Eelgrass disease dynamics:

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

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Contents

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

PLoS One 9:e92448 (2014)

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

Marine Ecologic Progress Series 571:97–108 (2017)

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

prepared for submission

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*)?
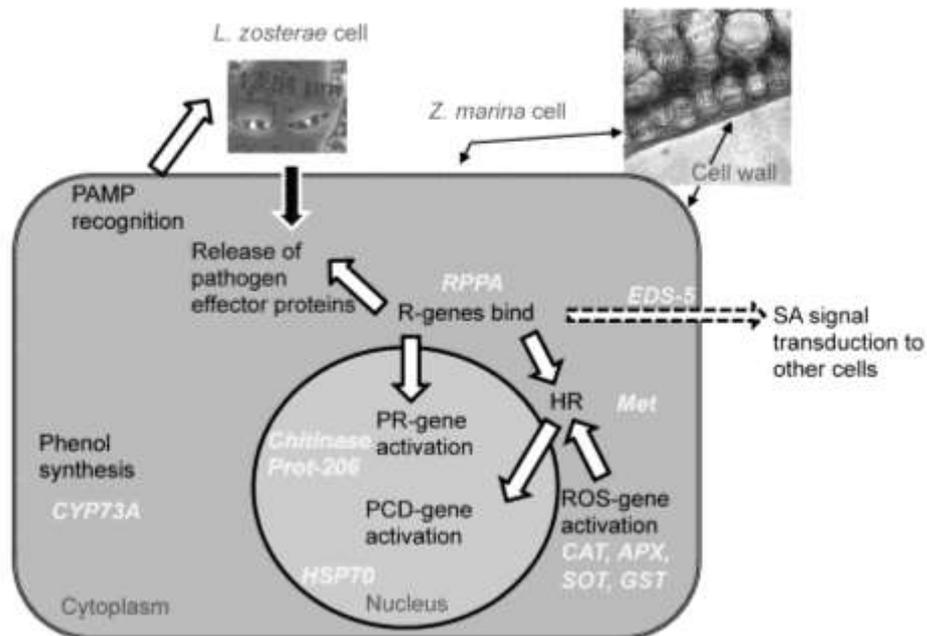
Figure 1. Defense mechanism of *Zostera marina.*

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

<u>*Labyrinthula zosterae* isolation and cultivation</u>

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.
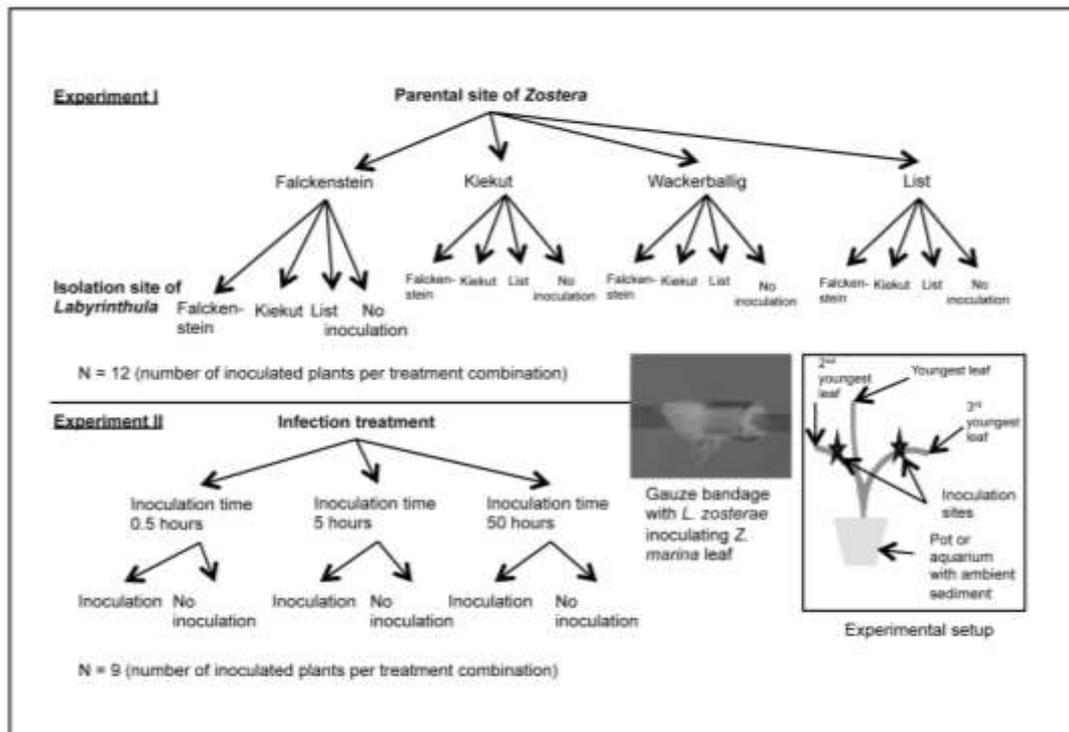

Figure 2. Experimental design and setup of experiment I and II.

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$_2$O 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 marina* (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).
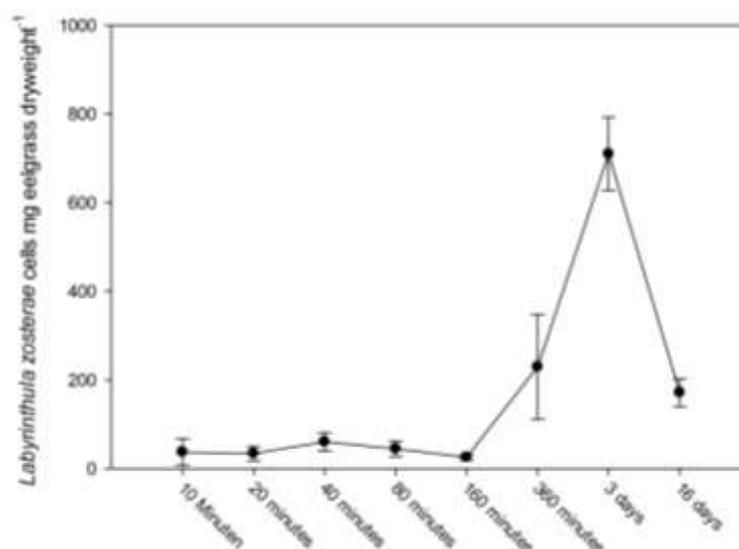

Figure 3. Abundance of *Labyrinthula zosterae* cells per mg *Zostera marina* leaf sample (dry weight) depending on inoculation time during experimental *L. zosterae* infection. Results are partly from experiment I and II, means with standard error bars.

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]:

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

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´-CTCTTCTAGCACGCAATTGG-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).

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

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

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

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.
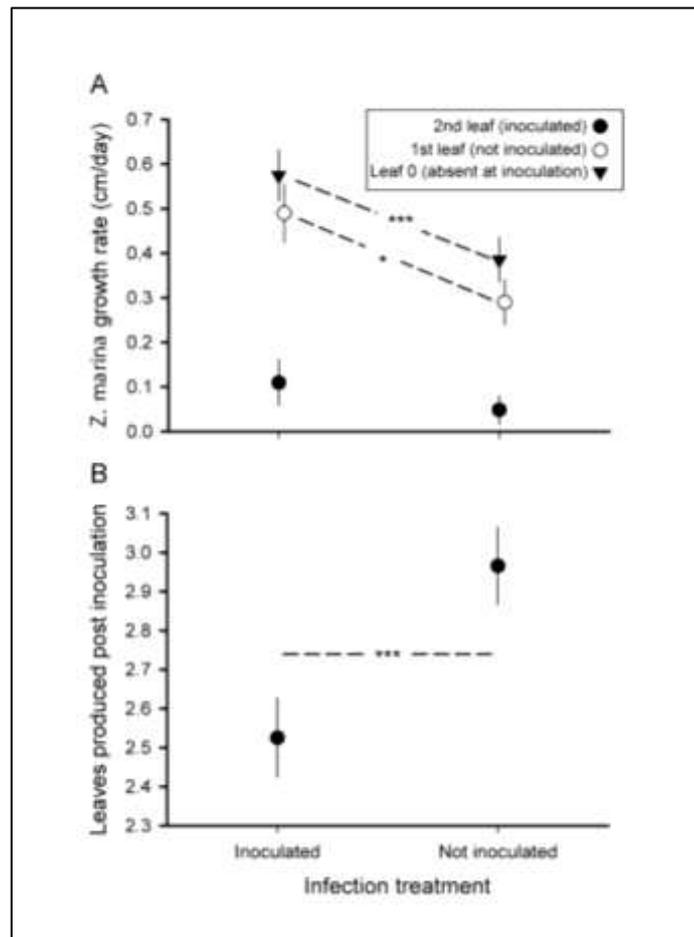
Figure 4. Growth (**a**) and leaf production (**b**) of *Zostera marina* leaves 2-4 weeks after experimental infection with *Labyrinthula zosterae*. 2nd leaf = inoculated 2nd oldest leaf of each *Zostera marina* shoot (growth measured 1st to 2nd week post inoculation), 1st leaf = youngest leaf at inoculation, 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).
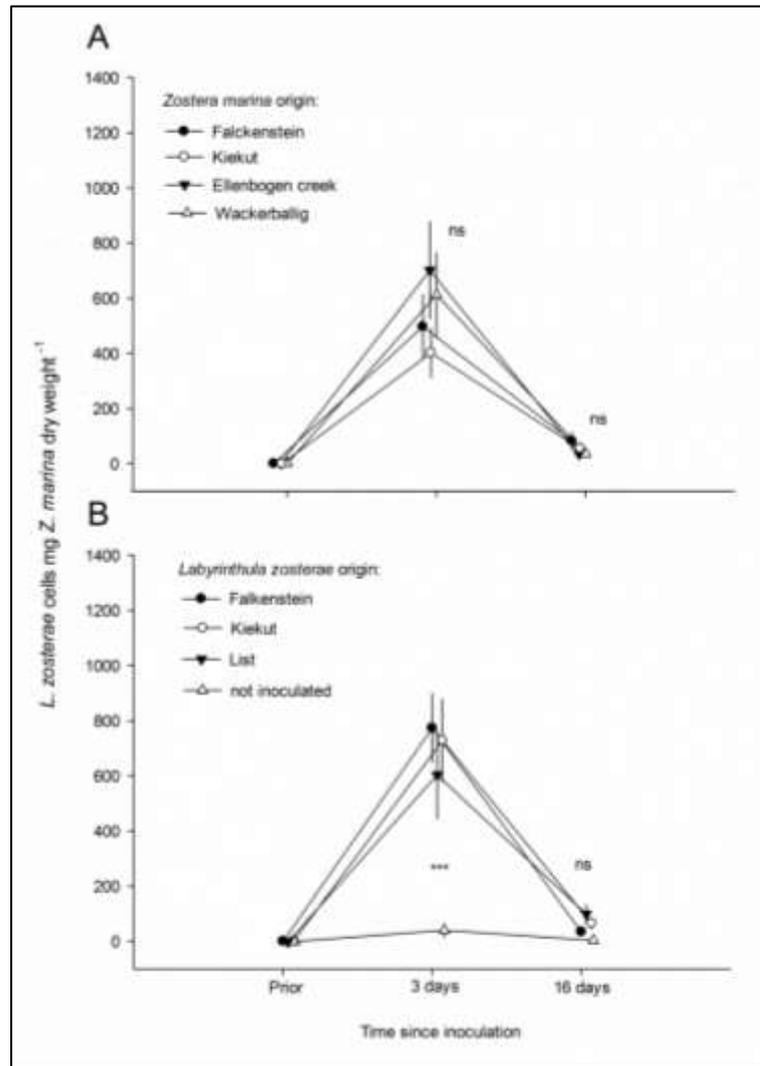
Figure 5. Abundance of *Labyrinthula zosterae* cells per mg *Zostera marina* leaf sample (dry weight) after experimental inoculation depending on the parental site of *Z. marina* (a) and the isolation site of *L. zosterae* (b). *** indicates significant differences at $p<0.01$, ns= not significant, means with standard error bars.
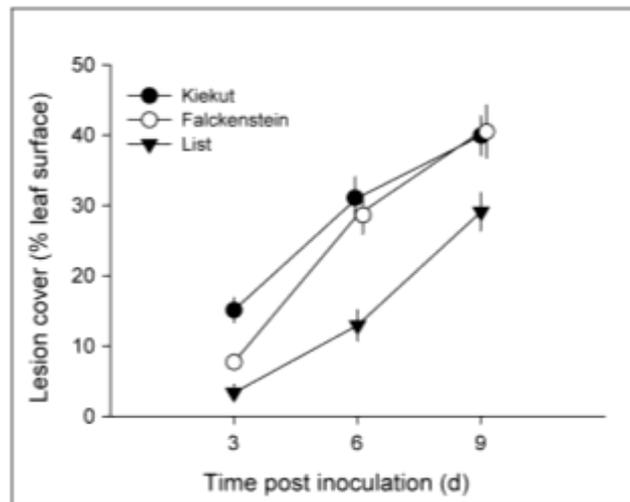
Figure 6. Spread of lesions on *Zostera marina* 2nd oldest leaves of different origin after experimental inoculation with *Labyrinthula zosterae,* *** indicates significant differences at p<0.01, means with standard error bars.

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/X^2	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).
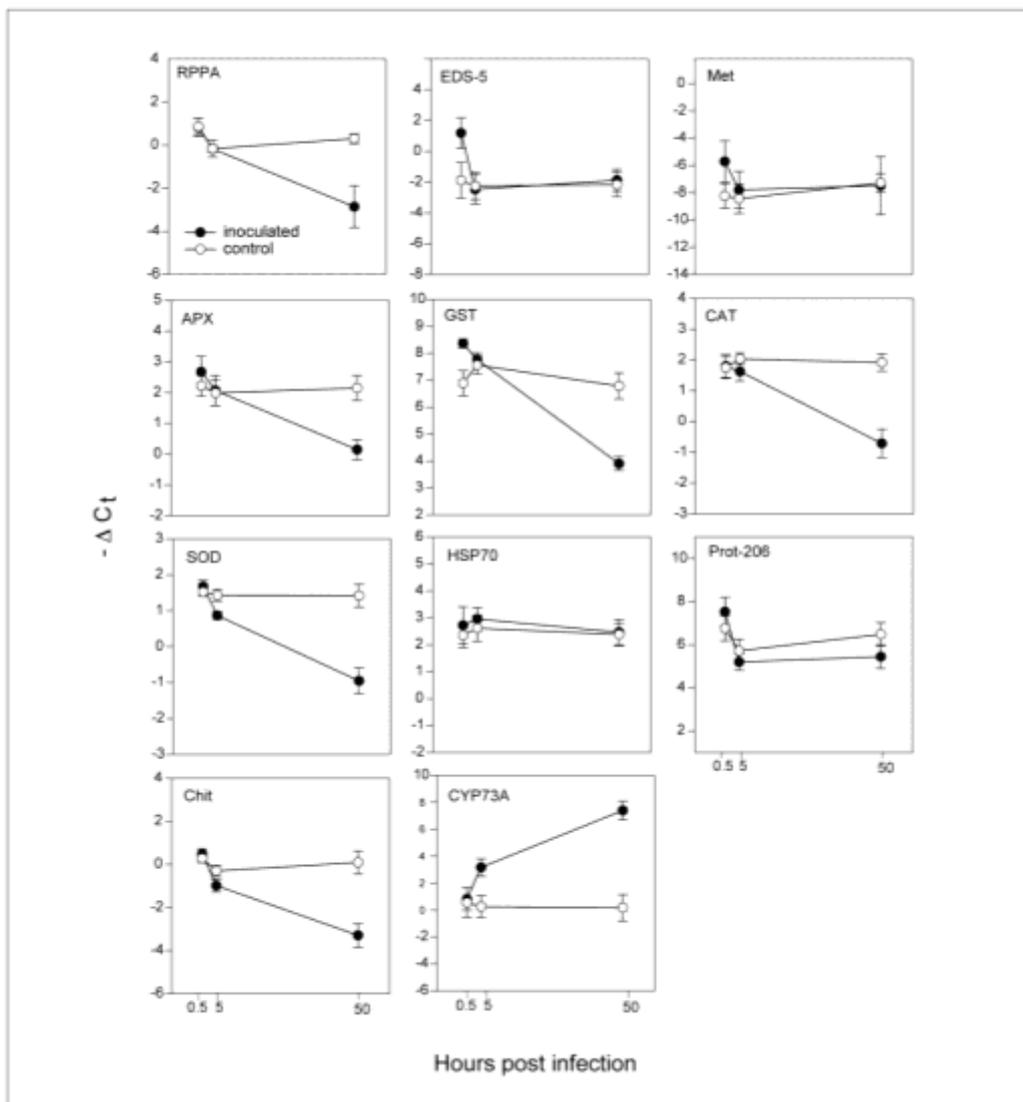

Figure 7. Gene expression of *Zostera marina* defense genes after experimental infection with *Labyrinthula zosterae*. I = inoculation treatment with *L. zosterae*, NI = no inoculation. Results have been normalized to *eIF4A* housekeeping gene. $-\Delta C_t$: log 2 scale. * indicates significant differences 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.

Chapter 3

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

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Prepared for submission

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.

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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54

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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 _____