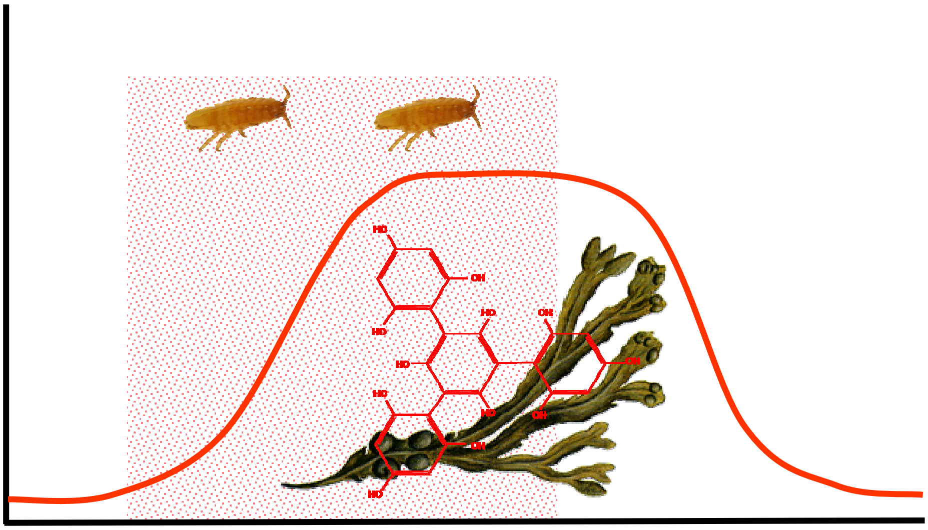
Defense induction in marine macroalgae:

Its prevalence, capabilities and limitations



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Zusammenfassung

Herbivorie beeinflusst wesentlich die Biomasse von Makroalgen, ihre Abundanzen und ihre Verbreitung. Insbesondere in marinen Systemen konsumieren Herbivore oft 50 – 100% der Algenproduktion. Jedoch spielen Algen nicht nur eine passive Rolle in ihren Interaktionen mit Herbivoren, sondern haben verschiedene Strategien entwickelt, um der Herbivorie zu begegnen. Eine Anpassung ist die Produktion von Sekundärmetaboliten mit Fraßhemmender Wirkung. Diese Metabolite können entweder kontinuierlich in aktiven Konzentrationen gebildet werden (konstitutive Verteidigung), oder die Produktion wird durch einen externen Reiz ausgelöst (induzierte Verteidigung). Induzierte Verteidigung ist von Vorteil, wenn die Verteidigung den Algen metabolische Kosten verursacht, der Fraßdruck von Konsumenten zeitlich variabel ist und eine erhöhte Variabilität der Verteidigung ihre Effektivität erhöht. Bei terrestrischen Pflanzen sind die Verbreitung und die Mechanismen der induzierten Verteidigung bereits ausgiebig dokumentiert worden. In Makroalgen sind jedoch die Verbreitung von induzierter Verteidigung und auch die induzierenden Reize kaum bekannt. Auch die Interaktionen mit anderen Faktoren wie Stress und die zeitliche Dynamik von Verteidigungsinduktion in Makroalgen sind unbekannt.

Diese Arbeit untersuchte das Auftreten von induzierter Verteidigung und die Art der induzierenden Reize bei den Algenarten *Fucus serratus*, *Fucus evanescens* (Phaeophyceae), *Delesseria sanguinea*, *Phyllophora pseudoceranoides* und *Furcellaria lumbricalis* (Rhodophyta) (Kapitel I). Diese perennierenden Arten sind in der westlichen Ostsee weit verbreitet und bilden wichtige Habitate für viele assoziierte Organismen. Zudem wurde die Effektivität von den beiden Induktionsreizen direkter Fraß und Fraß an benachbarten Algenindividuen untersucht.

Alle untersuchten Rotalgen, *D. sanguinea*, *P. pseudoceranoides* und *F. lumbricalis,* reagierten auf direkten Fraß mit induzierter Verteidigung. Bei den Braunalgen *F. serratus* und *F. evanescens* konnten auch induzierte Effekte in Bezug auf Fraßverteidigung nachgewiesen werden, diese waren jedoch nicht konsistent über verschiedene Fraßversuche. Diese Ergebnisse unterstützen neuere Studien, die belegen, dass Verteidigungsinduktion in marinen Makroalgen ein verbreitetes Phänomen ist.

Meine weiteren Studien beschäftigten sich mit dem Blasentang *Fucus vesiculosus*. Diese Algenart ist eine der wichtigsten strukturierenden Algenarten der Ostsee, jedoch ist seit den 1970ern ein stetiger Rückgang der Populationen in der Ostsee zu verzeichnen. Es wird angenommen, dass dieser Rückgang Konsequenz von Eutrophierungsprozessen ist und mit Lichtlimitation und Veränderungen von Konkurrenz- und Prädationsdrücken einhergeht. Diese Annahmen stützen sich jedoch bisher auf Feldbeobachtungen ohne durch experimentelle Daten unterstützt zu werden.

Diese Studie zeigte, dass *F. vesiculosus* in der Kieler Förde schon ab 2 m Wassertiefe Licht-limitiert ist und die physiologische Tiefengrenze zwischen 4 – 6 m Tiefe liegt. Epibionten verstärkten den Effekt der Lichtlimitation. Diese reduzierte Tiefenverbreitung und der damit einhergehende Verlust immenser Biomasse könnten den Effekt von Herbivoren weiter verstärken und somit Verteidigungsmechanismen noch wichtiger machen.

Sowohl die Algenphysiologie, als auch Algen-Herbivor Interaktionen können durch abiotische Faktoren beeinflusst werden. Jedoch wurden Interaktionen von abiotischem Stress und Fraßverteidigung, insbesondere Verteidigungsinduktion, bisher kaum untersucht. Klimamodelle sagen für die Ostsee eine zukünftige Erhöhung der Wassertemperatur von ca. 4°C voraus. Diese Wassertemperatur entspräche derer, die *Fucus* an seiner südlichen Verbreitungsgrenze noch toleriert und kann somit physiologischen Stress darstellen. Um die Auswirkungen von reduziertem Lichtangebot und erhöhten Temperaturen zu untersuchen, führte ich einen zwei-faktoriellen Versuch durch, der nachwies, dass ein reduziertes Lichtangebot bei momentan vorherrschenden Temperaturen (15°C) keinen Effekt auf die Verteidigungsinduktion von *Fucus* hat, obwohl die Wachstumsraten signifikant reduziert wurden. Eine Temperaturerhöhung auf 20°C verhinderte jedoch jegliche Verteidigungsinduktion. Dadurch ist diese Studie eine der ersten, die nachweisen konnte, dass abiotische Stressfaktoren Einfluss auf Algen-Herbivor Interaktion nehmen und somit auch indirekt Populationen von Makroalgen beeinflussen können.

Die Koevolution von Verteidigung bei Algen und die Anpassungen der Herbivoren an die Verteidigung können zu sehr hoher Artspezifität dieser Interaktionen führen. Die zeitliche Dynamik von Verteidigungsinduktion- und deren Reduktion kann deshalb sehr spezifisch an die Herbivorenart angepasst sein. Ein Versuch dieser Arbeit löste sehr akkurat die zeitliche Dynamik der Verteidigungsinduktion von *F. vesiculosus* in Antwort auf Fraß der Meerassel *Idotea baltica* auf. Der Zeitraum bis zur effektiven Verteidigungsinduktion und die Abnahme der induzierten Verteidigung nach Beendigung des Fraßdrucks wurden quantifiziert und zeigten, dass *Fucus* nach zehn Tagen Fraßdruck von *Idotea* Verteidigung induziert. Bereits 2 - 4 Tage nach Beendigung des Fraßes wurde die Verteidigung wieder reduziert. Diese Ergebnisse weisen deutlich darauf hin, dass Verteidigung sparsam und nur nach längeren Fraßperioden eingesetzt wird, um substanziellen Verlust von Biomasse zu vermeiden.

Die vorliegende Arbeit bietet neue Erkenntnisse im Bereich der Prävalenz von Verteidigungsinduktion bei Makroalgen, der Variabilität von induzierter Verteidigung und den Interaktionen von Verteidigung mit biotischen und abiotischen Faktoren. Sie stellt zudem ein mögliches Szenario vor, wie sich die Klimaveränderungen auf die Interaktionen von *F. vesiculosus* und des Konsumenten *I. baltica* auswirken können und somit indirekt Einfluss auf die *Fucus*-Populationen der Ostsee nehmen können.

Summary

Herbivory is an important biotic factor affecting algal biomass, abundance, and distribution. Especially in marine habitats, herbivores often consume 50 -100% of the macroalgal production. Algae are not only passive player in this interaction but have evolved a variety of strategies to cope with herbivory. One strategy is the production of chemical metabolites that function as defense against consumers. These metabolites can either be continuously present at bioactive concentrations (constitutive defense), or be produced ‘on demand’, i.e. when appropriate cues indicate presence of consumers (inducible defense). Ecological theory postulates that inducible defense should be favoured when the defense incurs costs to algal fitness, when the presence of herbivores is variable or when increased chemical variability enhances its effectiveness. In vascular plants, the occurrence and mechanisms of inducible defense are well documented. However, the prevalence of defense induction, the cues triggering induction, the interactions with other factors like stress, and the temporal dynamics of defense induction in macroalgae are generally not well understood.

This thesis investigated the prevalence of defense induction and the nature of the inducing cues in the macroalgal species *Fucus serratus*, *Fucus evanescens* (Phaeophyceae), *Delesseria sanguinea*, *Phyllophora pseudoceranoides*, and *Furcellaria lumbricalis* (Rhodophyta) (Chapter I). These species are widely distributed perennial seaweeds of the western Baltic and represent important habitats for many associated species. Also, the efficiency of two proposed induction cues, direct grazing and waterborne cues, were investigated.

All tested red algae, *D. sanguinea, P. pseudoceranoides* and *F. lumbricalis*, induced defense in response to direct grazing. The brown algae *F. evanescens* and *F. serratus* also showed induced effects but these were not always significant in different types of feeding assays. This study supports recent findings that defense regulation in marine macroalgae is a rather common phenomenon, especially in the Baltic Sea.

My further experiments focused on the bladderwrack *Fucus vesiculosus*. Its populations in the Baltic have declined strongly from the 1970s. This decline has been attributed to eutrophication leading to light limited conditions and associated changes in competitive interactions and grazing pressure. However, these statements are based on observational data without support of experimental evidence. This study demonstrated that *F. vesiculosus* in the Kiel Fjord is already light-limited in 2 m water depth and its lethal depth limit lies between 4 - 6 m depth (Chapter II). Epibionts enhance the negative effect of reduced light conditions. The reduced depth distribution and consequently the massive biomass loss of the *Fucus*-population in the Baltic may be enhanced by the detrimental effects of herbivores which stresses the ecological importance of algal resistance to consumption.

Algal physiology and alga-herbivore interactions can be affected by abiotic factors. However, the interactions of environmental stress and defense, in particular defense induction, have not been assessed so far. Climatic models predict a water temperature increase of ca. 4°C for the Baltic. This would lead to water temperatures comparable to those at *Fucus'* southern distribution limit. They can, thus, be expected to represent a physiological stress for *Fucus*. To assess the interactive effects of reduced light and increased temperatures on the defensive performance of *F. vesiculosus*, I conducted a two-factorial experiment, which showed that reduced light conditions reduced growth rates significantly but had no effect on inducible defense at ambient temperatures (15°C), and that the predicted summer warming suppressed induced defensive responses completely. This study is one of the first that shows that abiotic stress can affect alga-herbivore interactions and therefore indirectly macroalgal population dynamics.

The coevolution of algal defense and herbivore tolerance of the defenses can result in high species specificities of the interactions. Temporal dynamics of induction and relaxation of defense may therefore be tuned the typical activity patterns of locally important herbivore species. This project identified the temporal dynamics of antifeeding defense regulation in a marine macroalga, *F. vesiculosus* in response to its main consumer, the isopod *Idotea baltica*. Time lag of induction and relaxation of induced feeding resistance were assessed. *F.*

vesiculosus induced defense 10 days after the onset of grazing by the isopod *Idotea baltica.* Defenses were relaxed within 2 - 4 days after cessation of grazing. Thus, defense seems to be deployed sparingly and only induced in longer periods of high grazing pressure to avoid substantial loss of tissue.

This thesis gives new insights in the prevalence and the variability of induced resistance and its interactions with other biotic and abiotic factors, a study field which this thesis has started to examine. Also, I present a possible scenario what effects the predicted climate changes may have on the interactions of *F. vesiculosus* and its consumer *I. baltica* and how these changes may consequently affect the *Fucus*-populations in the Baltic.

General Introduction

Strategies in macroalgal chemical defenses

In marine littoral habitats, herbivory is an important factor affecting macroalgal biomass, abundance and distribution (Duffy & Hay 2000; Lotze *et al.* 2001). Consumption by herbivores can typically remove a high percentage (50-100%) of macroalgal production (Cyr & Pace 1993; Hay 1991), which is on average thrice higher than in terrestrial communities (Cyr & Pace 1993). Beside the direct biomass loss by consumption, grazing can increase tissue loss by weakening and breakage of the thallus (Toth & Pavia 2006), increase pathogenic infections and decrease growth rates due to loss of meristematic tissue (Honkanen & Jormalainen 2002; Schmitz & Lobban 1976). Thus, grazing can be a major selective agent for macroalgal traits.

There are a number of hypotheses to explain and understand patterns of plant resistance to herbivory (reviewed in Cronin 2001; Stamp 2003). The most general framework on the evolution of plant defenses is provided by the optimal defense hypothesis (ODH) (Feeny 1976; Rhoades 1979). The ODH asserts that “organisms evolve and allocate defenses in a way that maximizes individual fitness” (Rhoades 1979:12) and that defenses are costly in terms of fitness because they divert resources from other needs. This theory aims to explain between-species, within-species and within-individual variation in defense and encompasses both evolutionary and ecological time scales (Cronin 2001).

If defenses are costly, natural selection will act to optimize the allocation of resources to defense. This can include within-plant allocation of defense in direct proportion to the vulnerability and the value of the tissue, or defense allocation with regard to temporal variation in grazing pressure. Especially chemical defenses have proven to be very variable at different scales (Karban & Baldwin 1997). If chemical defenses are permanently expressed (‘constitutive’), resources are required for synthesis, maintenance and storage even when grazers are absent and the benefits of protection are not realised. Therefore, the ODH predicts that, if grazing pressure is variable, defenses are produced at active concentrations only upon an indication of an increased grazing risk ('induced defense').

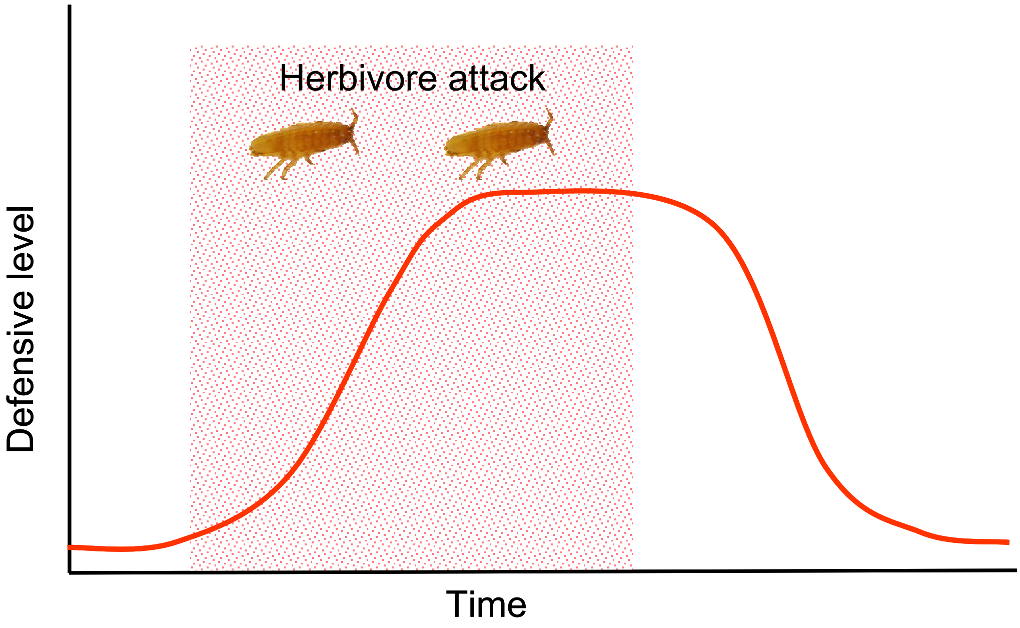

Figure 1: Defense induction and relaxation in response to grazing.

Inducible defenses are thought to be an adaptation to minimize costs in environments where grazing pressure varies temporally because they are generated only on demand and relax when grazing ceases (Karban & Baldwin 1997; Tollrian & Harvell 1999) (Figure). Additional advantages of inducible defense are a reduced risk of self-intoxication or making the potential prey a "moving target" which should reduce the risk of co-evolutionary adaptations (Agrawal & Karban 1999). In order to be effective, induced defenses depend on reliable cues that predict pending attacks. A reliable cue can be the predator attack itself, since most algae tolerate partial predation. Especially mesograzers, which are the dominant herbivores in temperate regions, often feed on individual macroalgae sufficiently long periods for a chemical response to be produced (Hay 1996). While induction of defense in response to grazing is well documented for terrestrial plants (Karban & Baldwin 1997), there were only few examples in marine macroalgae, when I started these studies in 2004 (e.g. Cronin

& Hay 1996; Pavia & Toth 2000; Rohde *et al.* 2004). Defense induction in response to airborne chemical cues was also well documented in terrestrial plants (Bruin *et al.* 1995; Shonle & Bergelson 1995). In contrast there were only two examples of waterborne chemical cues inducing chemical defense in seaweeds (Rohde et al. 2004; Toth & Pavia 2000).

To test whether this results from a research bias (Cronin 2001), one objective of the present study was to investigate the prevalence of defense induction and the nature of the inducing cues in widely distributed perennial seaweeds of the western Baltic (Chapter I). I chose the macroalgal species *Fucus serratus*, *Fucus evanescens* (Phaeophyceae), *Delesseria sanguinea*, *Phyllophora pseudoceranoides* and *Furcellaria lumbricalis* (Rhodophyta), because they are widely distributed perennial seaweeds of the western Baltic and represent important habitats for many other local species. In particular, I compared the efficiency of the inducing cues direct grazing and waterborne cues originating from feeding on neighboring individuals.

Defense induction is postulated to be of selective advantage in environments where grazing pressure is variable and defenses incur metabolic or ecological costs. While some studies demonstrated the prevalence of defense induction in marine macroalgae in recent years (reviewed in Toth & Pavia 2007), we know very little about the interactive impacts of stress and herbivory on marine algae. Factors like nutrient concentrations, light and desiccation may affect concentrations of defensive metabolites (reviewed in Cronin 2001; Hay 1996). Since responses of plants and herbivores are dependent on abiotic factors (Chapin *et al.* 1987), it is crucial to study theses interactions to understand the complex responses of populations to changes in environmental conditions and species' abundances.

Thus, a further objective of this project was to investigate how abiotic stress affected the defense capacity of the bladderwrack *Fucus vesiculosus,* one important macroalgal species in the Baltic Sea. In the Western Baltic, *F. vesiculosus* is the only perennial, canopy-forming brown alga, which makes it a key species in creating habitat structure and shelter for invertebrate and fish fauna. Eutrophication and associated changes in competitive interactions and grazing pressure led to a decline of *F. vesiculosus* from the 1970s (reviewed in Korpinen *et al.* 2007a). Competition with filamentous epiphytic macroalgae and sessile fauna, that benefit from eutrophication, results in high epibiont loads on *Fucus* (Wikstrom & Kautsky 2004). Grazing on *Fucus* can be temporally very high. Grazers can consume up to 80% of juvenile *Fucus* (Korpinen *et al.* 2007b) and extensive grazing damage of entire *Fucus*-belts occurs during mass occurrence of the isopod *Idotea baltica* (Engkvist *et al.* 2000). Consequently, both epibiosis and herbivory can generate strong selection for resistance.

One major effect of epibionts is the pre-emption of light and nutrients. Light is a main factor regulating depth distribution of algal species (Nielsen *et al.* 2002). Local depth limits of growth of *F. vesiculosus* correlate with local light attenuation in the water column (Bäck & Ruuskanen 2000). Decreased light penetration is therefore a probable reason for the increased limitation of algae to shallow depths (Kautsky *et al.* 1986), and decreased light may also stress the photosynthetic potential and consequently the performance of all algal metabolic pathways. However, the realised depth distribution of *F. vesiculosus* is in most regions of the Baltic much shallower than the physiological depth limit allows. The reasons for this are unstudied, but biotic factors (herbivory, epibionts) are likely further stressors that decrease the realised depth limit of *Fucus*.

Although the causal chain of eutrophication leading to reduced light penetration via denser phytoplankton and more algal epiphytes, which reduces overall photosynthetic carbon gains and ultimately results in a shallower depth distribution of *Fucus* is intuitive and generally accepted, it has rarely been confirmed by experimental evidence. Therefore, I tried to investigate experimentally the depth distribution limit *in situ* for *F. vesiculosus* and to estimate how epibionts may further reduce its physiological and ecological performance, what may also affect defensive mechanisms (Chapter II).

Beside light stress, other abiotic factors may affect algal-herbivore interactions. Abiotic interactions with herbivore resistance had so far only been demonstrated with regard to nutrient conditions (e.g. Hemmi *et al.* 2004); the effect of light and temperature on defensive responses have not been assessed. Climate models predict a significant warming of the water temperature due to climate change within the next 50 years. The surface temperatures of the Baltic may increase around 4°C, depending on the model used (HELCOM 2006). Since *F. vesiculosus* is a cold temperate species and adapted to cooler waters, this warming effect may have negative effects on its physiology and consequently on the defensive performance.

I investigated the direct impact of abiotic stress factors on the inducible defense of *F. vesiculosus* is assessed (Chapter III). Low light levels and the predicted temperature increase were chosen as stress factors, and their effects on the defense induction of *F. vesiculosus* were investigated. I hypothesized that light limitation and a temperature increase will negatively impact the defensive performance of *F. vesiculous*.

Also, the ecological implications of variability and dynamics of inducible defense received little attention in studies on both terrestrial as well as marine plant-herbivore interactions (Toth & Pavia 2007). Consequently, very little is known about the inertia of the response, i.e. the time lag from the onset of grazing to the deployment of an effective defense and the duration of relaxation, e.g. the time that defenses are upheld after grazing ceases.

The response speed, i.e. the time requested to induce and reduce chemical defense after the onset and cessation of grazing is an essential feature in the interaction between prey and grazer. Because the deployment of chemical defenses may incur costs and may enhance the risk of detection, if specialised herbivores use defensive cues as signals, these drawbacks should be counterbalanced by the advantage of limiting the loss of supportive, photosynthetic or reproductively important tissue. I therefore expected that regulation speed relates to the motility of the grazer species and the damage per unit time it typically inflicts in order to reduce high biomass loss (Chapter IV). Based on the observation that mesograzers –in particular the locally very common isopod *Idotea baltica* – may inflict substantial biomass loss in a short while, I hypothesize that defense in *F. vesiculosus* will be induced within a few days. On the other hand, if defense is costly as often assumed but rarely proven (Ianora *et al.* 2006), the relaxation time of induced defenses is expected to be short as well (Stamp 2003).

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Chapter I

Antifeeding defense in Baltic maroalgae:

Induction by direct grazing vs. waterborne cues

Abstract

Algal distribution is often controlled by herbivory. However, algae may react to herbivore attack by inducing defenses. The prevalence of defense induction and the cues triggering induction are generally not well understood. We examined the capacity of defense and the mechanisms of regulation in five common perennial macroalgal species from the Baltic Sea, *Furcellaria lumbricalis, Delesseria sanguinea, Phyllophora pseudoceranoides, Fucus serratus* and *Fucus evanescens*. Specifically, we investigated whether direct feeding, and/or waterborne cues from feeding on neighboring conspecifics decreased the palatability of the tested algae.

Direct feeding by the local isopod *Idotea baltica* triggered the induction of chemical defense in *F. lumbricalis, D. sanguinea, P. pseudoceranoides*, *F. serratus* and *F. evanescens*. Conversely we did not find any evidence for waterborne cues associated with feeding to trigger defense induction in neighboring conspecifics.

This study supports recent findings that defense regulation in marine macroalgae is a rather common phenomenon, especially in the Baltic Sea.

Introduction

Many investigations have demonstrated that seaweeds produce metabolizes that serve as defenses against consumers (Amsler & Fairhead 2006; Hay & Fenical 1988). However, the production of defensive metabolites may be costly because it requires resources that otherwise could be allocated to other metabolic processes like growth or reproduction (Coley *et al.* 1985).

Chemical defense can either occur in a constitutive form, i.e. permanently expressed on a high level, or generated 'on demand' (Harvell & Tollrian 1999; Karban & Baldwin 1997). Saving resources is discussed as major advantage of inductive defense. There are additional possible benefits of defense induction: (i) a higher temporal and spatial variability in plant chemistry may reduce herbivore and pathogen adaptation in ecological as well as in evolutionary times, and (ii) because any metabolite may serve as a signal for detection, a 'flickering' chemical defense represents an unreliable cue for consumers, pathogens or epibionts, and (iii) the risk of self-intoxification by defense compounds is reduced (e.g. Agrawal & Karban 1999; Miner *et al.* 2005). On the community level, the regulation of defense may dampen population fluctuations and stabilize the system (Verschoor *et al.* 2004). Inducible defenses are a type of phenotypic plasticity, the ecological (Miner et al. 2005) and evolutionary (Pigliucci 2005) importance of which have recently been highlighted. Inducible defenses may evolve in all organisms that are under selective pressure by predators or pathogens. However, the evolution of inducible defense against herbivory requires special ecological conditions (reviewed in Harvell & Tollrian 1999; Miner et al. 2005; Pigliucci 2005). Herbivore pressure must be variable, since constant grazing pressure would select for a constitutive defense. Reducing defense should enhance fitness in the absence of consumers. And, finally, a reliable cue for triggering induction must exist. Direct grazing of local herbivore species is the most obvious cue that plants could use to increase their resistance. On the other hand, chemicals released by grazed neighbors can provide a reliable - and earlier - hint to induce defenses (Tollrian & Harvell 1999). Numerous studies on terrestrial plants have demonstrated this 'talking tree' phenomenon, where plants react to air-born cues from grazed neighbors by increased resistance against herbivores (Bruin *et al.* 1995). In contrast to the numerous studies on defense induction in terrestrial organisms, similar investigations for perennial marine seaweeds are scarce (reviewed in Cronin & Hay 1996; Karban *et al.* 1997; Tollrian & Harvell 1999). Recently, however, evidence was provided that inducible defense in seaweeds may be a quite common phenomenon (Ceh *et al.* 2005; Macaya *et al.* 2005; Pavia & Toth 2000; Rohde *et al.* 2004; Weidner *et al.* 2004). Water-born cues as triggers for induced resistance were reported from only three species of brown algae *Ascophyllum nodosum* L.*, Fucus vesiculosus* L. *and Glossophora kunthii* C. Agardh (Macaya et al. 2005; Rohde et al. 2004; Toth & Pavia 2000). However, defense induction in response to waterborne cues may be of selective advantage if the grazer feeding on a neighbor represents a real risk, i.e. is mobile and not specialized on the neighbor species and if tissue loss may be reduced by increasing resistance in time.

The Baltic Sea is an ecosystem where defense induction should be a selectively favored trait. In contrast to tropical regions, where macrograzer are the most significant grazers (Bolser & Hay 1996), in the Baltic Sea gastropods and small crustaceans are the main consumers of macroalgae (Kangas *et al.* 1982). The isopod *Idotea baltica* Pallas is the most important grazer of the littoral vegetation and its abundance fluctuates considerably due to seasonal changes, but also due to small-scale aggregations that result in high *Idotea*-concentrations in some areas , while others remain less affected (Kangas et al. 1982; Kotta *et al.* 2006). Consequently, the herbivore pressure is very patchy in space and time, but can be considerable (Kangas et al. 1982; Kotta et al. 2006). Additionally, light conditions under water are often poor due to high turbidity (Bäck & Ruuskanen 2000; Lehvo *et al.* 2001). This leads to a strong energy limitation in many algal species.

In the current theoretical framework (Miner et al. 2005; Pigliucci 2005) these two system properties – variability of grazing pressure and energy limitation - should favor the evolution of inducible defenses.

The macroalgal species *Fucus serratus* L., *Fucus evanescens* C. Agardh (Phaeophyceae), *Delesseria sanguinea* Hudson, *Phyllophora pseudocera-*

noides S.G. Gmelin, and *Furcellaria lumbricalis* Hudson (Rhodophyta) are widely distributed perennial seaweeds of the western Baltic and represent important habitats for many other species. Any factor, e.g. defense regulation, ensuring their persistence in the face of energy limitation and variable consumption pressure is of system-wide relevance. We investigated the existence of defense induction and the nature of the inducing cues in these species. The induction efficiency of two cues, direct feeding and waterborne cues from feeding on neighbors was compared.

Material and Methods

Study site and Organisms

Algae were collected from rocky shores in the Kiel Fjord (54°26’N, 10°11’E), except *Furcellaria lumbricalis* which was sampled in the Flensburg Fjord (54°47’N, 9°49’E), Individuals of *Fucus serratus* and *Fucus evanescens* were collected in depths from 0.2-0.7 m, *Delesseria sanguinea and Phyllophora pseudoceranoides* from 6-7 m and *Furcellaria lumbricalis* in 3 m depth.

All algae were removed by hand from the substratum, transferred in coolers to the laboratory, cleaned from all visible epibionts and maintained in aerated seawater until used (within 24 hours).

The isopod *Idotea baltica* Pallas, as the most important mesograzer in the Baltic Sea (Kangas et al. 1982) was collected as herbivore for the feeding assays from sea grass beds in the Kiel Fjord, maintained in 300 l aerated flow-through tanks and fed with a variety of local algal species and fish.

Induction experiments

All experiments were run in a constant temperature room (15°C) from August 2004 to June 2005 at the Leibniz Institute of Marine Sciences (IFM-GEOMAR) in Kiel, Germany. Ambient seawater from the nearby Kiel Fjord was UV-sterilized (UV water steriliser 500, hw Wiegandt GmbH), stored in a 150 l tank and from there individually supplied (0.25 $L \bullet h^{-1}$) to 2.9 l aerated plastic aquaria. Light was provided by fluorescent tubes (OSRAM FLUORA 36 W/77 25X1) giving a total irradiance of 65.5 ± 2 $\mu mol\ photons \bullet m^{-2} \bullet sec^{-1}$.The aquaria were divided by a vertical plastic mesh (1 mm) into equally sized upstream and a downstream compartments.

We applied two different treatments to test for defense induction. Direct feeding, where three isopods were allowed to feed on the test algae; neighbor feeding, where in the upstream compartment three isopods fed on algal pieces of the same species as the test algae while the latter were kept without grazer.in the downstream compartment. To neutralize genetic variation only one algal individual was used for each replicate. Depending on the size of the algal species only apical pieces of ca. 10cm length (*F. evanescens,* n=15*; F. serratus,* n=15) or whole plants (*D. sanguinea,* n=7*; P. pseudoceranoides,* n=7*; F. lumbricalis,* n=13) were used. Each genotype was divided in nine equal pieces and distributed in the downstream compartment of three aquaria as follows: two pieces in an aquarium with three isopods in the same compartment to allow feeding on the test-algae (direct feeding), two pieces in an aquarium where three isopods and two pieces of another algal individual of the same species were kept in the upstream compartment to exert feeding on neighboring plants (neighbor feeding) and the remaining five pieces were kept in an aquarium without any grazer as controls. After 14 days of treatment, defense induction was assessed by two different feeding assays.

In a first approach, treated algal pieces (direct or neighbor feeding) were offered together with an untreated (control) piece to three isopods. A second piece of control alga was kept in an additional aquarium to serve as a control for autogenic weight changes. Feeding assays were run for three days in aerated 2.9 l aquaria.

All algal pieces were weighed before and after the feeding assay The biomass consumed was calculated as H_0 x (C_f/C_0)-H_f, where H_0 and H_f were pre-assay and post-assay wet weights of the algae in the feeding trials and C_0 and C_f were the equivalent weights of the growth control, with C_f/C_0 representing autogenic change in mass (Sotka *et al.* 2002).

In the second assay, we used artificial food to test for preferences based on chemical or nutritional differences only. Algal pieces were freeze-dried and ground to a fine powder and then reconstituted in agar. Agar was heated to boiling point in a microwave oven (0.18 g Agar agar + 2.5 ml distilled water), allowed to cool to ca. 50°C and mixed with the moistened algal powder (0.5 g of powdered alga + 2 ml distilled water). The mixture was poured over a mosquito net (mesh size 1 mm) and flattened between two layers of wax paper (Hay *et al.* 1994). The net was cut into squares of 15 x 15 mm and treatments were labeled by clipping the corners in different shapes. The reconstituted tissues were offered to two isopods in 2-way choice assays (treated vs. control alga). Feeding assays were terminated when either half or more of one agar piece was consumed or when the assay had run for 48 hours. We quantified consumption by counting the number of empty meshes.

Bioassays were analyzed by Wilcoxon test using directed P-values (p_{Dir}) with δ/α=0.8 (Deal *et al.* 2003; Rice & Gaines 1994) because our hypothesis was one-sided (algae with a grazing history being less palatable) but we wanted to preserve the possibility to also support the alternative.

Since all assays were run as two choice preference tests with a treated alga (direct feeding or neighbor feeding) versus a control alga, the results are represented as effect size = A_t - A_c., i.e. differences between amounts eaten of treated alga (A_t) and control alga (A_c)

Feeding assays run with intact algal pieces are affected by all differences between differently treated algal pieces (chemical, structural, or nutritional). In contrast, reconstituted food features only chemical and nutritional properties. Negative values of the calculated effect size represent higher feeding rates on the controls than on the treated pieces, i.e. induced resistance in the treated pieces.

Results

None of the tested algae, *Fucus evanescens, Fucus serratus, Delesseria sanguinea, Phyllophora pseudoceranoides*, and *Furcellaria lumbricalis,* induced resistance in response to neighbor grazing (Wilcoxon, $p_{(dir)}$>0.05; Figure 1-5).

While with *F. evanescens* defense induction in response to direct feeding was not significant in the intact algae assay ($p_{(dir)}$=0.1), the corresponding reconstituted food assay showed this effect with significance ($p_{(dir)}$=0.042, Figure 1). *F. serratus* exhibited induced defense in response to direct feeding in the intact algae assay ($p_{(dir)}$=0.0012), but this activity was not significant in the reconstituted food assay ($p_{(dir)}$=0.17, Figure 2). The tested red algae showed a more consistent pattern. All three species, *D. sanguinea* (Figure 3), *P. pseudoceranoides* (Figure 4) and *F. lumbricalis* (Figure 5) showed induced resistance in response to feeding in both types of feeding assays ($p_{(dir)}$≤0.05).

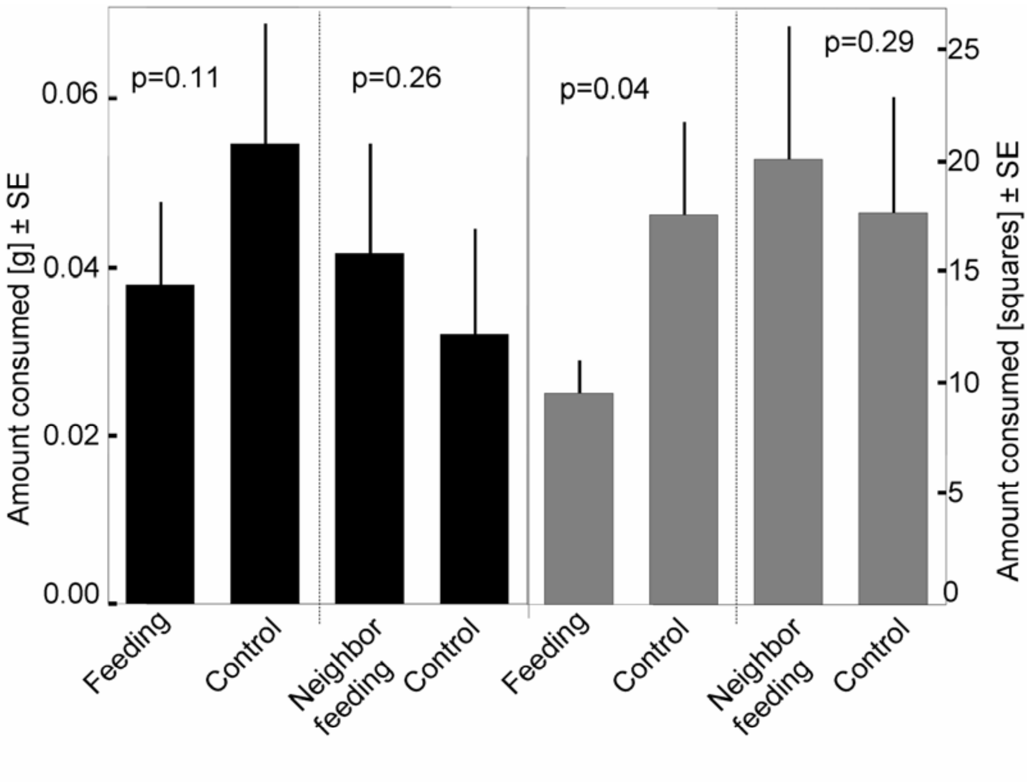

Figure 1: Feeding assays with *Fucus evanescens*. Two-choice assays were run with algae that in the treatment phase were grazed (Feeding), or subjected to neighbor grazing (neighbor feeding), or ungrazed algae (control), resp. All feeding assays were run with intact algal pieces (black bars) and with reconstituted food (grey bars).

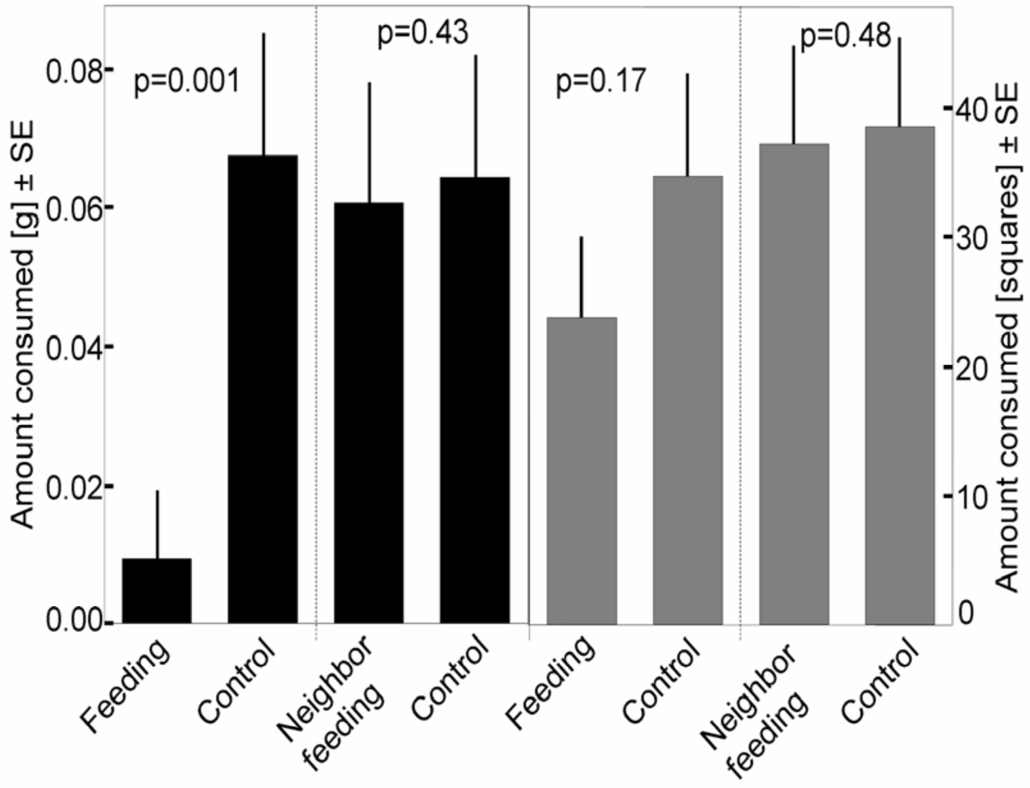

Figure 2: Feeding assays with *Fucus serratus*. For further explanations see Figure 1.

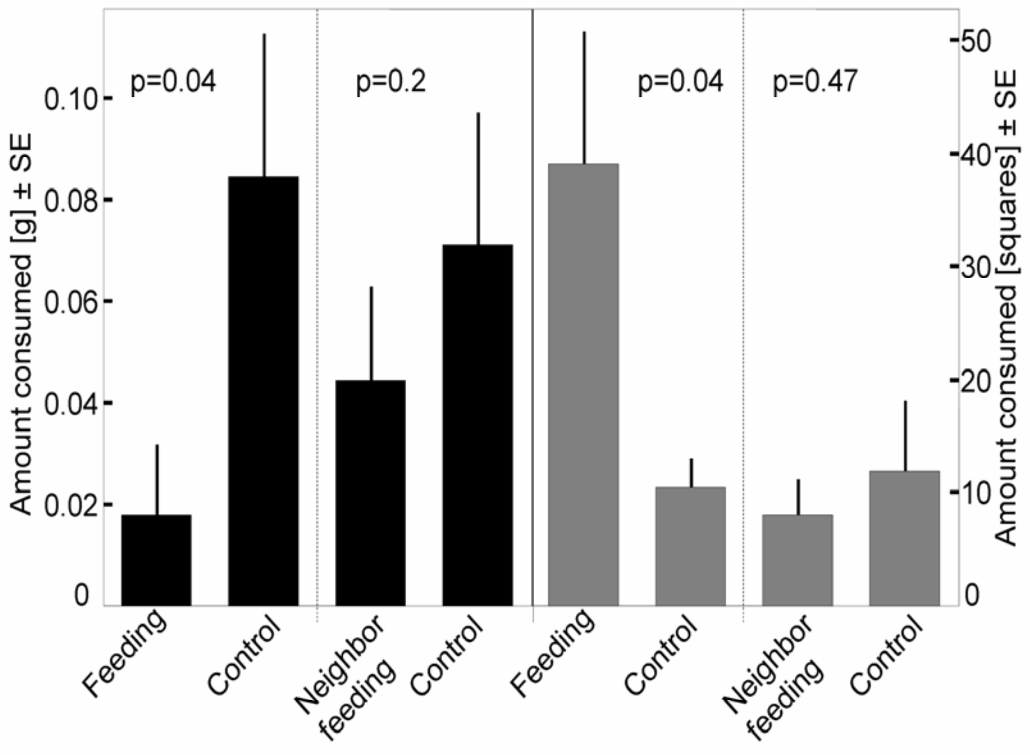

Figure 3: Feeding assays with *Delesseria sanguinea*. For further explanations see Figure 1.

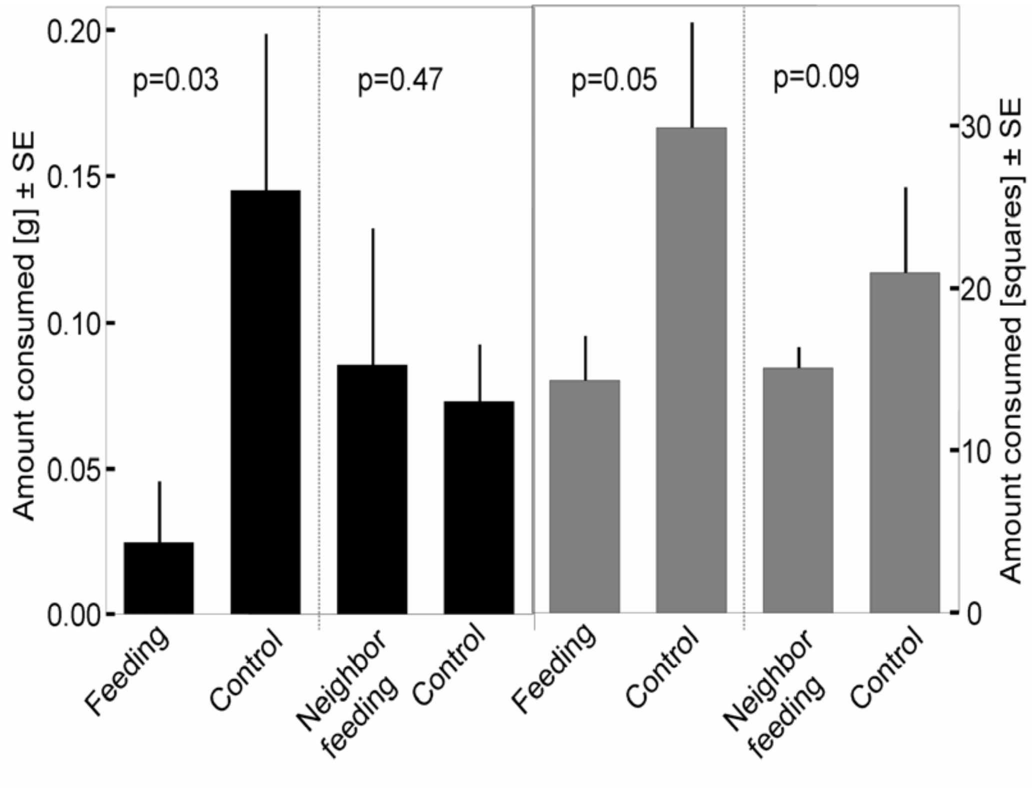

Figure 4: Feeding assays with *Phyllophora pseudoceranoides*. For further explanations see Figure 1.

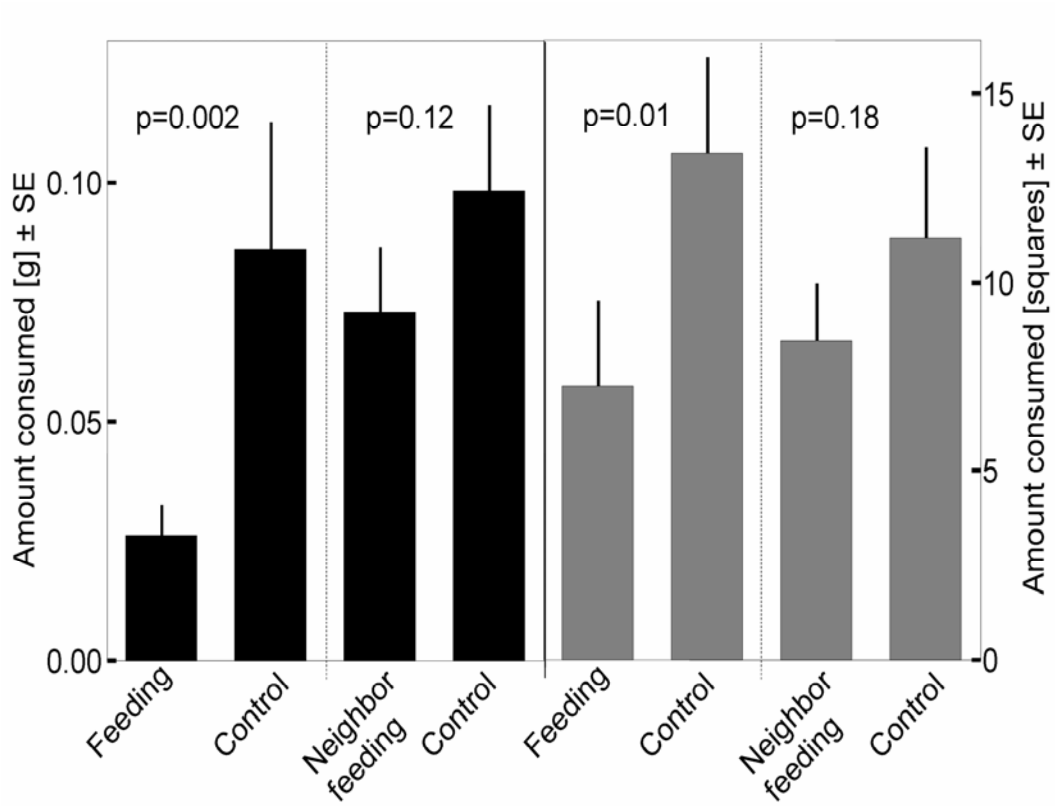

Figure 5: Feeding assays with *Furcellaria lumbricalis*. For further explanations see Figure 1.

Discussion

All tested red algae, *Delesseria sanguinea, Phyllophora pseudoceranoides* and *Furcellaria lumbricalis*, induced defense in response to direct grazing. The brown algae *Fucus evanescens* and *Fucus serratus* also showed inductive effects but these were not always significant in both types of feeding assays. The trends, however, generally were consistent between the two tests. When intact algal assay and the reconstituted food assay produce similar results, this indicates a chemical rather than structural defense.

To our knowledge, there were no previous studies on anti-herbivore defense in the red algae *D. sanguinea, P. pseudoceranoides* and *F. lumbricalis*. Consequently, any information on the chemical identity or variability of defensive traits is lacking. However, Amsler et al. (2005) showed that the congeneric species *Delesseria lancifolia* and *Delesseria salicifolia* repelled the sea star *Odontaster validus* and the fish *Notothenia coriiceps* and that extracts of these algae were responsible for this effect. Also, extracts of a *Phyllophora* species, *P. antarctica*, deterred feeding by the sea urchin *Sterechinus neumayeri* (Amsler *et al.* 1998).

The brown alga *F. serratus* showed a clear induced defense in the intact algal assay, which was a similar but non-significant trend in the reconstituted food assay. Either a structural defense component was partly destroyed by the grinding for the reconstituted food assay, or, if grazing induced production of a chemical deterrent, this compound could have degraded during the production of the reconstituted food. Because this is the first study on anti-herbivore defense of *F. serratus*, we can not exclude any of these possibilities. However, all studies on the defensive potential of congeneric *Fucus* species found chemical defense (e.g. Deal et al. 2003; Hemmi *et al.* 2004; Peckol *et al.* 1996; Rohde et al. 2004; Van Alstyne 1988). This could support the hypothesis of degraded compounds in the reconstituted food assay.

In *F. evanescens* the results showed an opposite pattern: significant effects in the reconstituted food assay and a similar but insignificant trend in the intact algal assay. Effect strength is not substantially different, but the variability among replicates is lower in the reconstituted food assays. Apparently, the elimination of structural properties reduced among-individual variability sufficiently to make the effect significant.

In contrast to the direct feeding treatment, feeding on neighboring plants did not induce defenses in any of the tested algal species. The fact that direct feeding under the same experimental conditions induced defense indicates that our design should have had the power to detect increased resistance in algae downstream of grazed conspecifics if there was one. Furthermore, the applied flow from the upstream to the downstream compartment was relatively low (0.25 $L \cdot h^{-1}$) potentially exposing the seaweeds to higher concentrations of any putative inducing metabolite than in the natural environment, which could have increased the trigger strength of defensive responses. In contrast, the slow flushing of the experimental units may have prolonged the time between exudation of a cue and its reception by the target organism, which in turn could have increased the probability of the cue being degraded on the way. The very short distance between emitting and receiving plant may have compensated this effect. Finally, these two putative effects of slow flow –high concentration and degradation – may have neutralized each other. Using the exact same experimental setup in a previous study, we did detect defense induction by waterborne cues (produced by feeding on neighbours) in *Fucus vesiculosus* (Rohde et al. 2004). Defense induction in marine macroalgae in response to waterborne cues has previously only been shown in *Ascophyllum nodosum* (Toth & Pavia 2000), *F. vesiculosus* (Rohde et al. 2004) and *Glossophora kunthii* (Macaya et al. 2005). However, while *A. nodosum* responded to waterborne cues from *Littorina obtusata*, but not from cues from *Idotea granulosa*, *F. vesiculosus* responded only to feeding cues from *Idotea baltica*, not from *Littorina littorea*. That demonstrates that prey alga species may react differently to different species of co-occurring consumer species and that the induction potential of a given consumer may vary between prey species and habitats. Sotka et al. (2002) detected increased resistance in *Sargassum filipendula* in response to direct feeding but not in response to waterborne cues. Thereupon they postulated that induction from direct grazing was more important with regard to *S. filipendula* than from grazing on neighboring plants. However, only few studies have explicitly investigated induction by waterborne cues in marine macroalgae and any general statements are not yet justified. The results of this study, where none of five tested algal species responded to neighboring grazing, strengthens the impression that the importance of waterborne cues is at best second to that of direct grazing as a trigger for defense induction. All algal species tested in this study may form dense, sometimes monospecific stands in the Western Baltic. Therefore, the risk that herbivores switch between neighboring conspecifics is high and this would favor the evolution of a communication between plants to reduce the loss by herbivory. Furthermore, waterborne cues could be used as inducer for a systemic response within one seaweed-individual. In vascular plants this response can be triggered by an internal signal transport via conducting tissue. But most seaweeds have only poorly developed conducting tissues and long distance transport is mostly restricted to symplasmatic transports (Raven 1984; Raven 2003). A more general question is whether response to waterborne cues should only be restricted to within species communication. Regarding more generalist herbivores, as e.g. the isopod used in this study, *I. baltica*, waterborne cues from other grazed algal species could be of similar informational value. But even generalists do not feed indiscriminately, but often show clear preference gradients (Barker & Chapman 1990). Thus, cues emitted by neighbor grazing where the neighbor is a different and possibly more preferred prey species may not reliably indicate impeding danger. Signals from a less preferred alga, however, should be most ‘alarming’. Generalists are more common among marine mesograzers than among terrestrial insects (where the talking tree effect has been discovered)(Hay *et al.* 1987). We postulate that in the marine environment trans-specific signaling should exist and it should be most effective when the signals stem from a grazed species which ranges lower in the generalist’s preference range. Where macroalgal species occur in dense aggregations, however, communication within single species should be an even more reliable cue than grazing on other species. Despite these potential advantages, defense induction by waterborne cues from conspecifics was not found in the tested algal species. Trans-specific cues were not tested. Apparently, direct feeding is a good enough cue, and tissue loss between the onset of consumption and the induction of defenses is tolerable.

The other potential role of waterborne cues - systemic defense induction in an organism without internal signaling system - was not assessed. In all instances we offered the entire plant piece of a treatment to the grazers. Either defense had been induced also in the ungrazed portions of the thallus or the presence of both defended and undefended thallus parts did not ‘dilute’ the defense enough to make it undetectable.

To date, induction has been shown in only a handful of macroalgal species (reviewed in Amsler & Fairhead 2006). But the increasing number of studies in the last years (Ceh et al. 2005; Macaya et al. 2005; Pavia & Toth 2000; Rohde et al. 2004; Weidner et al. 2004) which report of defense regulation in marine macroalgae indicates that the apparent scarcity of defense induction in macroalgae may result rather from a research bias than from any evolutionary pattern.

Based on our regionally restricted data on the literature published to date, we suggest that defense regulation is selectively more advantageous in temperate than in tropical regions. At higher latitudes, slow-moving (meso-) grazer, not fishes, are the predominant herbivores (Bolser & Hay 1996; Floeter *et al.* 2005). Mesograzers feed more slowly and for a longer extent of time in a given place than fishes which makes induction - even with a certain lag - the appropriate defense against the former (Gaines & Lubchenco 1982; Hay 1996). Also, in temperate regions seasonality provides an additional source for variability in herbivore pressure, which is one prerequisite for the evolution of defense induction (Harvell & Tollrian 1999). However, a recent meta-analysis study could not detect latitudinal patterns (Toth & Pavia 2007). The other prerequisites, reliable cues and costs of metabolites, depend on the species involved and the identity of defensive compounds and are therefore not intuitively related to latitude. However, light limitation should be more frequent at higher latitudes due to short day and low sun angle in winter and dense plankton blooms in summer. Light limitation may make cost saving by regulatory defenses a selective advantage. Additional stresses like low salinity and extreme temperatures, typical for the Baltic environment, may incur additional physiological costs that make the putative costs-saving aspects of defense regulation even more advantageous. This could explain the apparently high prevalence of defense regulation in the tested algal populations from the Baltic.

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Chapter II

Decreased depth distribution of *Fucus vesiculosus* (Phaeophyceae) in the Western Baltic:

Effects of light deficiency and epibionts on growth and photosynthesis

Abstract

For many coastal areas of the world, a decrease in abundance and depth penetration of perennial macroalgae and seagrasses has been documented and attributed to eutrophication. A surplus of nutrients impairs perennial seaweeds in at least two ways: increased phytoplankton densities reduce the depth penetration of light and in addition filamentous seaweeds and microalgae growing epiphytically shade their perennial hosts.

A reduction of depth limit and total abundance has also been observed for the brown seaweed *Fucus vesiculosus* at many sites in the Baltic Sea. However, in most cases the mechanistic reason for the loss of *Fucus* has been deduced from observations rather than from experimental evidence.

Here, we present results of a two-factorial (water depth / light supply and epibionts) experiment that was run in Kiel Fjord, western Baltic, from August to October 2005. Performance of *F. vesiculosus* was recorded by growth and chlorophyll measurements, PI-curves and *in situ* measurements of the photosynthetic activity as the relative rate of electron transport (ETR). ETR and growth decreased with water depth. Chlorophyll *a* concentrations increased with reduced light intensities, but this apparently could not compensate for the light deficiency. Epibionts enhanced the negative effect of reduced light conditions on growth.

According to these findings we estimated the physiological depth limit of *F. vesiculosus* in Kiel Bight to lie between 4 and 6 m water depth.

Introduction

In many coastal areas of the world a shift from perennial benthic vegetation towards ephemeral macroalgae has been observed (e.g. Duarte 1995; Munda 1993; Schories *et al.* 1997). In the Baltic Sea this led to a decrease in abundance of former predominant *Fucus* species during the second half of the last century (e.g. Torn *et al.* 2006; Vogt & Schramm 1991). Among other reasons such as increased sedimentation, loss of appropriate habitats due to commercial harvesting of stones or increased grazing due to over-fishing of the mesograzers' predators (Eriksson & Johansson 2003; Eriksson & Johansson 2005; Kangas *et al.* 1982; Lehtinen *et al.* 1988; Nilsson *et al.* 2004; Pedersen & Snoeijs 2001; Salemaa 1987; Schaffelke *et al.* 1995; Vogt & Schramm 1991), nutrient enrichment seems to be the major reason for this shift (Torn et al. 2006). Enhanced nutrient supply favours filamentous and thin growth forms with a high surface to volume (SA:V) ratio at the expense of perennial forms with an often thicker and more complicated morphology (Karez *et al.* 2004; Pedersen & Borum 1996). Microalgae feature a particularly high SA:V, and thus epiphytic and, more important, phytoplanktonic microalgae are especially favoured by high nutrient supply. Measures of chlorophyll *a* and phyto-proteins in the water column indicate that phytoplankton densities almost doubled between 1958 and 1975 in Kiel Bight, southern Baltic Sea (Babenerd 1986; Babenerd & Zeitschel 1985), while water transparency measured as Secchi depth decreased (Sanden & Hakansson 1996). Higher light attenuation is assumed to be the main reason for the observed upward shift of the lower depth distribution limit for *Fucus* spp. (Kautsky *et al.* 1986; Schramm & Nienhuis 1996; Vogt & Schramm 1991). As a consequence, the total area available for *Fucus* populations is reduced, which probably explains most of the considerable loss. The historical and the current depth limits of *Fucus* are not uniform along the salinity gradient of the Baltic Sea (Torn et al. 2006). In Kiel Bight the depth limit of *Fucus* decreased from 10 to 2 m, while the total biomass decreased by 95% when data from 1988 and 1950 were compared (Vogt & Schramm 1991). Recent monitoring found 3.5 m to be the limit for single thalli in the same area (Führhaupter *et al.* 2003). A substantial decrease in *Fucus* should affect large portions of the ecosystem since it provides food for numerous herbivores, substrate for epibionts and shelter for many associated species, plays an important role in biogeochemical cycles (e.g. Rangeley & Kramer 1995) and provides other valuable ecosystem services (Rönnbäck *et al.* 2007).

In addition to attenuation by phytoplankton, epibiosis may have a considerable effect on light availability to the basibiont. All immersed surfaces inevitably become covered by inorganic, organic and organismal components settling from the water body. The general phenomenon is called ‘biofouling’, with ‘biofilm’ designating the slime composed of macromolecules and microorganisms, and ‘epibionts’ comprising the multicellular components of the biofouling. The consequences for the fouled organism may be manifold (Wahl 1989), both beneficial (e.g. associational defence, camouflage) or detrimental (e.g. weight increase, shading, competition for nutrients, co-consumption sensu Karez *et al.* 2000). Additionally, since most interactions between a benthic aquatic organism and its environment pass through its outer body surface, an epibiotic layer will invariably modulate these interactions.

Both micro- and macroepiphytes may compete with their algal host for sun light (Booth 1987; Oswald *et al.* 1984). At depths where light becomes limiting for a host alga, any kind of fouling may enhance energy limitation, potentially reduce the host alga’s fitness and ultimately push the species distributional limit upwards. At nutrient enriched coastal waters such as those of the Baltic Sea, filamentous macroalgae or periphytic microalgae as epiphytes on *Fucus* will be favoured (Ronnberg *et al.* 1992). In addition, many animals (epizoans) may use *Fucus* as substratum. These may be favoured by eutrophication or not; e.g. filter feeders may benefit from enhanced plankton supply (Clausen & Riisgard 1996).

Although the causal chain of eutrophication leading to reduced light penetration, which reduces overall photosynthestic carbon gains and ultimately results in a shallower depth distribution of *Fucus* is intuitive and generally accepted, it has rarely been confirmed by experimental evidence. Today the depth distribution of *Fucus* (especially of *F. vesiculosus*) in the Baltic Sea received new attention in conjunction with the implementation of the EU Water Framework Directive (WFD, Directive 2000/60/EC of the European Parliament). The WFD requests a 'good' ecological status to be obtained by 2015 and macroalgae are a mandatory quality element to assess ecological quality in coastal waters. Thus, in the future, member states around the Baltic Sea will strive to increase the depths distribution of *F. vesiculosus* as a key macroalga with regard to numerous ecosystem services (Rönnbäck et al. 2007, and references therein). The lower depth limit of *F. vesiculosus* will certainly be one of the main measures for the ecological quality.

The present study tries to investigate experimentally and *in situ* the depth distribution limit for *F. vesiculosus* and to estimate how epibionts may further reduce its physiological and ecological potential. We tested the following hypotheses: In Kiel Bight *F. vesiculosus* is light limited and therefore growth and photosynthesis will decrease with increasing depth. If light conditions alone are responsible for the depth distribution of *F. vesiculosus*, the physiological growth limit should be at approximately 3 to 4 m depth, the maximum depth the alga is currently found in Kiel Bight. The presence of epibionts should further decrease the growth of *F. vesiculosus*. To compensate for decreasing light, *Fucus* should increase its chlorophyll *a* content with depth.

Materials and Methods

Study site and experimental design

All specimens of *Fucus vesiculosus* were collected in the Kiel Fjord, western Baltic (54°22’ N; 10°09’ E), where *Fucus* forms dense, macroscopically almost monospecific stands on stones. We chose *Fucus* individuals of 7 to 10 cm length, growing on pebbles (3 to 5 cm in diameter) in depths of 0.2 to 1 m. Additional organisms on these stones and macroscopic epibionts were removed by hand leaving only one *Fucus*-individual per stone.

The experimental set-up was located at a pier in the inner Kiel Fjord (54°19.5’ N; 10°09’ E). Ten ropes with ground weights were lowered from the pier to a water depth of 7 m. The horizontal distance between the ropes was approx. 2 m. At depths of 1, 2, 3, 4 and 6 m PVC-gutters were fixed in the centre at the ropes. This experimental setup and the location prevented consumption by the most dominant local herbivores, the snail *Littorina littorea* and the isopod *Idotea baltica*. On each end of the gutters one pebble with an attached *Fucus*-individual was fixed with cable ties, resulting in two *Fucus* individuals per gutter that represented one replicate (see below). One end of each gutter was marked with an additional cable tie to allow the identification of each *Fucus* individual during the following experiment. To investigate the effect of epibionts on growth and photosynthesis of *Fucus*, one *Fucus*-individual of each gutter was cleaned manually every second week, while the other individual on the gutter was left uncleaned. The height and the structure of the pier prevented shading effects on the algae. The study was run from the 14th of August to the 13th of October 2005. During that time surface water temperature is highest, varying from 15 to 18°C. Thus, respiration costs in *Fucus* are highest and may limit depth distribution.

Light conditions

To obtain information about the ambient light conditions in the different water depths, we measured the daily solar irradiation (photosynthetically active radiation, PAR) at the water surface during the course of the experiment, by using solarimeter CM 11 (Kipp & Zonen B.V., The Netherlands). Additionally, around noon of five different days we measured light intensities in water depths from 0 - 6 m by using a Licor Li-192 underwater quantum sensor (LI-COR Bioscience, Bad Homburg, Germany) and calculated the local attenuation coefficient (k_d) as $k_d = -\ln(I_2 / I_1) / d$, where I_1 and I_2 are the irradiance intensity in depths 1 and 2, respectively, and d is the layer depth. The coefficient of all days was averaged. Irradiance decrease from air into the water column due to reflexions from the water surface was taken into account by subtracting 6.6 % of the irradiance values (Kirk, 1994). Thus, we could estimate the total fluence of PAR and the average irradiance in the depth range from 0 to 6 m.

Algal growth, chlorophyll *a* concentrations and epibiont load

At the beginning and in the end of the experiment the length of the longest fronds of all *Fucus* individuals were measured with a ruler (rounded to the nearest mm) to quantify growth.

To estimate chlorophyll *a* concentrations, a piece from every *Fucus* individual (0.4 to 1.5 g WW) was stored for one week in 10 ml N,N-dimethylformamide (DMF) in the dark at 7 °C after the experiment (i.e. after 8 weeks of exposure to the different depths). Then, chlorophyll *a* concentrations were estimated as:

$\text{Chl a}[g/l] = 12.7\ A_{664} - 0.08\ A_{630}$ (modified from Moran 1982).

At the end of the experiment all epibionts were removed from a middle piece (ca. 0.1 g DW) of the fouled *Fucus*, dried in an oven (60°C) to constant weight and weighed. The *Fucus*-piece was treated in the same way and the epibiont load was estimated as Epibionts [g DW] / Alga [g DW].

The effect of epibionts on growth were analysed by an analysis of covariance (ANCOVA) with epibionts as independent factor and depth as covariate. Differences in chlorophyll *a* concentrations between different depths and epibiont load between different depths were analysed by separate one-way ANOVAs.

Photosynthesis

Photosynthetic activities of the algae during the experiment were measured *in situ* with a Diving-PAM-fluorometer by SCUBA divers. As photosynthetic characteristics, we measured the effective fluorescence yield (ΔF / Fm') and calculated the relative rate of electron transport (ETR) on day 66 of the experiment. To calculate ETR, *in situ* irradiance was measured by the quantum sensor provided with the Diving PAM, previously intercalibrated with a Licor Li-192 underwater quantum sensor (LI-COR Bioscience, Bad Homburg, Germany). The fluorescence was estimated as the average of five consecutive measurements for each individual. To reduce the variability of the integrated light sensor, we used the mean of all measured light intensities at one depth to calculate the ETRs. Differences in photosynthesis were analysed via an analysis of covariance (ANCOVA) with epibionts as independent factor and depth as covariate.

To estimate the physiological condition of the algae at the start of the experiment, 20 individuals of the collected *Fucus* were used to determine the optimum quantum yield of photosynthesis (Fv/Fm) by PAM-fluorescence measurements (Diving-PAM, Walz GmbH, Effeltrich, Germany) according to (1998). Subsequently, we recorded a photosynthesis (ETR) vs. irradiance curve (PI-curve) for each individual with eight consecutive measurements of the effective quantum yield (ΔF / Fm', "yield") at increasing irradiances (ETR = yield x irradiance). The illumination series ranged from 8 to 470 µmol $m^{-2}s^{-1}$. To estimate acclimation of photosynthesis to the light regime at different depths, light curves were measured again at the end of the study with all remaining algae. Prior to the measurements, all *Fucus* were cleaned from macroscopic epibionts. Differences of photosynthetic performance (ETR) from applied illumination series of *Fucus* individuals exposed to different depths were analysed by a two-way ANOVA (factors: illumination and depth). To assess acclimation over time we compared PI-curves recorded prior and after the experiment as interpolated with the model of Eilers & Peeters (1988). Differences between the two groups of PI-curves (before, after) were checked by comparing the following parameters and their confidence intervals: initial slope *alpha*, light saturation point (I_k) and maximum electron transport rate (ETR_{max}). Because one independent curve was obtained for each *Fucus* individual, each parameter was replicated 20-fold (before the experiment), or 62-fold (after the experiment).

Results

Light conditions during the experiment

Incoming radiation (PAR) was measured above the water surface and the irradiance reaching the different depths was calculated using an attenuation coefficient of $k_d = 0.43\ m^{-1}$. Given this k_d-value, the 1%-depth of surface PAR was at 10.71 m for the period August to October 2005. Figure 1 shows the calculated mean irradiance and the total fluence of PAR that reached the different depths in the course of the experiment.

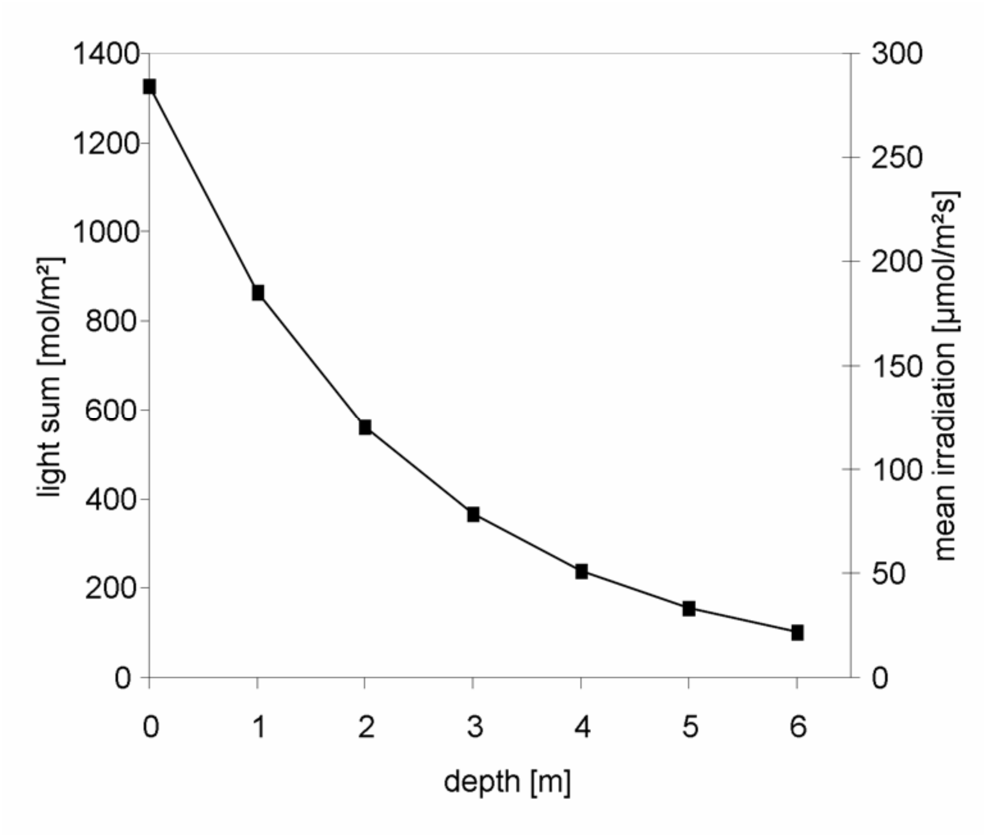

Figure 1: Light conditions during the experiment are presented as accumulated light sum measured continuously throughout the 8 weeks of the experiment and the mean irradiance per second calculated on 24 h basis.

Growth of *Fucus vesiculosus*

Growth rates were measured as increase in maximum length (Fig. 2). They decreased significantly following the depth gradient from 3.7 cm at 1 m depth to 1.7 cm at 4 m, while at 6 m depth the least growth (0.5 cm) was measurable (ANCOVA, F = 51.89, $p < 0.001$). Individuals covered with epibionts grew on average 2.0 cm, compared to the growth of clean *Fucus* of 2.7 cm. Epibiont load significantly decreased the growth of *Fucus* (ANCOVA, F = 9.07, p = 0.004) by an average of 26%. At 6 m depths, epibiont load was lowest (see below). Here, *Fucus* individuals with epibionts grew better than cleaned individuals. However, this may have been an artifact. These severely light-limited individuals started to disintegrate and may have lost tissue when epibionts were removed leading to an observed weight loss in almost half of the replicates.

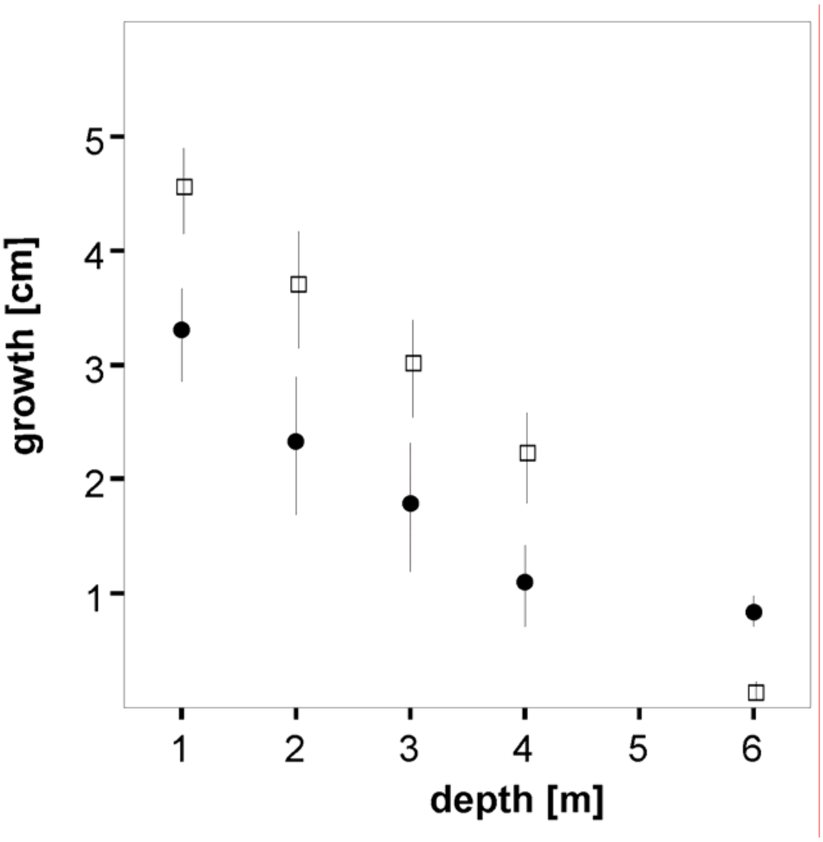

Figure 2: Growth rates (± SE) of *Fucus vesiculosus* during the experiment (black circles = fouled *Fucus*; white squares = cleaned *Fucus*; n = 7-10). The lengths of the longest fronds were recorded before and after the experiment (duration: 8 weeks) and the absolute increase was calculated.

Chlorophyll *a* concentrations

Epibionts had no effect on the chlorophyll concentrations (ANOVA, F=0.19, p = 0.66). Therefore, the data of both epibiont treatment levels were pooled for further analysis. With increasing depth from 1 to 4 m, the chlorophyll *a* concentrations in *Fucus* increased from 500 to 750 µg/g FW. At and below 4 m, chlorophyll *a* concentrations were significantly higher than at and above 2 m depth (ANOVA, p < 0.001, Tukey HSD posthoc, p < 0.05, Fig. 3).

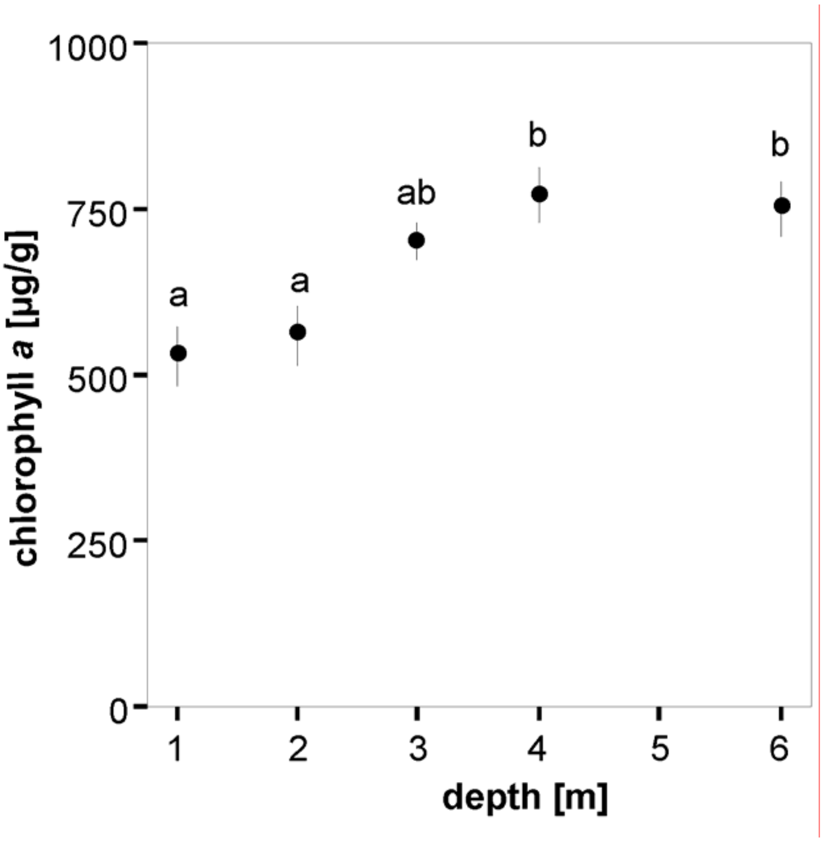

Figure 3: Chlorophyll *a* concentrations (± SE) in *Fucus vesiculosus* after 8 weeks. Letters indicate significant differences (ANOVA, p < 0.05). Data from fouled and cleaned *Fucus* did not differ and were therefore combined.

Epibiont load

The epibiont load did not differ between *Fucus* individuals exposed at depths of 1, 2, 3 and 4 m (13-23 g DW epibionts/g DW *Fucus*), but was significantly lower at 6 m depth (8 g DW epibionts / g DW *Fucus*) (ANOVA, F=5.33, p = 0.02, Fig. 4). However, the composition of the epibiotic community changed with depth. While on *Fucus* individuals from 1 m depth filamentous algae (*Enteromorpha* sp., *Ceramium* sp.) and polychaetes (*Polydora* sp.) dominated, the proportion of barnacles (*Balanus improvisus*) increased continuously with depth. *Fucus* individuals at 4 m depth were almost completely covered with barnacles, resulting in the high epibiont / *Fucus* dry weight ratios. At 6 m depth epibiont cover was clearly less than in the depth from 2 to 4 m and consisted mainly of *Polydora* sp.

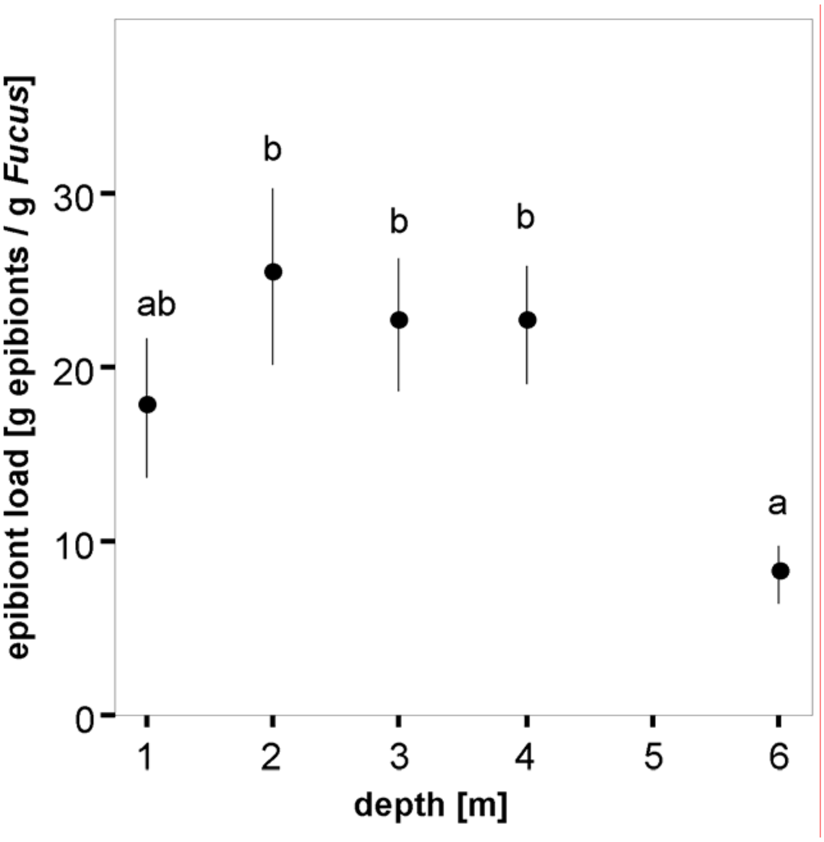

Figure 4: Epibiont load (± SE) on *Fucus vesiculosus* after 8 weeks of exposure to experimental water depths (n = 7-10) Letters indicate significant differences (ANOVA, $p < 0.05$).

Photosynthesis

Measurements of the *in situ* ETR after eight weeks did not reveal any effect of epibionts on the photosynthesis of *F. vesiculosus* (ANCOVA, F = 0.107, p = 0.74, Fig. 5). Along the gradient of decreasing *in situ* irradiance at increasing depths, photosynthetic electron transport decreased from 115 to 15 relative units.

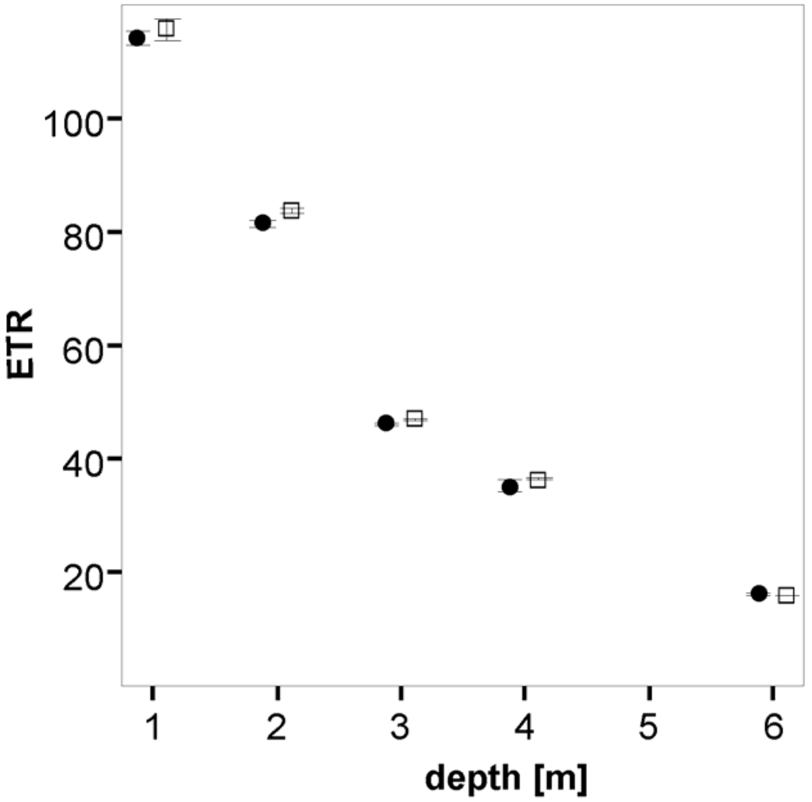

Figure 5: Photosynthesis in terms of the electron transfer rate (ETR, ± SE) of *Fucus vesiculosus* (black circles = fouled *Fucus*; white squares = cleaned *Fucus*; n = 7-10) 8 weeks after transplantation to the different depths (n = 7-10). Note that both treatments were placed at the same respective depth and are only shown juxtaposed to avoid overlap.

PI-curves recorded before and after the experiment did not indicate any acclimation of photosynthetic performance to the progressively reduced light regime at increasing depths (ANOVA, F = 2.05, p = 0.08, Fig. 6a & b). Such an adaptation would have been indicated by an increase in the initial slope *alpha*, a reduction in the light saturation point (I_k) and maximum electron transport rate (ETR_{max}). Interpolation with the model of Eilers & Peeters (1988) revealed an ETR_{max} of 85.9 ± 3.1 CI before and 81.8 ± 9.3 CI after the experiment. Mean *alpha* remained practically unchanged (0.65 ± 0.07 CI before and 0.64 ± 0.04 CI after the experiment) and I_k decreased only slightly from 135.0 µmol $m^{-2}s^{-1}$ ± 9.7 CI to 128.0 µmol $m^{-2}s^{-1}$ ± 3.9 CI. Thus, there were no significant differences in PI-parameters recorded before and after the experiment (Fig. 6a & b).

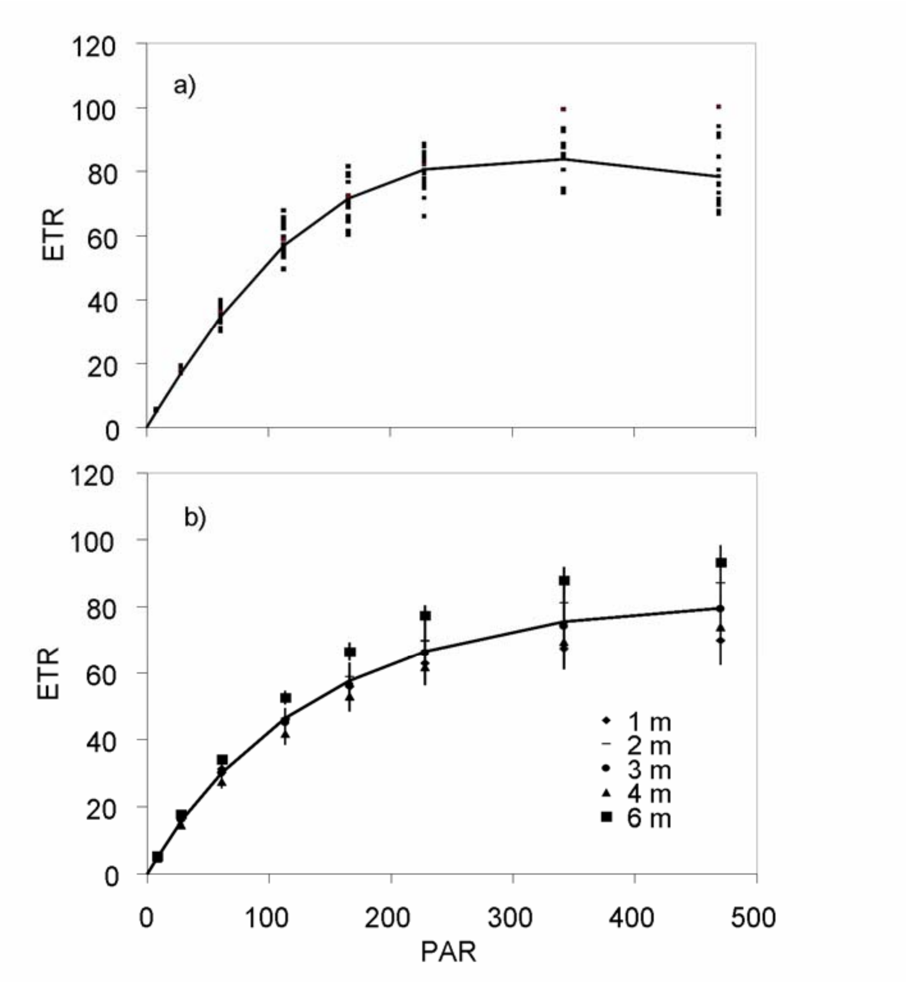

Figure 6: PI-curves (± SE) measured prior (a) and after (b) exposure to experimental different dephts (1 – 6 m).

Discussion

Results presented here shed light on the physiological base for upward-shifts in the lower distributional limit of *Fucus vesiculosus* in the Kiel fjord. Photosynthesis and growth decreased with decreasing light intensity to the extent that at 6 m depth growth was reduced to less than 1 mm per week. Since such growth rates are presumably too low to compensate for physical damage or consumption, the lower physiologically determined distribution limit of *F. vesiculosus* in the Western Baltic seems to be situated between 4 m and 6 m depth. We found no effect of epibionts on the actual photosynthetic rate, but fouled *Fucus* individuals grew significantly slower than unfouled organisms. The PI-curves measured after the experiment with transplanted algae revealed no differences in adaptation to different depths. Also comparisons of PI-curves measured before and after the experiment indicated only slightly lower ETR's at low light intensities after the experiment compared to the initial values. However, in response to reduced light availability along the depth gradient, the algae increased cellular chlorophyll *a* concentrations. Epibiont mass was lower at 6 m depth compared to the intermediate depths of 2 to 4 m.

Differences in light availability only resulted in minor changes in average light use characteristics of the experimental individuals. Thus, I_k values at about 130 µmol m^{-2} s^{-1} were found remarkably stable over time and depth in our study. Middelboe *et al.* (2006) found that even in the course of one year I_k values in *F. vesiculosus* were only weakly responding to changes in light availability. In a year round field study conducted at the shoreline of Zealand (Denmark, Baltic Sea) at 1m water depth, maximal variation in I_k values of *F. vesiculosus* was about approx. 40 µmol m^{-2} s^{-1} (range between approx. 100 and 140 µmol m^{-2} s^{-1}), despite strong variation in irradiance and temperature in the course of the seasons. In contrast, large seasonal variation in light compensation points was observed by Middelboe *et al.* (2006), i.e. from maximal values of 35 µmol m^{-2} s^{-1} in summer (June to August) down to 8 µmol m^{-2} s^{-1} in February. The plasticity to adjust compensation points to seasonal variation in light availability (e.g. by reducing respiration rates) allows positive net carbon gains under a wide range of radiation conditions either in the course of the year (Middelboe et al. 2006) or presumably also along the depth gradient (our study).

In our study no data on respiration and, thus, light compensation could be collected. During winter surface water temperature in Kiel Bight typically drops down to approx. 3° C and in the course of our study a maximum temperature of 18° C has been recorded. As respiration is highly temperature dependent, at least a transient shortage in energy supply may be likely for deep-water algae in the summer months, when *Fucus* may exhibit high respiration rates, and thus, increased compensation levels for photosynthesis. As indicated in Fig. 1, specimens kept at 6 m water depth were exposed to a mean irradiance of about 20 µmol m^{-2} s^{-1}, and, thus, below summer values for light compensation of 35 µmol m^{-2} s^{-1} reported for *Fucus* from Zealand (Middelboe et al. 2006).

In *F. vesiculosus* from the Gulf of Finland, summer values of light compensation points were determined at 25 µmol m^{-2} s^{-1} and light saturation of photosynthesis was recorded at 300 µmol m^{-2} s^{-1} (Bäck & Ruuskanen 2000). Based on data on photosynthetic performance and light attenuation the authors determined the depth for optimum growth of *F. vesiculosus* to be at < 3 m. At depth greater than 5 m light quantity was found to be insufficient to support growth. Due to the limitation of the PAM fluorescence method no information on compensation points could be deduced from our experiment.

However, the slightly differential shape of PI curves and chlorophyll *a* concentrations in individuals from different depths measured at the end of the experiment (Figs. 3, 6) indicate ongoing photo-acclimation in the different thalli of *Fucus*, in order to compensate light limitation with increasing depth. Irradiances just above the compensation point might be sufficient to maintain the integrity and functionality of the photosynthetic apparatus even during long periods of light limitation at the expense of growth. Even under exposure to irradiances below the compensation point, internal energy reserves (e.g. mannitol, Lehvo *et al.* 2001) might be used to temporarily compensate for variation in light availability. However, this strategy only allows survival of algae for a limited period of time under reduced light conditions. This process enables *F. vesiculosus* to survive below ice covers in the Gulf of Finland for several months (Lehvo et al. 2001). Thus, the duration of our experiment (eight weeks) might be too short to observe substantial changes in photosynthetic parameters by PAM fluorescence, as solely information on photosystem II functionality and not on variation in light compensation can be provided by this technique. Future transplantation experiments should include measurements of changes in respiration rates and thus compensation points.

Due to the large differences in light climate along the depth gradient and, thus, in *in situ* photosynthetic performance (Fig. 5), growth rates were strongly diminished. Epibiont load was shown to contribute to reduction in growth rates. Several causes may be responsible for these effects, either singly or in combination (reviewed in Wahl 1997). Most organisms reflect or absorb light to some extent and, thus, reduce the amount of light reaching the substratum they grow on - *Fucus* in this case. Additionally, the access of *Fucus* to nutrients may be modified in three ways: (i) the thallus portion serving as attachment area is unavailable for nutrient uptake, (ii) epibionts tend to increase the boundary layer and, thus, hinder the exchange of water at the thallus surface, and (iii) epiphytes may compete with the algal basibiont for nutrients while epizoans may excrete nutrients benefitting the host alga. Taken together, the nutrient–reducing effects seem to dominate. The effect of epibiosis depends on the species composing the epibiotic community and on the degree of fouling. Epibiont mass was lowest in 6 m depth, relatively high at intermediate depths of 2 to 4 m and slightly lower again at 1 m depth. However, it is not always possible to extrapolate from epibiont mass to percent cover or the shading effect. Regular observations revealed that the fouling community changed along the depth gradient. While at 1 m depth ephemeral algal species (*Ceramium* sp., *Enteromorpha* sp.) dominated, barnacles constituted an increasing part of the fouling community from 2 to 4 m depths. Increasing portions of barnacles lead to increasing load ratios due to the heavy calcareous shells and percent cover (which was not measured) by epibionts may be more equally distributed between depths than suggested by dry weight. However, visual impression suggested that at 6 m depth, percent cover was lower than in all other depths. The fact that only at this greatest depth the effect of epibiosis on growth was null or even positive may have several reasons. Either shading no longer had an effect because even un-fouled algae were light-limited or at that depth nutrients were so plentiful that even the reduced water exchange at the thallus surface caused by epibionts did not entail a nutrient starvation.

If growth is an adequate proxy for algal fitness, then the stress (e.g. by light limitation) exerted by epibionts at the density and composition found here is equivalent to a downward shift by 2 depth meters. Indeed, at all depths between 1and 4 m, the growth rates of epibiont-free *Fucus* were equivalent to the growth rates of fouled *Fucus* situated 2 m deeper (Fig. 2). The most probable effect of epibionts on growth was shading, because physiologically different epibiont species (e.g. autotrophic and heterotrophic epibionts) had similar impacts on growth. Consequently, if the alga does not control fouling at its surface, the stress of diminishing light with increasing depth is enhanced through shading by epibionts provoking a further upward shift in the distribution of *Fucus*.

The physiological depth limit of *F. vesiculosus* suggested by our study lies between 4 and 6 m. However, today in Kiel Bight the actual depth limit seems to be at approximately 3.5 m with only single plants are found at that depth and the main *Fucus* belt is normally not found below 2 m (Führhaupter et al. 2003 and own observations). The absence of *Fucus* between its physiological depth limit and the observed depth limit may partly be explained by the additive light reduction effect caused by epibionts. Occasionally, single plants can be found considerably deeper (Dirk Schories, pers. comm. and monitoring programmes in northern Germany), but it is unclear if these are local effects of enhanced light transparency.

Fucus depth limit thus seems to be a suitable metric for the assessment of ecological quality for the WFD, since its reduction seems to indicate the current high levels of eutrophication. However, our study emphasises that growth at a certain depth may be further reduced by epibionts and that it is not only epiphytes, which may also be directly connected to high nutrient levels (e.g. Karez et al. 2004), but also or mainly animals whose indicative value for water quality is unclear. An additional important factor, not surveyed in our study, is salinity, which shows a strong gradient along the shores of the Baltic Sea and to which the deeper depth limits in the inner Baltic Sea (the 'downward process' after Waern 1952) is related. If depth limit should be used to assess water quality, it is crucial to better understand the influence of these (and more) factors on local *Fucus* depth limits in the Baltic Sea, and thus more experimental rather than observational evidence along gradients of light and salinity is needed.

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Chapter III

Effects of temperature and light on defense regulation in *Fucus vesiculosus*

Abstract

Herbivory is an important biotic factor affecting algal biomass, abundance and distribution. Many algal species reduce biomass loss due to herbivores by defensive metabolites. Defense can be constitutive, i.e. constant, or only produced on herbivore attack as inducible defense. While much research effort has focussed on the prevalence of defense induction, the interactions of environmental stress and defense, in particular defense induction, have not been assessed so far. Algal-herbivore interactions can be affected by abiotic factors, especially since distinct changes in the climatic conditions due to global warming are predicted. The brown alga *Fucus vesiculosus* is known to induce defense in reponse to grazing. Eutrophication in the Baltic has progressively diminished the light availability for *Fucus* during the last decades, and climate models predict a temperature increase within the next century. To assess the interactive effects of these environmental conditions on the defensive performance of *F. vesiculosus,* we conducted a two-factorial experiment. As a further response variable, effects on the density of epibiotic bacteria on *Fucus* were assessed.

We found no effects of reduced light conditions on inducible defense at ambient (15°C) temperatures. Growth rates were significantly reduced at lower light intensities. Epibacterial numbers were significantly higher at higher light intensities. The predicted temperature increase to 20°C prevented induced defensive responses independently of light intensities. Our study is one of the first to show that abiotic stress can affect algal-herbivore interactions and can therefore indirectly affect macroalgal communities.

Introduction

Fucus vesiculosus (hereafter *Fucus*) is the main macroalgal constituent of the upper littoral zone in the western Baltic (Kangas *et al.* 1982). However, during the last four decades *Fucus* populations in this region evinced a dramatic decline in spatial range and biomass (reviewed in Torn *et al.* 2006). Two main reasons for this *Fucus* decline are currently under discussion. Increased eutrophication led to increased phytoplankton concentrations which enhanced light attenuation in the water column and reduced irradiation of the benthos (Kangas et al. 1982), Low light stress may decrease the photosynthetic potential in algae and consequently the performance in most life history traits. Also, the abundance of the isopod *Idotea baltica* increased as a result of increased availability of filamentous algae that serve as food for young isopods (Kangas et al. 1982; Salemaa 1987; Schaffelke *et al.* 1995). Larger grazer populations mean higher grazing pressure on *Fucus* when alternative preys, i.e. filamentous algae, are at their seasonal low.

In addition to low-light stress and enhanced grazing, *Fucus* in the Baltic Sea may also experience high-temperature stress in the future. Climate models predict a significant warming of the surface water temperature in this region by approximately 3°C in winter and 6°C in summer within the next 50 years, depending on the model used (HELCOM 2006). Since *F. vesiculosus* as a cold temperate species is adapted to cooler waters, this warming may have negative effects on its physiology. The southernmost *F. vesiculosus* population lives in Portugal with summer sea temperatures from 16-20°C. In the Baltic, sea temperatures in summer can reach 20-22°C. However, in the Baltic Sea average summer surface temperatures vary between 16-18°C. With the predicted warming of 4°C, the *Fucus* populations in the Baltic will in the future more often and over longer periods experience temperatures similar to those of their southern distribution limit: Even at sub-lethal temperatures, warming may jeopardize the alga’s persistence if it reduces its competitiveness or defense capacities.

Herbivory is an important biotic factor affecting algal biomass, abundance and distribution (reviewed in Lubchenco & Gaines 1981; Van Alstyne *et al.* 2001). Its effects on algal individuals may last over relatively long periods because grazed individuals are rarely killed (Dethier *et al.* 2005). The capacity to resist to some degree to harmful grazers and microorganisms is considered as an important trait of multicellular organisms. Such resistance may be either constitutive (stable in intensity) or regulated, e.g. enemy-induced: molecular signals indicate the presence or activity of potential enemies to the cell and activate defensive mechanisms (Amsler & Fairhead 2006). Several theories postulate selective advantages of induced defense (Agrawal & Karban 1999). Defense induction is suggested to be advantageous in environments where consumption pressure is variable (Karban & Baldwin 1997). The gene-to-gene model predicts a reduced risk of enemy adaptation to facultative defensive traits (Keen 1990). The cost allocation model postulates a trade-off between an investment into defense and its benefit (Cronin 2001). Compared to constitutive resistance, regulated defenses do not cause defense related costs under enemy-free conditions. In the absence of grazers, defense is down-regulated and, thus, energy is saved or exposure to autotoxic defensive compounds is reduced (Heil 2002). However, when an initial damage is required for induction this biomass loss represents an additional cost as compared to constitutive defense systems. It is notable, though, that while numerous studies demonstrated defense induction in marine macroalgae in recent years (reviewed in Toth & Pavia 2007), none could rigorously demonstrate incurring costs.

Many algal species reduce biomass loss by defensive metabolites that decrease feeding rates of herbivores, and such defensive traits may be constitutive or enemy-induced.

However, relatively little is known about how abiotic stress may modulate alga-herbivory interactions. Factors like nutrient concentrations, light and desiccation may affect concentrations of defensive metabolites (reviewed in Cronin 2001; Hay 1996). Since responses of plants and herbivores are depending on abiotic factors (Chapin *et al.* 1987), we need to study these interactions to understand the complex responses of communities to changes in environmental conditions and species' abundances.

Some studies on marine macroalgae investigated the relation between defenses and environmental factors like UV-radiation (Cronin & Hay 1996b; Pavia *et al.* 1997) or nutrients (e.g. Pavia & Brock 2000; Yates & Peckol 1993) with mostly positive effects of nutrients and light on chemical deterrents. To our knowledge there are no studies that investigated the effects of temperature on feeding deterrents in marine macroalgae.

In addition to grazers, macroalgae also need to resist to pathogens and epibionts. The aquatic environment favours the development of biofilms on algal surfaces. These usually contain a wide array of heterotroph and photoautotroph microorganisms, which use the alga as a substrate and often also as a nutrient source. While anti-settlement activities of epibacteria have also been described (Kanagasabhapathy *et al.* 2006), many biofilms on macroalgae contain taxa that promote macrofouling through production of settlement cues for spores or larvae (Steinberg & DeNys 2002; Weinberger *et al.* 2007), as well as opportunistic pathogens that have the capacity to decompose algal cell walls (Küpper *et al.* 2002). However, macroalgae are obviously capable to cope with these fouling-promoting and pathogenic organisms under most circumstances. Whether the capacity of *Fucus* to keep epibiotic biofilms at bay is jeopardized by environmental stress is a further objective of this study.

The defensive properties of *F. vesiculosus* received much scientific attention. Already Geiselman & McConnell (1981) demonstrated the antifeeding properties of *F. vesiculosus.* Thereafter, several studies further investigated its defensive mechanism. We therefore know, that *F. vesiculosus* induces defenses in response to grazing (Rohde *et al.* 2004) and that induced resistance by simulated herbivory is not affected by nutrient enrichment (Hemmi *et al.* 2004; Peckol *et al.* 1996). The effects of other biotic stress factors like temperature and light on the chemical defense of *F. vesiculosus* have not yet been studied. Enemy-regulated resistance against microorganisms has not been described for *Fucus* yet and it is largely unknown, how this alga defends itself against detrimental microorganisms.

This study aims to investigate the effects of light and temperature on the induction of anti-grazing defense in *F. vesiculosus*. We hypothesize that the putative stress inflicted by light reduction and a temperature increase will affect the defensive performance of *F. vesiculous*. We also monitored densities of the bacteria associated with *F. vesiculosus* in order to compare stress- and grazing effects on epibacterial densities.

Material and Methods

All *Fucus vesiculosus* individuals were collected in March 2006 from a rocky shore in the Kiel Fjord, western Baltic (54° 26’ N, 10° 11’E), where they form dense, almost monospecific stands. Algae were transferred in coolers from the subtidal zone (0.2-0.7 m) to the laboratory and maintained in aerated seawater until required (within 12 hours). The isopod *Idotea baltica* was chosen as herbivore for our experiment, because it has previously shown to induce defenses in *Fucus* (Rohde et al. 2004).

The induction experiment was run in two constant temperature rooms (CTR) (15°C and 20°C) at the Leibniz Institute of Marine Sciences (IFM-GEOMAR) in Kiel, Germany. In each room the experimental setup consisted of a flow-through system of 40 transparent plastic aquaria (2.9 L). Ambient water was obtained from the nearby Kiel Fjord and sterilised with UV light (UV water steriliser 500, hw Wiegandt GmbH). Then it was stored in a tank (150 L) before supply to the aquaria, which were individually regulated with roller clamps to provide a flow rate of 0.25 L h^{-1}. In each room two light regimes were installed. Light was provided by fluorescent tubes (OSRAM FLUORA L 36 W/77 25X1), which were mounted in parallel above the aquaria. For the high light level, reflectors were installed above the fluorescent tubes. For the low light level, no reflectors were installed and window screen was mounted between the lights and the aquaria, given a total irradiance of 75 ± 2 µmol m^{-2} s^{-1} and 30 ± 2 µmol m^{-2} s^{-1}, respectively. The light/dark period was 12/12 hours.

20 *Fucus* individuals, of equal size and without severe grazing damage or epibionts, were collected on the starting day of the experiment (one algal individual was comprised of the tissue descending from a single holdfast). The indi-

viduals were randomly evenly distributed to both CTRs and each divided in into 16 comparable apical pieces (1 – 2 g wet weight). Each replicate in each CTR consisted of two pairs of aquaria, one pair under high light levels and one pair under low light levels. The *Fucus* pieces from one individual were randomly distributed to the two pairs of aquaria for each replicate and let acclimatised for three days under the given conditions.

After this acclimatisation, one *Fucus* piece was taken from each aquarium to sample the bacterial biofilm. One sample was taken to estimate total cell numbers and one sample was used to investigate the bacterial community composition. For both samples algal pieces were rinsed in 0.2 µm filtered seawater and an area of 23.8 mm² was wiped off with sterile cotton swabs. In order to sample defined surface areas, pieces of overhead projector foil bearing a circular 19.6 mm² hole were used as screens during the wiping procedure. After wiping, the ends of the cotton swabs were stored in autoclaved 1.5 mL Eppendorf caps. The swab for determination of total cell numbers was fixed by addition of 1 ml sterile seawater containing 5% formaldehyde, while the swab for community composition analysis was frozen (-20°C). For quantification, bacteria were suspended from the swabs by vortexing for 30 seconds at full speed. Aliquots of the resulting suspension were filtered quantitatively upon blackened nitrate cellulose filters (0.2 µm pore width, DHI, Hørsholm, Denmark) as described by Zimmermann & Meyer-Reil (1974), stained with 4’6-diamidino-2-phenylindole (DAPI, Invitrogen, Karlsruhe, Germany) and assessed by epifluorescence microscopy as described by Coleman (1980).

After the acclimatisation, the feeding treatment was applied. One aquarium of each aquarium-pair served as control where no grazers were introduced and in the other one five *Idotea* were allowed to feed on the *Fucus* pieces for the following 14 days. One algal piece from the control aquaria was carefully blotted dry and weighed to the nearest 0.001 g to measure growth rates during the following treatment phase.

After this treatment phase, one *Fucus* piece of each aquarium served to take samples of the biofilm again (s. above) and all remaining pieces were used for the feeding assay to test for induced defences as follows:

Algal pieces from both the control and the treatment from the same replicate and conditions were carefully blotted dry, weighed and transferred to a 2.9 L aquarium containing seawater, to which three *Idotea* were added. Additional algal pieces from each pair of aquariums were also weighed and transferred to another aquarium to serve as controls for autogenic changes in mass. Algal pieces were reweighed after three days and the biomass consumed was calculated as $H_0 \times (C_f/C_0) - H_f$, where H_0 and H_f were pre-assay wet weights of the algae in the feeding trials and C_0 and C_f were the weights of the growth controls, i.e. C_f/C_o represents autogenic changes in mass (Sotka *et al.* 2002).

Statistical analysis

Differences in consumption in the feeding assay were analysed by pairwise Wilcoxon test. *Fucus* growth rates were calculated as percentage biomass change per day. Prior to a one-way ANOVA with temperature and light as fixed factors, data were arcsin-transformed to obtain normality (Kolmogorov-Smirnov) and homogeneity of variance (Cochran). Wilcoxon tests were performed as post hoc tests and results were Bonferroni-corrected.

Changes in cell densities of the bacterial communities associated with *F. vesiculosus* during the treatment phase were expressed as ratios of cell numbers detected after and before the treatment. Differences among treatments were analyzed by Kruskal-Wallis ANOVA and Nemenyi-test. In addition, a four-way ANOVA was conducted with Box-Cox-transformed data ($\lambda = 0.21$). This transformation resulted in only 35 out of 40 cases in normality (Kolmogorov-Smirnov) and homogeneity of variance (Cochran). A significance level $p = 0.001$ was therefore chosen, in order to reduce the risk of first order error.

Results

Feeding assays

Two choices feeding assays with algae that were kept at 15°C revealed that algae that were exposed to *Idotea*-grazing were significantly less palatable than control algae (Fig. 1). This effect was consistent over both low light ($p = 0.04$) and high light ($p = 0.01$) conditions. Under higher temperatures (20°C) no significant differences in palatability persisted between grazed and control algae, neither under the high, nor under the low light regime ($p > 0.05$).

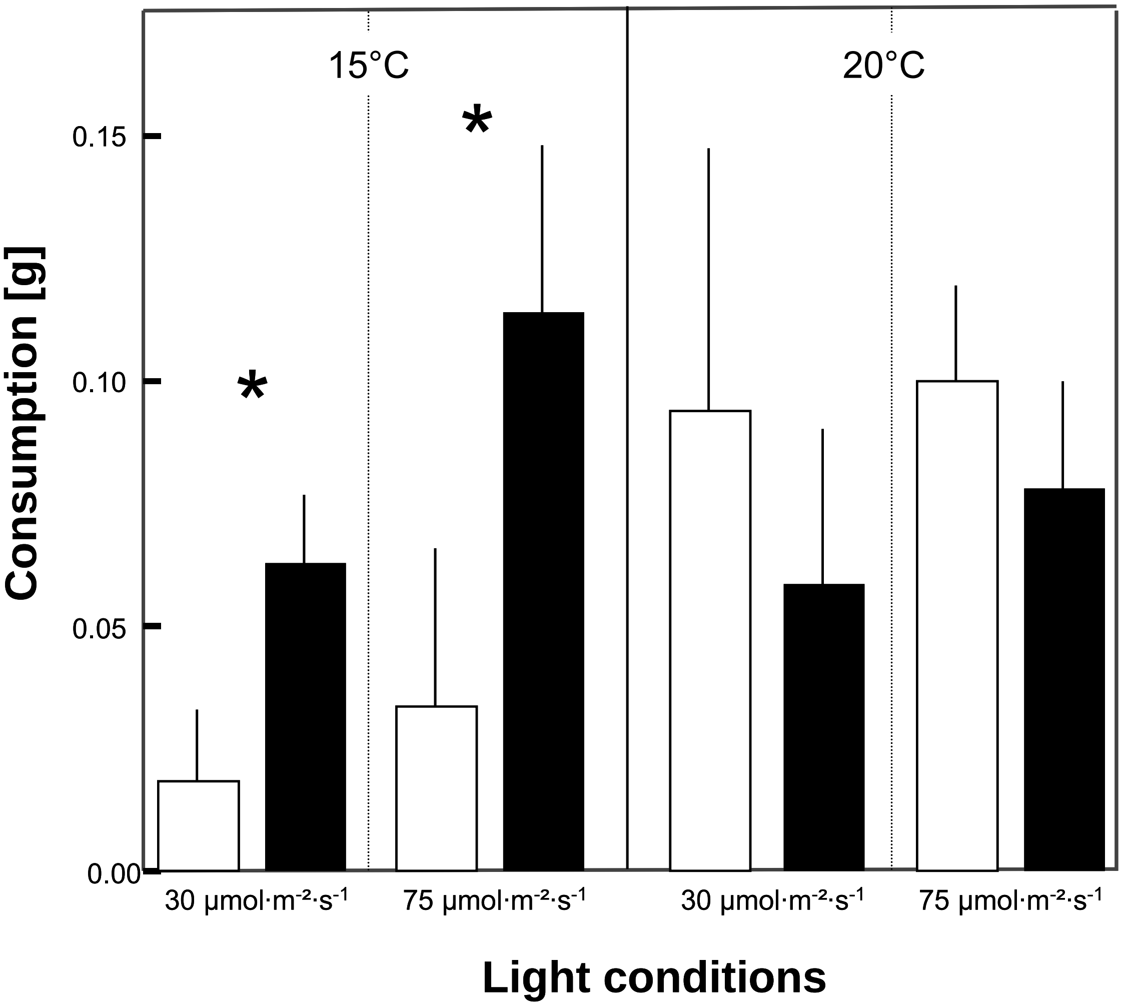

Figure 1: Results of the feeding assays after exposure of *F. vesiculosus* to different light and temperature treatments. Shown are the consumption rates of *I. baltica* on formerly grazed (white bars) and ungrazed (black bars) *F. vesiculosus*. (* = controls were significantly more palatable than treated algae, Wilcoxon, $p < 0.05$).

Growth rates

Growth rates of *Fucus* were measured for the treatment phase as changes in biomass (Fig. 2). ANOVA revealed that light affected growth significantly ($p < 0.001$), while temperature had no effect ($p = 0.88$). At 15°C *Fucus* grew significantly more (Wilcoxon, $p = 0.002$) under the high light regimes (0.7%/d) than under the low light conditions (2.8%/d). However, this effect was not present at 20°C, where we found no significant differences (Wilcoxon, $p = 0.28$) between the growth of *Fucus* from different light conditions (1.4%/d at low light, 2.2%/d at high light). Additionally, comparing the growth rates from the same light regime but different temperatures, temperature increase from 15 to 20°C did no alter significantly the growth of *Fucus* (Wilcoxon, $p > 0.05$).

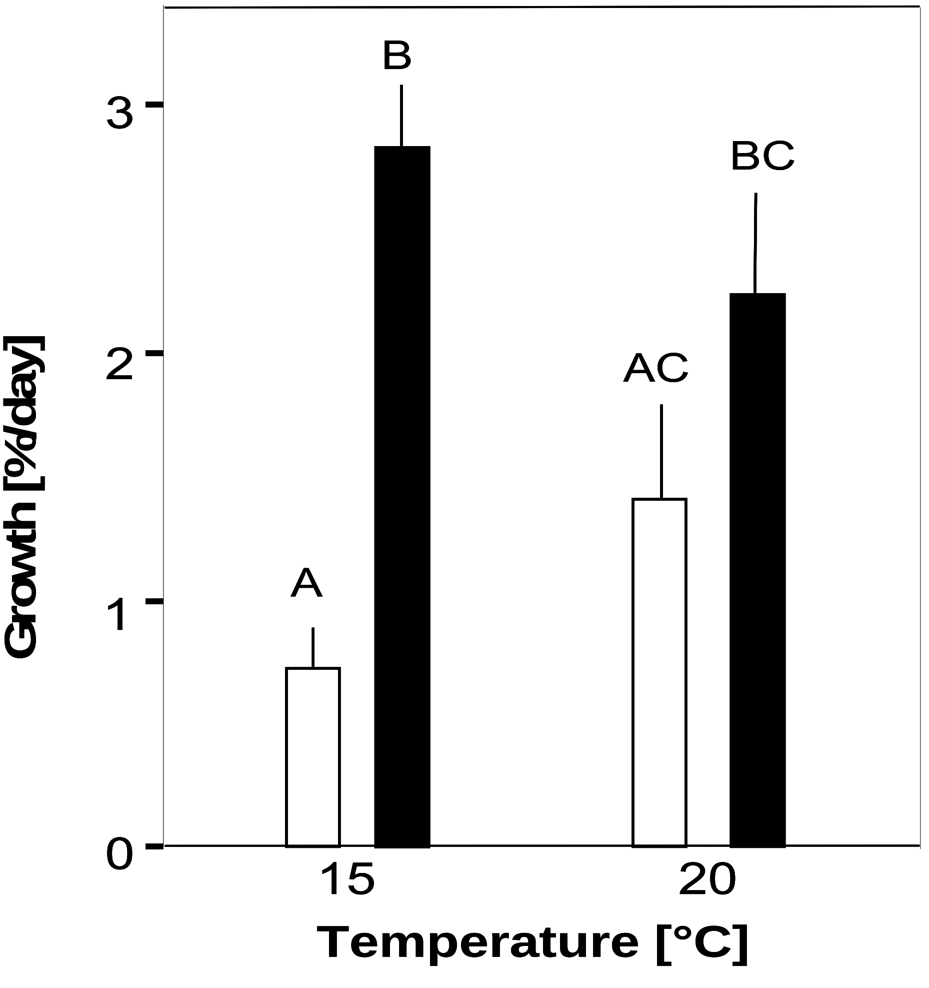

Figure 2: Growth rates of *F. vesiculosus* during the treatment phase (white bars = low light [30 µmol m^{-2} s^{-1}]; black bars = high light [75 µmol m^{-2} s^{-1}]). Growth was measured as changes in biomass. Different letters represent significant differences in growth, Wilcoxon, $p < 0.01$)

Epibacteria

Significant increases in bacterial numbers were observed during the treatment phase under all conditions except at 20°C in combination with low light (Fig. 3). The intensity of increases varied widely within all treatments and significant differences among treatments were not detected (Nemenyi-test, p = 0.05; Tukey-test, p = 0.001). A four-wayANOVA (Tab. 1) revealed that the variability resulted primarily from different behaviour of the five tested algal individuals, which behaved in three significantly different ways (Tukey-test, p = 0.001). Significant effects of light (stronger increases in high light) and temperature (stronger increases at 15 °C) were also detected, as well as significant interactions among all three factors (Tab. 1). Only the grazing treatment did not significantly affect bacterial development.

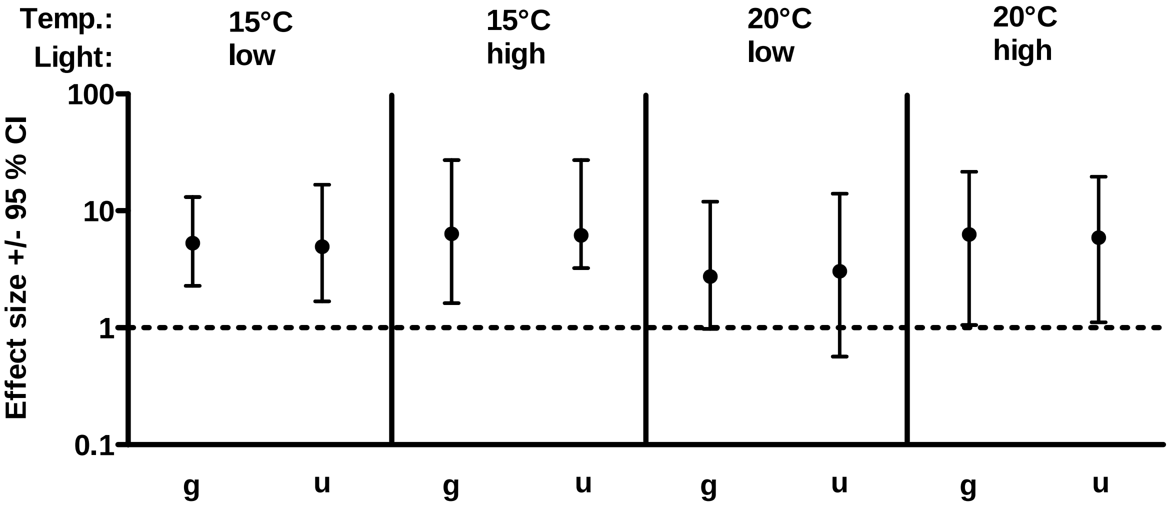

Figure 3: Development of the densities of bacteria associated with *F. vesiculosus*. The effect size was calculated as the ratio of bacterial numbers after and before the treatment incubation. Geometric mean +/- 95 % CI (n = 5). Values above 1 indicate increases during the incubation, error bars not including 1 indicate significant increases. g = grazed, u = ungrazed.

Table 1: Results of four-way-ANOVA conducted with Box-Cox transformed ratios of bacterial cell densities after and before the treatment incubation. T = Temperature, L = Light, G = Grazing, I = Individual; SS = sum of squares, Df = degrees of freedom, MQ = mean of squares, F = F-value p = level of significance.

	SS	Df	MQ	F	P
Constant	287.9199	1	287.9199	51967.76	**< 0.000001**
T	0.0655	1	0.0655	11.82	**0.000748**
L	0.2009	1	0.2009	36.26	**< 0.000001**
G	0.0001	1	0.0001	0.01	0.910796
I	0.5313	4	0.1328	23.97	**< 0.000001**
T*L	0.0609	1	0.0609	10.99	**0.001134**
T*G	0.0013	1	0.0013	0.23	0.63301
L*G	0.0013	1	0.0013	0.23	0.632187
T*I	0.5459	4	0.1365	24.63	**< 0.000001**
L*I	0.1136	4	0.0284	5.13	**0.00065**
G*I	0.02	4	0.005	0.9	0.46402
T*L*G	0.0015	1	0.0015	0.27	0.601378
T*L*I	0.2873	4	0.0718	12.96	**< 0.000001**
T*G*I	0.0315	4	0.0079	1.42	0.229715
L*G*I	0.0175	4	0.0044	0.79	0.533435
T*L*G*I	0.0399	4	0.01	1.8	0.130999
Error	0.8865	160	0.0055		

Discussion

To understand population dynamics of marine plants, we need to investigate the responses to abiotic conditions along with biotic interactions like herbivory. Numerous terrestrial studies demonstrated interactive effects of environmental and biotic factors on plant performance, but corresponding marine work is scarce (see discussion in Dethier et al. 2005). Our study clearly demonstrated that the predicted warming of summer surface temperature by 5°C prevents induction of anti-grazing defense in *Fucus vesiculosus*, while high light levels resulted in increased numbers of associated microorganisms.

Interestingly, we found no effect on the defensive level in response to stress by decreased light intensities. Nonetheless, the low light treatment effectively exerted stress on *Fucus*, since it reduced growth. In *F. vesiculosus* from the Gulf of Finland, summer values of light compensation points were between 25 - 35 µmol m^{-2} s^{-1} and light saturation of photosynthesis was detected at 300 µmol m^{-2} s^{-1} (Bäck & Ruuskanen 2000). Light saturation was also measured for *Fucus* individuals from the same population used in this study and estimated between 350 – 500 µmol m^{-2} s^{-1} (Rohde *et al.*, in press). Therefore, our applied light treatments are relatively low light conditions, since the high light level allows around 70% of the maximal photosynthesis of *F. vesiculosus* (Bäck & Ruuskanen 2000) and the low light level is approximately the compensation point. The predicted temperature increase did not affect growth rates but inhibited antigrazing defense induction.

There have been some studies on inducible defensive mechanisms of *F. vesiculosus*, showing that clipping (Hemmi et al. 2004; Van Alstyne 1988; Yates & Peckol 1993) or natural herbivory (Rohde et al. 2004) can induce herbivore resistance. However, whether environmental conditions modulate defense induction has not been assessed so far. To our knowledge, the only study on *F. vesiculous* that investigated interactions among abiotic factors on herbivore deterrence dealt with nutrient concentrations: Hemmi and coworkers (2004) have shown that nutrient enhancement did not affect the inducible resistance of *F. vesiculous*. The lack of a correlation between resource supply and defense is supported by our study. Lower light levels, decreasing physiological performance resulting in lower growth rates, did not correlate with decreased defenses.

Previous studies on other brown algae examining interactions among abiotic factors on seaweed chemical metabolites gave inconclusive results. Cronin and Hay (1996a) found no effect of nutrients and a positive effect of light on C-based metabolites in *Dictyota ciliolata* and no effects at all of light and nutrients on phlorotannin concentrations in *Sargassum filipendula*. Phlorotannin concentrations in *Ascophyllum nodosum* did not vary in response to nutrient enrichment. However, light had a positive effect in natural populations, but not in a manipulative experiment (Pavia & Toth 2000), while desiccation and UV-B radiation also positively affected phlorotannin concentrations (Pavia & Brock 2000).

While resistance can increase in response to grazing, White (1984) suggested that stress may increase the susceptibility to herbivores by increasing the concentrations of nitrogenous compounds that herbivores use as feeding cues. His hypothesis of increased stress resulting in increased susceptibility to herbivores is supported by our results with regard to temperature stress, but not to light stress. However, in this study we did not measure nitrogen concentrations. Renaud and co-workers (1990) found that the susceptibility of the red alga *Gracilaria tikvahiae* to urchin grazing decreased as a function of its previous desiccation that was paralleled with decreasing protein content. This indicates that higher consumption rates were based on higher nutritional value. However, the same study indicated that increased stress by desiccation of the brown alga *Padina gymnospora* resulted in highly increased herbivory rates. Since the preference for unstressed algae was not accompanied with concurrent change in tissue protein content and assays with extracts of stressed and unstressed algae showed the same results, it strongly suggests that chemical defense decreased with increasing stress.

Increasing densities of seaweed-associated microorganisms must not necessarily result from weakened defense. They can also be due to increased availability of nutrient resources. *F. vesiculosus* and other seaweeds continuously generate DOM (dissolved organic matter), which constitutes a nutrient source for microorganisms (Bell & Mitchell 1972). During the cultivation of seaweeds in relatively small water bodies such as aquaria with limited water exchange, DOM may accumulate and allow for accelerated growth of microorganisms. Algal excretion of DOM generally increases with photosynthesis, and this may possibly explain the observed particularly marked increases in high light. We did not find any differences in epibacterial cell numbers between control and herbivore-defended *Fucus*. This is a strong indication that herbivore defense is not related to antimicrobial defense. Our data also negate that the amount of surface bacteria affects the susceptibility to herbivores.

We hypothesised that low light intensities will decrease the defensive performance of *F. vesiculosus*. This hypothesis is in accordance with environmental stress theories which propose that defensive characters decrease in response to environmental stress (Rhoades 1985). Our hypothesis also corresponds with resource-based theories, such as the Growth-Differentiation-Balance-Hypothesis (Herms & Mattson 1992; Loomis 1953), which proposes that with increasing light, secondary metabolites will increase proportionally with growth (Stamp 2003). Also the Carbon-Nutrient-Balance-Hypothesis (Bryant *et al.* 1983) predicts that lower light levels will reduce the excess carbon production and, consequently, increase the availability of assimilated nitrogen for defense (Stamp 2003). However, no nitrogen-containing defense metabolites have so far been found in brown algae, despite an extensive effort (Amsler *et al.* 2001). We would therefore expect that the low light level in our experiment should decrease defenses. This is not supported by our results.

These unexpected results may be affected by the fact that we measured inducible, not constitutive defense, since inducible defense is a resource-saving mechanism and therefore may have potentially evolved in plants that often experience resource limitation. In any case, our results show that defense induction in *F. vesiculosus* is not directly affected by the light conditions used in our study, indicating that the metabolic process that produces the defensive compounds is not directly correlated to growth.

Interestingly, an increased temperature affected *F. vesiculosus* in a different way. No defense induction took place independent of light intensity. We proposed that 20°C exert stress on *F. vesiculosus* because this is the highest temperature that we find for longer periods within its natural distribution range

(Lüning 1990). Since at laboratory conditions, *F. vesiculosus* is capable to photosynthesise until 28°C (Lüning 1984), the upper temperature limit would allow it to penetrate further south into warmer waters, but some other factors must limit its distribution. If the decreased defensive performance at 20°C is also present in natural communities, which still has to be studied in the field, we can hypothesize that a reduced resistance to herbivory may be an important factor that limits the southern distribution of *F. vesiculosus*.

In conclusion, we found that a temperature increase jeopardized the capacity defense induction. This is especially noteworthy since a temperature increase is predicted for the Baltic Sea and therefore the prevalent alga-herbivore interaction may be affected in the future. Additionally, we could demonstrate that defense induction was not affected by light intensities and consequently not correlated with growth, which is predicted by several plant defense hypotheses.

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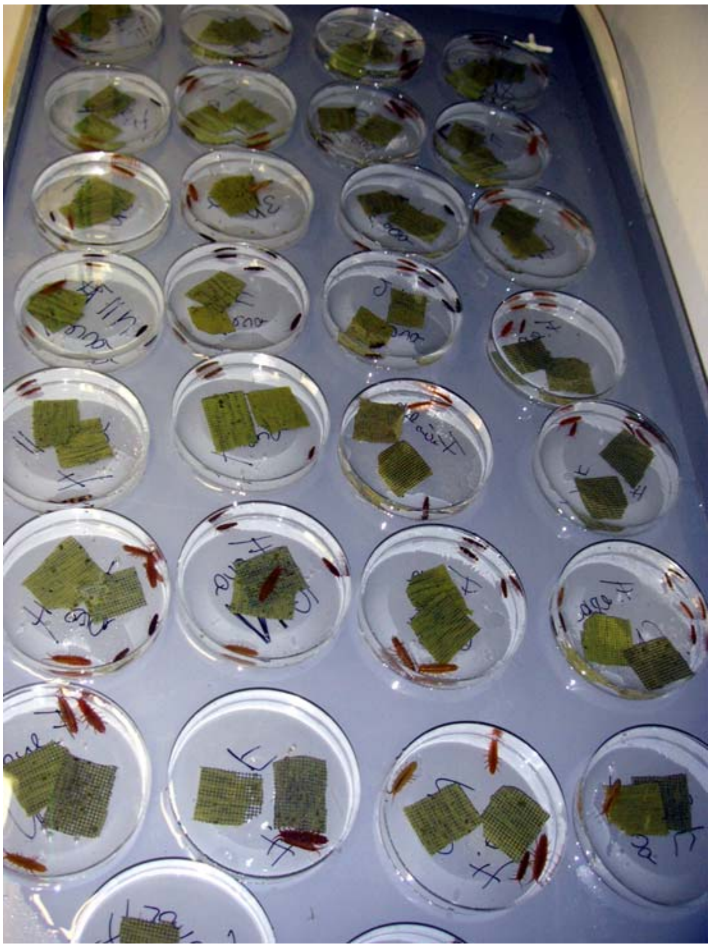
Chapter IV



Temporal dynamics of regulated defense in a marine macroalga:

Time lag of induction and reduction in *Fucus vesiculosus*

Abstract

Inducible resistance to herbivory in marine algae received much attention in recent years. However, while many studies on terrestrial plants identified eliciting signals and the response cascade to resistance, the knowledge on inducible marine macrolagal defense is lagging far behind and is often restricted to the mere recognition of its presence. This study is the first to accurately quantify the temporal dynamics of antifeeding defense in a marine macroalga, *Fucus vesiculosus* from the Baltic Sea. Time lag of induction and reduction of induced feeding resistance were assessed via feeding assays, because the identity of defensive compounds is still unknown. Our results demonstrate that *F. vesiculosus* induced defense 10 days after the onset of grazing by the isopod *Idotea baltica.* Defenses were relaxed within 2-4 days after cessation of grazing. Thus, defense seems to be deployed sparingly and just long enough to avoid substantial loss of tissue.

Introduction

By the 1970s, the Optimal Defense Hypothesis postulated that defensive requirements should be produced and allocated in a way that maximizes individual fitness (Rhoades 1979). Since then a multitude of alternative or complementary defense models have been suggested (Cronin 2001; Ianora *et al.* 2006). These models mostly explain the evolutionary occurrance of defense. At a finer temporal resolution and at the intra-specific level, however, defenses may also vary. Plasticity theory predicts that in environments where herbivore pressure is variable and the costs and/or risks of defense production are high, inducible defenses should have a selective advantage over constitutive defenses (Long & Trussell 2007; Zangerl & Bazzaz 1992). Besides saving resources, advantages of inducible defenses include reduced reliability as a signal for specialised herbivores (‘moving target’) or reduced risk of self-intoxication (reviewed in Karban & Baldwin 1997). Defense induction is a well-studied phenomenon in terrestrial plants. For decades, terrestrial ecologists have identified inducible defensive compounds, the elicitors and the signal cascade that convey this information (reviewed in Karban & Baldwin 1997; Tollrian & Harvell 1999; Zangerl 2003). In contrast, defense induction in marine macroalgae was believed to be scarce (but see Cronin & Hay 1996; Van Alstyne 1988; Yates & Peckol 1993). Studies on marine macroalgae that demonstrated inducible defense used mesograzers as inducers, since it is hypothesized that their feeding will cue induction of chemical defense in benthic prey, because they feed over temporal and spatial scales that allows induced defense to be effective (Hay 1996). The first two examples of induced defensive responses in seaweeds both involved mesograzers that could graze for days and weeks on a plant without killing it (Cronin & Hay 1996; Van Alstyne 1988). Recently, however, several studies suggested that defense induction in marine habitats may be more common than expected (Ceh *et al.* 2005; Hemmi *et al.* 2004; Macaya *et al.* 2005; Pavia & Toth 2000; Rohde *et al.* 2004; Weidner *et al.* 2004).

In contrast to investigations in the terrestrial environment, studies on marine macroalgal defense regulation are generally restricted to identify the mere existence of the capacity for defense induction. Only few studies include questions about the species specificity of induced defenses (Pavia & Toth 2000; Rohde et al. 2004), and environmental conditions were only considered in terms of nutrient supply to investigate cost-related allocations (e.g. Hemmi et al. 2004). The speed of algal response to the onset and the end of grazing are virtually unstudied. Also, the ecological implications of variability and dynamics of inducible defense received little attention in studies on both terrestrial as well as marine plant-herbivore interactions (Toth & Pavia 2007).

To date, studies on induced defenses in marine algae concentrated on brown algae (reviewed in Amsler & Fairhead 2006) with a chemical focus on phlorotannins (Boettcher & Targett 1993; Geiselman & McConnell 1981; Pavia & Toth 2000; Steinberg 1988). Meanwhile, the antifeeding role of phlorotannins has been discussed controversially. Inactivity against grazers (Deal *et al.* 2003; Pavia *et al.* 1997; Steinberg & van Altena 1992; Van Alstyne *et al.* 2001), or even feeding-enhancing effects (Jormalainen & Honkanen 2004) are described.

Research effort along several lines is requested for a better understanding of the ecology of inducible chemical defenses in marine communities: a taxonomically wider screening of the prevalence of inducible defenses to identify phylogenetic patterns (but see Toth & Pavia 2007), a widening of the chemical scope beyond phlorotannins to produce a more complete picture of chemical diversity, and an investigation of the intraspecific variability of inducible defenses as a consequence of intrinsic (physiology, life history stages, genetics) or extrinsic causes (environmental conditions, inducing agents such as predators) and of the ecological implications of this variability. In the present study, we start this endeavour by the last focus and examine the lag times of induction and reduction in a brown algal species.

The bladder wrack *Fucus vesiculosus* is an important constituent of many temperate littoral communities, especially so in the Baltic (Kangas 1982). Already Geiselman and McConnell (1981) demonstrated the antifeeding properties of *F. vesiculosus,* and the first documentation of inducible antifeeding defense in macroalgae was done on the genus *Fucus* (Van Alstyne 1988). Thereafter, several studies investigated further the defensive mechanism of *F. vesiculosus* often with regard to phlorotannin concentrations (Hemmi et al. 2004; Peckol *et al.* 1996; Rohde et al. 2004; Yates & Peckol 1993).

In this species, as in most others, nothing is known about the inertia of the response, i.e. the time lag from the onset of grazing to the deployment of an effective defense and the duration of relaxation, e.g. the time that defenses are upheld after grazing ceases. In most studies, an induction period of at least 14 days was allowed before the first probing (Pavia & Toth 2000, 2-4 weeks; Rohde et al. 2004, 2 weeks; Van Alstyne 1988, 2 weeks; Yates & Peckol 1993, 2 weeks). Generally, it remains unclear on which basis this induction period was chosen. Hemmi *et al.* (2004), by a unique clipping-induced resistance in *F. vesiculosus* that was upheld for 10 days, and Hammerstrom *et al.* (1998) demonstrated that clipped kelp species increased their phlorotannin concentration within 1-3 days and returned to pre-treatment levels within 10 days after treatment. In a former study, we did not detect induction after 3 days of feeding (Rohde et al. 2004). The response speed, i.e. the time requested to induce and reduce chemical defense, after the onset and cessation of grazing, is an essential feature in the interaction between prey and grazer. Because the deployment of chemical defenses may incur costs and may enhance the risk of detection, these drawbacks should be counterbalanced by the advantage of limiting the loss of supportive, photosynthetic or reproductively important tissue. We therefore expect that regulation speed relates to the motility of the grazer species and the damage per unit time it typically inflicts. Based on the observation that mesograzers – and in particular the locally very common isopod *Idotea baltica* – may inflict substantial biomass loss in a short while, we hypothesize that defense in *F. vesiculosus* will be induced within a few days. On the other hand, if defense is costly as often assumed but rarely proven (Ianora et al. 2006), the relaxation time of induced defenses is expected to be short as well (Stamp 2003).

Material and Methods

Study site and Organisms

All *Fucus* individuals were collected from a rocky shore in the Kiel Fjord (54°26’N, 10°11’E) at a depth of 0.5-1 m. All algae were removed by hand from the substratum, transferred in coolers to the laboratory, gently cleaned from all visible epibionts and maintained in aerated seawater until used (within 24 hours).

The isopod *Idotea baltica* Pallas, as the most important mesograzer in the Baltic Sea (Kangas *et al.* 1982) was collected from sea grass bedsin the Kiel Fjord, maintained in a 300 L aerated flow-through tank and fed with a variety of local algal species and fish.

Induction experiments

The experiment was run in a constant temperature room (15°C) at the Leibniz Institute of Marine Sciences (IFM-GEOMAR) in Kiel, Germany, in September 2006. Ambient seawater from the nearby Kiel Fjord (salinity: 15-17) was UV-sterilized (UV water steriliser 500, hw Wiegandt GmbH), stored in a 150 L tank and from there individually and continuously supplied (0.25 $L{\bullet}h^{-1}$) to 2.9 L aerated plastic aquaria. Light was provided by fluorescent tubes (OSRAM FLUORA 36 W/77 25X1) giving a total irradiance of 65.5 ± 2 $\mu mol\ photons{\bullet}m^{-2}{\bullet}sec^{-1}$.

40 plastic aquaria were used in this experiment; half of the aquaria were assigned to the treatment group (with grazers), the other half to the control group (without grazers). Treatment and control aquaria were randomly arranged in pairs of two in view of the final pair-wise feeding assays which were used to detect differences in palatability (as a proxy for defense). To reduce the variability caused by genetic differences, only one algal individual provided all the thallus pieces needed within any subset of 4 aquaria which represented the two treatment levels (grazed, ungrazed) in the two experiments (induction, reduc-

tion). Since the level of replication was ten, we used apical parts of ca. 10 cm length (each 2 - 2.5 g wet weight) from ten large individuals.

Experiment 1: Induction speed

Pre-treatment phase: to avoid any carry-over of unknown factors in the field, prior to the experiments all algae were maintained in running, filtered seawater without grazers for 14 days.

On day 0 of the treatment, five isopods were added to each of the ‘treatment’ aquaria while the control aquaria remained without grazers. Subsequently, on days 2, 4, 6, 9 and 14 one piece of alga at a time was removed from each aquarium and immediately frozen at -20°C. With each piece of alga removed, we withdrew one isopod from the corresponding aquarium to maintain grazing pressure per unit prey at a constant level. Feeding loss during the treatment phase was not measured. However, all removed algal pieces showed an increasing abundance of grazing marks with increasing treatment duration (ca. <0.5% on day 2 and 15% on day 14).

Experiment 2: Reduction speed

Conditioning phase: All algae in the treatment aquaria were exposed to grazers for 14 days, whereas no grazers were added to the control aquaria. Two weeks are sufficient for grazed algae to induce defenses and for non-grazed algae to reduce any carry-over effects from prior field conditions (Rohde et al. 2004). The removal of all grazers at the end of the conditioning phase marked the beginning of the reduction phase.

During the reduction phase, one piece of *Fucus* was collected from each aquarium on days 0 (removal of grazers), 2, 4, 6, 9 and 14. All algal samples were immediately frozen (-20°C). At the end of the two parallel experiments (induction and reduction), defense level was assessed by pair-wise comparison of palatability between treatment and control algae.

Feeding assays

To avoid physiological and chemical changes of the algae during the 24h-feeding assays, we used reconstituted food. This procedure only tests for chemical food quality and ignores structural properties of the algae. From previous studies we know, however, that antifeeding defense of *Fucus* is chemically mediated (Rohde et al. 2004). Frozen algal pieces were freeze-dried and ground to a fine powder with mortar and pestle, and this algal powder was then reconstituted in agar. First, the agar was heated to boiling in a microwave oven (0.18 g Agar + 5 ml distilled water), then allowed to cool to ~ 50°C, and subsequently mixed with the moistened alga powder (0.25 g of powdered alga + 1 ml distilled water). The mixture was poured over a mosquito mesh (mesh size 1 mm) and flattened between two layers of wax paper (Hay et al. 1994). The net was cut into squares of approximately 20 x 20 cells and treatments and controls were identified by clipping the corners in different shapes. These pieces of reconstituted food were offered in a two-way choice to three isopods for 24 h. From each treatment we counted the number of net cells consumed. To reduce the variability caused by differences between grazer individuals, we prepared three assays for each experimental replicate, calculated the relative feeding preference in percent and ran the statistical analysis using the mean preferences of each replicate.

Preferences were analyzed by the Wilcoxon test using directed P-values (pDir) with $\delta/\alpha=0.8$ (Rice and Gaines 1994; Deal et al. 2003) because our hypothesis was one-sided (algae with a grazing history being less palatable) but we wanted to preserve the possibility to also support the alternative.

Results

In the induction experiment, the isopods did not differentiate between non-grazed algae and grazed algae on days 2, 4, 6 and 9 (pDir >0.17, see Fig. 1).

In the induction experiment, the isopods did not differentiate significantly between non-grazed algae and grazed algae on days 2, 4, 6 and 9 (pDir >0.17, see Fig. 1). However, from day 6 onward a tendency to prefer ungrazed over grazed algae gradually increased in strength and became a significant discrimination by day 14 (pDir =0.003, see Fig. 1).

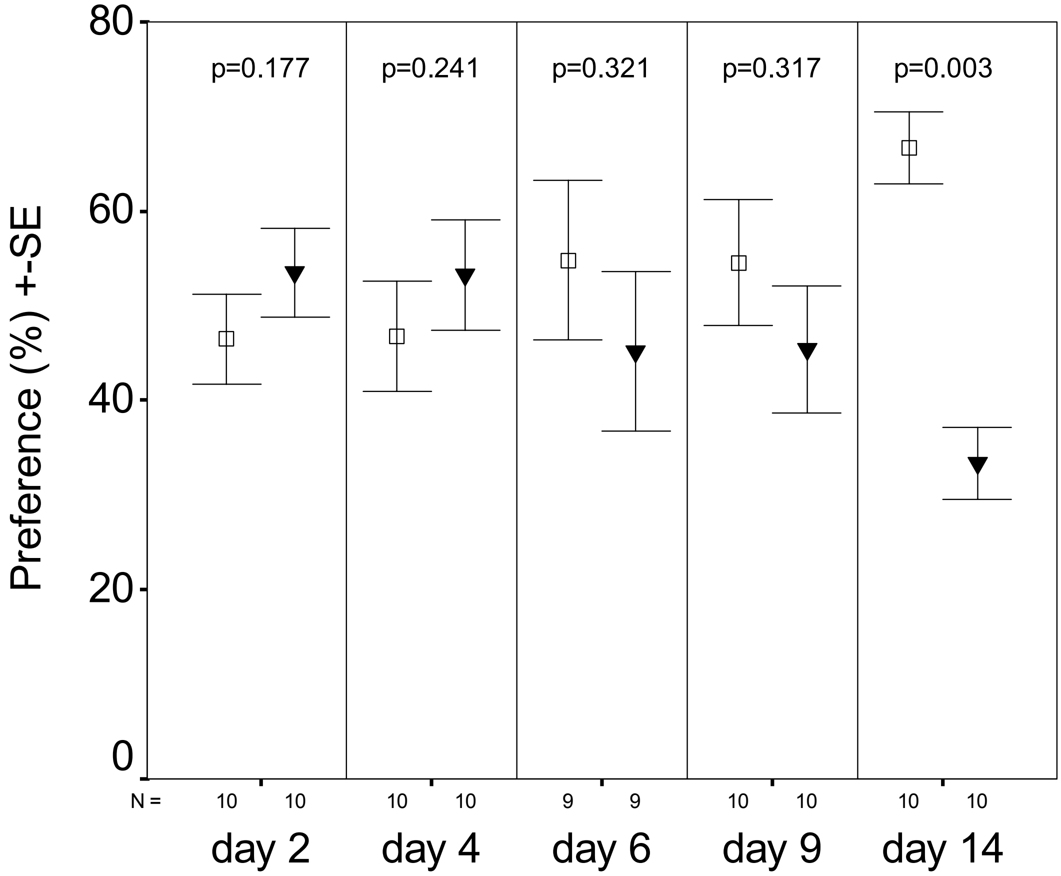


Figure 1: Feeding preference of the mesograzer *I. baltica* between *F. vesiculosus* which had not been exposed to previous grazing (white squares) or had been exposed to previous grazing for variable duration (black triangles).

In the reduction experiment, algae which had previously been exposed to grazers for 14 days were significantly less consumed than control algae (pDir=0.005), confirming the results of the induction experiment. 3 to 4 days after the removal of the grazers, this difference in palatability started to diminish. The significance of this difference disappeared by day 4 (pDir=0.08, see Fig. 2), and on day 6 after the cessation of grazing even the trend had disappeared (pDir=0.24, see Fig. 2). In view of this clear result, no more assays were performed with the samples from days 9 and 14.

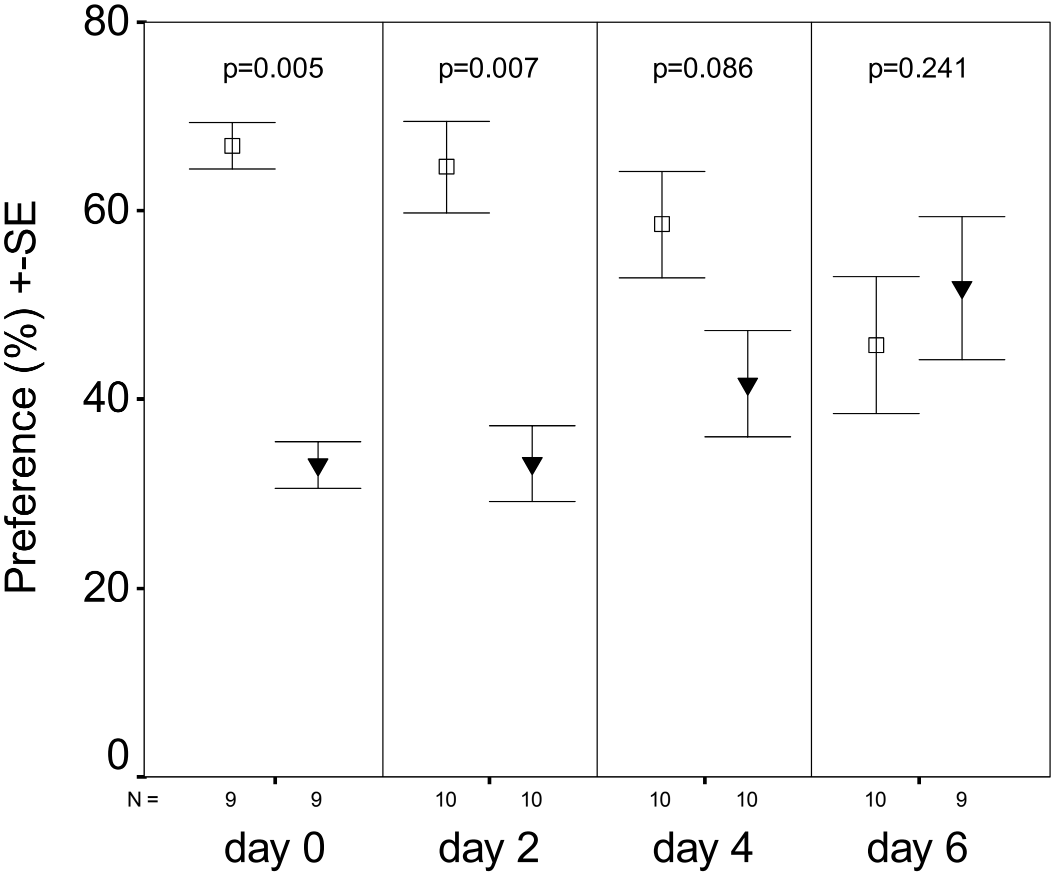


Figure 2: Feeding preference of the mesograzer *I. baltica* between *F. vesiculosus* which had not been exposed to previous grazing (white squares) or had been exposed to previous grazing for 14 days (black triangles).

Discussion

This study shows that *F. vesiculosus* induced defense against the isopod *I. baltica* after a grazing period of 9-14 days. Resistance was upheld for 2 to 4 days after cessation of grazing. The fact that this effect was detected in reconstituted food assays implies that the induced resistance to feeding was of chemical rather than morphological nature.

To our knowledge, this is the first study to demonstrate the temporal dynamics in the defense regulation of macroalgae. Consequently, direct comparison with the defense dynamics in other species is difficult. Grazing on the brown alga *Ascophyllum nodosum* from the Swedish west coast induced higher phlorotannin concentrations and herbivore resistance within 14 days (Pavia & Toth 2000), but not within 26 days on the US east coast (Long & Trussell 2007). Van Alstyne (1988) and Yates and Peckol (1993) induced higher phlorotannin concentrations in *Fucus* spp. within 14 days. However, none of these studies investigated at which moment during this period induction actually appeared. Peckol et al. (1996) analysed this phenomenon with more temporal resolution. They found that after clipping, the phlorotannin concentration in *F. vesiculosus* could increase within 3 days and decrease again after 7 days. However, in this study only phlorotannin concentrations were measured without a direct assessment of resistance against herbivores. Hemmi *et al*. (2004) induced resistance in *F. vesiculosus* by simulated herbivory (clipping) that was detectable (by grazing preference tests using *I. baltica*) within 2 and 10 days after the clipping event. Resistance was not correlated with phlorotannin concentrations in this study, indicating a defensive compound other than phlorotannins, which is supported by a study of Deal et al. (2003) who found a galactolipid responsible for feeding resistance rather than phlorotannins (but see Kubanek *et al.* 2004). The induction period of 2 days in the study of Hemmi et al. (2004) and the maintenance of resistance for 8 consecutive days are not supported by our findings. We found that a minimum of 10 days of grazing by *I. baltica* was required to induce defense and this defense was only upheld for 2-4 days after cessation of grazing.

This longer induction period and the shorter maintenance as compared to the data of Hemmi et al. (2004) may be due to the different nature of the induction cue, clipping *vs.* natural grazing. It is also possible that a critical amount of tissue must be lost before induction occurs, a threshold reached instantaneously by clipping but only progressively by isopod grazing. Interestingly, clipping induced no resistance in *F. vesiculosus* in our earlier experiment (Rohde et al. 2004). As a third possible reason for the differing test results, the experimental conditions with potentially limiting light intensities may have slowed down the induced response. However, *F. vesiculosus* grew well under these light conditions (1 - 2.5 %d-1, Rohde et al. 2004), which exceeded highly growth rates of *F. vesiculosus* in a outdoor study with natural sunlight (<0.7%d-1, Lehvo *et al.* 2001). The fast growth may be an indication that *F. vesiculosus* was not energy limited in our set-up. Finally, it is possible that the different treatments (clipping versus grazing) induced different defensive compounds with different temporal patterns or with different threshold concentrations with regard to isopod deterrence. Phlorotannins – controversially discussed as antifeedants in wrack - were not measured in this experiment. But phlorotannins as other polar compounds dissolve out in hours from agar matrices (Jormalainen *et al.* 2005). Since in our tests using reconstituted food the activity was detectable over days the deterrent compound is unlikely to be phlorotannin (or it is a very large polymer) and other polar compounds.

Since *I. baltica* is the major herbivore of *F. vesiculosus* in the Western Baltic and the only species that can cause significant biomass losses (Kangas et al. 1982), one might expect inducible defenses of the prey species to be evolutionarily attuned to the activity patterns of this herbivore. On a large scale, grazing by *Idotea* exhibits a seasonal pattern with a gradual increase in late spring and early summer, a conspicuous peak in fall, followed by a decrease in early winter (Salemaa 1979 and personal observations). At a smaller scale, *I. baltica* is a highly mobile herbivore and grazing pressure may locally vary in the course of hours or days. Defensive dynamics may be adapted to cope with these fast and short-term herbivore attacks of migrating isopod groups to limit tissue damage in times when grazing is maximal. Since induction and reduction of defense are regulated by the onset and cessation of grazing, respectively, defense levels will automatically adjust to the seasonal demands. The observed 10-day time lag of induction suggests that resistance is not induced in response to herbivore encounters of shorter duration. Possibly, the tissue loss inflicted by weak or short grazing is tolerable. Surprisingly, induced resistance relaxed already after 2-4 days. If induction last several times longer than reduction, then in an environment of sporadic grazing events defenses should be shut off most of the time. However, we did not examine whether defense once induced may be upheld by very moderate grazing pressure. Neither did we test whether an induction shortly after a reduction happens more rapidly.

The evolution of defense induction is often supposed to be driven by cost-saving advantages (but see Pohnert *et al.* 2007). The pattern of slow induction and fast reduction of defenses found in this study may support this hypothesis. While costs were not measured in this study, earlier experiments found no growth reduction (which could be indicative of energy limitation) in response to induced resistance (Hemmi et al. 2004; Rohde et al. 2004). An alternative explanation for the seemingly sluggish resistance dynamics may be that the metabolites responsible for resistance have primarily evolved for other processes like wound healing and resistance occurs only as a side effect (Hemmi et al. 2004). Any anti-biotic activity limiting infection of grazer inflicted wounds should, however, be deployed in the course of hours, not days. Also, if the active metabolites detected by our assay are part of a protective syndrome, their dynamics may be influenced by additional functions and triggers (Agrawal 2007). It is also possible, that defenses are produced in pulses short enough to deter grazer but not long enough to make it rewarding and feasible for the grazers to physiologically adapt to them. A short pulse of defense may also benefit *F. vesiculosus* by dispersing future tissue loss within the individual and/or the population and thereby reducing the risk of breakage of the whole fronds, which would greatly magnify the biomass loss (Hemmi et al. 2004).

We summarize that *F. vesiculosus* induced resistance 10 days after the onset of natural grazing by *I. baltica* and reduced resistance after 2-4 days after cessation of grazing. Further studies may focus on the natural variability of isopod grazing pressure, on the interaction between grazing intensity and induction speed, and on the intensity or frequency of grazing events necessary to maintain defenses at an active level after their initial induction.

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Synthesis and Conclusions

Studies on herbivore resistance in marine seaweeds have shown that differences in the susceptibility to grazers contribute to (a) driving ecological specialization (Hay 1992), (b) affecting species' distribution and community organization (Estes & Steinberg 1988; Lubchenco & Gaines 1981), and (c) determining feeding patterns and digestive efficiencies (Horn 1989; Pennings & Puglisi 1996; Targett *et al.* 1995). Understanding plant chemical defenses is thus fundamental for a wide array of ecological and evolutionary topics (Hay 1996).

Numerous investigations have demonstrated that seaweeds, like terrestrial plants, produce a large variety of secondary metabolites with highly variable chemical structures and many of them function as feeding deterrents (McClintock & Baker 2001). While much of the earlier studies on algal chemical defenses were focused on differences among species and higher order taxa, as well as on biogeographic comparisons (Hay 1996; Hay & Steinberg 1992; Van Alstyne *et al.* 2001), there has been a recent shift towards intraspecific variation and dynamic responses (Ianora *et al.* 2006).

Despite the increasing number of studies on induced responses in seaweeds, induction of defense has only been shown in a handful of macroalgal species (reviewed in Amsler & Fairhead 2006).

The Baltic Sea is an ecosystem where defense induction can be a selectively favored trait. In contrast to tropical regions, where macrograzer are the most significant grazers (Bolser & Hay 1996), in the Baltic Sea gastropods and small crustaceans are the main consumers of macroalgae (Kangas *et al.* 1982). The isopod *Idotea baltica* is the most important grazer of the littoral vegetation and its abundance fluctuates considerably due to seasonal changes, but also due to small-scale aggregations that result in high *Idotea*-concentrations in some areas, while others remain less affected (Kangas et al. 1982; Kotta *et al.* 2006).

Consequently, the herbivore pressure is very patchy in space and time, but can be considerable (Kangas et al. 1982; Kotta et al. 2006). Additionally, light conditions under water are often poor due to high turbidity (Bäck & Ruuskanen 2000; Lehvo *et al.* 2001). This leads to a strong energy limitation in many algal species.

These two system properties – variability of grazing pressure and energy limitation - should favor the evolution of inducible defenses.

The present work has contributed substantially to a better understanding of the prevalence of inducible defense in seaweeds (Chapter I). Since all tested red algae, *Delesseria sanguinea, Phyllophora pseudoceranoides* and *Furcellaria lumbricalis*, induced defense in response to grazing and the brown algae *Fucus evanescens* and *Fucus serratus* also showed inductive effects I suggest: 1) that the scarcity of defense induction in macroalgae results rather from a research bias than from any evolutionary pattern; this is also supported by a recent meta-analysis of 41 seaweed species, which found defense induction a common phenomenon (Toth & Pavia 2007); 2) that defense regulation is of particular selectively advantage in the Baltic Sea. In the Baltic, the conditions reflect all prerequisites discussed for the evolution of inducible defense: a) mesograzer like gastropods and small crustaceans are the main consumers of macroalgae (Kangas et al. 1982), b) grazing intensity is very variable since herbivore abundances fluctuate considerably due to seasonal changes, but also due to small-scale aggregations (Kangas et al. 1982; Kotta et al. 2006), c) light conditions under water are often poor due to high turbidity (Bäck & Ruuskanen 2000; Lehvo et al. 2001), which leads to a strong energy limitation in many algal species.

Despite several potential advantages (see Chapter I), defense induction by waterborne cues from conspecifics was only found in *Fucus vesiculosus* (Rohde *et al.* 2004). Trans-specific cues were not tested. Apparently, direct feeding is a more effective induction cue, and tissue loss between the onset of consumption and the induction of defenses is tolerated by the tested algal species. However, further research on ecological roles of waterborne substances from macroalgae is needed and may provide interesting new insights into the interactions of algal communities.

The bladderwrack *F. vesiculosus* is an extensively studied macroalgal species with regard to chemical defense (Hemmi *et al.* 2004; Jormalainen *et al.* 2003; Peckol *et al.* 1996; Rohde et al. 2004; Yates & Peckol 1993). This species has declined in the Baltic and this decline has been attributed to eutrophication and associated changes in competitive interactions and grazing pressure (reviewed in Korpinen *et al.* 2007). Although the causal chain of eutrophication leading denser phytoplankton, reduced light penetration, more epiphytes, the thereof resulting reduction in overall photosynthetic carbon gainsfor benthic macroalgae, and ultimately the shallower depth distribution of *F. vesiculosus* is intuitive and generally accepted, it has rarely been confirmed by experimental evidence. This work demonstrated that stress by low light leads in *F. vesiculosus* in the Kiel Fjord to a physiological depth limit between 4 and 6 m depth (Chapter II). Epibionts enhanced the negative effect of reduced light conditions on growth. The reduced depth distribution and consequently the massive biomass loss of the *Fucus*-population in the Baltic may further increase the detrimental effects of herbivores and enhance a strong selection for resistance.

Reduced light has shown to affect *Fucus'* depth distribution and climatic models predict an increase in water temperature in the Baltic. While terrestrial plant ecologists have found complex interactions between abiotic stress and herbivory, we know very little about how herbivory and stress interact to affect key algal functions (Dethier *et al.* 2005). However, for the first time this study provides evidence that the global warming can have strong affects on *Fucus*-herbivore interactions (Chapter III). A temperature increase from 15°C to 20°C, which is predicted for the Baltic, prevented defense induction in *F. vesiculosus*, while reduced light had no effects on inducible defense. There were no differences in epibacterial cell numbers between control and defense-induced *Fucus*. This may indicate that herbivore defense is not related to antimicrobial defense. However, only cell numbers, not bacterial community composition was tested. My data also negate that the amount of surface bacteria affect the susceptibility to herbivores. The findings of this study point the way to the importance of knowing how herbivore-alga interactions will respond to a changing environment.

In plant herbivore interactions, both plants and herbivores generate selection pressure on each other's traits, which leads to a coevolution of these species. While herbivores select for defenses, the defenses on the other hand select for the herbivores' ability to overcome them (Thompson 2005 and references therein). Therefore, efficiency of defences and herbivores' ability to tolerate them should be regarded in the context of the evolutionary history of both parties. Consequently, high species specificity in the interactions is expected and also regularly found (Cruz-Rivera & Hay 2003; Targett & Arnold 2001). Only few studies included questions about the species specificity of induced defenses (Pavia & Toth 2000; Rohde et al. 2004). The defensive dynamics, i.e. the time requested to induce and relax chemical defense, is an essential feature in the interaction between alga and grazer. Because chemical defenses may incur costs and may enhance the risk of detection by specialised herbivores, these drawbacks should be counterbalanced by the advantage of limiting the loss of supportive, photosynthetic or reproductively important tissue. I therefore expected that regulation speed relates to the motility of the grazer species and the damage per unit time it typically inflicts. This study shows that *F. vesiculosus* induced defense against the isopod *I. baltica* after a grazing period of 9-14 days (Chapter IV). Resistance was upheld for 2 to 4 days after cessation of grazing. This is the first study to demonstrate the temporal dynamics in the defense regulation of macroalgae. Since *I. baltica* is the major herbivore of *F. vesiculosus* in the Western Baltic and the only species that can cause significant biomass losses (Kangas et al. 1982), one might expect inducible defenses of the prey species to be evolutionarily attuned to the activity patterns of this herbivore. The observed 10-day time lag of induction suggests that resistance is not induced in response to herbivore encounters of shorter duration. Possibly, the tissue loss inflicted by weak or short grazing is tolerable. Surprisingly, induced resistance relaxed already after 2-4 days. The fact that induction lasts several times longer than relaxation indicates that in an environment of sporadic grazing events defenses of *F. vesiculosus* are shut off most of the time and are only induced in longer periods of high grazing pressure.

This thesis gives new insights in the prevalence as well as in abiotic interactions and temporal dynamics of inducible defense in macroalgae. Initially, very few studies supported the existence of inducible defense in macroalgae, but this study and other observations started to accumulate observations on inducible responses (reviewed in Toth & Pavia 2007). Therefore, inducible responses have shown to be common and future studies should address more advanced hypotheses. Interesting and rewarding research areas within the field of induced defenses could be: the effects of induced responses on other organisms and community structure, metabolic or ecological costs of induced responses, and at last the variability of induced resistance and its interactions with other biotic and abiotic factors, a field which this thesis has started to examine.

If we attempt to predict future changes in the benthic community in the Baltic, we have to consider intensively the effects on the *F. vesiculosus*-population. *F. vesiculosus* is the only perennial, belt-forming brown alga in the Baltic, which makes it a key species in creating habitat structure. However, the *Fucus*-population the Kiel Bight has already decreased in biomass of up to 95% (Vogt & Schramm 1991) and future distributional developments of the population are unclear, especially if we take the predicted climate changes into consideration. *Fucus'* distribution is controlled by substrate and light availability and herbivores. Substrate availability will not change considerably, but light intensities have already decreased and banished *Fucus* from greater depths. Herbivores contributed to the *Fucus* decline by extensive grazing and this project strongly suggests that their impact will still increase in the future. The predicted temperature increase weakened the defensive responses of *Fucus* and may thus further support higher grazing rates. This effect can be amplified by the fact that higher temperatures can increase the metabolic rate and consequently the feeding rates by herbivores (e.g. Vernberg & Piyatiratitivorakul 1998). To conclude, the future distribution of *F. vesiculosus* in the Baltic is questionable, but grazers can have a tremendous negative impact that will still enhance the direct effects of anthropogenic changes.

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Origin of pictures

Sven Rohde: Front page; general introduction, Fig. 1; front page Chapter III & IV

www.algaebase.org: frontpage Chapter I (modified)

Martin Wahl: frontpage Chapter II

Curriculum Vitae

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07/2001 – 12/2001	Studium der Meeresbiologie an der James-Cook-University in Townsville, Australien
06/2002	Diplomprüfungen in den Fächern Zoologie, Biologische Meereskunde und Meereschemie
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seit 07/2004	Promotion am IFM-GEOMAR, Kiel

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Ich habe bisher ke inen anderen Promotionsversuch unternommen, und diese Arbeit hat weder ganz noch teilweise im Rahmen eines anderen Prüfungsverfahrens vorgelegen. Bei der Erstellung dieser Abhandlung habe ich mich an die Regeln guter wissenschaftlicher Praxis gehalten.

Kiel, 25. September 2007

Teile dieser Arbeit wurden bereits wie folgt veröffentlicht bzw. eingereicht:

Rohde, S., and Wahl, M. Antifeeding defense in Baltic macroalgae: Induction by direct grazing vs. waterborne cues. *Journal of Phycology* **In press**.

Rohde, S., Hiebenthal, C., Bischof, K., Karez, R., and Wahl, M. Decreased depth distribution of *Fucus vesiculosus* (Phaeophyceae) in the Western Baltic: Effects of light deficiency and epibionts on growth and photosynthesis. *European Journal of Phycology* **In press**.

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Rohde, S., and Wahl, M. Temporal dynamics of regulated defense in a marine macroalga: time lag of induction and reduction in *Fucus vesiculosus*. *Submitted to Oecologia.*