005).

Plant – symbiont interaction

A host's defense status (immunocompetence) can be critical to understand the link between environment and disease outbreak in a host - pathogen interaction. Higher plants evolved various strategies to withstand and fight pathogens. This includes physical barriers, like a waxy cuticle or cell wall apposition (Hardham et al. 2007, Underwood 2012), and chemical barriers in form of secondary plant compounds that may inhibit or kill microbes by intoxication (Bednarek & Osbourn 2009). Further, membrane-bound and intercellular receptors recognize potential pathogens by microbial-associated molecular patterns (MAMPS) or by virulence factors and trigger the expression of pathogenesis related genes to fight pathogens (Jones & Dangl 2006, Spoel & Dong 2012). Additionally, recognition can trigger a hypersensitive response which hinders the spread of biotrophic pathogens by induction of programmed cell death (Heath 2000, Glazebrook 2005) or induce an extracellular oxidative burst that repels microorganisms (Daudi et al. 2012).

Seagrasses adapted to the marine environment 100 million years ago, which released them from various frequent terrestrial plant pathogens. However, in the marine environment they are faced with high abundances of microorganisms belonging to diverse phyla. In order to prevent degradation by this plethora of microbes, seagrasses must possess efficient ways to defend themselves (see as well Kubanek et al. 2003). A wide variety of secondary compounds in seagrasses have been described, of which some were identified to inhibit growth of certain microbes (see Zidorn 2016). However, chemical defense in context of eelgrass wasting disease is poorly understood. Phenolic derivates, particularly coffeic acid, has been proposed to play a role in defending eelgrass against *Labyrinthula zosterae,* because caffeic acid concentration increase upon infection with *L. zosterae* (Vergeer & Develi 1997, Mckone & Tanner 2009). In the seagrass species *Thalassia testudinum* four synergistically acting metabolites were identified (flavone glycoside thalassiolin B, p-coumaric acid, p-hydroxybenzoic acid, 3,4-

dihydroxybenzoic acid and vanillin) that clearly inhibit *Labyrinthula sp.* growth (Trevathan-Tackett et al. 2015). However, phenolic acids isolated from *Zostera marina* have not been unambiguously proven to inhibit its pathogen growth (Vergeer & Develi 1997). Comparatively little is known about the protein based defense against *L. zosterae.* Hypothesis can be drawn however from genetic features of *Z. marina* (Olsen et al. 2016). The ability to overcome and reproduce after a pathogen attack will not only be shape by the defense mechanism, but additionally to the ability to tolerate a successful infection by a pathogen. This may include for foliar pathogens similar mechanisms as for grazers, namely relative high growth rates, pre-existing high carbohydrate storage in roots and the ability to shunt storage to the leaves after damage (Strauss & Agrawal 1999).

Zostera marina and its endophyte *Labyrinthula zosterae*

Seagrasses are a paraphyletic group of marine angiosperms that adapted to the marine environment about 100 million year ago (Les et al. 1997). As ecosystem engineers (*sensu* Jones et al. 1994) they build up an ecologic and economic highly valuable ecosystem in the shallow waters, providing a wide range of ecosystem services, supporting human health, food security and protection of the coasts (Costanza et al. 1998, Orth et al. 2006, Cullen-Unsworth et al. 2014). Highly recognized is the role of seagrasses to sequester carbon dioxide, estimated sequestration rates are 27.4 - 44 Tg C yr^{-1} on global scale (Duarte et al. 2005). Further, only recently it has been shown that seagrasses decrease abundance of pathogenic bacteria in the water column (Lamb et al. 2017). In addition, seagrass beds are nursery ground for many finfish and shell fish species (Heck et al. 2003), which is of great importance especially in coastal communities in developing countries that rely on traditional fisheries as food source. These examples illustrate that the conservation of seagrass beds is of great importance for human well-being on local as on global scale considering biodiversity and carbon dioxide sequestration (Cullen-Unsworth et al. 2014).

Nonetheless, seagrasses beds are declining at alarming rates. Rates of seagrass disappearance were estimated to have reached an annual loss of 7 % since 1990 (Waycott et al. 2009). Causes for the loss of seagrass are divers and include among other eutrophication, global warming, habitat destruction, but as well diseases (Orth et al. 2006).

The most prominent disease, which led to the biggest ever reported seagrass die-off, has been described as the ‘wasting disease’, which hit trans-Atlantic *Zostera marina* populations in the 1930s. Nowadays, it is widely accepted that the marine net-slime mold *Labyrinthula zosterae* is the agent of the so called 'wasting disease' (Muehlstein et

al. 1991, Sullivan et al. 2013). *L. zosterae* has been isolated and pathogenicity was confirmed according to Koch's postulates from eelgrass plants during a reoccurrence of the disease in the 1980s (Short et al. 1987, 1988, Muehlstein et al. 1988).
However, seagrasses are frequently inhabited by these marine net slime molds (Raghukumar 2002), which live as endophytes with or without provoking symptoms in the leaves of various seagrass species (Vergeer & den Hartog 1994, Bockelmann et al. 2012, Martin et al. 2016). *Labyrinthula* is a genus within the Labyrinthulomycota (also known as Labyrinthulamycetes or Labyrinthulea), an early diverging lineage within the straminopiles (Tsui et al. 2009). *Labyrinthula spp.* are colonial, characteristic are spindle shaped cells which are connected by an extra-cellular network (EN) that encloses cells by a membrane allowing intercellular communication. Further, this network is used for locomotion, anchoring and nutrition. Additionally, it might play as well a role for penetration of plant tissue (Muehlstein 1992). Cells glide within the EN on actin filaments (Preston & King 2005), which are secreted together with the EN through a specialized organelle, called the bothrosome (Porter 1969). *Labyrinthula spp*. exhibits an osmotrophic nutrition, it feeds on cell organelles like chloroplasts of its host plant (Raghukumar 2002). It has been isolated from old, decaying leaves (Vergeer & den Hartog 1994, Raghukumar 2002), where it lives presumably as saprophyte, and from younger leaf tissue where it actively spreads through the leaf tissue causing black to brown irregular necrotic lesions (Short et al. 1987). These symptoms have been described in the 1930s and 1980s wasting disease outbreak (Renn 1935, Short et al. 1988). Here, lesion spread rapidly within few days on the leaves, leading to leaf detachment. After new growth of leaves, lesions spread again along the plant causing once more leaf detachment. Finally the rhizome softened and after repeated loss of leaves the plant died (Muehlstein 1989).

This ecological highly relevant study system gains importance in light of global change. The incident of the eelgrass 'wasting disease' is often cited an example for an opportunistic pathogen (e.g. Burge et al. 2013). However, clear evidence that disease outbreak is triggered by environmental change is missing. Diverse environmental factors have been blamed to have caused increased susceptibility of eelgrass, as elevated temperatures (Rassmussen 1977) or extremes in precipitation (Martin 1954), reduced light intensity (Giesen et al. 1990) or a combination of different construction activities going along with increased turbidity (Den Hartog 1987). All factors were suggested based on correlative observation of disease occurrence and environmental anomalies. So far only few experimental infection experiments were conducted concerning salinity (Mckone & Tanner 2009). Recent molecular based studies show that *Labyrinthula zosterae* and two further *Labyrinthula spp.* are locally abundant in eelgrass meadows

without apparently causing population declines in northern Europe (Bockelmann et al. 2012, 2013). The question arises, what is the contemporary nature of the eelgrass - *L. zosterae* interaction and whether *L. zosterae* represents a threat for eelgrass beds in this area, especially if conditions become disadvantageous for eelgrass individuals. The aim of my thesis was thus to characterize the nature of the contemporary interaction between eelgrass and *L. zosterae.* I further aimed to investigate how environmental factors alter the plant - protist interaction. Assuming different performance under changing environmental conditions, I hypothesized depending on the respective environmental condition a rather mutualistic or pathogenic role of *L. zosterae* in its plant host.

Thesis outline

While a range of correlative field studies have been carried out to investigate the nature of eelgrass - *L. zosterae* interaction and influences of environmental conditions (e.g. Hily et al. 2002; Bull et al. 2012; Groner et al. 2014, 2016a), I took a different approach in this thesis. I investigated the plant - protist interaction in a manipulative set-up using seed grown naïve eelgrass plants in indoor wet-lab facilities at Geomar (Kiel). This approach allows in contrast to correlative field studies explicitly to test hypotheses. To the best of my knowledge, this is the first time that eelgrass plants were raised from seeds to investigate wasting disease interactions. By using naïve eelgrass plants, I secured that plants had the same infection experience, same age and had lived through the same environmental conditions, as conditioning and acquired resistance might bias the results (Ryalls et al. 1996). I performed a series of experimental infections. Therefore *L. zosterae* isolates were isolated each time anew and kept as short as possible in cultivation to prevent adaptation to lab conditions. Thereby, over all the here presented studies 6 independent *L. zosterae* isolates were tested. I set-up the experiments in tanks of 300 L - 600 L with natural seawater and sub-replicated eelgrass plants within one tank. In order to address this sub-replication, I applied in the statistical procedures linear mixed model which allows defining random factor, i.e. tank here. In some cases, where this procedure was not possible, I averaged respective values over a tank, and analyzed these values.

In the following I will give a short outline of my thesis which contains three chapters, each displaying a manuscript.

The **first chapter** of this thesis addresses the question how virulent are *Labyrinthula zosterae* isolates from the south-western Baltic and North Sea in interaction with

eelgrass and whether virulence and infectivity varies with origin of *L. zosterae* isolate or eelgrass plants. Further, I investigated whether gene expression of putative eelgrass defense genes changes as response towards infection with *L. zosterae*. Therefore, infection was verified and quantified by *L. zosterae* specific RT-qPCR (Bergmann et al. 2011) together with assessment of wasting disease symptoms. Additionally, eelgrass leaf growth parameters response upon infection was assessed. In this experiment inoculated eelgrass plants grew faster than not inoculated plants. This led to the hypothesis that *L. zosterae* infection might facilitate eelgrass growth.

I followed up on this idea in the **second chapter**. Here, the research question was whether under nutrient limitation *L. zosterae* would facilitate eelgrass growth by enhanced internal recycling of nutrients compared to not inoculated eelgrass plants. The alternative exclusive hypothesis was that nutrient limitation would enhance *L. zosterae* infection in eelgrass plants. To test this, I fully crossed nutrient level (high and low) with *L. zosterae* inoculation (yes/no). Again infection dynamics (*L. zosterae* cell densities and symptom development) and eelgrass growth parameters were assessed over 21 days. As molecular responses are mostly faster than physiologic responses and can thus help to uncover processes occurring in the plant (Macreadie et al. 2014). Therefore, I further performed targeted gene expression analysis to assess the molecular response of the plant host including primary and secondary metabolism, putative defense genes and general stress genes.

Finally, in the **third chapter** I ask how the interaction between eelgrass and *L. zosterae* responds if individuals are exposed to multiple stressors. I hypothesized that under the influence of low light stress, heat stress and increased salinity *L. zosterae* will increase host damage. Additionally to previously measured host growth parameters, I investigated carbohydrate storage. Further, in cooperation with Stina Jakobsson-Thor from Gothenburg University, we assessed chemical host defense by measuring the inhibition capacity of eelgrass extracts on *L. zosterae* growth.

Chapter 1

Current European *Labyrinthula zosterae* are not virulent and modulate seagrass (*Zostera marina*) defense gene expression*

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Abstract

Pro- and eukaryotic microbes associated with multi-cellular organisms are receiving increasing attention as a driving factor in ecosystems. Endophytes in plants can change host performance by altering nutrient uptake, secondary metabolite production or defense mechanisms. Recent studies detected widespread prevalence of *Labyrinthula zosterae* in European *Zostera marina* meadows, a protist that allegedly caused a massive amphi-Atlantic seagrass die-off event in the 1930s, while showing only limited virulence today.

As a limiting factor for pathogenicity, we investigated genotype x genotype interactions of host and pathogen from different regions (10-100 km-scale) through reciprocal infection. Although the endophyte rapidly infected *Z. marina*, we found little evidence that *Z. marina* was negatively impacted by *L. zosterae*. Instead *Z. marina* showed enhanced leaf growth and kept endophyte abundance low. Moreover, we found almost no interaction of protist x eelgrass-origin on different parameters of *L. zosterae* virulence / *Z. marina* performance, and also no increase in mortality after experimental infection.

In a target gene approach, we identified a significant down-regulation in the expression of 6/11 genes from the defense cascade of *Z. marina* after real-time quantitative PCR, revealing strong immune modulation of the host's defense by a potential parasite for the first time in a marine plant. Nevertheless, one gene involved in phenol synthesis was strongly up-regulated, indicating that *Z. marina* plants were probably able to control the level of infection. There was no change in expression in a general stress indicator gene (*hsp70*). Mean *L. zosterae* abundances decreased below 10% after 16 days of experimental runtime. We conclude that under non-stress conditions *L. zosterae* infection in the study region is not associated with substantial virulence.

* Please note that the displayed *L. zosterae* cell number differ by the factor 100 to the published version, as they were corrected here, see Submitted Erratum at the end of the manuscript (page 45).

Introduction

In the recent past, microorganisms, associated with multi-cellular organisms, have been receiving increasing attention as a driving factor in ecosystems (e.g. [1]). Endophytes in plants can change host growth and shoot production [2] by altering nutrient uptake [3], secondary metabolite production or defense mechanisms [4]. Moreover, endophytes can be parasites and thereby play a crucial role in ecosystems by controlling the dynamics of host populations, by regulating host abundances and, thus, by contributing to ecosystem stability [5]. In the marine realm, emerging diseases caused by microorganisms, have been recognized as causes for species extinction, regime shifts or altered community structure [6,7]. How two species interact, whether the host benefits or is degraded by the microbe depends mainly on two factors: the effectiveness of the defense reaction of the host and the pathogenicity of the microorganism.

In this study we investigated the interaction of the most abundant seagrass in the northern hemisphere [8], *Zostera marina*, with the endophytic protist *Labyrinthula zosterae*, which caused the world´s largest reported seagrass die-off event. Seagrasses form one of the most valuable coastal ecosystems on earth [9]. They are marine flowering plants, which form huge meadows, providing food, shelter and settlement substrate for many organisms. Being the foundation species of one of the most productive ecosystems [10], they sequester 15% of the total marine consumed CO_2 and represent thereby an important sink and storage of atmospheric CO_2 [11]. Seagrass meadows contribute to coastal protection [12], play a key role in nutrient cycling [13] and add to water clarity by reducing current velocity and by increasing sedimentation [14]. Seagrasses are sensitive to reduced light availability due to eutrophication [15] or increasing water turbidity [16]. Since anthropogenic impact on this sensitive ecosystem is still increasing, seagrass populations are declining worldwide [16,17].

In the 1930s, the so called ‘wasting disease’ affected *Z. marina* populations along the Atlantic coasts of North America, the European Atlantic, the North and Wadden Sea and the Baltic Sea, affecting eelgrass populations in France, Great Britain, The Netherlands, Germany and Denmark (for review see [18,19,20]). During the ‘wasting disease’ epidemic more than 90% of the Atlantic coast eelgrass populations disappeared [19] after repeatedly developing expanding black or brown lesions on the leaf blades that finally resulted in a disintegration of the rhizome and death of the plants. The eelgrass loss had a tremendous impact on the eelgrass associated fauna (reviewed by [19]). Recovery of the *Z. marina* populations was slow [21] and in some areas eelgrass never

recovered, e.g. the western Wadden Sea [22]. In the 1980s, a reoccurrence of the ‘wasting disease’ was reported from New Hampshire and Maine [21,23,24].

Already in the 1930s, Renn [25] proposed a marine slime mold, *Labyrinthula sp*., as the agent of the 'wasting disease'. In 1988 Muehlstein *et al.* [26] confirmed, by applying Koch´s postulate, *Labyrinthula zosterae* to be the causative agent of the wasting disease.

Recent studies detected widespread prevalence of the protist *Labyrinthula zosterae* in European eelgrass (*Zostera marina*) meadows [27], demonstrating that *L. zosterae* is still an integral part of the eelgrass ecosystem. The *L. zosterae*-strains currently occurring in northern European eelgrass meadows apparently cause neither massive disease symptoms nor die-offs. The primary objective of this study was to better understand the *Z. marina – L. zosterae* interaction, by gaining information about the host’s defense mechanisms as well as local co-adaptations of both, host and microbe. This insight may also enable us to explain the actual absence of the disease and to predict the risk of future lethal epidemics in seagrass beds.

Nothing is known about pathogen defense in *Z. marina* specifically, but in general, flowering plant defense reactions against pathogens are evolutionary conserved [28] and can be understood as a cascade with different layers (Fig. 1). First, physical (e.g. wax cuticle or cell walls) and biochemical barriers (e.g. antimicrobial enzymes or secondary metabolites) inhibit pathogen growth [29]. One important group of secondary metabolites are phenolic acids and their derivates, which have various functions, for examples antioxidant capacity [30] and antimicrobial function [31]. Accumulation of phenolic compounds probably also plays a role in the interaction between Z*. marina* and *L. zosterae*, since higher concentrations of phenolic acids, mainly caffeic acid, were detected in infected as compared to healthy plants [32].

Secondly, receptors at the cell surface recognize slow evolving pathogen (or microbe) associated molecular patterns (PAMPs=MAMPs, e.g. bacterial flagellin or fungal chitin), which induce a basal defense [33]. However, some pathogens can overcome this defense induction by inhibiting the pathway through release of effector proteins into the host tissue. As a counter response, most plants demonstrate cytoplasmic or membrane-localized receptors (so called resistance-genes or R-genes), that bind directly to pathogen-released effectors or to damaged host cell fragments [34]. Upon binding to the receptor, reactions are triggered that can induce a hypersensitive response (HR) and the expression of a set of pathogenesis-related proteins [35]. HR is mediated by metacaspases and other factors, such as hydrogen peroxide concentration. In HR, the

infected cell undergoes a programmed cell death (PCD or apoptosis), which limits the reproduction and spread of the pathogen within the host tissue [36]. As a final level of defense, pathogenesis-related genes (PR-genes) are expressed such as chitinases, defensins or beta-1,3-glucanase, which work against pathogens in various ways [37].
During induction and regulation of plant defense reactions, plant hormones spread information about infection throughout the plant, which might lead to systemic resistance. In general, Salicylic acid (SA) seems to be the dominant hormone in biotrophic pathogen interaction, while Jasmonic acid (JA) and Ethylene (ET) have been found to be involved more frequently in necrotic interaction [38].

In regard to the lack of virulence of today's *L. zosterae* infection, several explanations are possible. First, the genotypes of the protist currently present may generally show low or no virulence. This was tested by experimentally inoculating naïve *Z. marina* raised from seeds with *L. zosterae*. Second, plant genotypes may be adapted to local protist genotypes (in particular in historical wasting disease areas) preventing virulence effects. Hence, we investigated the host – pathogen co-adaptation in different populations on a regional spatial scale by applying a reciprocal infection design to test infectiousness and pathogenicity. Third, we characterized the defense reaction of *Z. marina* after infection with *L. zosterae* by measuring the gene expression of 11 defense related genes that were identified using *Z. marina* EST library sequences [39] via comparison of gene models of terrestrial model plants at different time intervals post infection. We choose genes from different levels of the defense cascade (Fig. 1). We aimed to answer the following research questions:

1. How virulent is *Labyrinthula zosterae* in the study area (measured as lesion development, leaf growth and leaf production by *Zostera marina*; Experiment I: experimental inoculation of the eelgrass hosts with *L. zosterae*)?
2. Are there differences in infectiousness and virulence between *Zostera marina* hosts and *Labyrinthula zosterae* endophytes with different origin, which may explain local persistence of host and pathogen (Experiment I: Reciprocal inoculation of eelgrass hosts and endophyte with *L. zosterae,* both with different origin)?
3. Does infection of *Zostera marina* by *Labyrinthula zosterae* lead to enhanced expression of defense related genes (Experiment II: Defense gene expression in *Zostera marina*)?

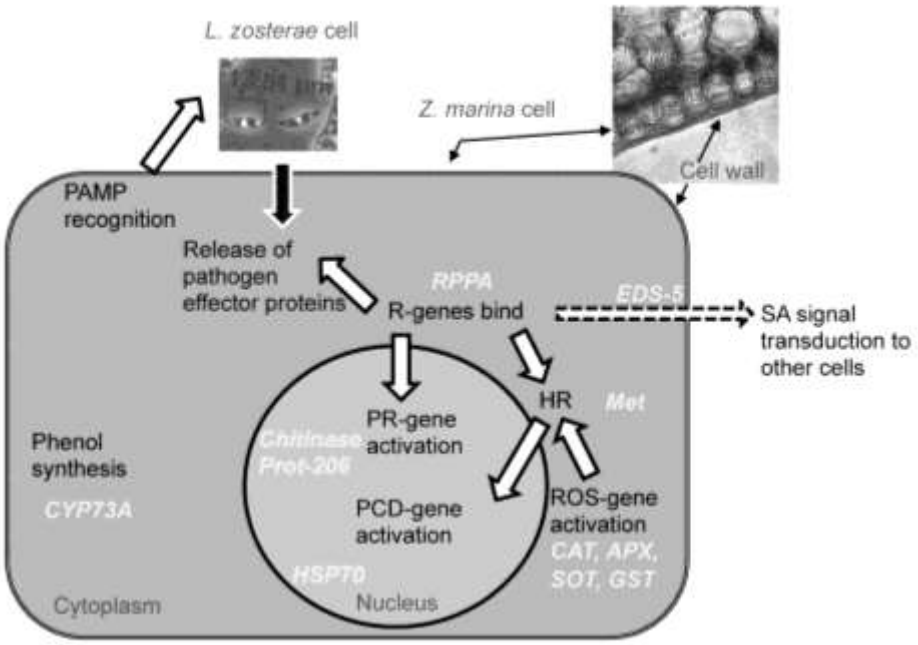

Figure 1. Defense mechanism of *Zostera marina.*

Material and Methods

Seed collection, germination and cultivation of *Zostera marina*

In order to raise *L. zosterae* naïve plants for experiment I, we collected about 100 flowering shoots with seeds from each of three subtidal populations along the north-western German Baltic (Wackerballig in Flensburg Fjord, Kiekut in Eckernförde Bay and Strande in Kiel Fjord) in July 2010 (Table 1). No specific permissions were required for these locations/activities, since GEOMAR research activities along the coasts and shelf areas in the Baltic Sea are permitted when adhering to the general guidelines for the operation of research vessels. Our field studies did not involve endangered or protected species. In October 2010, another 100 flowering shoots were collected from a subtidal population of *Zostera marina* in List on the island of Sylt in the German Wadden Sea (Table 1). Sampling at Ellenbogen Creek was permitted by the nature conservation authority and Mr. Diedrichsen, the owner of this private property. Collected flowering shoots were immediately transported in water containers to GEOMAR Kiel and stored floating in mesocosms, in filtered seawater at 21°C and with the respective sampling site's salinity until seeds were ripe.

Ripe seeds were stored at 5°C for stratification (September-November 2010: Baltic seeds; November 2010-January 2011: Wadden Sea seeds). Subsequently, *Zostera*

marina seeds were sown in plastic aquaria filled with ambient sediment and submerged in mesocosms with ambient sea water (15 psu) at 10° - 12°C and with 12 hours light (~600 µE m^{-2} s^{-1}).

When seedlings reached a size of 10 - 15 cm in March - April 2011, 6 seedlings were transferred to each plastic aquarium holding sediment of 25 cm thickness, submerged in 50 x 50 x 100 cm aerated containers with a 1:1 mixture of Kiel Fjord Sea and North Sea water (25 psu). Each seedling received ~0.02 g Nitrate and ~0.009 g Phosphate (Plantacote Mix 4M, Manna, Germany). Temperature was raised to 17 °C and a light: dark regime of 15 : 9 was applied to mimic early summer conditions. One third of the water was exchanged every week.

Zostera marina seeds for experiment II were collected in an eelgrass population close to Strande (Table 1) in June 2011. No specific permissions were required for these locations/activities (see above). The procedure was identical to the first experiment. Seeds germinated between December 2011 and February 2012. In March 2012, *Z. marina* seedlings were planted into aquaria. Temperatures were continuously increased from 12 °C in March to 18 °C in August. The light period was extended from 12 hours in March to 16 hours in August.

Table 1. Sampling sites of *Zostera marina.*

Area	Location	Geograph. coordinates	Sampling date	Salinity (psu)	Sampled
Experiment I					
Sylt, Wadden Sea, Germany	List	N 55.0410 E 08.4130	October 2010 August 2011	>30	Flowering shoots, leaves for isolation of *L. zosterae*
Flensburg Fjord, Germany	Wackerballig*	N 54.7557 E 09.8668	July 2010 August 2011	15-17	Flowering shoots, leaves for isolation of *L. zosterae*
Eckernförde Bay, Germany	Kiekut	N 54.4483 E 08.7106	July 2010 August 2011	15-17	Flowering shoots, leaves for isolation of *L. zosterae*
Kiel Fjord, Germany	Strande	N 54.4330 E 10.1699	July 2010	15-17	Flowering shoots
Kiel Fjord, Germany	Falckenstein	N 54.3954 E 10.1935	August 2011	15-17	Leaves for isolation of *L. zosterae*
Experiment II					
Kiel Fjord, Germany	Strande	N 54.4330 E 10.1699	June 2011 July 2012	15-17	Flowering shoots, leaves for isolation of *L. zosterae*

*Leaves for isolation of *L. zosterae* were harvested from plants infected in experiment I and kept in mesocosms until March 2012

Labyrinthula zosterae isolation and cultivation

For isolation of *L. zosterae* for experiment I, we sampled leaves from vegetative *Zostera marina* shoots at the seed sampling sites List, Kiekut and Falckenstein. *Labyrinthula zosterae* was isolated and cultured on seawater-agar-medium as previously described [18]. In preparation of the infection procedure, we autoclaved medical gauze compresses (Lohman und Rauscher, Germany). Five squares of gauze (1.5 x 1.5 cm) were placed in a circle on each seawater medium plate. We then inoculated the centre of these plates with *L. zosterae* cells, resulting in an identical distance of all gauze pieces to the inoculated *L. zosterae* culture. After 5 days the gauzes were overgrown by *L. zosterae.* Four different strains of *L. zosterae* were used for each original site (see below). *L. zosterae* DNA from one gauze piece of each culture was extracted (see below) and subjected to real-time quantitative PCR analysis (rt-QPCR, see below) for the determination of inoculation concentration of *L. zosterae*. Inoculation concentration was 1,531,000 ± 324,000 *L. zosterae* cells/square of gauze.

In experiment II the isolation of *L. zosterae* cultures for infection was identical to experiment I. Here, we sampled *Z. marina* leaves from Strande (Table 1) in July 2012

and received three different *L. zosterae* strains. The gauze bandages used for inoculation were rectangular and smaller (1.5 x 0.75 cm, 601,700 ± 85,300 *L. zosterae* cells/square of gauze) in this case.

Experiment I: Reciprocal infection of host and endophyte with different origin

Experimental design

Before the start of the experiment on August 25th, 2011, 48 plastic aquaria (15 x 25 cm) were filled with 10 cm of ambient, sterilized sediment. Six *Zostera marina* seedlings from one of the four parental sites (experimental factor 1, Fig. 2) were planted in each aquarium, resulting in 12 aquaria per parental site. Each seedling received slow-release fertilizer (see above) again and was given six weeks for settlement. After that, one aquarium from each parental side was placed in each one of 12 mesocosms. The latter were filled with 600 L of a mixture of Kiel Fjord and North Sea water resulting in a salinity of 25 psu at a temperature of 18 - 19 °C. During the experiment 1/3 of the water was exchanged every week and temperature and salinity were controlled every other day. The light period was 16 hours.

For infection, the second and third oldest leaf of each *Z. marina* shoot was wrapped with a gauzed bandage containing *Labyrinthula zosterae* from different isolation sites (second experimental factor, Fig. 2, Table 1) for 24 hrs. All plants in aquaria of the same mesocosm received bandages from the same isolation site, resulting in three mesocosms with four aquaria and 72 plants per isolation site. Plants in the remaining three mesocosms were not infected. The second and third oldest leaf of three of the six plants was wrapped with non-infected bandage to control for an effect of the bandage itself. After one day all bandages were removed and infection success was determined by the appearance of lesions on the leaf surface.

The size of the lesions was determined by estimating the fraction of the leaf that had turned black in five classes (0%, >0-10%, >10-25%, >25-50%, >50-75%, >75-100%). We assessed lesion size one, two, three, six and nine days after infection on the second oldest leaf. Lesions on the third oldest leaf were estimated one, two, three, six days after infection. At day three the leaf 3rd was harvested and dried for *L. zosterae* determination by rt-QPCR. Furthermore, we measured leaf length of the third oldest, second oldest and youngest leaf at the start of the experiment and at day six. After harvesting the third oldest leaf, leaf length of the second oldest (as far as it was present and not naturally shed), youngest and all newly appearing leaves was measured after 10, 17 and 32 days.

On day 32 after infection, the first leaf that appeared post infection was harvested and analyzed by rt-QPCR for *L. zosterae* infection.

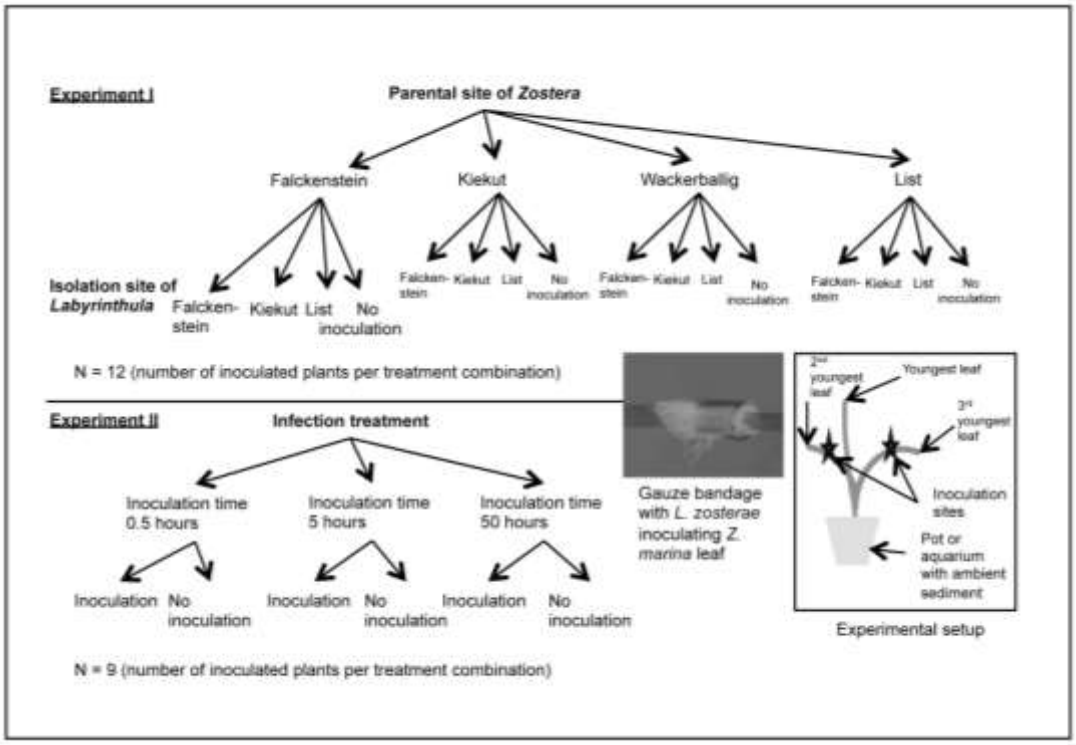

Figure 2. Experimental design and setup of experiment I and II.

DNA-extraction and real-time quantitative PCR assay (rt-QPCR)

After sampling, the harvested leaves were air dried. Approximately 2 - 4 mg dried leaf material from 2 - 3 cm above and below the region where infective gauze bandage had been placed was first ground in a ball mill (Retsch, Germany) at maximal speed (4 x 8 min.). DNA extractions of *L. zosterae* were performed with an Invisorb spin tissue mini kit (Invitek, Berlin, Germany) following the manufacturer's instructions. To enhance extraction efficiency and to ensure that even low amounts of target DNA were carried through the filter absorption steps, 1 µL (containing ~500 ng) of UltraPure salmon sperm DNA solution (Invitrogen, Life Technologies, USA) was added to each extraction to saturate silica columns with DNA. Target DNA was purified using a one-step PCR inhibitor removal kit (Zymo Research, USA).

To determine *Labyrinthula zosterae* cell number, we followed a TaqMan based rt-QPCR assay as described in Bockelmann *et al*. [18] with a fluorescently-labeled ITS probe.
In one reaction we used 10 µL TaqMan universal Master Mix (Applied Biosystems, now Life Technologies) in a 20 µL reaction volume: 2 µL 1:10 diluted template DNA, 2.4 µL

(40.8 nM) of the two primers, 2.4 µL Milli-Q H_2O and 0.8 µL probe (50 nM), respectively. The thermo-cycling program on a Step-One QPCR machine was 2 min at 50°C and 10 min at 95 °C, followed by 48 cycles at 95 °C for 15 s and 1 min at 60 °C.

Data analysis and statistics

Lesion size was estimated as percent data and had to be arc sine transformed to achieve variance homogeneity.

$$\text{Cell number} = \sin\text{^-}1\sqrt{\text{Lesion size}/100}$$

Growth rates for individual leaves were calculated as

(Shoot length$_{t2}$ – Shoot length$_{t1}$) / Number of days between measurements

Growth rates and leaf production (number of new leaves produced post infection) data were log transformed.

All samples analyzed by rt-QPCR were tested in triplicate and the standard deviation of triplicates never exceeded 0.5 units of cycle threshold (Ct). Only CT values <39 were considered.

Standard curves using preparations of *Labyrinthula zosterae* with known cell numbers attained correlation coefficients between r^2 = 0.97 and 0.99 and a detection limit of ~0.01 cells. Abundance as the number of *L. zosterae* cells in each milligram (dry weight) *Zostera marina* sample was calculated from the linear regression of the standard curve (Standard cell number against mean Standard Ct calculated from all rt-QPCR reactions; 150 cells = 22.493 Ct ± 0.060 SE, 15 cells = 27.080 Ct ± 0.080 SE, 0.5cells = 32.215 Ct ± 0.125 SE).

$$\text{Cell number} = (-a + b*(de\log(Ct)))/w*10$$

where a = intercept, b = slope and w = sample dry weight. Cell number has to be multiplied by 10 because the samples were diluted 1:10 prior rt-QPCR.

Statistical analysis was based on a general linear model and done by 2-way analysis of variance (implemented in software JMP 9, SAS Institute, USA). “Parental site” of *Zostera marin*a (Kiel Fjord, Eckernförde Bight, Flensburg Fjord and Sylt) and “Isolation site” of *Labyrinthula zosterae* (Kiel Fjord, Eckernförde Fjord, Sylt and no infection) were independent factors in the model. The control treatments were analyzed as a forth level of the factor isolation site. Dependent factors were “lesion size”, “growth rate / day”, “leaf production” and “*L. zosterae* cells / mg *Z. marina* dry weight”. Table 3 summarizes the results of the statistical analysis.

Experiment II: Defense gene expression in *Zostera marina*

The objective of the second experiment was to analyze the *Zostera marina* defense reaction in a target-gene approach. In a pilot experiment, we first tested the abundance of *L. zosterae* within *Z. marina* leaves after different inoculation times in order to investigate how much time the protist needs to enter an eelgrass leaf. *Zostera marina* and *Labyrinthula zosterae* were both collected from an eelgrass population in the Eckernförde Bay (Table 1). The plants were either cultured from seeds (see above) or sampled in February 2012, when *L. zosterae* prevalence in the population showed to be minimal [18]. *Labyrinthula zosterae* cultures were isolated from *Zostera marina* plants, which had been infected in experiment I and had been cultivated in our mesocosm facility thenceforth. On April 24th and 25th the 2nd and 3rd youngest leaves of each plant were infected and sampled. We tested incubations of 10, 20, 40, 80, 160 and 320 minutes. To control for accidental infection prior to the experimental infection treatment, we took samples from all plants before infection treatment. Cell numbers of *Labyrinthula zosterae* per mg *Zostera marina* dry weight were obtained and tested in the same way as described for experiment I (see above). This pilot study revealed that the first plants were infected after 10 minutes. After 5:20 hrs, cell numbers started to increase. By combining these results with the cell numbers from experiment I, we found a maximum after 3 days and decreasing cell numbers thereafter (Fig. 3).

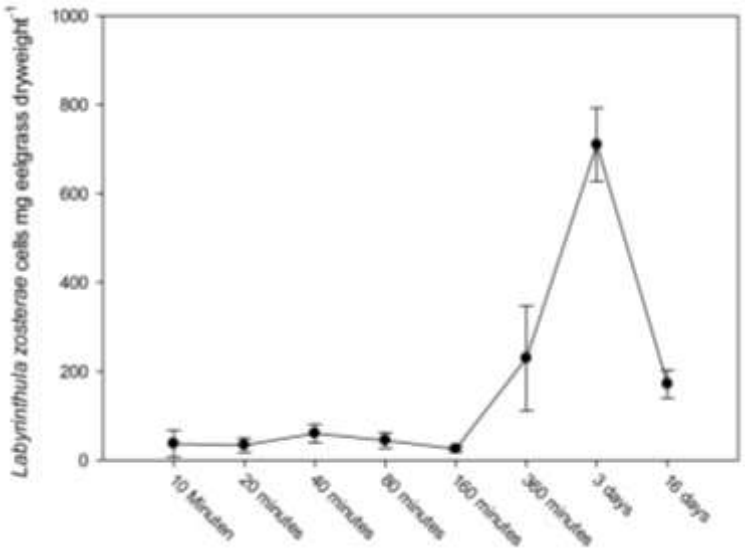

Figure 3. Abundance of *Labyrinthula zosterae* cells per mg *Zostera marina* leaf sample (dry weight) depending on inoculation time during experimental *L. zosterae* infection. Results are partly from experiment I and II, means with standard error bars.

Experimental design

When the experiment started on August 15th, 2012, plants were 6 to 9 month old. Single plants were transplanted to 6 L plastic buckets filled with a 10 cm layer of sieved sandy sediment (mesh size 1000 µm) one week before the start of the experiment. To improve growth of *Z. marina* in the new sediment, each plant was fertilized as described above. Temperature was 19°C, salinity 15 - 17 psu. Nine buckets were placed in each of 6 mesocosms filled with ~ 600 L of seawater. In three of the six mesocosms plants were infected by using gauze bandages overgrown by *L. zosterae* (see above, Fig. 2). Plants were inoculated for different time intervals: either 0.5 hrs, 5 hrs or 50 hrs (experimental factor). Three mesocosms served as controls, in which plant leaves were wrapped with non-infected gauze bandages stored in seawater medium plates.

RNA extraction and reverse transcription

After incubation, a ~4 cm leaf blade including the infection site as well as 1 cm above and below the infection site was cut and wiped with sodium hypochlorite (0.5 %) to sterilize the surface. Plant tissue samples were immediately frozen in liquid nitrogen and ground with a mortar and pestle. To ensure a rapid RNA isolation, samples were taken in two time series shortly after each other.

We isolated RNA with the Invitrap Spin Plant RNA Mini kit (Stratec Molecular, Germany). Homogenized samples were kept 15 – 30 min in RP-lysis buffer under constant shaking. We then followed the instruction by the company. To determine the concentration of the RNA, we used a spectrophotometer (NanodropND-1000 from peQLab, Germany). RNA was transcribed to cDNA using QuantiTectReverse Transcription Kit (Qiagen, USA). Approximately 80 ng of RNA was inserted per transcription reaction. The kit contained a DNA wipe-out step to prevent gDNA contamination. As a control, we took a non-reverse transcript sample to test later in the rt-QPCR for gDNA contamination.

Selection of genes and primer design

Using the rt-QPCR assay, we tested 11 genes of which five genes have been previously described [40,41]. These genes are encoding a heat shock protein and four ROS scavenging enzymes, which are known to be sensitive to biotic as well as abiotic stress. Six additional genes were identified based on homology search with known gene models from rice and *Arabidopsis* using the expressed sequence tags (EST) library database Dr. ZOMPO [39]. We chose genes that were associated with the plant pathogen defense cascade (Table 2) and made sure that these were homologous and complete when compared to other model plants using alignments. The housekeeping gene eIF4A served

as reference gene for later normalization of rt-QPCR results [40]. Using the software PerlPrimer [42], primers were designed and tested for identical sequences against the EST library of *Z. marina*. Primer efficiencies (PE) were tested using a 5 fold dilution series (1:10 – 1:810) in three replicates. Efficiency E was > 1.7 and R^2 0.87 – 0.99. PE was calculated according to Rasmussen *et al.* [41]:

$$E = 10\text{^}(-1/\text{slope})$$

Table 2. *Zostera marina* genes for gene expression analysis and their predicted function.

Symbol	Gene	Predicted function	Sequence
RPPA	NB-ARC domain-containing disease resistance gene	Immune receptor	F 5´-GCATCACATCGATATCTGATTCTTT-3 R 5´-CTGTGGTAATTTCGACCCATC-3´
EDS 5	Enhanced disease suceptibility-5	Signal molecule in SA pathway	F 5'-GATTGGGATGTGGATATGTTCTC-3' R 5'-GGATGTAGAAATGCCGAGGA-3'
Met-1	Metacaspase	Regulation HR	F 5'-CATTCCTTGTGCTTGAAAGTC-3' R 5'-ACCCTTATAGAATCCCAACGA-3'
APX*	L-ascorbate peroxidase 2 (cytosolic)	ROS regulation	F 5'-GGTGATTTCTACCAGCTTGC-3' R 5'-GATCCGCACCTTGGGTA-3'
CAT*	Catalase II	ROS regulation	F 5'-ACAAAATTCCGTCCGTCA-3' R 5'-GTCCTCAAGGAGTATTGGTCCTC-3'
GST*	Glutathione S-transferase	Detoxification	F 5'-CATGAATCCATTCGGACAAG-3' R 5'-CAGCAAGGTGAGTAAGGTCAG-3'
SOD*	Superoxide dismutase (mitochondrial)	ROS regulation	F 5'-ATGGGTGTGGCTTGCTTA-3' R 5'-ATGCATGCTCCCATACATCT-3'
HSP70**	Heat shock protein 70	Folding and unfolding of other proteins	F 5´-ACCGTCTTTGATGCGAAGC-3´ R 5´-CAGAAAATTGCTTATCTTCTCCCTTA-3´
Prot-206	Disease resistance-responsive protein 206	Pathogenesis-related protein	F 5´-CTCTTCTAGCACGCAATTTGG-3´ R 5´-CCGAAAATGTCTCCTTCGAG-´3
Chit	Chitinase 1-like protein	Pathogenesis-related protein	F 5´-AAACAGCCATCAGCACATGA-3´ R 5´-GTCAGCAAATCCCTGTCCAC-3´
CYP73A	Trans-cinnamate 4-monooxygenase	Enzyme for phenol synthesis	F 5'-ATATCCACCTTGTCCATTCCC-3' R 5'-CTGACTTCCGATACTTGCCT-3'
eIF4A*	Eukaryotic initiation factor	Eukaryotic translation initiation factor	F 5'-TCTTTCTGCGATGCGAACAG-3' R 5'-TGGATGTATCGGCAGAAACG-3'

SA = salicylic acid. HR = hypersensitive response. ROS = reactive oxygen species, * from Winters et al. 2011, ** from Bergmann et al. 2010

Real-time quantitative PCR-Assay (rt-QPCR)

Rt-QPCR was conducted in a StepOne Plus (Applied Biosystems, USA). In one reaction we used 10 µL SYBR green fast master mix (Applied Biosystems, USA) as provided by the company, 0.8 µL of primer reverse (final concentration 200 nM), 0.8 µL primer forward (final concentration 200nm (0.4 µL in case of EDS-5 and Met), 4.4 µl HPLC H_2O (4.8 µL in case of EDS-5 and Met) and 4 µL of cDNA sample, 1:20 diluted. Cycling temperatures were 95°C 3 min (once), 95°C 20 sec, 60°C 20 sec, 72° 30 sec, 42 cycles. On each plate we used a balanced design of infected and control samples to correct for plate variation. Furthermore each plate contained the reference gene and a negative control as well as a no-template and a no-reverse transcript control (taken after genomic DNA digestion to control for genomic DNA contamination) sample.

Data analysis and statistics

All samples were tested in triplicate and the standard deviation of triplicates never exceeded 0.5 units of cycle threshold (Ct).
To obtain a relative measure for transcript amounts, we calculated - ΔC_t values (1). Fold changes in gene expression were calculated according to equation (2) and (3).

$$\Delta C_t = C_t \text{ Target Gene} - C_t \text{ Reference Gene} \quad (1)$$

$$\Delta\Delta C_t = -\Delta C_t \text{ treated sample} - (-\Delta C_t \text{ control sample}) \quad (2)$$

$$\text{Fold change} = 2^{\Delta\Delta Ct} \quad (3)$$

Statistical analysis was based on - ΔC_t values in a general linear model -Delta Ct as response variable and Infection and Incubation Time (0.5, 5 or 50 hours) as independent variables. For statistical differences between incubation time levels, we conducted a Tukey post-hoc test. All statistical tests used here, were performed with the software R (R Development Core Team [43]). An overview of the results of statistical analysis is given in Table 4.

Results

Experiment I: Reciprocal infection of host and endophyte with different origin

Across all experimental factors, lesion development after 24 hours indicated that infection had been successful in 187 out of 210 experimental *Zostera marina* plants (89%) inoculated with *Labyrinthula zosterae*. After 48 hours, 18% of the inoculated 3rd oldest leaves were covered by lesions. Three days post inoculation (after 72 hours), lesion size had doubled to 36%. Lesion progression was slightly slower on the 2nd oldest leaf, where only 24% of the leaf surface was black after 3 days. However, lesions continuously increased thereafter resulting in a lesion cover of 36% after 7, 46% after 9 and 60% after 16 days. After 10 days, black spots (6 ± 1%) appeared on the youngest leaf (at inoculation), increasing to 10 ± 1% after 16 days. Mortality of *Z. marina* during the experiment was very low and similar to the natural mortality in our experimental set-up. Four out of 262 plants in total (3.1%) died by the end of the experiment after 16 days (3.1%), resulting in 249 plants left.

Infected plants grew better than uninfected controls and showed enhanced growth of the younger leaves that were either uninfected or formed after the infection (Fig. 4a, Table 3). Furthermore infected plants produced fewer new leaves across all origins (Fig. 4b, Table 3). We found no genotype x genotype (host origin x protist origin) interactions on any of the response variables. However, there were some main effects of the factor genotype on lesion development.

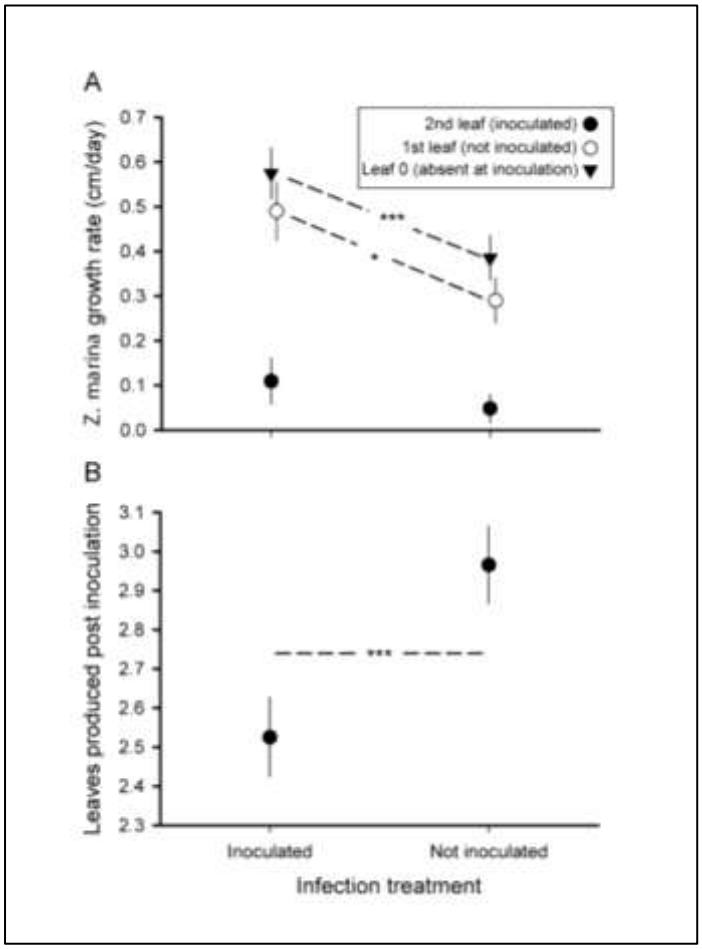

Figure 4. Growth (**a**) and leaf production (**b**) of *Zostera marina* leaves 2-4 weeks after experimental infection with *Labyrinthula zosterae.* 2nd leaf = inoculated 2nd oldest leaf of each *Zostera marina* shoot (growth measured 1st to 2nd week post inoculation), 1st leaf = youngest leaf at inoculation, not inoculated (growth measured 1st to 4th) week post inoculation), leaf 0 = leaf not yet present at inoculation, therefore not inoculated (growth measured 3rd to 4th week post inoculation). * indicates significant differences at $p<0.05$, *** indicates significant differences at $p<0.01$, ns= not significant, means with standard error bars.

Infected *Z. marina* plants from different origin did not differ in *L. zosterae* abundance (*L. zosterae* cells/mg *Z. marina* dry weight, Fig. 5a), leaf production or leaf growth. Origin of the *L. zosterae* culture also did not lead to significant differences in the parameters mentioned above (Fig. 5b). Seven days after infection, abundance of *L. zosterae* across all origins was reduced to low levels (Fig. 5a, b, Table 3). However, origin of the *L. zosterae* culture significantly impacted lesion progression. Infection with *L. zosterae* originating from List eelgrass beds lead to the development of significantly smaller lesions than Baltic protists (Fig. 6, Table 3).

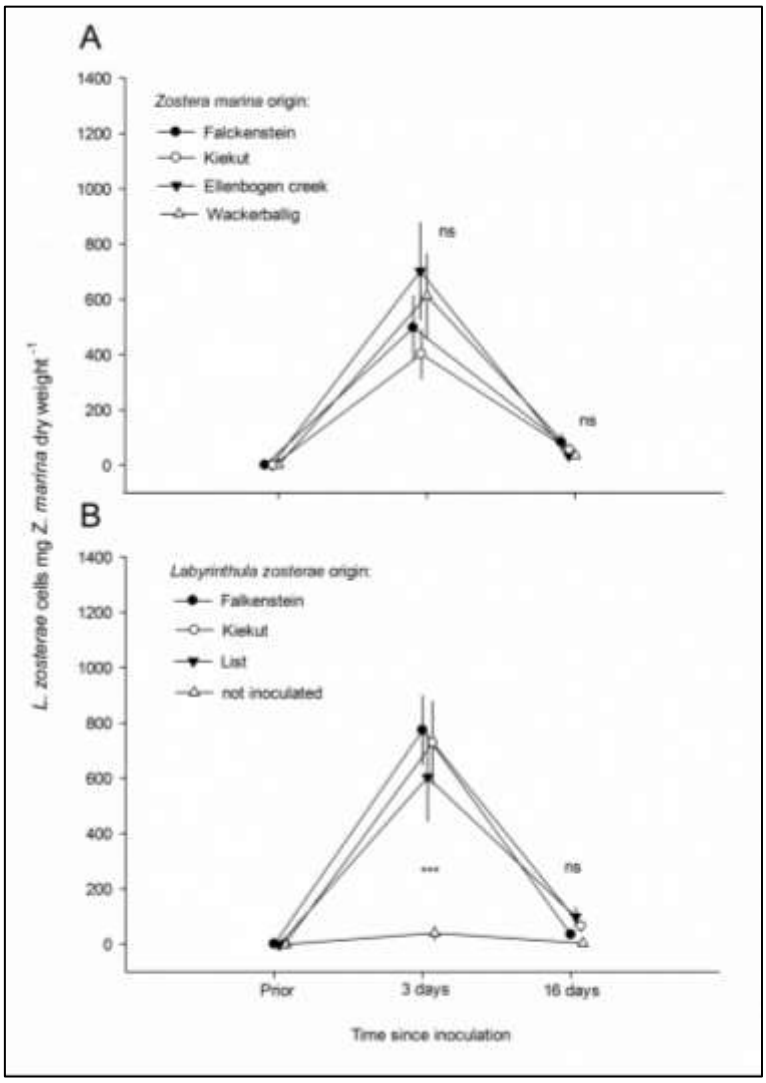

Figure 5. Abundance of *Labyrinthula zosterae* cells per mg *Zostera marina* leaf sample (dry weight) after experimental inoculation depending on the parental site of *Z. marina* (a) and the isolation site of *L. zosterae* (b). *** indicates significant differences at p<0.01, ns= not significant, means with standard error bars.

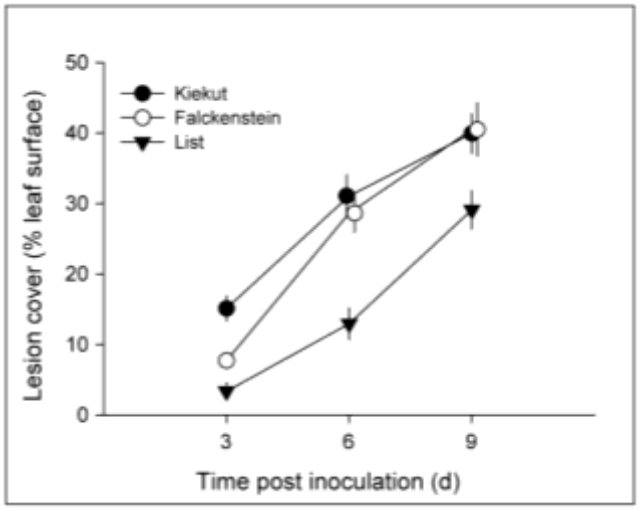

Figure 6. Spread of lesions on *Zostera marina* 2nd oldest leaves of different origin after experimental inoculation with *Labyrinthula zosterae,* *** indicates significant differences at p<0.01, means with standard error bars.

Table 3. Experiment 1: Statistical analysis of differences in *Labyrinthula zosterae* abundance, lesion size, growth rate and leaf production after inoculation of *Zostera marina* with *L. zosterae* compared with uninoculated plants.

Response variable	**Factor**	**Df**	**SS**	**F/X²**	**P**	**Residual SS**
L. zosterae abundance*	*Z. marina* origin *L. zosterae* origin	3 3		6.39 46.47	0.09 **<0.0001**	
Lesion size leaf 3§	*Z. marina* origin *L. zosterae* origin *Z.m* ori..x *L.z*. ori.	3 3 9	0.32 9.77 0.28	3.81 119.27 1.15	**0.01** **<0.0001** 0.33	6.74
Lesion size leaf 2§	*Z. marina* origin *L. zosterae* origin *Z.m* ori..x *L.z*. ori.	3 3 9	0.45 11.67 0.77	2.49 63.81 1.41	0.06 **<0.0001** 0.18	14.56
Growth rate *Z.m.* leaf 2‡	Inoculated vs. not inoculated	1	0.13	0.15	0.697	106.33
Growth rate *Z.m.* leaf 1‡	Inoculated vs. not inoculated	1	1.44	5.40	**0.021**	61.70
Growth rate *Z.m.* leaf 0[3]	Inoculated vs. not inoculated	1	6.57	9.10	**0.003**	159.62
Leaves produced post infection‡	Inoculated vs. not inoculated	1	0.87	16.64	**0.0003**	15.47

*=Wilcoxon Test, §= lesion size 3 days post inoculation, 2-way-ANOVA, ‡=1-way-ANOVA

Experiment II: Defense gene expression in *Zostera marina*

Contrary to expectations, in 6/11 defense genes, expression levels were down-regulated upon experimental infection. In relation to a housekeeping gene eIF4A, $-\Delta C_t$ was significantly lower in plants infected with *L. zosterae* for RPPA, APX, GST, CAT and SOD (Fig. 7, Tab. 4) with levels from 5 to 12-fold. Four genes showed no difference in expression in comparison to the housekeeping gene. In contrast, the expression of CYP73A which is involved in phenol synthesis increased almost 80-fold upon infection (Fig. 7).

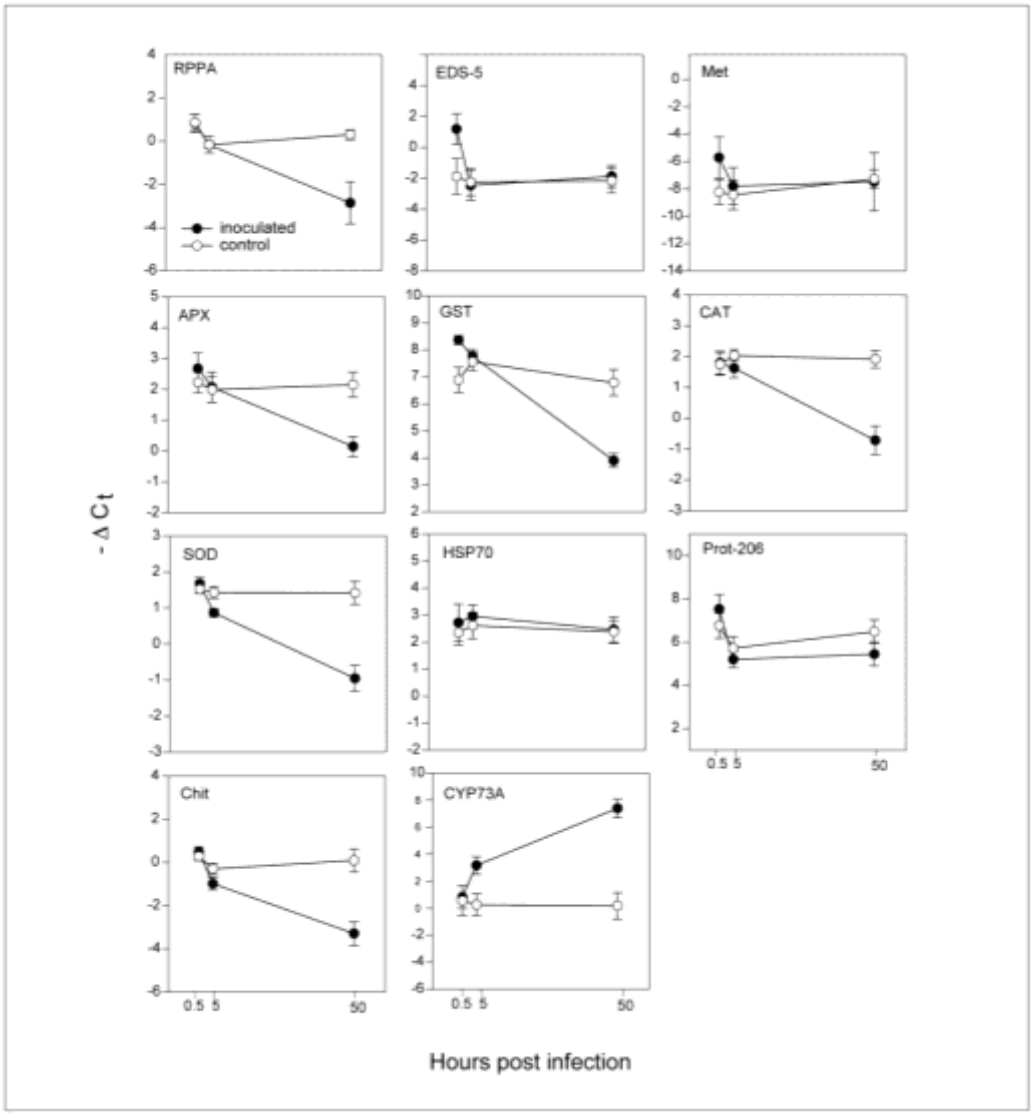

Figure 7. Gene expression of *Zostera marina* defense genes after experimental infection with *Labyrinthula zosterae.* I = inoculation treatment with *L. zosterae*, NI = no inoculation. Results have been normalized to *eIF4A* housekeeping gene. $-\Delta C_t$: log 2 scale. * indicates significant differences at $p < 0.5$, ns = not significant. ***RPPA:*** NB-ARC domain-containing disease resistance receptor gene. **EDS-5**: Enhanced Disease Susceptibility 5. **Met**: Metacaspase ***APX:*** L-ascorbate peroxidase. **GST**: Glutathione S-transferase. **CAT**: catalase II. **SOD:** superoxide dismutase. **HSP70**: heat shock protein 70. **Prot-206*:*** Disease resistance-responsive protein 206. **Chit**: Chitinase. **CYP73A**: Trans-cinnamate 4-monooxygenase, means with standard error bars.

Table 4. Experiment II: Statistical analysis of gene expression in *Zostera marina* after inoculation with *Labyrinthula zosterae* depending on inoculation time.

	Infection				**Inoculation time**				**Infection x incubation time**				**Residual**
Gene	**df**	**SS**	**F**	**p**	**Df**	**SS**	**F**	**p**	**df**	**SS**	**F**	p	**SS**
RPPA*	1	5.25	4.99	**<0.05**	2	16.32	7.76	**<0.02**	2	17.29	8.22	**<0.02**	35.77
EDS-5	1	11.95	1.87	ns	2	33.20	2.59	ns	2	21.50	1.68	ns	211.33
Met	1	11.83	0.99	ns	2	8.63	0.36	ns	2	12.14	0.51	ns	393.00
GST	1	184	0.89	ns	2	6505.80	15.79	**<0.01**	2	6040.60	14.66	**<0.01**	7210.60
APX	1	1.66	1.24	ns	2	8.23	3.06	ns	2	11.45	4.26	**<0.05**	49.73
CAT	1	45.84	12.79	**<0.02**	2	41.89	5.85	**<0.02**	2	60.30	8.41	**<0.02**	129.07
SOD	1	147.75	21.88	**<0.01**	2	185.26	13.71	**<0.01**	2	213.69	15.82	**<0.01**	270.17
HSP70	1	0.82	0.45	ns	2	0.34	2.00	ns	2	0.17	0.05	ns	70.76
Prot-206	1	0.86	0.37	ns	2	22.85	4.99	**<0.05**	2	6.55	1.43	ns	93.95
Chit	1	13.41	16.59	**<0.01**	2	19.00	11.75	**<0.01**	2	21.03	13.01	**<0.01**	33.15
CYP73A	1	120.15	21.77	**<0.01**	2	81.72	7.40	**<0.02**	2	84.70	7.67	**<0.01**	215.21

*=See Table 3 for gene descriptions

Discussion

To the best of our knowledge, we are one of the first to apply controlled infection of naïve *Z. marina* plants raised from seeds (also see [44]). Our experiments show that infection with present-day *L. zosterae* genotypes from North Sea /Baltic Sea in a non-stressful environment is not associated with the detrimental effects on *Z. marina* described for the wasting disease. Mortality levels were low and not significantly different from controls although the infectivity of the endophyte was high. Moreover, endophyte abundances inside plant tissue remained low, and decreased progressively to low levels after experimental infection, which is typical for permanent non-lethal infections [45].

The development of lesions covering significant parts of the leaf was correlated with a significant increase in growth rate of the un-inoculated younger leaves of the same shoot. Similar plant – endophyte interactions that lead to increased growth and shoot production and ultimately result in enhanced survival of the host as a consequence of infection are known from many terrestrial grass species [2,46,47,48]. The mechanisms underlying this effect are for example enhanced nutrient use efficiency for nitrogen and phosphorus [3,4,49]. Endophyte-infected terrestrial grasses also exhibit fundamental changes in their secondary metabolites including a range of alkaloids [50,51] and phenolic compounds [4,52]. Phenols produced by endophyte-infected grasses can not only be a reaction upon infection but for example be released through root exudates leading to an increase in P availability [52]. Along these lines, the observed ~80 fold increase in CYP73A transcript in our study (Fig.7) could be a direct result of host manipulation by *L. zosterae*. In addition to changes in nutrient availability, indirect beneficial effects for *Z. marina* could also be a reduction of herbivory by grazing invertebrates [53,54,55], which may be induced by enhanced phenolics or by infection with other microbes such as marine fungi, bacteria or viruses [31]. Furthermore, polyphenols probably control endophyte abundance by their antimicrobial function [30]. The repellent function of difference phenolic acids (e.g. caffeic acid) has previously been shown for *Z. marina* [32,56,57]. Moreover, phenolic compounds are also regarded as carbohydrate storage molecules in situations with nitrogen limitation [58]. Working with the subtropical seagrass *Thalassia testudinum*, Steele et al. [59] identified a correlation between infection with *Labyrinthula* sp. and the concentration of phenolic acids in plant tissue. The authors interpreted this as a consequence of over-accumulation of carbon resources in the regions above the leaf lesions (across which assimilate flow was disrupted) rather than an induced defense reaction by the plant.

The results of our transcription analysis further revealed that different layers of the host´s pathogen defense were not activated: Neither R-genes (RPPA), PR-genes (Chitinase and Prot-206), genes involved in HR (Metacaspase) or signal transduction through SA (EDS-5) nor ROS scavenger genes (APX, CAT, SOD, GST) showed enhanced transcription after infection of *Z. marina* with *L. zosterae*. RPPA, Chitinase and all measured ROS scavenger genes even showed a significant 5-15-fold down-regulation (Table 4). Moreover, expression of the general stress indicator gene HSP70 was not changed due to infection (Fig.7). This indicates that the plants were not generally stressed upon the experimental inoculation procedure. This is the first report of any marine plant that describes such immune modulation of the host defense by a potential parasite, here a protist.

Many pathogens have evolved mechanisms to manipulate host response by suppressing defense reaction e.g. through effector proteins [34,60,61]. One example, where several pathogenesis related (PR) genes and other genes from the defense cascade are down-regulated after infection with *Phytophthora citricola*, is Fagus *sylvatica* [62]. The author concluded that *P. citricola* escaped recognition by the host, probably by repressing it. How such an effector might work, has recently been shown by de Jonge et al. [63]. The LysM effector Ecp6 in *Cladosporium fulvum* binds Chitin and prevents thereby a Chitin-triggered host response. Comparably, *L. zosterae* might release a related effector that oppresses immune induction in *Z. marina*. In our study, the tested resistance-gene immune receptor (RPPA, involved in recognition of pathogens), as well as the pathogenesis-related proteins (Chitinase and Prot-206 from the base of the signal cascade) are non-differential or lower expressed in infected plants, supporting this theory.

Another indication that the endophyte manipulates the defense reaction of *Z. marina* is the down regulation of ROS scavenging genes (SOD, CAT, APX, GST). ROS is a crucial signal for HR and other pathogenesis related defense mechanisms and does therefore play an important role in plant-pathogen interaction [64]. The observed down regulation of ROS scavenging genes (SOD, CAT, APX and GST) in *L. zosterae* infected eelgrass, especially SOD which catalyzes the dismutation of superoxide (O_2-) to oxygen and hydrogen peroxide might imply that the eelgrass does not recognize *L.zosterae*. Robb *et al.* [65] observed a comparable down regulation of host antioxidant enzymes in the tolerant interaction between the tomato strain *Lycopersicon esculentum* and the pathogen *Verticillium dahliae,* concluding that no oxidative burst occurs in these plants. Alternatively, the down-regulation of antioxidant enzymes could also result in an accumulation of reactive oxygen species (ROS) resulting in damage of plasma- and compartment-membranes and macromolecules [66]. In consequence, plant cell

exploitation and symplastic movement of *L. zosterae* might be facilitated through non-functional cell components [67].

Although *L. zosterae* has no severe impact on *Z. marina* in our study area today, it is very well possible that this may change as shown in many other examples of host-microbe associations [68,69]. Survival of eelgrass strongly depends on the leaf turn-over rate: As long as new leaves grow faster than old leaves decay, the survival is assured. But if growth will be reduced through abiotic or biotic stressors, leaf mortality may outbalance leaf growth. Predominant general stressors for *Z. marina* are increasing water temperatures in the face of global climate change and reduced light availability caused by eutrophication [16,17,22,41,70]. Potentially, these stressors could alter the actually non-virulent relationship between eelgrass and its endophyte towards pathogenicity.

We can conclude that under our non-stressful experimental conditions, *L. zosterae* infection in the study region is not associated with the detrimental effects on *Z. marina* described for the wasting disease. Although infectiousness of the endophyte was high, we found no evidence that *Z. marina* is negatively impacted by *L. zosterae* infection. Instead *Z. marina* seemed to profit through enhanced leaf growth and kept endophyte abundance low possibly as a consequence of high concentrations of phenolic acids. We hypothesize that under adverse conditions (e.g. high water temperatures, low light availability) imposing stress on *Z. marina*, the protist-plant relationship may become pathogenic.

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Submitted Erratum to PlosOne

Article Title	Current European Labyrinthula zosterae Are Not Virulent and Modulate Seagrass (Zostera marina) Defense Gene Expression
Original Article DOI Example: pone.1234567	pone.0092448
Description of the Error(s) Include any relevant information like updated ID numbers (e.g. grant numbers, DOIs, URLs, etc.)	We regret an error concerning the presentation of absolute cell numbers of *Labyrinthula zosterae* in planta. Due to a calculation error, all *Labyrinthula zosterae* cell numbers must be multiplied by a factor of 100.
How did this error occur?	Different people did the analysis of cell numbers. While handing over the analysis from one person to a second one, a calculation error in absolute cell numbers of *Labyrinthula zosterae* in planta occurred, because the second person used μg instead of μmol/l.
How do the error(s) affect the results, conclusions, and overall scientific understanding of your study?	All statistical analyses, comparisons and conclusions are still valid.

Chapter 2

Moderate virulence caused by the protist *Labyrinthula zosterae* in ecosystem foundation species *Zostera marina* under nutrient limitation

Janina Brakel, Thorsten B. H. Reusch, Anna-Christina Bockelmann

Abstract

The nature of many microbe–host interactions is not static, but may shift along a continuum from mutualistic to harmful depending on the environmental conditions. In this study, we assessed the interaction between the foundation plant eelgrass *Zostera marina* and the frequently associated protist *Labyrinthula zosterae.* We tested how an important environmental factor, nutrient availability, would modulate their interaction. We experimentally infected naive eelgrass plants in combination with 2 nutrient levels (fertilized and non-fertilized). We followed *L. zosterae* infection, eelgrass growth parameters and host defense gene expression over 3 wk in large 600 l tanks. Inoculation with *L. zosterae* and nutrient limitation both reduced eelgrass growth. These effects were additive, whereas no interaction of nutrient treatment and *L. zosterae* inoculation was detected. Gene expression levels of 15 candidate genes revealed a reduced expression of photosynthesis-related genes but an increased expression of classical stress genes such as *Hsp80* in inoculated plants 2 d post-inoculation. However, we found no effects on plant mortality, and plants were able to clear high infection levels within 3 wk to ambient background levels of infection as assessed via specific RT-qPCR designed to quantify endophytic *L. zosterae*. Thus, we found no evidence that *L. zosterae* is a facultative mutualist that facilitates eelgrass growth under nutrient-limiting conditions. We suggest that the interaction between contemporary *L. zosterae* genotypes and *Z. marina* represents a mild form of parasitism in northern Europe because the damage to the plant is moderate even under nutrient limitation stress.

Introduction

Many host–microbe interactions may change along a continuum between parasitism and mutualism depending on the prevailing environmental conditions and life stage (Bronstein 1994, Newton et al. 2010). Environmental stressors may alter the physiology of host and microbes in different ways and thus modulate their interaction. If keystone or foundation species are involved, interactions at the host–microbe level may produce changes that affect the entire ecosystem (Harvell et al. 2002, Burge et al. 2013). It has recently been shown that host–microbial interactions may also determine ecosystem productivity and diversity, in particular in seagrass-dominated systems (Van Der Heijden et al. 2008, Mendes et al. 2013).

In this study, we focus on the foundation species *Zostera marina* L. (eelgrass). Eelgrass belongs to the seagrasses, a polyphyletic group of marine angiosperms that populate soft-bottom habitats in all climate zones except the polar regions, and provide critical ecosystem functions and services (Costanza et al. 1997, Cullen-Unsworth et al. 2014). Along with a great diversity of microorganisms such as fungi and bacteria (e.g. Sakayaroj et al. 2010, Garcias-Bonet et al. 2012), seagrasses are frequently colonized by endophytic net slime mold of the genus *Labyrinthula* (Vergeer & Den Hartog 1994, Garcias-Bonet et al. 2011, Bockelmann et al. 2012, 2013). *Labyrinthula spp.* live within leaf tissue of diverse seagrass species, where they may exist asymptomatically (Raghukumar 2002), or produce necrotic lesions in case of pathogenic outbreaks for which the specific triggers are still unknown. Repeated seagrass die-offs have been associated with *Labyrinthula spp.* and are collectively summarized under the somewhat unclear label of ‘wasting disease’ (Sullivan et al. 2013). In the 1930s, the largest ever recorded seagrass die-off was reported all across the Northern Atlantic, supposedly caused by infection with the protist *Labyrinthula zosterae* (Short et al. 1987), resulting in drastic ecological consequences such as the reduction of associated fish, shellfish and crustacean populations (Muehlstein et al. 1991).

Contrary to the situation depicted above, there is increasing evidence that *Labyrinthula spp.* may also coexist with their host without disease symptoms (Bockelmann et al. 2013, Martin et al. 2016). For example, a field survey in northern European eelgrass meadows revealed high abundances of *L. zosterae* in contemporary eelgrass meadows without any observable mortality (Bockelmann et al. 2013). Furthermore, experimental infections of eelgrass with *L. zosterae* revealed low virulence of *L. zosterae* genotypes in eelgrass populations from the Western Baltic Sea and the Wadden Sea, while inoculation even induced higher growth rates in *L. zosterae*-infected eelgrass plants when grown under

ambient Western Baltic Sea conditions (Brakel et al. 2014). These experimental results demonstrate that we have still not identified the exact nature of the protist–host plant relationship, at least of contemporary *L. zosterae* genotypes.

Investigation of the host–microbe interaction while manipulating different environmental conditions may reveal insight into the continuum between parasitism and mutualism (Webster et al. 2008), and can thus lead to a better understanding of which factors influence virulence, pathogenicity and host defense. In this study, we investigated the influence of nutrient levels on the eelgrass–protist interaction. It has been shown that nutrient availability affects the interaction of several plant species to bacterial, fungal or viral pathogens by either enhancing or inhibiting infection (Hoffland et al. 2000, Snoeijers et al. 2000, Lacroix et al. 2014). Seagrasses, including our focal species *Z. marina*, may also suffer from nutrient limitation (Bulthuis & Woelkerling 1981, Reusch et al. 1994), even although eutrophication is one of the main causes for seagrass disappearance worldwide (Orth et al. 2006). The effect of nitrogen deficiency is well documented for *Z. marina*, and includes a reduction in growth rates, biomass production and shoot length (Short 1987). During summer in particular, when growth and biomass productivity are highest, nitrogen deficiency becomes substantial in shallow, nutrient-poor silicate sediments (Pedersen & Borum 1993), emphasizing the potential relevance of internal nitrogen recycling for eelgrass.

The core hypothesis of this work was that degradation processes driven by *L. zosterae* will alleviate nutrient limitation in *Z. marina*, ultimately enhancing eelgrass growth and vegetative shoot production. *Labyrinthula zosterae* prefers older eelgrass leaves at the third position counting from the meristematic leaf forming zone (Bockelmann et al. 2013) while *Labyrinthula* species in general exude a wide range of enzymes enabling the degradation of organic compounds and display an absorptive mode of nutrition (Raghukumar & Damare 2011). Also, the sister group aplanochytrids are efficient degraders of mangrove litter (Bremer 1995, Leander et al. 2004). A potentially commensal or mutualistic role of *Labyrinthula spp.* has been suggested previously (Vergeer & Denhartog 1994, Raghukumar 2002), but experimental data are lacking. Alternatively, as described above, nutrient limitation is a well-described stressor and may weaken eelgrass growth and production. Therefore, our second hypothesis was that nutrient limitation enhances detrimental effects of *L. zosterae* inoculation.

In order to test our hypotheses, we designed a tank experiment that combined 2 nutrient levels with *L. zosterae*-inoculated and sham-inoculated *Z. marina* plants from the Western Baltic Sea. We measured several response variables: (1) quantification of *L.*

zosterae infection by wasting disease index and *L. zosterae* abundance measurement (by RT-qPCR) in eelgrass leaves, (2) growth quantification by measuring leaf production, leaf growth rate and above- and belowground biomass and (3) host defense exploration by gene expression analysis of target defense genes.

Materials and Methods

Zostera marina and *Labyrinthula zosterae* origin and cultivation

In order to control the infection level of our experimental plants, we raised the *Zostera marina* plants from seeds. Seeds were collected in 2 eelgrass beds in the Western Baltic Sea near Kiel (54.39°N, 10.18°E) and Flensburg (54.75°N, 9.87°E), Germany. To ensure vernalization, we incubated seeds for 12 wk at 5°C submerged within the sediment. The emerging seedlings were raised for 1.5 yr within large 600-l tanks under semi-continuous water flow with Baltic seawater (approximate salinity 15 psu) as previously described (Brakel et al. 2014).

Labyrinthula zosterae cultures were isolated from necrotic leaves of *Z. marina* plants collected at the east side of the island of Sylt, North Sea (55.04°N, 8.41°E), in August 2013. We isolated and cultivated L. zosterae cultures on seawater medium agar plates as described in Bockelmann et al. (2012). Isolated L. zosterae cultures were inspected under 100× magnification and cells were identified based on their typical spindle-shaped form. Species identity of *L. zosterae* was also confirmed by species-specific real-time qPCR, which was developed on a portion of the internally transcribed spacer (ITS) of the rDNA gene; these were 100% equal to virulent strains (GenBank accession nos.: JN121409, JN121410) (Bergmann et al. 2010). We chose not to infect healthy eelgrass plants and re-isolate L. zosterae strains before the experiment according to Koch's postulate as this would have selected for the most aggressive *L. zosterae* genotype. Instead, we wanted to maintain genetic diversity and keep cultures as short as possible in culture after isolation. We proliferated the *L. zosterae* culture at 25°C for 2 wk to obtain sufficient material for the inoculation.

Experiment design and setup

In a 2 × 2 factorial design we combined the factors nutrient level (fertilized/unfertilized, where based on earlier studies [Reusch et al. 1994, Worm & Reusch 2000] we assume that unfertilized plants were nutrient limited) and *L. zosterae* inoculation (yes/no). The treatments were arranged in 6 tanks, 3 containing plants with high and the other 3

containing plants with low nutrient levels. Each tank was divided into 2 subareas containing either inoculated or sham-inoculated plants. Each subarea contained 6 plants, which were arranged at a distance of 40 cm to prevent leaf contact between plants. We subdivided the tanks into 2 sections to separate infected from healthy plants by installing a wall that prevented direct leaf contact. Water circulation between both sides was allowed through a 10 × 1 cm opening at the bottom of the tanks. *Zostera marina* shoots were planted individually in 6 l plastic buckets containing sandy sediment to a height of 15 cm. The sediment contained little organic material (<2%) and was collected in the vicinity of the sampling site. It was incubated overnight at 80°C before planting, to limit inadvertent microbial activity (including *L. zosterae*) in the sediment. The buckets with the plantings were submerged in 50 cm of water into 600 l tanks containing filtered Baltic seawater from Kiel Fjord, of which 300 l was exchanged every other week. Within the time of the experiment, the salinity increased from ambient 13.5 psu to 18 psu due to water evaporation, which is within the range of natural salinity variation in Kiel Fjord (Hiebenthal et al. 2012). Light was provided by 2 halogen metal vapor lamps with a light intensity of ~600 µmol photon s^{-1} m^{-1} in a 16 h light:8 h dark cycle. Water temperature was kept at 20.7°C (±0.9°C). Salinity and temperature were measured 3 times weekly.

We fertilized the plants every third week using a mixture of 2 types of coated fertilizer (slow and immediate release 1:1; Plantacote Mix 4M, Manna) (Worm & Reusch 2000) (for concentrations, see Table 1). Fertilizer pellets were placed individually 2 cm deep in the sediment at 2 cm distance to the plants. The low nutrient treatment plants were physically handled in the same way, without adding fertilizer. Nutrient concentrations (NH_4^+, NO_2^+/NO_3^- and PO_4^{3-}) of pore water and water column were measured twice, after establishment of the plants in the sediment and before inoculation treatment of eelgrass plants (Fig. 1). About 40 ml of pore water was sampled using a syringe with a perforated tip that was pushed 5 cm deep into the sediment.

Table 1. Fertilization steps and the estimated nutrient concentration by the mixed fertilizer Plantacote Mix 4M (Manna) for the high nutrient treatment. Date = dd.mm.yyyy

Date	Fertilizer type	Number of pellets per plant	Corresponds to NO_2^+/NO_3^- and NH_4^+ (mg)	Corresponds to PO_4^{3-} (mg)
15.07.2013	Coated slow releasing fertilizer	2	12.06	8.04
05.08.2013	Immediately available fertilizer	1	3.156	2.104
30.08.2013	Immediately available fertilizer	1	3.156	2.104
19.09.2013	Immediately available fertilizer	2	6.312	4.208

We verified that eelgrass would be nutrient limited in the treatments that received no fertilizer. The measured ammonium concentrations of pore water in the unfertilized treatments in our study of 7.5 µmol l^{-1} (SE ± 1.4 µmol l^{-1}) were shown in a previous study to be limiting in the Western Baltic Sea (Reusch et al. 1994). The ammonium levels of 41.5 µmol l^{-1} (SE ± 15.6 µmol l^{-1}) in the fertilized treatments represent natural nutrient-rich conditions, with natural ammonium concentrations measured in an eelgrass meadow in geographic vicinity ranging from 29 to 50 µmol l^{-1} and 21 to 29 µmol l^{-1} between May and September, with and without mussels (*Mytilus edulis*), respectively (Worm & Reusch 2000).

After 7 wk of establishment in nutrient-poor or -rich sediment, we inoculated eelgrass leaves with *L. zosterae*. For inoculation, sterile gauze pieces were first placed on the surface of an agar plate covered with *L. zosterae* culture for 5 d until they were overgrown by *L. zosterae*. A 1 × 2 cm piece of the *L. zosterae*-infested gauze was then gently fixed onto the middle section of the 2nd and 3rd oldest eelgrass leaves for 24 h. Control treatments were treated similarly with sterile gauze pieces incubated on agar medium plates without *L. zosterae* culture. The gauze on average transferred 2.14±0.197 × 10^5 (n = 6, ±1 SE) *L. zosterae* cells to the leaf surface as determined by RT-qPCR as described in the next section.

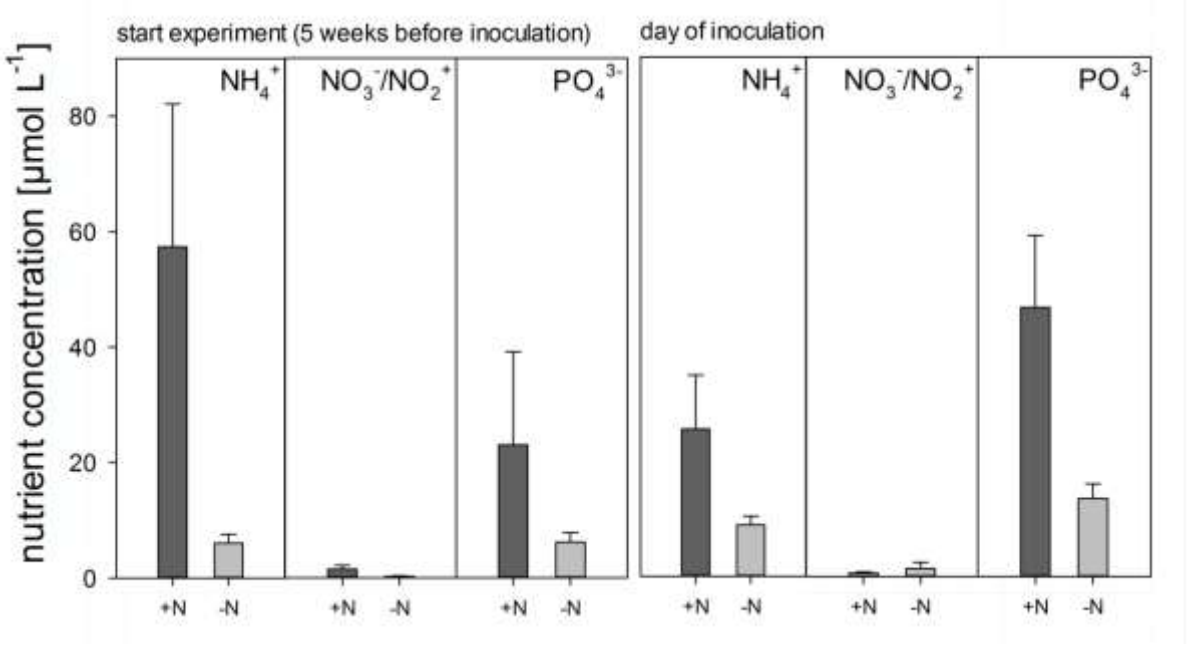

Figure. 1. Mean (+SE) nutrient concentration in sediment pore water (n = 12) measured at the start of the experiment (5 wk before inoculation) and at the day of inoculation of eelgrass *Zostera marina* plants with *Labyrinthula zosterae*. N+: fertilized; N–: unfertilized

Wasting disease symptoms and *Labyrinthula zosterae* quantification

As one of the most widely observed symptoms, an *L. zosterae* infection produces black lesions covering the eelgrass leaves. We quantified lesion surface according to the wasting disease index (Burdick et al. 1993), which estimates the relative area of lesion coverage using 6 classes (0%, >0–10%, >10–25%, >25–50%, >50–75% and >75–100%). We estimated wasting disease index at 1, 2, 3, 5, 7, 9, 12, 14, 16 and 20 days post-inoculation (dpi).

We also quantified *L. zosterae* abundance in *Z. marina* leaf tissue by real-time quantitative PCR assay in accordance with Bockelmann et al. (2013), amplifying a species-diagnostic region of the ITS region of *L. zosterae*. For sampling, each harvested leaf was divided longitudinally. One section was dried for later *L. zosterae* quantification while the other half was immediately stored in RNA-later for gene expression measurements (see below). One half of each plant was harvested 2 dpi, sampling a leaf of 2nd rank, while the second half of each plant was harvested 20 dpi, sampling similarly a leaf of 2nd rank. For *L. zosterae* quantification, dried leaf pieces (3–15 mg dry weight [DW]) were ground in a ball mill with a stainless steel bead (Retsch) and DNA was extracted with Invisorb Spin DNA Extraction Kit (Stratec Molecular). One microliter of salmon sperm (Life Technologies) was added to saturate silica columns with unspecific

DNA. Target DNA was purified using a one-step PCR inhibitor removal kit (Zymo Research). RT-qPCR was performed on a StepOne Plus q-PCR machine (Applied Biosystems). In a reaction, we mixed 10 µl TaqMan universal Master Mix (Life Technologies), 2.4 µl of forward and reverse primer (final concentration 40.8 nM), 2.4 µl Milli-Q H_2O, 0.8 µl fluorescently labeled probe (50 nM) and 2 µl 1:10 diluted template DNA. The thermo-cycling protocol was 2 min at 50°C and 10 min at 95°C, followed by 48 cycles at 95°C for 15 s and 1 min at 60°C. Each sample was run in technical triplicate. Cycle threshold (C_T) was calculated with a fixed threshold of 0.05. We ran on each q-PCR plate 3 standard DNA solutions of known *L. zosterae* cell numbers of 0.5 cells (C_T: 33.51 ± 0.12 SE), 15 cells (C_T: 27.75 ± 0.12) and 150 cells (C_T: 23.49 ± 0.03). C_T values above 39 were not considered. Standard deviation was calculated for all samples; if it exceeded 0.5, samples were excluded from further analysis.

Eelgrass response variables

We followed leaf growth, leaf and shoot production of individually marked eelgrass shoots over 11 wk. Recognition of individual leaves was realized by pricking the tip of the respective leaf with a syringe needle (diameter 0.5 mm). We counted new leaves and novel side shoots once a week. Leaf length was measured with a ruler from the leaf tip to leaf base to the nearest 0.5 cm. We noted that leaf growth decreased with increasing leaf age. In the first week after appearance, *Z. marina* leaves showed strongest growth rates of mean 1.7 cm d^{-1}. During the second week, leaf growth strongly decreased due to age to levels of 0.6 cm d^{-1}. No growth could be detected once a leaf was older than 17 d. Therefore, we compared only leaves of the same age, irrespective of the date on which measurement were performed.

At the end of the experiment, we excavated all plants including their rhizome. We freeze-dried the material and weighed it to the nearest 1 mg.

Targeted gene expression assay

In order to assess molecular defense reaction of eelgrass plants, we measured levels of gene expression of 5 immune genes (see Table 5 for full names): *RppA, pl 206, CLT1, Metacasp* and *CYP73A* (Brakel et al. 2014); 4 redox and detoxification genes: *GST, SOD, APX* and *CAT* (Winters et al. 2011); and 2 general stress genes: *Hsp70* and *Hsp80* (Bergmann et al. 2010). Additionally, we included 4 genes of primary metabolism to investigate molecular physiologic response upon nutrient and inoculation treatment: *Chl_synth*, *STS, RuBisCo* and *FBiA* (Salo et al. 2015). Gene expression values were normalized with the housekeeping gene eIF4A. Gene expression was measured with a

Fluidigm Biomark (HD Systems) on a 96.96 Dynamic Array IFC chip according to published protocols (Salo et al. 2015). Assays of each gene were run in 4 technical replicates.

Data analysis

All statistical analyses were performed with R version 3.1.2 (R Core Development Team 2014). To evaluate the effect of nutrient treatment, inoculation and the factor interaction on all response variables other than gene expression, we used linear mixed models of the R package lme4 (Bates et al. 2015). We ran models with both factors and their interaction and reduced the model if possible based on Akaike's information criterion (AIC). We included, according to the nested split-plot design, the terms 'tank' and 'inoculation nested in tank' as random factors to the model. If the model output revealed that the variation by 'inoculation nested in tank' was negligible ($<10^{-10}$ %), we reanalyzed the dataset excluding the non-significant random term. Nevertheless, results of the full model are shown in Tables S1 and S2 in the Supplement at www.int-res.com/articles/suppl/m571p097_supp.pdf. In order to achieve variance homogeneity, cell numbers of *L. zosterae* were square root transformed and biomass data were log transformed.

To analyze gene expression values of the 15 target genes, we used a 2-step approach. First, we performed a permutational multivariate ANOVA (PERMANOVA) on $-\Delta C_T$ values for samples collected 2 and 20 dpi for each time point separately. If the PERMANOVA results revealed a significant pattern of dissimilarity, we performed univariate analyses for each single gene.

We averaged repeated measures across plants from the same split-unit, as it was not possible to include a random factor into such a model type. Tank was included as a random factor into the analysis, because inoculated and sham-inoculated plants shared the same water body (tank). A PERMANOVA was performed using the R package vegan (Oksanen et al. 2016), based on Euclidean distances and 9999 permutations. In order to illustrate the results for gene expression, a heat map (including a dendrogram based on mean values) based on average gene expression values ($-\Delta C_T$) was created within the R package gplots (Warnes et al. 2009).

All primary data have been deposited in the data repository PANGAEA under the doi: https://doi.pangaea.de/10.1594/PANGAEA.869864.

Results

Wasting disease symptoms and *Labyrinthula zosterae* quantification

Characteristic symptoms for wasting disease, namely black lesions on the leaf area, were visible 24 h post-inoculation. Lesion development on the 2 leaves was highly correlated (t = 61.51, df = 402, p = <0.001). Therefore, we calculated the average index value of leaf 2 and leaf 3 for each plant and analyzed these together. Within the first 20 dpi, black lesions did not differ significantly between nutrient-limited and fertilized plants, although there was a slight trend for nutrient-limited plants to develop symptoms faster (p = 0.10; Table 2, Fig.2). Most inoculated leaves were 50–75% covered in necrotic lesions after 20 dpi for both nutrient treatments.

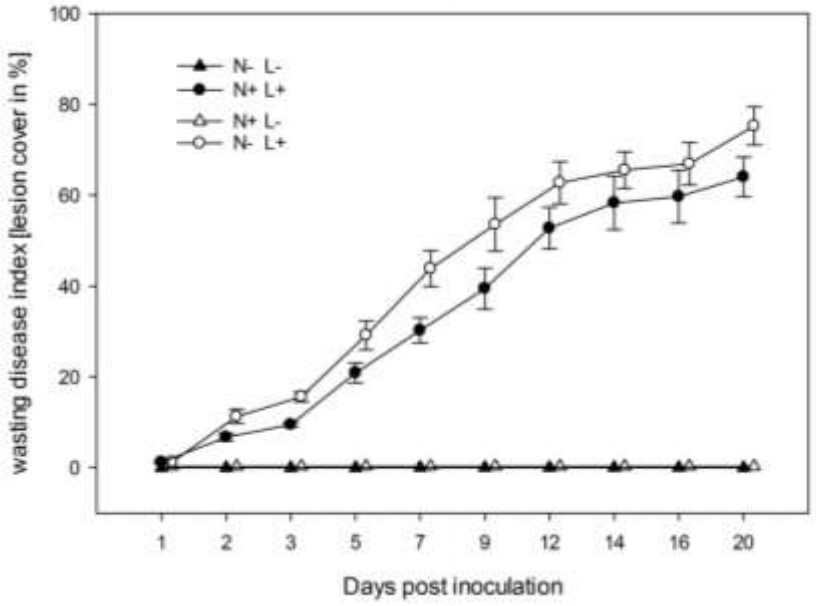

Figure 2. Time course of the wasting disease index (WDI) estimated from the 2nd and 3rd youngest eelgrass leaves. As lesion coverage of 2nd and 3rd leaves was highly correlated (t = 61.51, df = 402, p < 0.001), we show averages of the 2nd and 3rd leaves. The WDI refers to % leaf area covered b y symptomatic necrotic lesions and was estimated in 6 categories. Depicted are mean (±SE) values of estimated index data. N+: fertilized; N–: unfertilized; L+: inoculation treatment with *L. zosterae*; L–: inoculation control = sham inoculated.

Parallel to the wasting disease index, we measured *Labyrinthula zosterae* cell abundance at 2 time points, 2 and 20 dpi. Fertilized and inoculated plants 2 dpi carried on average 12,730 cells mg^{-1} eelgrass leaf DW, while the corresponding value in

unfertilized plants was about double (23,108 cells mg^{-1} eelgrass leaf DW). Owing to the large variance, these differences were not significant ($p = 0.15$; Table 2). A baseline of 41 (±9.1 SE) *L. zosterae* cells mg^{-1} eelgrass leaf DW was detected without experimental inoculation, which attained only 0.2% of values found in the inoculation treatments. After 20 dpi, *L. zosterae* cell abundance was measured in the newly grown leaf which had not been inoculated initially, but which formed at the day of measuring the 2nd rank. Measured values in these leaves did not exceed the baseline level considerably, on average 65 (±64.9 SE) *L. zosterae* cells mg^{-1} eelgrass leaf DW were detected in the inoculated, fertilized plants and 9 (±8.6 SE) cells mg^{-1} eelgrass DW in inoculated, unfertilized plants (see Fig. 3).

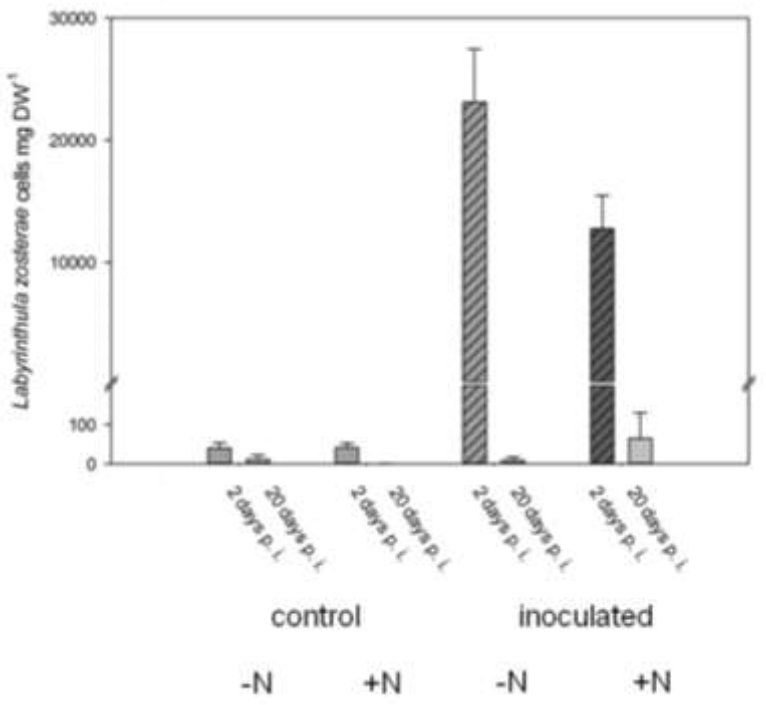

Figure 3. Mean (+SE) *Labyrinthula zosterae* cell numbers detection via Taqman based RT-qPCR in *Zostera marina* leaves (2nd youngest leaf) 2 and 20 days post-inoculation (dpi) with and without *L. zosterae* inoculation. N+: fertilized; N–: unfertilized. dpi: days post inoculation.

Eelgrass growth and biomass production

Neither inoculation with *Labyrinthula zosterae* nor nutrient limitation resulted in enhanced eelgrass shoot mortality. Each plant produced on average 2.0 (±0.13 SE) side shoots throughout the experiment (Fig.4A). The number of side shoots was increased as a result of nutrient addition ($p = 0.02$; Table 2) by 65% compared to non-fertilized plants. Inoculation with *L. zosterae* did not influence the production of side shoots, or the interaction of inoculation and nutrient treatment. We compared dry weight of eelgrass plants from different treatments as a proxy for biomass production. Biomass was significantly reduced by low nutrient level ($p = 0.02$; Table 2), and further, there was a trend that inoculation treatment reduced biomass, though this was not significant ($p = 0.06$; Table 2). Biomass increased with fertilization by 25% (Fig. 4B). Biomass was not affected by the interaction of nutrient and inoculation. We compared leaf growth rates from leaves of the same age in order to correct for different leaf growth rates correlating to leaf age. Leaf growth rates were 26.7% higher (1.9 versus 1.5 cm d^{-1}) in sham-inoculated leaves compared to those inoculated with *L. zosterae* ($p = 0.03$; Table 2). Unfertilized and inoculated plants grew slowest, with growth rates of 1.3 cm d^{-1}, but the cumulative effect of a lack of nutrient addition along with inoculation treatment was purely additive, as no significant statistical interaction was detectable (Fig. 4C, Table 2). During 3 wk of experimental growth post-inoculation, plants had produced between 0 and 4 leaves. However, the number of newly formed leaves did not respond to fertilization, inoculation treatment or the interaction between both factors (Fig. 4D, Table 2).

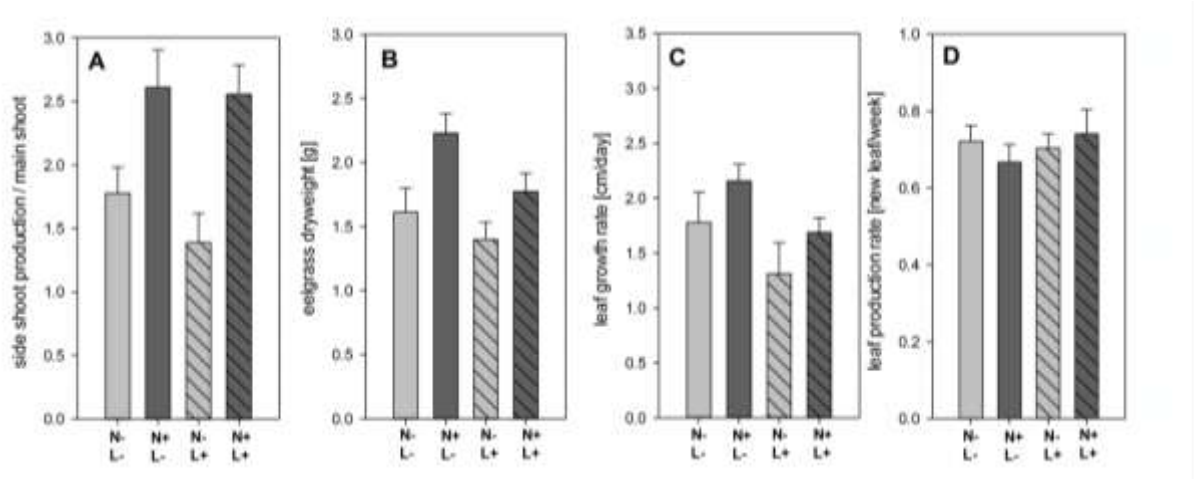

Figure 4. *Zostera marina* growth responses to nutrient and inoculation treatment. N+: fertilized; N–: unfertilized; L+: inoculation treatment with *L. zosterae*; L–: inoculation control. (A) Mean (+SE) number of side shoots per plant sprouting from main plant 20 d post-inoculation (n = 18). (B) Biomass of *Z. marina* (dry weight) 20 d post-inoculation. (C) Leaf growth rate of youngest leaf after inoculation corrected by leaf age (mean ± SE). (D) Leaf production rate post-inoculation.

Table 2. Results of a linear mixed model ANOVA for *Labyrinthula zosterae* concentration, wasting disease index (WDI) and eelgrass growth, based on AIC model selection. Significant results are shown in bold ($p < 0.05$); dpi = days post inoculation, DW = dry weight.

	Variable	*F*	Df	p	Variance	SD
Labyrinthula cells (2 dpi) (cells mg^{-1} eelgrass DW)	Nutrient	<0.001	1,8	0.984		
	Inoculation	73.608	1,4	**0.001**		
	Nutrient×Inoculation	3.056	1,4	0.155		
	Tank				39.2	6.261
	Tank(Inoculation)				218.8	14.792
WDI – Leaf 2 & 3 (categorical index)	Nutrient	0.020	1,60	0.888		
	Inoculation	351.864	1,58	**<0.001**		
	Nutrient×Inoculation	2.726	1,58	0.104		
	Day	400.078	1,401	**<0.001**		
	Plant ID				0.094	0.307
Leaf growth rate (cm d^{-1})	Nutrient	1.959	1,4	0.234		
	Inoculation	4.842	1,41	**0.033**		
	Tank				0.035	0.189
Biomass (g)	Nutrient	12.264	1,4	**0.025**		
	Inoculation	3.589	1,65	0.063		
	Tank				<0.001	<0.001
Shoot production (no. sideshoots main $shoot^{-1}$)	Nutrient	17.105	1,4	**0.014**		
	Inoculation	0.845	1,65	0.361		
	Tank				<0.001	<0.001
Leaf production (no. leaves main $shoot^{-1}$)	Nutrient	0.037	1,4	0.856		
	Inoculation	0.335	1,65	0.565		
	Tank				<0.001	<0.001

Quantification of gene expression levels in 15 target genes

At 2 dpi, multivariate gene expression patterns differed strongly between inoculated and non-inoculated leaves (PERMANOVA $p = 0.002$; Table 3), but were unaffected by nutrient limitation. There was also no interaction detectable between inoculation and nutrient treatment (Table 3). Leaves harvested 20 dpi, containing only very few *L. zosterae* cells inside, did not differ in gene expression pattern between inoculated and sham-inoculated plants (Fig. 5, Table 3).

In the univariate analysis, at 2 dpi, 10 out of 15 genes were differentially expressed in inoculated versus sham-inoculated plants ($p < 0.05$). Genes that encode proteins involved in detoxification of reactive oxygen species (*CAT, GST, SOD*) were downregulated 2.0-, 3.3- and 2.9-fold, respectively, in inoculated versus sham-inoculated leaves ($p < 0.001$, $p = 0.007$ and $p < 0.001$, respectively; Table 4). Of the known stress genes, *Hsp70* was 1.6-fold downregulated ($p = 0.042$, Table 4), while *Hsp80* was 13-fold upregulated ($p = 0.003$, Table 4). Two of 4 genes involved in primary production were downregulated as a consequence of inoculation. These were RuBisCO ($p < 0.001$; Table 4) and Chlorophyll synthase ($p < 0.001$). Furthermore, the immune genes Chitinase ($p < 0.001$; Table 4) and the receptor RPPA ($p = 0.008$) were downregulated 3.4- and 2.8-fold, respectively. The highest change in gene expression was observed in *CYP73A*, which encodes the enzyme trans-cinnamate 4-monooxygenase, involved in phenol synthesis, and was upregulated 45-fold in inoculated leaves ($p < 0.001$; Table 4). Most genes did not show an interaction between nutrients and inoculation in their response, with the only exception of *GST*, which had the lowest expression in inoculated and nutrient-limited plants ($p = 0.059$; Table 4). A linear regression of *GST* gene expression values and *L. zosterae* abundance showed a significant negative correlation ($R^2 = 0.379$, $F_{1,29} = 20.777$, $p < 0.001$) of *GST* expression and *L. zosterae* cell numbers.

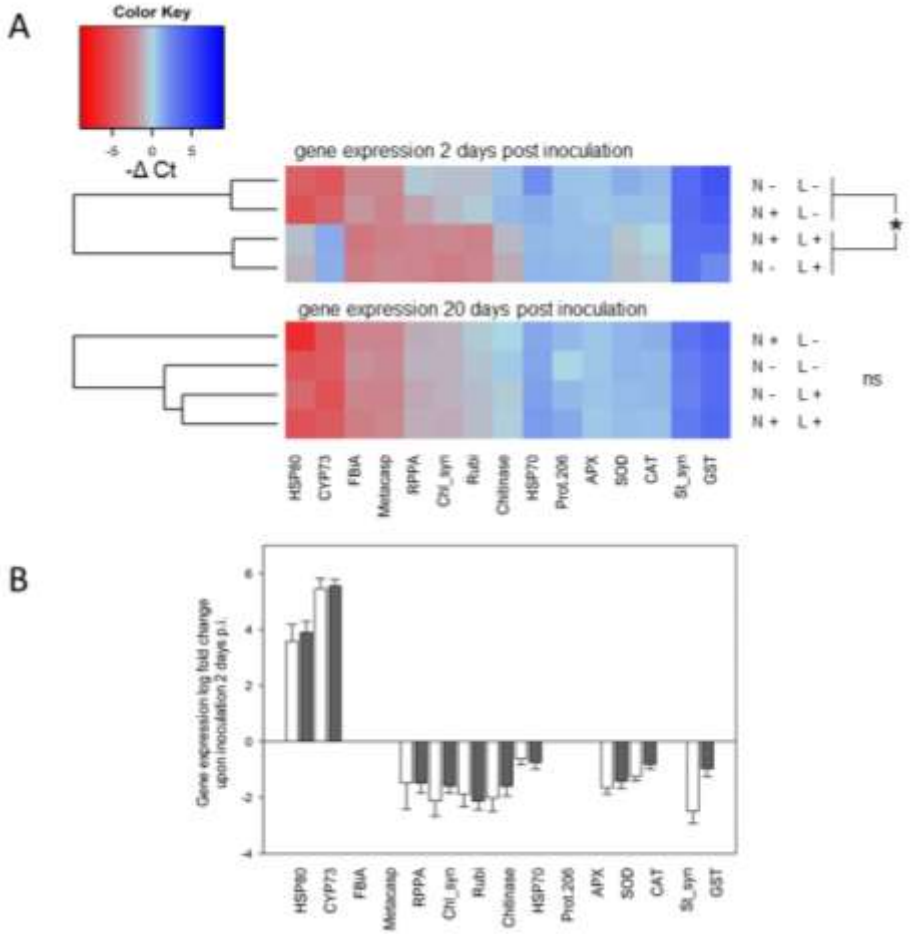

Figure 5. (A) Mean values of relative gene expression ($-\Delta C_T$) of 15 targeted genes depicted in a heat map 2 and 20 d post-inoculation (dpi). N+: fertilized; N–: unfertilized; L+: inoculation treatment with *Labyrinthula zosterae*; L–: inoculation control. *p < 0.05 in PERMANOVA (see Table 3); ns: not significant. (B) Mean (±SE) log fold change upon inoculation with *L. zosterae* 2 dpi for individual genes. Bars are only shown when fold change upon inoculation was significant (see Table 4). White bars: unfertilized plants; grey bars: fertilized plants (n = 9). C_T = cycle threshold.

Table 3. Results of PERMANOVA analysis based on Euclidean distances for gene expression pattern of 15 target genes in relation to nutrient treatment, inoculation, their interaction and tank for 2 and 20 d post-inoculation (dpi). p-values are based on 9999 permutations. Significant results are shown in bold ($p < 0.05$).

	Variable	Df	SS	*F* model	Pr (>*F*)
2 dpi	Nutrient	1	6.940	0.754	0.510
	Inoculation	1	170.221	18.486	**<0.001**
	Tank	4	38.892	1.056	0.461
	Nutrient×Inoculation	1	26.196	2.845	0.097
	Residuals	4	36.833		
20 dpi	Nutrient	1	19.864	2.041	0.161
	Inoculation	1	27.626	2.838	0.094
	Tank	4	41.780	1.073	0.459
	Nutrient×Inoculation	2	1.488	0.153	0.968
	Residuals	4	38.936		

Table 4. Results of linear mixed model ANOVA for gene expression of target genes 2 days post-inoculation for designated predictors by AIC model selection. For gene abbreviations, see Table 5. Significant results are shown in bold ($p < 0.05$).

Gene	Variable	*F*	df	P	Var	StdDev
SOD	Nutrient	3.811	1,13	0.072		
	Inoculation	42.390	1,27	**<0.001**		
	Nutrient×Inoculation	3.684	1,27	0.065		
	Tank				<0.001	<0.001
GST	Nutrient	0.800	1,8	0.397		
	Nutrient×Inoculation	6.862	1,4	0.059		
	Tank				0.000	0.000
	Tank(Inoculation)				0.113	0.336
APX	Nutrient	0.089	1,4	0.741		
	Inoculation	1.160	1,28	0.291		
	Tank				<0.001	<0.001
CAT	Nutrient	0.541	1,4	0.503		
	Inoculation	23.570	1,28	**<0.001**		
	Tank				<0.001	<0.001
Hsp80	Nutrient	0.117	1,4	0.729		
	Inoculation	29.574	1,5	**0.003**		
	Tank				0.00	0.00
	Tank(Inoculation)				0.529	0.728
Hsp70	Nutrient	3.126	1,6	0.130		
	Inoculation	8.364	1,4	**0.042**		
	Nutrient×Inoculation	2.223	1,4	0.210		
	Tank				0.245	0.138
	Tank(Inoculation)				0.019	0.138
STS	Nutrient	0.223	1,4	0.661		
	Inoculation	0.446	1,4	0.510		
	Tank				0.003	0.056
FBiA	Nutrient	<0.001	1,4	0.979		
	Inoculation	4.978	1,4	0.076		
	Tank				0.00	0.00
	Tank(Inoculation)				0.063	0.250
Chl_synth	Nutrient	0.058	1,4	0.822		
	Inoculation	35.551	1,29	**<0.001**		
	Tank				1.832	1.354
RuBisCO	Nutrient	0.003	1,4	0.448		
	Inoculation	26.589	1,29	<**0.001**		
	Tank				0.00	0.00
Metacasp	Nutrient	0.662	1,4	0.462		
	Inoculation	0.124	1,28	0.728		
	Tank				0.00	0.00
CTL1	Nutrient	0.707	1,4	0.448		
	Inoculation	26.589	1,28	**<0.001**		
	Tank				0.00	0.00
RppA	Nutrient	0.933	1,4	0.389		
	Inoculation	8.112	1,28	**0.008**		
	Tank				<0.001	<0.001
pl 206	Nutrient	0.024	1,4	0.883		
	Inoculation	0.058	1,5	0.820		
	Tank				1.385	0.883
	Tank(Inoculation)				1.385	1.177
CYP73A	Nutrient	0.412	1,4	0.557		
	Inoculation	131.918	1,26	**<0.001**		
	Tank				0.00	0.00

Table 5. Information about target genes.

Gene code	Gene name	Function	Source
SOD	Superoxide dismutase (mitochondrial)	Antioxidant	Winters et al. 2011
GST	Glutathione S-transferase	Detoxification	Winters et al. 2011
APX	L-ascorbate peroxidase 2 (cytosolic)	Antioxidant	Winters et al. 2011
CAT	Catalase II	Antioxidant	Winters et al. 2011
Hsp80	Heat shock protein 80	Molecular chaperone	Bergmann et al. 2010
Hsp70	Heat shock protein 70	Molecular chaperone	Bergmann et al. 2010
STS	Starch synthase	Enzyme, starch biosynthesis	Salo et al. 2015
FBiA	Fructose biphosphate aldolase	Enzyme, fructose metabolism	Salo et al. 2015
Chl_syn	Chlorophyll synthase	Enzyme, chlorophyll synthesis	Salo et al. 2015
RuBisCO	Ribulose-1,5-biphosphate carboxylase/oygenase	Enzyme, photosynthesis	Salo et al. 2015
Metacasp	Metacaspase	Regulation hypersisitive response	Brakel et al. 2014
CTL1	Chitinase 1-like protein	Pathogenesis-related protein	Brakel et al. 2014
RppA	NB-ARC domain-containing disease resistance gene	Immune receptor	Brakel et al. 2014
pl 206	Disease resistance-responsive protein 206	Pathogenesis-related protein	Brakel et al. 2014
CYP73A	Trans-cinnamate 4-monooxygenase	Phenol synthesis	Brakel et al. 2014
eIF4A	Eukaryotic initiation factor	Reference gene	Ransbotyn et al. 2006

Discussion

In this study, we assessed how nutrient limitation affects the interaction between *Zostera marina* and *Labyrinthula zosterae*. *Labyrinthula zosterae* infection reduced eelgrass growth, as did nutrient limitation. The observed effects were purely additive, as we found no interaction among our nutrient addition treatment and *L. zosterae* inoculation. Thus our working hypothesis, namely, that rapid degradation and mineralization of decaying leaves was enhanced via the decompositional activity of *L. zostera*, which then may have alleviated nutrient limitation, was not supported. There was no evidence that *L. zosterae* is a facultative mutualist and facilitates eelgrass growth under nutrient-limiting conditions. In line with earlier experiments (Brakel et al. 2014), we found little evidence for enhanced plant mortality, while there were small negative effects on growth. Accordingly, plants were able to clear high inoculation levels within 3 wk to ambient background levels of infection.

Several response variables demonstrated moderate negative effects of *L. zosterae* on the eelgrass host. Eelgrass leaf growth was reduced by *L. zosterae* infection. Furthermore, we noted a 13-fold elevation of gene expression of the known stress indicator gene *Hsp80* in inoculated eelgrass plants, indicating that plants indeed suffered metabolic stress upon inoculation. Hsps not only react upon heat stress (Bergmann et al. 2010), but also play an essential role in various plant stress responses including pathogen attacks (Park & Seo 2015). The gene expression levels of chlorophyll synthesis and a subunit of *RuBisCO* were reduced in inoculated plants, which may explain why photosynthesis was reduced in infected plants. Inhibition of photosynthesis through *Labyrinthula spp.* infection has been shown before (Ralph & Short 2002, Olsen & Duarte 2015). Although we found no interactive effects of nutrient limitation and inoculation on eelgrass responses, both stressors acted in an additive way and reduced biomass production. We cannot exclude that this additive effect does not have other more long-term consequences—for example, reduced winter survival—which could not be addressed in our short-term (3 wk) experiment.

We found no evidence for a mutualistic interaction via a mechanism of enhanced nutrient recycling of *L. zosterae*, at least when assessing leaf growth rates and vegetative shoot recruitment. However, we cannot exclude that *L. zosterae* infection provides eelgrass with other fitness advantages over uninfected plants, for example, via herbivore deterrence or increased resistance against other pathogens or abiotic stressors. *Labyrinthula* spp. infection causes the accumulation of phenolic compounds surrounding the infected leaf section (Vergeer et al. 1995, Steele et al. 2005). In line with those earlier

findings, we found elevated expression of the enzyme CYP73A, an essential enzyme for the phenol pathway, in this and an earlier study (Brakel et al. 2014). These phenolic compounds might reduce herbivory rates to which seagrass plants are subjected (Steele & Valentine 2015), thus infected seagrass may suffer less from grazing than uninfected plants as an indirect beneficial effect of *L. zosterae* presence.

Although *L. zosterae* cell densities were higher than measured values in the field (Bockelmann et al. 2013), this did not result in increased mortality, similar to previous experiments. Survival was high in both nutrient treatments, supporting previous results that the virulence of contemporary Northern European *L. zosterae* genotypes is low (Brakel et al. 2014). It remains to be seen which environmental stressors, if any, may trigger pathogenicity and virulence on the side of the protist. In the coral–dinoflagellate symbiosis, it is well established that adverse environmental conditions, such as extreme sea surface temperature and/or ocean acidification, may turn a mutualistic relationship into a harmful one (Glynn & D'Croz 1990, Brown 1997). Further work should therefore be directed towards identifying those combinations of conditions that determine the position along the commensal–parasite gradient in the *L. zostera–Z. marina* interaction.

As an alternative explanation, the low virulence genotypes currently encountered in northern Europe may differ from the highly virulent *Labyrinthula sp.* that caused the wasting disease in the 1930s. So far, investigated *L. zosterae* strains from the East and West Atlantic, Pacific and Mediterranean show a very high similarity in ITS and 18S sequence (99.3 and 99.4% identity, respectively), including strains that differed significantly in virulence assays (Martin et al. 2016). We speculate that there is additional hidden diversity that we cannot address with the current genetic markers because they do not address functional genes.

In conclusion, we have characterized the interaction between *L. zosterae* and its plant host under one set of varying environmental conditions (i.e. nutrients). We conclude that the interaction is rather parasitic in nature, although with a low virulence of the endophytic protist under ambient conditions. Although we did not find a mutualistic interaction, a recent report on the importance of mutualistic interactions in seagrass beds (van der Heide et al. 2012) underlines the importance of microbial interactions for the persistence of seagrass beds. Future experiments should address more realistic combinations of stressors, such as warming, light and nutrient limitation combined to further characterize the nature of the *Labyrinthula–Zostera* interaction.

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Chapter 3

Multifactorial stressor experiment reveals strong interaction of temperature and salinity on eelgrass - protist interaction

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Abstract

Marine infectious diseases can decimate populations and thereby impact ecosystem stability and services, especially if foundation or key stone species are affected. Here, we investigate the interaction between the seagrass foundation species *Zostera marina* (eelgrass) and its endophyte *Labyrinthula zosterae*. *L. zosterae* is claimed to be the agent of the eelgrass wasting disease, which caused a large eelgrass die-off throughout the northern Atlantic in the 1930s. The omnipresence of *L. zosterae* in eelgrass stands today raises the question of potential risk for sudden wasting disease outbreak, if unfavorable conditions for the host arise.

In a fully-factorial experiment, we exposed *Z. marina* plants to combinations of *L. zosterae* infection, heat stress, light limitation and different salinity levels and followed eelgrass wasting disease dynamics over 3 weeks, along with several eelgrass fitness associated traits such as leaf growth, mortality and carbohydrate storage. We also investigated if stressors affected the chemical defense ability of the plant, by evaluating the inhibition capacity of eelgrass extracts on *L. zosterae* growth.

Contrary to our expectation, inoculation with *L. zosterae* did not reduce fitness associated traits, such as leaf growth or mortality, under any condition. Inhibition capacity of eelgrass extracts was similarly not reduced by the stressors. However, we detected a strong interaction between salinity and temperature on pathogenicity, namely *L. zosterae* was not able to infect eelgrass under high temperature (27°C) and low salinity (12). This work corroborate the idea that contemporary *L. zosterae* isolates do not represent an immediate risk for eelgrass beds in the south-western Baltic, however we stress that other genotypes of the pathogen might behave differently.

Introduction

The oceans are impacted by human induced stressors in all parts of the world with an unprecedented intensity; this includes global warming, ocean acidification, pollution, and eutrophication (Halpern et al. 2008). Anthropogenic stressors affect species directly, but may also affect complex species interactions by disrupting or changing the type or strength of the interaction (Araújo and Luoto 2007; Tylianakis et al. 2008; Van der Putten et al. 2010; Yang and Rudolf 2010; Birrer et al. 2012). Over a long period co-evolved fine-tuned host – microbe interactions may be disrupted or interactions can switch, e.g. commensals can turn pathogenic, or mutualists turn commensals. It has been argued that under proceeding global warming disease prevalence will increase (Harvell et al. 1999, 2002). Opportunistic pathogens are more likely to cause a disease, if changed environmental condition compromise host immune competence or increase pathogen reproduction rates (see review Burge et al. 2013). Some host - microbe - environment interactions are better understood than others, e.g. a strong correlation between warming and disease occurrence has been detected for several coral diseases such as Caribbean yellow band disease on colonies of the star coral *Montastraea faveolata* (Harvell et al. 2009). While for other host-pathogen system the influence of environmental factors is less clear.

One less investigated system is the seagrass 'wasting disease' interaction, a marine flowering plant inhabited by a unicellular protist living within the leaf tissue. Seagrasses are foundation species that build up extensive meadows in shallow coastal waters, providing a variety of valuable ecosystem services (Costanza et al. 1998; Cullen-Unsworth et al. 2014; Lamb et al. 2017). Seagrasses are declining globally due to e.g. eutrophication, coastal construction, global warming, and invasive species (Orth et al. 2006; Waycott et al. 2009; Short et al. 2011). Though, the largest reported seagrass die-off was caused by the so called ‘wasting disease’ and struck the amphi-Atlantic population of eelgrass (*Zostera marina* L.) in the 1930s (Den Hartog 1987). During this disease, necrotic lesions rapidly spread on eelgrass leaves followed by leaf detachment and final death of eelgrass shoots (Muehlstein 1989). These lesions are associated with an infection of the marine net slime mold *Labyrinthula zosterae* (Short et al. 1987; Muehlstein et al. 1988). *L. zosterae* and other *Labyrinthula spp.* are endophytes that inhabit leaves of various seagrass species (Martin et al. 2016), feeding on plant cell organelles by osmotrophy (Muehlstein 1992, Raghukumar 2002). While some species occur asymptomatically, *L. zosterae* has been described frequently as a pathogenic form inducing wasting disease symptoms. However, *L. zosterae* is widespread in eelgrass meadows in the Atlantic and Pacific Ocean without being associated with mass-

mortalities (Bockelmann et al. 2012, 2013; Martin et al. 2016); it remains somewhat unclear what led to the mass-mortality in the 1930s.

One theory is that the outbreak of wasting disease in the 1930s was favored by unfavorable conditions for eelgrass. Extremes in temperature (Rassmussen 1977) or precipitation (Martin 1954), reduced light intensity (Giesen et al. 1990) or a combination of different construction activities like the building of the Afsluitsdijk in the Netherlands or Hindenburgdamm to the island Sylt, Germany (Den Hartog 1987) were claimed to have favored susceptibility for *L. zosterae* infection.

One factor that drives *L. zosterae* virulence, but does not explain the 1930s incidents, is salinity. During the 1930s, eelgrass meadows which were situated in areas with inflow of fresh water remained with little or no disease (Pokorny 1967), and several marine *Labyrinthula spp.* have been found to be sensitive to very high and low salinity levels. *Labyrinthula zosterae* isolated during the reoccurrence of the wasting disease in the 1980's did not grow at salinities below 10 (Muehlstein et al. 1988). Further, lesion expansion on eelgrass leaves caused by *L. zosterae* positively correlated with salinity at salinity levels between 5 and 40 (McKone and Tanner 2009).

Unlike salinity, light and temperature have been investigated less for their influence on seagrass wasting disease dynamics. Wasting disease occurrence in temperate eelgrass populations is strongest during the warmest period of the year (Hily et al. 2002; Bockelmann et al. 2013), which might indicate a positive influence of warmer temperatures. Olsen and co-authors isolated *Labyrinthula spp.* from *Posidonia oceanica* and *Cymodocea nodosa* in the Mediterranean and investigated influence of temperature on wasting disease symptoms in the respective seagrass species. Here, high summer temperatures limited the spread of necrotic lesions in both seagrass species (Olsen et al. 2014; Olsen and Duarte 2015). The influence of light availability on wasting disease dynamics has only been poorly investigated. Vergeer et al. (1995) report that light limitation increased wasting disease occurrence on eelgrass in a pilot experiment with little replication, but unfortunately did not follow up on it.

How environmental factors alter eelgrass resistance against *L. zosterae* and thus potentially alter the host-microbe interaction is poorly understood. Seagrasses are known to produce a wide range of secondary metabolites that can inhibit growth of microorganisms (see review Zidorn 2016). Leaf extracts from eelgrass have been shown to inhibit *L. zosterae* growth effectively (Jakobsson-Thor et al. in revision), indicating that eelgrasses possess a potent chemical host defense against *L. zosterae*. Phenolic acids have been suggested to function as a defense against *L. zosterae* (Buchsbaum et al. 1990; Vergeer et al. 1995; Vergeer and Develi 1997). In particular caffeic acid concentrations were increased in plants displaying wasting disease symptoms (Vergeer

and Develi 1997). However, whether the induction of phenolic acids function as a defense remains controversial and needs to be investigated further (Vergeer and Develi 1997; Groner et al. 2016).The production of phenolic acid has been investigated under the influence of different light intensities, temperatures and salinities (Vergeer et al. 1995; McKone and Tanner 2009). Phenolic acid concentration in eelgrass were decreased under low light levels and high temperature (Vergeer et al. 1995), but were not affected by salinity (McKone and Tanner 2009), though how this affects eelgrass susceptibility for wasting disease has to the best of our knowledge not been investigated.

Against the background of high abundances of *L. zosterae* in contemporary eelgrass meadows and proceeding global environmental change, it raises the question how environmental stressors change the interaction between eelgrass and *L. zosterae*. Very few studies so far have investigated the effect of multiple stressors on seagrass – *Labyrinthula sp.* interaction (Bishop 2013, Jakobsson-Thor unpubl data), and little is therefore known about interactive effects of environmental stressors on *Labyrinthula* sp. infection. Environmental factors are often coupled to each other. For instance, development of floating algal mats, limiting light penetration in the water, is much more likely, if temperatures are elevated. The interaction of two or more environmental factors might be additive, synergistic or antagonistic (Holmstrup et al. 2010; Gunderson et al. 2016). Thus, though seldom realized, complicated multifactorial experimental designs are critical to evaluate responses to simultaneously occurring stressors, as it is impossible to draw conclusions from single factor experiments.

Here, we investigate how salinity, temperature, light and any interaction of these stressors affect pathogenicity, virulence and chemical host defense of the eelgrass - *Labyrinthula zosterae* interaction.

Our study site, the south-western Baltic Sea, is characterized by strong variation in salinity. These are ruled by inflow events of fully marine seawater through the strait of the Skagerrak, and eastern winds that carry low saline water from the eastern parts of the Baltic. Salinity therefore varies between 12 and 25 (Hiebenthal et al. 2013). Eelgrass meadows carry high abundances of *L. zosterae* (Bockelmann et al. 2013), and *L. zosterae* strains isolated so far are characterized by low to moderate virulence under ambient environmental conditions (Brakel et al. 2014, 2017).

Here, we raised naïve eelgrass plants and experimentally inoculated them with the proposed agent of the wasting disease. We hypothesize that light limitation and heat stress and increased salinity increase pathogenicity and virulence and reduce host resistance. Further, we hypothesize that there are synergistic effects between light limitation, heat stress and salinity.

Material and Methods

Cultivation of eelgrass and *Labyrinthula zosterae*

We raised eelgrass plants from seeds to ensure similar infection experience of the plants. Seeds were collected from two field locations in July 2014 (south-western Baltic sea: Strande N 54.434, E 10.170, Eckernförde N 54.449, E 9.871). After ripening, seeds were vernalized for 75 days at 6°C. Seeds germinated at 8°C and grew for half a year within our indoor culturing facilities. For more details see supplementary material.

In August 2015, *Labyrinthula zosterae* cultures were isolated according to published protocols (Bockelmann et al. 2012) from eelgrass leaves displaying typical wasting disease symptoms (Fig. 1A) harvested at one of the sites where seeds had been collected the summer before (Strande: N 54.434, E 10.170). Briefly, eelgrass leaves were surface sterilized, cut into small pieces and incubated on seawater growth medium plates. We repeatedly tried to isolate *Labyrinthula zosterae* on plates of salinity of 15 (average salinity on sampling site) to prevent pre-adaptation to either of the salinity treatments. However, isolation attempts failed. Therefore, we decided to isolate *L. zosterae* as described before on plates with a salinity of 25 (see Bockelmann et al. 2012), but as soon as *L. zosterae* cultures appeared, these were transferred to either a seawater growth medium plates salinity 25 covered with liquid seawater growth medium of salinity 12, or seawater growth medium plates of salinity 12 covered with liquid medium of salinity 25, resulting in a gradient of salinity on the plates which should support potential diversity of *L. zosterae* genotypes by providing a salinity gradient. In this manner *Labyrinthula zosterae* cultures were proliferated for 2 weeks to obtain sufficient culture material for inoculation treatment. After isolation, colony morphology was inspected with a dissecting microscope at 60× magnification for the typical colony form of *Labyrinthula sp.* (Fig.1B). Later sequencing of the 18S rDNA confirmed 100% identity to *L. zosterae* strains from the Atlantic (GenBank acc. no xxx).

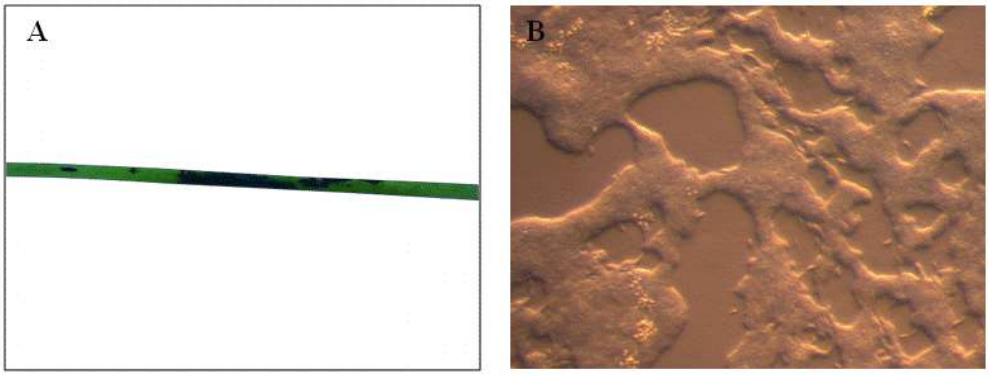

Figure 1. A- Eelgrass leaf displaying black necrotic lesions typical for wasting disease. B- *Labyrinthula zosterae* isolate (Str-7) at 60× magnification.

Experimental design, experimental conditions and treatments

Eelgrass plants were planted in boxes (16 x 26 x 16 cm) filled to a height of 8 cm with autoclaved natural sediment from the Baltic Sea (N 54.394, E 10.190), consisting of sediment from within the eelgrass bed and from a nearby beach. In each box we planted 3 different clones marked by zip ties with an ID tag for recognition. Wet weight of each individual was noted and we distributed eelgrass plants evenly to the boxes. 2 boxes were submerged in one 300 L tank. Water within the tanks was aerated and circulated with a rate of 670 L h^{-1}. Titan heating elements (Schego, Germany) and a temperature controlling system (Biotherm, Hobby-Aquaristik, Germany) adjusted temperatures throughout the experiment in each tank individually. Metal-halide lamps (Philips, Master Green Power 400W, light intensity at height of seagrasses ~195 µmol photons s^{-1} m^{-2}) provided light in 16:8 hours light/dark cycle corresponding to summer conditions.

The experimental factors heat stress (yes/no), light availability (shaded/full light), salinity (12/25), and inoculation with *Labyrinthula zosterae* (yes/no) were fully crossed. All treatment combinations of heat, salinity and inoculation were replicated 3 times in individual tanks (2 x 2 x 2 x 3 = 24 tanks). Light treatment was nested within tanks, while one side of the tank was covered by a shading panel, the other half of the tank received full light intensity. Each tank side contained one box with 3 genetically distinct eelgrass plants as described above, representing sub-replicates, see Fig. 2.

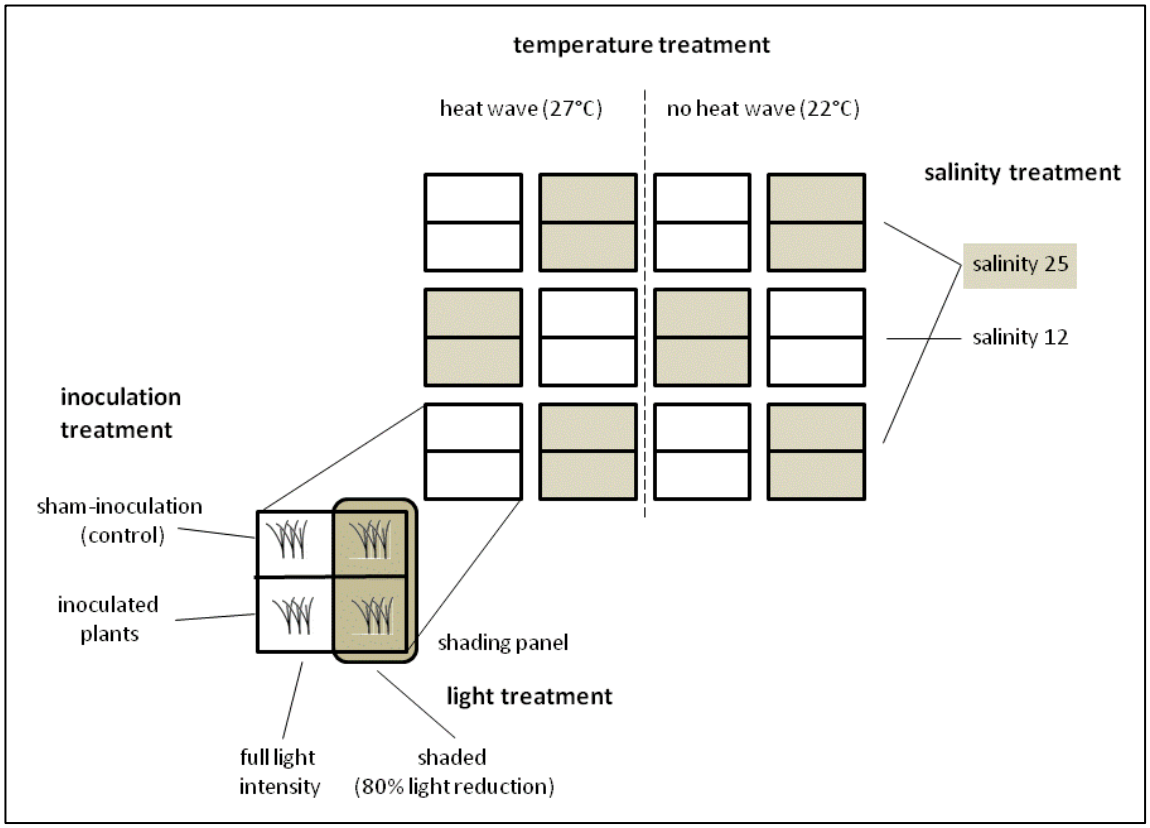

Figure 2. Experimental design - Distribution of tanks with according temperature, salinity, light and inoculation treatment.

To provoke heat stress, we simulated a high summer heat wave, which attained a temperature of 27°C, and held it for 10 days. Length of the heat wave corresponded to the heat wave of 2003 in central Europe (Reusch et al. 2005). In order to expose eelgrass to an extensive stressor the temperature amplitude exceeded the heat wave of 2003 by 1°C, taking into account that the surviving eelgrass plants from 2003 might have an increased temperature tolerance compared to before 2003. The applied temperature treatment represent an extreme whether event, which is expected to become more frequent in the future due to climate change (IPCC 2014). Especially shallow waters and estuaries where *Zostera marina* occurs may warm up on warm summer days. Ambient treatment had a temperature of 22°C (see Fig. 3).

Parallel to the temperature treatment, we applied a shading treatment. A shading panel, prepared by aluminum foil, was installed over ½ of the tank. Shading reduced light intensity by 80% from ~195 µmol photons m^{-2} s^{-1} to ~40 µmol m^{-2} s^{-1} resembling light reductions under drifting algal mats (Rasmussen et al. 2012).

As previously described seagrass – *Labyrinthula sp.* interaction is strongly influenced by salinity (McKone and Tanner 2009; Trevathan et al. 2011), and we chose to conduct our experiment under the extremes of natural occurring salinities of our study region, which

varies between 12 and 25 (Hiebenthal et al. 2013). To obtain water of salinity of 12 and 25 we mixed filtered (5 µm) Baltic Sea water of approximately salinity 15 and added either artificial sea salt (Seequasal GmbH) or deionized water and tap water (1:1) to reach salinity 12 or 25 respectively. Half of the water volume was exchanged every second week during the experiment. Before the water exchange, we let water mix and warm to the according temperature for half a day. Temperature and salinity were measured daily.

Inoculation by *L. zosterae* was performed as described in Brakel et al. (2014) on the second day of shading and heat wave exposure. In short, sterile medical gauze pieces of 1.5 x 1.5 cm size were placed on seawater growth medium plates with or without *L. zosterae* culture material. Gauze pieces were incubated on these plates for 5 days until *L. zosterae* culture had overgrown the gauze pieces. These were then attached carefully with plant wire for 48 hours to the leaf of the second and third rank of the eelgrass shoots.

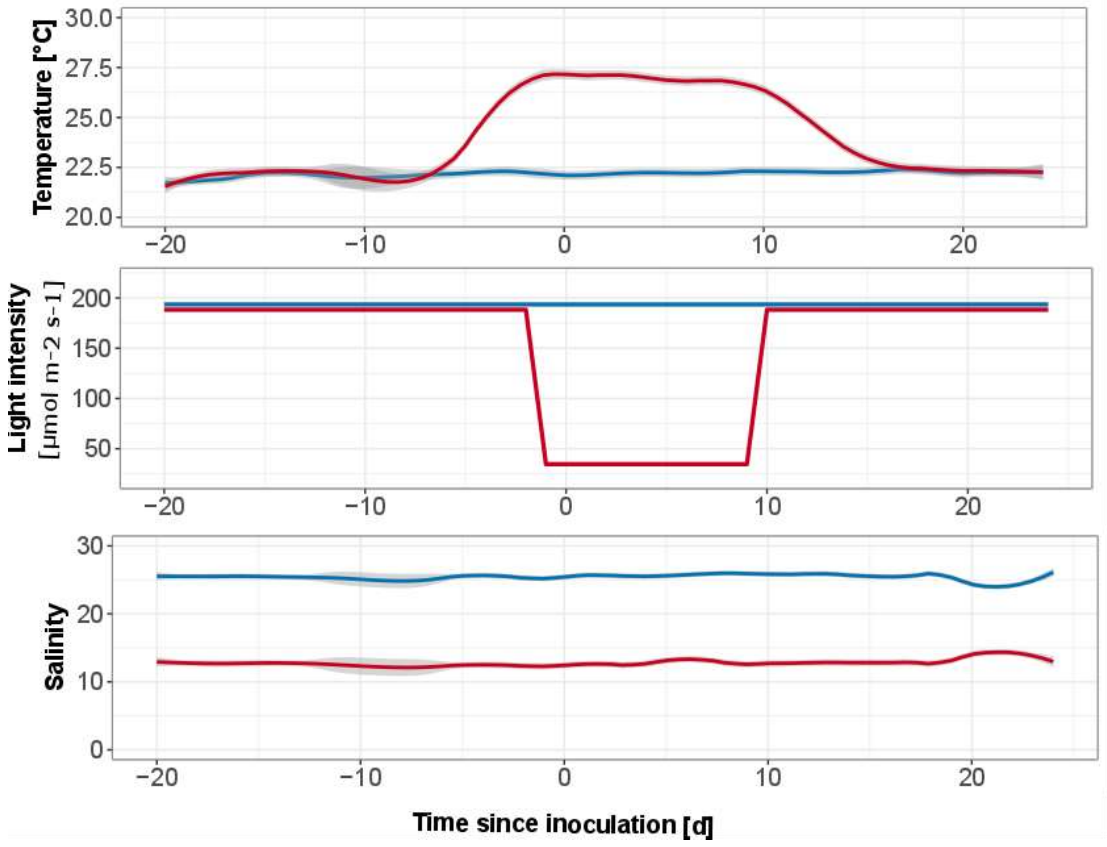

Figure 3. Experimental time course depicting temperature (°C), light (µmol m^{-2} s^{-1}) and salinity (no unit) values for high and low treatment of respective factor. Black line indicates the day when eelgrass plants were inoculated with *Labyrinthula zosterae* (or sham-inoculation as control).

Sampling and response variables

Wasting disease symptoms and numbers of *Labyrinthula zosterae* cells in leaf tissue were determined at the second and eighth day post inoculation of the leaf of third and second rank, respectively. Typical wasting disease symptoms are black necrotic irregular lesions on eelgrass leaves. Percent lesion coverage on the leaves was determined by measuring the length of the necrotic black lesions and the maximum leaf length with a ruler. To quantify *L. zosterae* cells within leaf tissue, we harvested a leaf piece from 5 cm below to 5 cm above inoculated site. Leaf pieces were air dried, weighed and powdered by a ball mill (Retsch, Germany). We extracted DNA with Invisorb® DNA Plant HTS 96 Kit (Stratec Molecular, Germany) following the manufacturer's instructions with the modification to add 500 ng μL^{-1} Salmon sperm DNA (Life Technologies, USA) to saturate silica columns with non-targeted DNA. Real time quantitative PCR (RT-qPCR) was performed as described in Bockelmann et al. (2013) by a Taqman probe based assay. A standard was prepared of the same *L. zosterae* strain used for inoculation. This standard solution was included on each RT-qPCR plate; 0.5 cells (C_T: 36.22 ± 0.26), 15 cells (C_T: 30.52 ± 0.12) and 150 cells (C_T: 25.64 ± 0.37). As DNA extraction procedure differed to previous published studies, we compared extraction efficiencies and intercalibrated *Labyrinthula zosterae* cell numbers to the previously performed DNA extraction procedure. The displayed results here are the adjusted *L. zosterae* cell values.

Eelgrass leaf growth rate was determined during the stress phase and after the stress phase. Therefore, leaf length of all leaves was measured twice seven days apart. To obtain a measurement of how loss of photosynthetic tissue by lesion expansion and reduced growth due to stressors added up, we calculated what we called 'net growth rate' by subtracting leaf growth rate by lesion expansion rate.

We further counted the shoot mortality and number of new side shoots per box throughout the experiments. Plant dry weight (as a proxy for biomass production), measurement of soluble carbohydrates and chemical defense capability of leaf extracts could only be measured at the end of the experiment. Therefore, 24 d post inoculation (45 d total) all main shoots were carefully excavated, cleaned from sediment, freeze-dried for 72 hours and plant dry weight was determined. Leaf tissue was further grounded and homogenized for analysis of soluble carbohydrates and extraction of chemical compounds for the *L. zosterae* growth assay (see below). For starch measurement in the belowground tissue, a part of the rhizome (without roots) between first and third internode was grounded.

Carbohydrates (sucrose and starch in leaves and only starch in rhizome) were extracted according to the method described in Huber and Israel (1982) with a few modifications. Briefly, sucrose was extracted by boiling the plant material in 90% (v/v) MeOH four times.

Starch was recovered subsequently from methanol-insoluble plant residuals. These residuals were incubated for 12 hours with 0.1 NaOH. Starch concentrations were determined in a photometric assay with anthron at 640 nm, relating sample absorbance to a standard curve (Yemm and Willis 1954), while sucrose was determined in a photometric assay with resorcinol measuring absorbance at 486 nm (Huber and Israel 1982).

To investigate if eelgrass shoots produce inhibitory compounds against *L. zosterae*, and whether the inhibitory effect differs between eelgrass shoots subjected to different environmental stressors, 6.5 mg of the aboveground biomass of each shoot was extracted in methanol and dichloromethane 1:1 for 1h on a shaker table. The plant material was filtered away and solvents were evaporated under nitrogen gas.

The inhibitory effects of the eelgrass extracts on *L. zosterae* was tested in a modified *L. zosterae* growth assay described by Martin et al. (2009). Each sample was re-dissolved in 1 mL liquid growth media (seawater growth medium without agar) containing 1% dimethyl sulfoxide, resulting in a 1/13 volumetric concentration of the leaf. The samples were transferred to 6 well plates (Ø = 35 mm), and *L. zosterae* plugs (Ø = 7 mm) taken from cultures growing on agar plates were placed in the center of each well. Wells containing growth media without eelgrass extracts (n = 18) served as negative controls. The well plates were wrapped with Parafilm and incubated at 25°C in the dark. After 14 h the *L. zosterae* colony growth was marked, photographed and the total area of the colony was measured using ImageJ software.

Statistical analysis

To evaluate *Labyrinthula zosterae* cell numbers, lesion coverage, net growth and inhibition capacity of eelgrass extracts we applied a linear mixed model of the package lme4 (Bates et al. 2014) within R version 3.1.2. To account for sub-replication within the tank, we defined ‘aquaria’ (box with planted eelgrass shoots) nested in ‘tank’ as a random factor. Salinity, temperature, light intensity and inoculation were assigned as fixed factors allowing for all possible interactions. We performed an ANOVA (type III sums of squares). If interactions were not significant, we subsequently tested ANOVA (type II sums of squares). To achieve normality data of *L. zosterae* cell numbers and lesion coverage the data was log transformed. We inspected normality, homogeneity of residuals visually examining qq-plots.

Fitness associated parameters (leaf growth rates, survival of eelgrass main shoot, production rate of side shoots, eelgrass dry weight as a proxy for biomass production, sucrose and starch concentration in leaves and rhizome) were interrelated and not independent. To reduce type 1 error we analyzed the parameters by multivariate

analysis. A PERMANOVA was conducted, using Euclidian distances and 9999 permutations of the package vegan (Oksanen et al. 2016). As PERMANOVA does not handle random factors, we calculated the mean values of samples from the same aquarium. For PCA graphs we performed z-scaling (z-score = (sample value – mean) / standard deviation).

Primary data has been deposited in the data repository PANGAEA under the doi: xxx.

Results

Abundance and pathogenicity of *Labyrinthula zosterae*

In order to assess the infection success and the ability to produce disease symptoms under different environmental treatments, we evaluated *Labyrinthul*a *zosterae* cell numbers in leaf tissue in parallel with the measurement wasting disease symptoms (lesion coverage) on inoculated leaves 2 days and 8 days post inoculation.

In contrast to our initial hypothesis, *L. zosterae* cell numbers were neither elevated at higher temperature imposing heat stress on the plants, nor under the shading treatment imposing low light stress. We detected on average 17.6 x 10^3 and 4.9 x 10^3 *L. zosterae* cells mg leaf dry weight^{-1} in inoculated plant tissue at 2 and 8 days post inoculation, respectively. *L. zosterae* cell numbers differed between salinity treatments. At both sampling points a salinity of 12 was associated with significantly reduced *L. zosterae* cell numbers compared to 25 (ANOVA, $F_{1,9} = 8.32$, $p = 0.02$; $F_{1,11} = 10.41$, $p < 0.01$; 2 and 8 days post inoculation respectively, Tab. 1, Fig. 4A). On average 9.3 x 10^3 ± 4.5 x 10^3 and 3.5 x 10^3 ± 0.9 x 10^3 (ME ± 1SE) *L. zosterae* cells mg leaf dry weight^{-1} were detected at salinity 12 over all treatments at 2 and 8 d post inoculation respectively. This corresponds to a 52% and 18% decrease respectively compared to plants at salinity 25. However, the strongest effect on *L. zosterae* cell numbers had the interaction between salinity and temperature treatment. Under low salinity (12) and high temperature (27°C) *L. zosterae* was rare within seagrass leaves [31.90 ± 6.98 and 25.65 ± 17.84 (ME ±1SE) cells mg leaf dry weight^{-1}] and did not exceed the background abundance of the inoculation control of 21.88 ± 6.65 and 38.33 ± 10.53 *L. zosterae* cells mg leaf dry weight^{-1} for 2 and 8 days post inoculation, respectively. Thus, control treatments without experimental inoculation attained 0.2% of the *L. zosterae* abundance found on average in the inoculated treatments.

Necrotic lesion coverage followed largely the trends of *L. zosterae* cell numbers. Similarly to *L. zosterae* cell numbers lesions were neither elevated significantly at higher temperature imposing heat stress on the plants, nor under the shading treatment imposing low light stress. Smaller lesions developed at salinity 12 compared to salinity

25 (ANOVA, $F_{1,8}$ = 6.48, p = 0.03, Tab. 1, Fig. 4) 2 d post inoculation. Lesion coverage was 55% smaller in plants at salinity 12 and 22°C compared to plants at salinity 25 at the same temperature. At low salinity level (12) and high temperatures no visible necrotic lesions developed (Fig. 4). The interaction of temperature and low salinity significantly affected lesion coverage 8 days post inoculation (ANOVA, $F_{1,8}$ = 6.47, p = 0.03, Tab. 1). Highest lesion coverage was detected on light limited plants at 27°C and salinity 25 with on average 45.42 ± 13.51% (ME ±1SE) coverage, however the interaction between temperature, salinity and light was not significant (Tab. 1). Lesion length and *L. zosterae* cell numbers positively correlated at both time points (Spearman rank correlation, 2 days post inoculation: rs = 0.92, N = 63, p < 0.001; 8 days post inoculation: rs = 0.83, N = 68, p < 0.001)

We then calculated how fast lesions would spread along the leaf during the stress treatment (Fig. 5B). These ranged on average per treatment between 0.55 cm d^{-1} (full light, salinity 12, 22°C) - 1.73 cm d^{-1} (shaded, salinity 25, 27°C). To examine the added effect of necrotic lesion and reduced growth due to the stress treatment we calculated a net growth rate value by subtracting leaf growth rate by lesion expansion rate (Fig. 5C). While in most treatments leaf growth rates exceeded lesion expansion rate this was not the case in shaded heat stressed plants at salinity 25, where the average net growth rate was negative (-0.41 ± 0.68 cm d^{-1} (ME ±1SE)). There was a trend that light intensity affected net growth positively ($F_{1,7}$ = 4.34, p = 0.08).

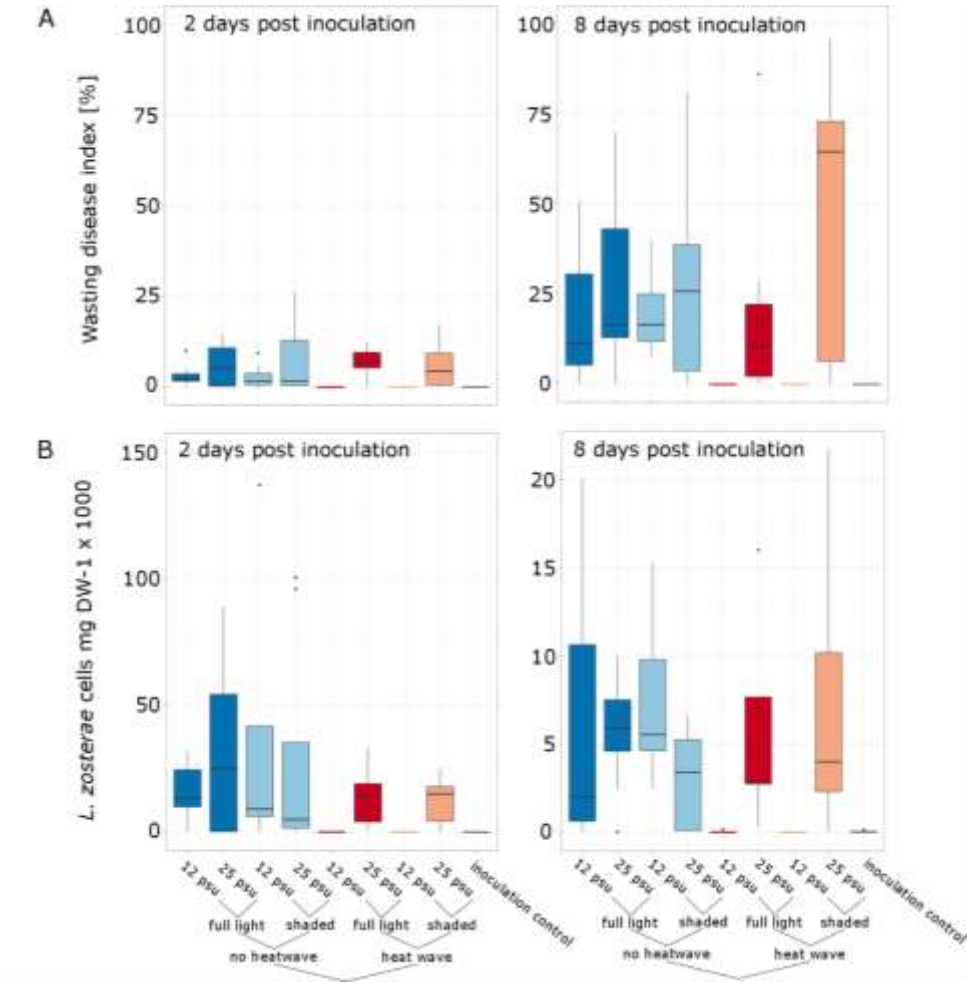

Fig. 4 A - Necrotic lesion area (wasting disease symptoms) as percentage of total leaf area 2 d and 8 d post inoculation. Inoculation control includes samples without *L. zosterae* inoculation from all environmental treatments. **B** - *Labyrinthula zosterae* cells mg dry weight leaf tissue^{-1} determined by Taqman based RT-qPCR 2 d and 8 d post inoculation in 10 cm leaf pieces taken from 5 cm below and above the inoculation site. Inoculation control includes samples without *L. zosterae* inoculation from all environmental treatments.

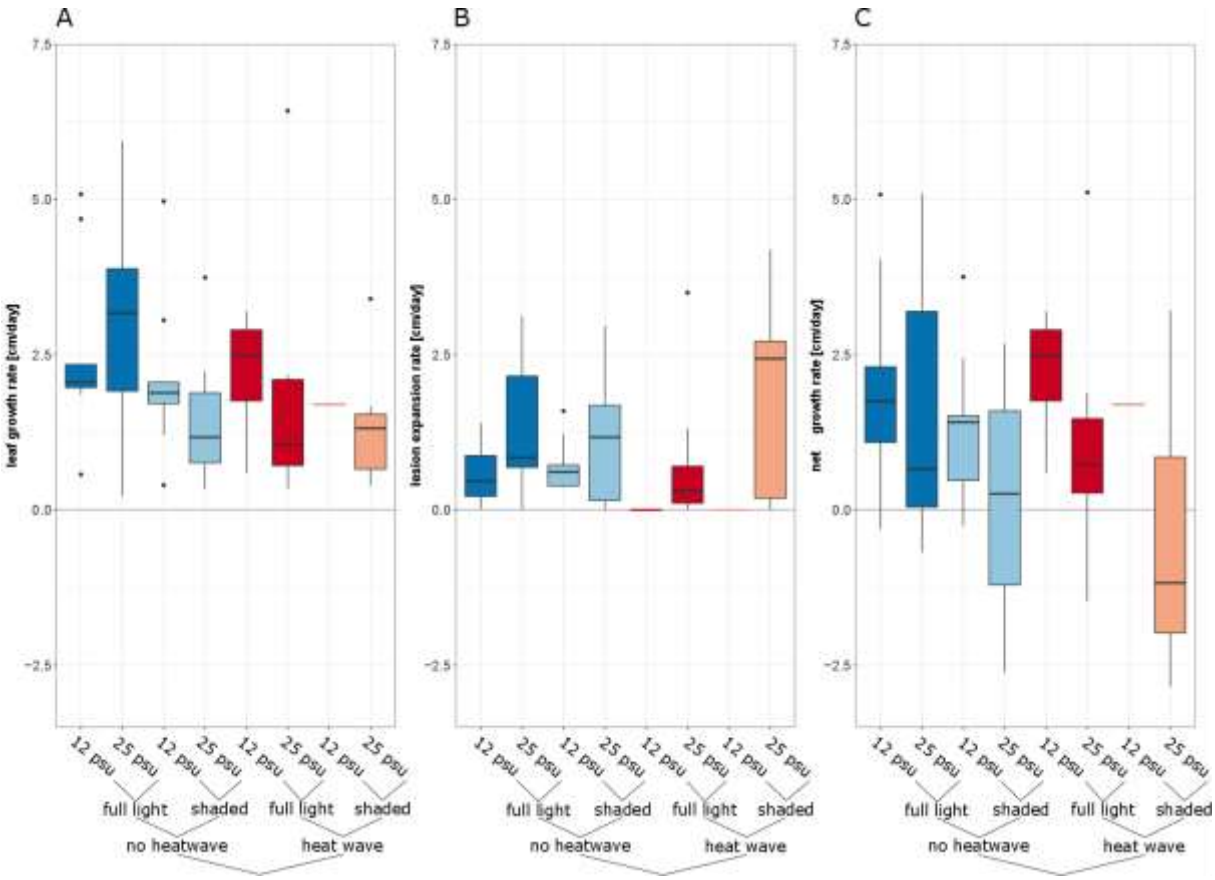

Figure 5. A Leaf growth rates of inoculated plants according to the respective stress treatment in cm d^{-1} during the stress treatment. **B** Expansion rate of necrotic lesion over the inoculated leaf in cm d^{-1}. **C** Net leaf growth rate calculated by subtracting the leaf growth rate by the lesion expansion rate in cm d^{-1}.

Table 1. Generalized linear mixed model results for *Labyrinthula zosterae* cells mg dry weight leaf tissue^{-1} and lesion coverage [%] on leaves 2 d and 8 d post inoculation according to experimental treatments. Only inoculated plants were considered.

		F	Df	P
L. zosterae cell # 2 d post inoculation	Salinity	8.32	1	**0.02**
	Light	0.19	1	0.69
	Temperature	0.21	1	0.66
	Salinity : Light	0.12	1	0.74
	Salinity : Temperature	6.78	1	**0.03**
	Light : Temperature	0.32	1	0.60
	Salinity : Light : Temperature	0.07	1	0.80
L. zosterae cell # 8 d post inoculation	Salinity	10.41	1	**<0.01**
	Light	<0.01	1	0.96
	Temperature	0.12	1	0.74
	Salinity : Light	0.03	1	0.86
	Salinity : Temperature	5.20	1	**0.04**
	Light : Temperature	0.44	1	0.53
	Salinity : Light : Temperature	0.73	1	0.42
Lesion coverage [%] # 2 d post inoculation	Salinity	6.48	1	**0.03**
	Light	0.29	1	0.60
	Temperature	1.34	1	0.27
	Salinity : Light	0.01	1	0.91
	Salinity : Temperature	2.81	1	0.13
	Light : Temperature	0.27	1	0.61
	Salinity : Light : Temperature	0.57	1	0.47
Lesion coverage [%] # 8 d post inoculation	Salinity	7.47	1	**0.02**
	Light	0.64	1	0.45
	Temperature	8.14	1	**0.02**
	Salinity : Light	0.15	1	0.71
	Salinity : Temperature	6.47	1	**0.03**
	Light : Temperature	0.30	1	0.59
	Salinity : Light : Temperature	1.68	1	0.22

Inhibition capacity of eelgrass extracts

As a proxy for host defense status we assessed inhibition capacity of eelgrass extracts against *L. zosterae* growth in liquid medium. We were most interested whether the inhibition capacity would differ between eelgrass shoots subjected to environmental stressors. To facilitate this, eelgrass extracts were diluted to a 1/13 volumetric concentration of the leaf. Nevertheless, diluted extracts of all eelgrass shoots inhibited *L. zosterae* growth significantly compared to the control medium without eelgrass extracts (ANOVA, $F_{1,140} = 251.06$, $p = < 0.001$). Inhibition capacity compared to the control ranged between 20.0% and 90.2%. Temperature treatment significantly influenced the inhibition capacity (ANOVA, $F_{1,29} = 4.65$, $p = 0.04$). Extracts from eelgrass shoots that were exposed to the heat wave treatment inhibited *L. zosterae* growth 7% more strongly than extracts from non-heat stressed plants (Fig. 6). Exposure to one of the other treatments (inoculation, light or salinity), or any interaction of these, did not influence the inhibiting capacity of *Z. marina* extracts (see Tab. 2 and Fig. 5).

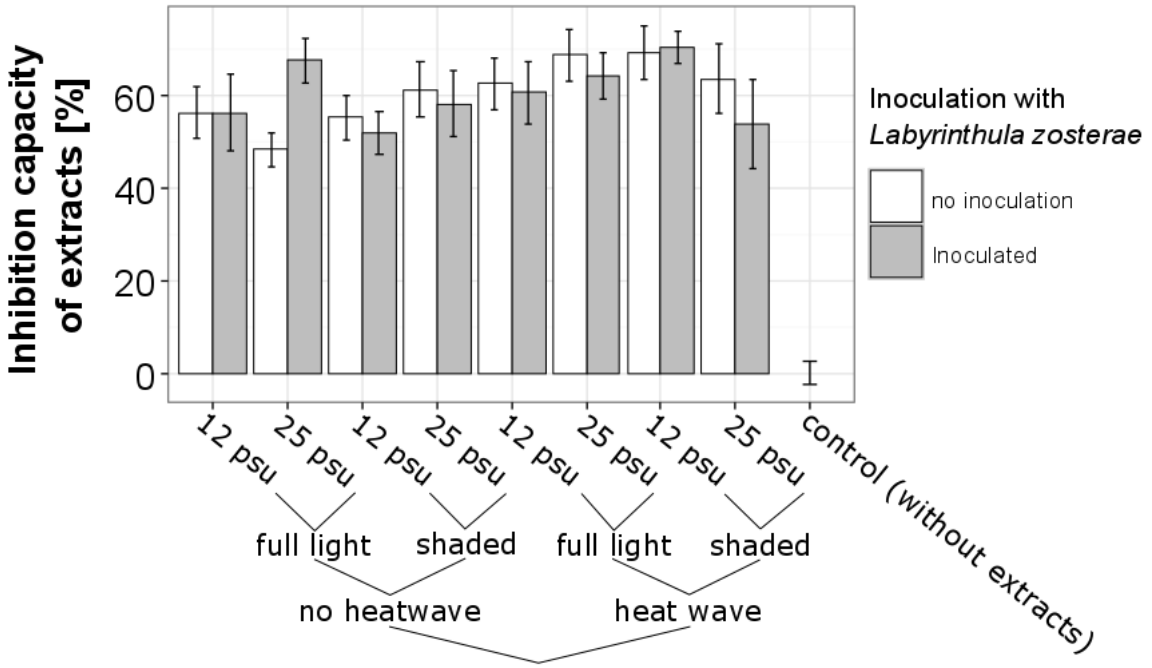

Figure 6. Inhibition capacity of eelgrass extracts on *Labyrinthula zosterae* growth according to experimental treatments to which eelgrass shoots were exposed during the experiment. Control represents *L. zosterae* growth in liquid growth medium without eelgrass extracts. *L. zosterae* growth assays were performed for all treatments at 25°C in the dark for 14 h.

Table 2. Generalized linear mixed model results for eelgrass extract inhibition capacity on *Labyrinthula zosterae* growth according to experimental treatments from which eelgrass shoots were exposed to during experiment. Random factor: tank nested in aquarium.

	F	**Df**	**P**
Salinity	0.56	1	0.46
Light	0.30	1	0.59
Temperature	4.65	1	**0.04***
Infection	0.25	1	0.62
Salinity : Light	0.98	1	0.34
Salinity : Temperature	1.27	1	0.27
Light : Temperature	1.95	1	0.19
Salinity : Inoculation	0.12	1	0.73
Light : Inoculation	0.12	1	0.73
Temperature : Inoculation	3.20	1	0.08
Salinity : Light : Temperature	2.17	1	0.16
Salinity : Light : Inoculation	0.10	1	0.76
Salinity : Temperature : Inoculation	1.52	1	0.23
Light : Temperature : Inoculation	0.85	1	0.37
Salinity : Light : Temperature : Inoculation	0.22	1	0.65

Eelgrass fitness associated parameters

To assess the damage *L. zosterae* infection causes in eelgrass under different environmental treatments, we assessed several eelgrass fitness associated parameters: leaf growth rates, survival of eelgrass main shoot, production rate of side shoots, eelgrass dry weight as a proxy for biomass production, sucrose and starch concentration in leaves and rhizome. Heat stress treatment had the strongest effect on eelgrass fitness associated parameters (PERMANOVA: $F_1 = 3.31$, $p = 0.03$, Tab. 3, Fig. 7). Heat stressed eelgrass and not heat stressed eelgrass clustered in the PCA on axis 1 and 2 (26.3% and 22.2% variation) along a diagonal line opposed to each other in the upper right and downer left corner. Vectors of leaf growth rate, survival and leaf sucrose concentration pointing towards the non heat stressed plants in the downer left corner indicate that eelgrass plants subjected to heat stress had lower growth rates, survived less frequently and had lower leaf sucrose concentration. In contrast, biomass, eelgrass vegetative shoot production and starch content (leaf and rhizome) contributed only little to the clustering.

Light limitation had the second strongest effect on eelgrass fitness associated parameters (PERMANOVA: $F_1 = 2.88$, $p = 0.05$, Tab. 3, Fig. 7). Parallel to plants under heat stress light limited plants cluster more to the upper right corner, while plants with full light cluster more in the downer left corner. An exception is the non inoculated plants under full light and heat stress, which cluster furthest in the right upper corner, indicating highest mortality, smallest leaf growth and lowest leaf sucrose. There was a trend for an interactive effect of inoculation treatment and shading (PERMANOVA: $F_1 = 2.37$, $p = 0.07$). Salinity or the interaction of salinity with the other treatments did not significantly affect fitness associated parameters (Tab. 3).

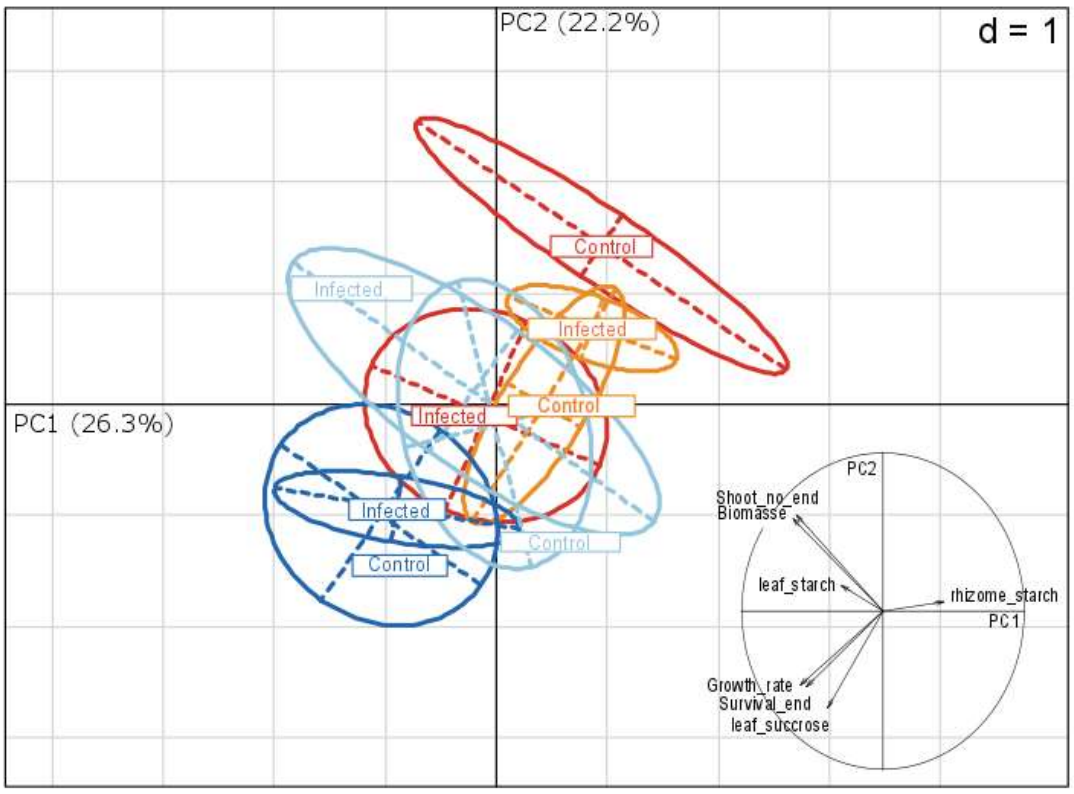

Figure 7. PCA graphs of eelgrass fitness associated measures (leaf growth rate, side shoot production, survival rate, root starch concentration, leaf starch concentration, leaf sucrose concentration, plant dry weight). Depicts are PC 1 on the x-axis, PC 2 on the y-axis. Circle in the downer right corner indicating the direction and strength of measured fitness associated parameters. Salinity treatment is not depicted as no significant effect or interaction was detected by the PERMANOVA (Tab. 3), dark red = heat stressed & full light, light red = heat stressed & shaded; dark blue = ambient temperature & full light; light blue = ambient temperature & shaded.

Table 3. PERMANOVA results of eelgrass fitness associated measures (leaf growth rate, side shoot production, survival rate, root starch concentration, leaf starch concentration, leaf sucrose concentration, plant dry weight) according to experimental treatments.

	Df	**SS**	**MS**	**F model**	**R^2**	**P-value**
Temperature	1	4.87	4.87	3.31	0.06	**0.03 ***
Inoculation	1	0.51	0.51	0.34	<0.01	0.77
Salinity	1	2.00	2.00	1.36	0.02	0.25
Light	1	4.23	4.23	2.88	0.05	**0.05 .**
Temperature : Inoculation	1	0.60	0.60	0.40	<0.01	0.72
Temperature : Salinity	1	1.42	1.42	0.96	0.01	0.39
Temperature : Light	1	2.72	2.72	1.85	0.03	0.15
Inoculation : Salinity	1	0.56	0.56	0.38	<0.01	0.74
Infection : Light	1	3.49	3.49	2.37	0.04	**0.07 .**
Salinity : Light	1	2.09	2.09	1.42	0.02	0.23
Temperature : Inoculation : Salinity	1	1.02	1.02	0.69	0.01	0.52
Temperature : Inoculation : Light	1	1.02	1.02	0.69	0.01	0.52
Temperature : Salinity : Light	1	1.52	1.52	1.03	0.02	0.36
Inoculation : Salinity : Light	1	2.08	2.08	1.41	0.02	0.24
Temperature : Inoculation : Salinity : Light	1	0.36	0.36	0.25	<0.01	0.85
Residuals	32	47.05	1.47		0.62	
Total	47	75.61			1.00	

Discussion

It is still contentious whether or not marine diseases are increasing in the face of global warming and increasing anthropogenic pressure on marine ecosystems (Harvell et al. 2002; Lafferty et al. 2004). Here, we present one of the first studies investigating the combined effect of multiple stressors on any marine host-pathogen system, which focused on the eelgrass - *Labyrinthula zosterae* interaction, which can provoke wasting disease in eelgrass. This host-pathogen system is hypothesized to have dramatic consequences for an entire ecosystem, if virulence increases in the interaction, as has happened presumably in the 1930s when the trans-Atlantic population of eelgrass disappeared to a huge extent (Muehlstein 1989).

Here, we tested the combined effect of elevated temperature and shading imposing heat stress and low light stress on eelgrass plants, and salinity level on the eelgrass - *Labyrinthula zosterae* interaction. Neither of the conditions turned the protist *L. zosterae* to lethal pathogen in the time span of our experiment. An increase of virulence would be anticipated for changing environmental conditions, if the environmental stressor favors the potential pathogen and/or reduce the host defense status (e.g. due to heat stress: Roth et al. 2010).

In our experiment we found neither of these two scenarios. Contrary, we detected a previously not known synergistic interaction between high temperatures and low salinity level which resulted in a containment of wasting disease symptoms accompanied with only marginal numbers of *Labyrinthula zosterae* cells within eelgrass leaf tissue. We argue that this was caused by a sensitivity of *L. zosterae* to the prevailing conditions. A similar decrease of wasting disease symptoms was reported for *Posidonia oceanica* and *Cymodocea nodosa* in the Mediterranean after experimental infection with a *Labyrinthula sp.* isolate from its respective host for temperatures above 28°C (Olsen et al. 2014; Olsen and Duarte 2015). In-vitro cultivations of these *Labyrinthula sp.* isolates revealed that temperatures of > 28°C decreased its reproduction. Similarly, strongly declined reproduction rates in low saline medium and inability to produce ectoplasmic network has been found for several marine *Labyrinthula spp.* (Young 1943; Pokorny 1967; Muehlstein et al. 1988; Martin et al. 2009). Thus, we suspected that the *L. zosterae* isolate in our experiment could sustain low salinity and high temperature alone, but a combination of both stressors probably led to reduced performance, which affected the ability to infect the eelgrass host. Whether or not the ectoplasmic network plays an essential role for the infection process is not known. It seems reasonable that the ability to attach and move along the host plant is essential to infect host plant. A careful study of the infection process by microscopy under different abiotic conditions might be informative to reveal infection mechanisms and its dependency of abiotic conditions.

Different to the Mediterranean, where high summer temperatures reduce pathogen pressure, our results show that under favorable salinities (25) the tested *Labyrinthula zosterae* isolate can sustain and efficiently infect eelgrass at temperatures of 27°C. This means that in temperate regions, where 27°C are at the extremely warm end of possible sea surface temperatures, *L. zosterae* could potentially thrive, yet in our experiments this did not translate to high virulence.

Further, we investigated the inhibition capacity of eelgrass leaf extracts on *L. zosterae* to assess chemical defense of the host. Neither of the treatment combinations was able to reduce the inhibition capacity considerably (Fig. 6). Though we do not know how inhibition capacity in the assay relates to in-planta defense capacity, it is a first indication that neither low light stress, nor heat stress nor the combination of them reduces the chemical defense. Detected eelgrass chemical defense might be one reason that hinders the spread of *L. zosterae* throughout the plant under non-stressful conditions as well as under stressful conditions. Our result also corroborate studies of the inhibitory effect of eelgrasses collected from different salinity regimes on the Swedish west coast that found no effect of salinity regime origin (Jakobsson-Thor et al. in revision). In contrast to studies on phenolic acids, which has been hypothesized to be a chemical defense against *L. zosterae* (Vergeer et al. 1995), the inhibitory effect on *L. zosterae* by *Z. marina* compound extracted in our study did not decrease by light limitation nor elevated temperatures. These results question the exclusive role of phenolic acids as defense compounds against *L. zosterae* in eelgrass.

However besides host defense, as well the ability to tolerate infection will influence the host fitness. An important tolerance mechanism might be the compensation of necrotic tissue due to leaf growth. Under the treatment combination of low light intensity, high temperature and high salinity level necrotic lesion expansion exceeded leaf growth rates. The consequence is thus, a net loss of photosynthetic active tissue. In our experiment we applied shading for only 10 days. Afterwards, leaf growth rates recovered. It seems reasonable, that if lesion expansion exceeds leaf growth over longer time periods, even low pathogenic *L. zosterae* genotypes will become detrimental and lead to severely declining eelgrass population. Thus, we believe that eelgrass meadows under chronically light limitation are especially sensitive to *L. zosterae* infection.

Concerning the eelgrass wasting disease in the 1930s, it seems unrealistic that light stress occurred in parallel in eelgrass populations on the European and American Atlantic coast. Light limitation probably has favored eelgrass die-off in some regions as reported from the Netherlands (Giesen et al. 1990). However, it seems unlikely that light limitation has been the initial and exclusive reason to trigger such a large-scale trans-Atlantic eelgrass die-off. One of the other hypotheses discussed seems more likely: A

very virulent form of the genus *Labyrinthula* evolved and disappeared when most host plants were wiped out. Further, the most susceptible eelgrass plants were eliminated from the populations in the 1930s, resulting in plants presently (more) resistant to the pathogen *L. zosterae*. Alternatively, and not mutually exclusively, another yet unknown pathogen infected eelgrass additionally to *L. zosterae.* Other species besides *Labyrinthula spp.* have been suspected to cause eelgrass die-off, e.g. Van der Werft inspected eelgrass from a die-off event 1980s in the Netherlands and suspected an endophytic green algae, Chaetophoraceae, to have caused the die-off (Nienhuis 1994).
To which extent the result observed here can be generalized or are an attribute to the specific isolated *L. zosterae* strain in this study is unclear. It has been shown, that *Labyrinthula sp.* isolates can differ in ability to infect eelgrass plants (Groner et al. 2014; Martin et al. 2016). We characterized our isolate by sequencing the 18S rDNA to confirm 100% matching base pairs to pathogenic forms of the genus *Labyrinthula*. The study by Martin and co-authors which connected phylogeny to pathogenicity assays and included strains from various regions was an essential first step (Martin et al. 2016). However, in order to uncover the nature of eelgrass and *Labyrinthula zosterae* interaction we need to go further and investigate genetic diversity and connectivity in combination with pathogenicity and virulence assays at a fine scaled genetic resolution including isolates from the entire northern hemisphere.

In conclusion, this work contributes to the idea that contemporary *L. zosterae* isolates in the south-western Baltic do not represent an immediate risk for eelgrass meadows even under stressful condition, with the exception that under chronic light stress we cannot exclude that lesion spread might exceed leaf growth rates which in the long run will be detrimental. However, other *L. zosterae* genotypes might behave differently. Thus, behavior of other *L. zosterae* genotypes and other *Labyrinthula spp.* should be assessed in future studies. Nevertheless, rapid evolution of microparasites with short generation time, host switching, or invasion of allopatric strains might generate virulent eelgrass pathogens and always represent a danger.

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Synthesis

A series of experimental infections with naïve eelgrass plants revealed that contemporary *Labyrinthula zosterae* isolates from the south-western Baltic and the North Sea are associated with low to absent levels of virulence. Even under a combination of stressful conditions, in particular a 10 day period of low light and heat stress (Chapter 3), or nutrient limitation (Chapter 2), *L. zosterae* infection in eelgrass was not detrimental to plants. This is in stark contrast to reports from the 1930s that describe eelgrass decay by necrotic lesions within a few days (Renn 1935, 1937). Thus, my thesis contributes to the idea that contemporary *L. zosterae* isolates from the south-western Baltic and the North Sea do not represent an acute threat to eelgrass beds in this region. As in any host-pathogen interaction, the difference to the historic data may either arise due to 1) resistance evolution of the host plant, and/or 2) partial loss of *L. zosterae´*s virulence factors. I briefly discuss the available evidence for both scenarios. Furthermore, I shortly elaborate the environmental influence on the plant - protist interaction and finally give a broader outlook on marine plant - microbe associations.

Identity and possible evolution of the pathogen

Genetic background of the microbe was characterized in my studies by 18S rDNA sequence (Chapter 3) and diagnostic sites from the inner-transcribed spacer sequence (ITS) (see Bergmann et al. 2011) (Chapter 1 & 2), confirming identity to what was called by Martin and co-authors (2016) 'haplotype 1', the putative species that has been described in the context of wasting disease (= *L. zosterae*). Yet, the oldest available sequences from *L. zosterae* were recovered from two isolates picked in the year 2001 (NCBI Genbank, August 2017). Thus, whether or not the current 'haplotype 1' (= *L. zosterae*) is indeed the descendent that led to the wasting disease incident in the 1930s and/or the 1980s remains open. Only very few putative species of the genus *Labyrinthula* are known to induce symptoms in seagrasses. Until today, *L. zosterae* is the only known putative species that is able to induce symptoms in *Zostera spp.* (Martin et al. 2016). However, this finding might be a bias of little research effort. If available, investigation of historic DNA in conserved eelgrass samples from the moment and place of disease might shed some light on the identity of the occurring strains in the 1930s. Investigations of historic DNA from herbaria samples in the pathosystem potato - *Phytophthora infestans* revealed that the genotype that caused the Irish famine in the 19th century is very distinctive from today's occurring genotypes (Yoshida et al. 2014). *Labyrinthula spp.*

probably inhabited eelgrass even before the wasting disease in 1930s indicated by necrotic lesions found on old herbaria eelgrass specimen (Den Hartog 1989). Thus caution will be necessary when interpreting the results of historic samples, as the solely presence of *Labyrithula spp.* during disease will not imply causality here.

Currently *L. zosterae* has been verified by molecular identification from eelgrass beds in the Northern Pacific (east coast), Northern Atlantic (east and west coast), Baltic Sea and the Mediterranean (Bockelmann et al. 2013; Martin et al. 2016). As a differentiation along these large geographic scales is currently impossible via currently used molecular markers, the design of new molecular markers with highly improved resolution seems mandatory. These will allow investigating the genetic based variation of virulence and protist behavior, as well as their geographical distribution. One way to investigate this would be to perform a restriction-site associated DNA (RAD) tag sequencing study of *L. zosterae* isolates sampled over the entire northern hemisphere, together with a standardized virulence assessment procedure.

Though highly speculative, one could hypothesize, that the identified low virulence level of *L. zosterae* in the south-western Baltic and intertidal system of the North Sea is associated to the frequent changes in salinity in the study region. One would expect an association between varying salinity level and low virulence, if low virulence towards eelgrass is a trade off to a greater salinity tolerance or if for other reasons a low salinity environment selects for individuals with lower virulence. Such trade-offs have been reported already in various host - pathogen systems, for example in *Phytophthora infestans* showing reduced growth rates due to adaptation to increased temperatures (Yang et al. 2016).

Acquired resistance of the host

Plant resistance is shaped by the integration of diverse traits, which can be very distinct, e.g. secondary metabolite production, induction of hypersensitive response upon recognition by resistance-genes (R-genes) or the expression of antimicrobial peptides (Bednarek and Osbourn 2009; Daudi et al. 2012; Spoel and Dong 2012). Analysis of the genome of *Zostera marina* reveals the absence of diverse genes that are associated with pathogen resistance in terrestrial plants. As an example, a relatively small number of R-genes, chitinases, and flavenoid synthesizing enzymes are encoded in the *Zostera marina* genome compared to terrestrial ancestors. Furthermore, genes of the ethylene signaling pathway are lacking (Olsen et al. 2016). The question arises, how eelgrass

deals with the great abundance of marine microorganism, including potential pathogens like *Labyrinthula zosterae,* as they are missing a considerable number of genes, which in terrestrial angiosperms are responsible for pathogen resistance. I investigated the differential gene expression of a small number of potential host defense genes upon inoculation with *Labyrinthula zosterae* (Chapter 1 & 2). A large proportion of these targeted genes were differentially expressed 50 hours post inoculation. Most targeted genes were down regulated, revealing a re-shaping of the expression pattern upon infection with *L. zosterae,* but did not elucidate further molecular interaction. The applied approach is limiting in several aspects: 1) only a small number of genes can be investigated and 2) due to the targeting approach only genes with an a priori hypothesis are addressed. Furthermore, whether or not expressed genes mediate indeed resistance remains obscure. Genes involved in fast evolving gene-for-gene interactions between co-evolving host and pathogen may lose or gain their effectiveness fast depending on the prevailing pathogen genotypes (Rausher 2001). A well-studied example for the gene-for-gene model in plant - pathogen evolution are the complementary resistance-genes (host) and avirulence-genes (pathogen) (Jones and Dangl 2006). The high specificity of these complementary genes is illustrated by the fact that even small changes in the nucleotide sequence of one gene can switch a non-susceptible to a susceptible host - pathogen interaction. This has been shown for tomato - *Cladosporium fulvum* interaction by a single nucleotide change in the avirulence gene C*f*4 (Joosten et al. 1994). R - genes are mediating resistance mostly in interactions with biotrophic pathogens, resistance to necrotrophic pathogen is achieved by other mechanisms (Glazebrook 2005). These are less well understood, however, WRKY transcription factors that regulate cross talking of signalling pathways seem to play an essential role for resistance to necrotrophs (Zheng et al. 2006; Birkenbihl and Somssich 2011).

To identify potential resistance mediating genes in *Z. marina* against *L. zosterae* one approach would be to perform a well-designed differential gene expression study that analyzes the full host-transcriptome at different infection stages. This approach will raise new hypothesis of which genes may be associated to the defense of *L. zosterae.* Finally, one would need to assess effectiveness of these genes e.g. by gene silencing and assessing the susceptibility to *L. zosterae* infection.

Besides resistance mechanism, tolerance might play an important role in the *Zostera marina* - *Labyrinthula zosterae* interaction. The increased leaf growth rates in Chapter 1 may be an adaptive trait for tolerating *L. zosterae* infection. Evolutionary theory predicts that contrary to resistance traits that underlie a frequency dependent selection tolerance traits will become fixed over time in a population (Roy & Kirchner 2000). Thus, I suspect

that the fitness response to *L. zosterae* infection of an eelgrass genotype will be the integration of its resistance traits which are probably polymorphic in a population, and its tolerance traits which are more likely to be fixed in the population.

Influence of the environment on eelgrass - protist interaction

There is an ongoing discussion, how global environmental change affects host – pathogen interaction and whether disease outbreaks will increase in the future (Harvell et al. 1999, 2002, 2009, Lafferty et al. 2004, Ward & Lafferty 2004, Lafferty 2009). My findings for the eelgrass - *Labyrinthula zosterae* interaction emphasize the complex nature of host - pathogen - environment interactions. Response to environmental parameters was shaped by the chemical defense capacity, host leaf growth compensation capacity and fitness of the microbe, which were partially affected in opposed directions. While general stress on the host did not affect chemical defense, eelgrass leaf growth was negatively affected which might reduce tolerance to *L. zosterae* infection (though not visible in our short stress period). Further, I detected a synergistic effect of high temperature and salinity on *L. zosterae* performance. These results underline the need of studying of individual systems and the necessity of complex experimental designs that test relevant interactive effects of environmental factors (see as well Holmstrup et al. 2010; Gunderson et al. 2016).

A new look onto marine plants - the seagrass holobiont

Plants are colonized by a plethora of microbes and viruses. It is widely recognized that plant - microbe symbiosis is essential for plants to withstand in their environment and that microbes contribute to the well-being of its host, e.g. mycorrhizae providing nutrients, or some fungal endophytes increasing stress resistance (Vandenkoornhuyse et al. 2015). Consequently the displayed host phenotype is not only a product by itself, but arises from interaction with all its associated microbes. Recognizing this unit of host and associated microbes, the term “holobiont” has been shaped (e.g. Bourne et al. 2009; Bordenstein and Theis 2015; Theis et al. 2016). Applying the holobiont model, disease can be understood not only as the result of a single interaction between host and pathogen, but rather as the result of a shift in a microbial community, where a diverse microbial community gets displaced by one where the pathogen is dominating (Egan & Gardiner 2016).

Recent studies focused on the description of seagrass associated microbial communities (Cúcio et al. 2016, Rotini et al. 2017, Fahimipour et al. 2017), recognizing the diversity of associated microorganisms and speculating about their potential functions. In chapter 2 of this thesis I hypothesized that *L. zosterae* facilitates eelgrass growth by enhanced internal nutrient recycling. Though no indication for such facilitation could be detected, further features of *L. zosterae* infection remain to be investigated. It is a wide open and worthwhile question as to which role associated microorganism play in the context of e.g. seagrass recruitment, nutrient uptake, pathogen and grazer defense or resistance to abiotic stressors and thus help to preserve seagrass stands. While seagrasses are declining at alarming rates, knowledge about beneficial microbial associations might help to successfully lead reestablishment of seagrass meadows.

Conclusion

In conclusion, this is to the best of my knowledge, the first systematic characterization of a *Labyrinthula spp.* - seagrass interaction where seagrass plants were reared from seeds to control prior infection experience. My thesis supports the idea that contemporary *L. zosterae* isolates from the North Sea and the south-western Baltic reveal rather low virulence even under stressful environmental conditions to the plant host. It is not clear whether contemporary *L. zosterae* isolates are descendants from the 1930s, or whether these highly virulent isolates are extinct. However, high abundances of contemporary *L. zosterae* in eelgrass stands may represent a reservoir from where more virulent *Labyrinthula spp.* forms may evolve. My thesis gives a first insight which role associated microorganism can play in seagrass and contribute thus to a slightly improved picture of a seagrass holobiont.

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Appendix

The following supplement accompany the article

Does nutrient limitation alter marine plant – microbe interaction between ecosystem founding species eelgrass Zostera marina and the protist *Labyrinthula zosterae*?

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Supplementary table 1:

Results of the full linear mixed model ANOVA for *Labyrinthula zosterae* concentration, wasting disease index (WDI) and eelgrass growth parameters between nutrient treatment, inoculation and their interaction, with the random factors "Tank" and "Inoculation nested in Tank". Significant results are shown in bold ($p < 0.05$).

	Variable	F	df	Res-df	P	Var	StdDev
Labyrinthula cells (2 days p.i.) [cells mg eelgrass dry weight^{-1}]	Nutrient	<0.001	1	8	0.984		
	Inoculation	73.608	1	4	**0.001**		
	Nutr:Inoc	3.056	1	4	0.155		
	Tank					39.2	6.261
	Tank/Inoculation					218.8	14.792
WDI - Leaf 2 & 3 [categorical index]	Nutrient	0.012	1	9	0.915		
	Inoculation	220.498	1	4	**<0.001**		
	Nutr:Inoc	3.286	1	4	0.138		
	Day	223.761	1	399	**<0.001**		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
	Plant ID					0.133	0.364
Leaf growth rate [cm day^{-1}]	Nutrient	1.116	1	8	0.3256		
	Inoculation	1.872	1	4	0.2294		
	Nutr:Inoc	0.004	1	4	0.9562		
	Tank					0.000	0.000
	Tank/Inoculation					0.035	0.187
Biomass [g]	Nutrient	8.978	1	8	**0.017**		
	Inoculation	0.651	1	4	0.465		

	Nutr:Inoc	0.558	1	4	0.497		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
Shoot production [number of sideshoots main shoot^{-1}]	Nutrient	5.894	1	8	**0.041**		
	Inoculation	1.284	1	4	0.321		
	Nutr:Inoc	0.472	1	4	0.530		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
Leaf production [number of leaves main shoot^{-1}]	Nutrient	0.670	1	8	0.437		
	Inoculation	0.074	1	4	0.799		
	Nutr:Inoc	0.930	1	4	0.390		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000

Supplementary table 2:

Results of a linear mixed model ANOVA for gene expression –ΔCt values of 15 targeted genes between nutrient treatment, inoculation and their interaction. For gene abbreviations see Tab. 5. Significant results are shown in bold ($p < 0.05$).

	Variable	**F**	**df**	**Res-df**	**P**	**Var**	**StdDev**
SOD	Nutrient	3.8107	1	8	0.089		
	Inoculation	42.0630	1	4	**0.002**		
	Nutrient:Inoculation	3.6690	1	4	0.128		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
GST	Nutrient	0.800	1	8	0.397		
	Inoculation	25.531	1	4	**0.007**		
	Nutrient:Inoculation	6.862	1	4	0.059		
	Tank					0.000	0.000
	Tank/Inoculation					0.113	0.336
APX	Nutrient	0.115	1	8	0.744		
	Inoculation	0.920	1	4	0.390		
	Nutrient:Inoculation	0.008	1	4	0.931		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
CAT	Nutrient	0.009	1	8	0.928		
	Inoculation	15.879	1	4	**0.015**		
	Nutrient:Inoculation	0.789	1	4	0.425		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
HSP 80	Nutrient	0.567	1	8	0.473		
	Inoculation	11.227	1	4	**0.032**		
	Nutrient:Inoculation	0.530	1	4	0.507		
	Tank					0.000	0.000
	Tank/Inoculation					0.580	0.762
HSP 70	Nutrient	3.126	1	6	0.130		
	Inoculation	8.364	1	4	**0.042**		
	Nutrient:Inoculation	2.223	1	4	0.210		
	Tank					0.245	0.138

	Tank/Inoculation					0.019	0.138
Starch synthase	Nutrient	0.026	1	8	0.875		
	Inoculation	0.402	1	4	0.560		
	Nutrient:Inoculation	0.057	1	4	0.822		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
Fructose biphophate aldolase	Nutrient	0.360	1	8	0.565		
	Inoculation	0.938	1	4	0.388		
	Nutrient:Inoculation	0.674	1	4	0.458		
	Tank					0.000	0.000
	Tank/Inoculation					0.083	0.289
Chlorophyll synthase	Nutrient	0.091	1	8	0.771		
	Inoculation	22.135	1	4	0.009		
	Nutrient:Inoculation	0.545	1	4	0.501		
	Tank					1.750	1.323
	Tank/Inoculation					0.000	0.000
Rubisco	Nutrient	0.367	1	8	0.561		
	Inoculation	13.594	1	4	0.021		
	Nutrient:Inoculation	0.655	1	4	0.463		
	Tank					0.009	0.097
	Tank/Inoculation					0.000	0.000
Metacaspase	Nutrient	0.439	1	8	0.526		
	Inoculation	0.126	1	4	0.742		
	Nutrient:Inoculation	0.023	1	4	0.888		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
Chitinase	Nutrient	0.108	1	8	0.751		
	Inoculation	14.867	1	4	0.018		
	Nutrient:Inoculation	0.133	1	4	0.734		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
RPPA	Nutrient	1.810	1	8	0.217		
	Inoculation	7.270	1	4	0.058		
	Nutrient:Inoculation	0.870	1	4	0.404		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000
Prot-206	Nutrient	0.023	1	8	0.884		
	Inoculation	0.176	1	4	0.697		
	Nutrient:Inoculation	0.141	1	4	0.727		
	Tank					3.044	1.745
	Tank/Inoculation					0.253	0.503
CYP73A	Nutrient	0.564	1	8	0.472		
	Inoculation	68.406	1	4	0.001		
	Nutrient:Inoculation	0.199	1	4	0.679		
	Tank					0.000	0.000
	Tank/Inoculation					0.000	0.000

Description of author contributions

The chapters of this thesis are published (chapters 1 and 2) or prepared for submission (chapter 3) to peer-reviewed journals under multiple authorship. The following list describes my specific contribution to each publication.

Chapter 1:

Current European *Labyrinthula zosterae* are not virulent and modulate seagrass (*Zostera marina*) defense gene expression

Authors: Janina Brakel, Franziska Julie Werner, Verena Tams, Thorsten BH Reusch and Anna-Christina Bockelmann

published in PLoS One 9:e92448 (2014)

Contributions: Conceived and designed the experiments: ACB JB TBHR. Performed the experiments: JB FJW ACB VT. Analyzed the data: JB ACB TBHR. Contributed reagents/materials/analysis tools: FJW VT. Wrote the paper: ACB JB TBHR.

Chapter 2:

Moderate virulence caused by the protist *Labyrinthula zosterae* in ecosystem foundation species *Zostera marina* under nutrient limitation

Authors: Janina Brakel, Thorsten B. H. Reusch, Anna-Christina Bockelmann

published in Marina Ecologic Progress Series 571:97–108 (2017)

Contributions: Conceived and designed the experiment: JB and ACB, Performed the experiment: JB. Performed the analysis: JB and ACB. Interpreted results and wrote manuscripts: JB ACB TBHR.

Chapter 3:

Multifactorial stressor experiment reveals strong interaction of temperature and salinity on eelgrass - protist interaction.

Authors: Janina Brakel, Stina Jakobsson-Thor, Anna-Christina Bockelmann, Thorsten B. H. Reusch

manuscript prepared for submission

Contributions: Conceived and designed the experiment: JB, ACB, TBHR; Performed the experiment: JB; Performed the analysis: JB and SJT; discussed results and wrote manuscript: JB, SJT and TBHR.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel:

"Eelgrass disease dynamics: An experimental analysis of the eelgrass - *Labyrinthula zosterae* interaction"

selbstständig, mit der Beratung meiner Betreuer, verfasst habe. Die Dissertation ist in Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel und Quellen verwendet. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden. Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und ist mein erstes und einziges Promotionsverfahren.
Teile dieser Arbeit wurden in wissenschaftlichen Fachzeitschriften publiziert (Kapitel 1: "Current European *Labyrinthula zosterae* are not virulent and modulate seagrass (*Zostera marina*) defense gene expression" mit den Autoren: Janina Brakel, Franziska Julie Werner, Verena Tams, Thorsten BH Reusch and Anna-Christina Bockelmann ist publiziert in PLoS One 9:e92448 (2014). Kapitel 2: "Moderate virulence caused by the protist *Labyrinthula zosterae* in ecosystem foundation species *Zostera marina* under nutrient limitation" mit den Autoren: Janina Brakel, Thorsten B. H. Reusch, Anna-Christina Bockelmann ist publiziert in Marina Ecologic Progress Series 571:97–108 (2017)). Die Koautoren aller Kapitel befinden sich zu Beginn des jeweiligen Kapitels in der Autorenliste. Der Anteil der Autoren an den Manuskripten wird im Abschnitt „Author contributions“ erläutert.

Kiel, den 24.08.2017 _____