

Invasion Ecology of Marine Macroalgae:

The relevance of stress resistance for the invasion success of *Gracilaria vermiculophylla* and consequences of its spread

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Mareike Hammann
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Invasionsökologie mariner Makroalgen:

Die Bedeutung von Stressresistenz für den Invasionserfolg von
G. vermiculophylla und Folgen ihrer Ausbreitung

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Vorgelegt von
Mareike Hammann
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Abstract

Originating from East Asia, the perennial red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss has successfully invaded several temperate areas of the Northern hemisphere and continues to spread. In its new range, the seaweed tends to form local mass appearances and to dominate the native community. A high tolerance towards both abiotic and biotic environmental stressors could explain the invasion success of this species.

I therefore compared the stress resistance of *G. vermiculophylla* from six native populations from South Korea and China and eight invasive populations from Europe and NW-Mexico. In short-term experiments *G. vermiculophylla* individuals were exposed to 1) heat shock, 2) UV-C-radiation and 3) elevated copper concentrations in the water. In a long-term experiment the seaweed had to cope with depletion stress (darkness in combination with low temperature and dryness) for several months. All experiments were carried out twice - one time in the native range in Qingdao, China and one time in the invaded range in Kiel, Germany - to rule out local acclimation effects. In order to compare the resistance against herbivory individuals of native and invasive *G. vermiculophylla* populations were fed to snails from the native (*Littorina brevicula*) and the invasive (*Littorina littorea*) range.

Non-native populations of *G. vermiculophylla* and single native populations (originating from the Sea of Japan) were less palatable than native populations from other areas. Thus, specimens from the Sea of Japan may have been successful invaders due to their resistance toward herbivory. To elucidate mechanisms for feeding resistance, selected arachidonic acid derived metabolites and extracts of *G. vermiculophylla* containing oxylipins were tested for their bioactivity towards *L. brevicula* and *L. littorea*. The elevated feeding resistance is at least in part explained by the deterring effect of oxylipins that are higher concentrated after wounding of non-native than of native individuals of the seaweed. In the case of the heat shock experiment in contrast to the feeding experiment, all natives – irrespective of the area of origin – showed a considerably poorer performance than all invasives, which suggests that heat shock resistance may have been selected early during the process of invasion. The resistance towards UV-C radiation was not significantly different among native and invasive populations. With respect to long term depletion stress and copper toxicity resistance, different results were obtained in the two testing sites suggesting that acclimation may possibly affect those capacities in *G. vermiculophylla*.

When testing invasion theories it might thus be very important to investigate 1) invasive and source populations of the same species, 2) several populations that have been sampled wide across each range and to execute the experiments 3) in both the native and the introduced range of the invader.

In the Baltic Sea, *G. vermiculophylla* might threaten the habitat-forming native brown alga *Fucus vesiculosus* through direct competition for resources and by providing a shelter for mesograzers, which prefer to feed on *F. vesiculosus*. Mesocosm-experiments were conducted over one year in the Kiel Fjord in order to test the direct and indirect effects of *G. vermiculophylla* on *F. vesiculosus*. High densities of *G. vermiculophylla* reduced both the growth of adult *F. vesiculosus* and the survival of *Fucus* germlings. Grazers associated with *G. vermiculophylla* also negatively affected *F. vesiculosus* adults. My results suggest that *G. vermiculophylla* impacts *F. vesiculosus* through direct competition for resources and by exposing it to higher grazer pressure.

Zusammenfassung

Die aus Ostasien stammende mehrjährige Rotalge *Gracilaria vermiculophylla* (Ohmi) Papenfuss hat erfolgreich zahlreiche Gebiete in den gemäßigten Breiten der nördlichen Hemisphäre neu besiedelt und breitet sich auch weiterhin aus. In den neu besiedelten Gebieten bildet die Alge oftmals lokale Massenvorkommen aus und neigt dazu, die heimischen Gemeinschaften zu dominieren. Eine hohe Toleranz gegenüber abiotischen und biotischen Umweltparametern könnte den Invasionserfolg dieser Art erklären.

Wir verglichen deshalb die Stressresistenz von sechs nativen Populationen von *G. vermiculophylla* aus Südkorea und China mit der Stresstoleranz von acht invasiven Populationen aus Europa und NW-Mexiko. In Kurzzeit-Experimenten wurden *G. vermiculophylla*-Individuen folgenden Stressoren ausgesetzt: 1) Hitzeschock, 2) UV-C-Strahlung, 3) erhöhte Kupferkonzentrationen im Wasser. In einem Langzeitexperiment musste die Alge mehrmonatigen Mangelstress (Dunkelheit kombiniert mit niedrigen Temperaturen und Austrocknung) erdulden. Alle Experimente wurden zweimal durchgeführt – einmal im nativen Gebiet in Qingdao, China und einmal im neu besiedelten Gebiet in Kiel, Deutschland, um lokale Akklimatisierungseffekte auszuschließen. Um die Resistenz gegenüber Herbivorie zu vergleichen wurden Individuen, die aus nativen und invasiven *G. vermiculophylla*-Populationen stammten, an Schnecken aus dem Ursprungsgebiet der Alge (*Littorina brevicula*) und aus dem neu besiedelten Gebiet (*L. littorea*) verfüttert.

Nicht-native *G. vermiculophylla*-Populationen und einzelne native Populationen (aus der Japanischen See) waren weniger schmackhaft als native Populationen aus anderen Gebieten. Der Invasionserfolg von Individuen aus der Japanischen See könnte daher durch ihre Resistenz gegenüber Herbivoren begründet sein. Um die Mechanismen aufzuklären, die für eine Fraßresistenz verantwortlich sein könnten, wurde die Aktivität von Oxylipin-Extrakten, die aus *G. vermiculophylla* gewonnen wurden, und die Aktivität ausgewählter Arachidonsäurederivate gegenüber *L. brevicula* und *L. littorea* getestet. Die erhöhte Fraßresistenz wird zumindest teilweise durch den abschreckenden Effekt von Oxylipinen erklärt, die nach Verwundung in nicht-nativen Algenindividuen in höheren Konzentrationen vorkommen als in nativen Individuen. Im Gegensatz zum Fraßexperiment überlebten beim Hitzeschockexperiment alle nativen – unabhängig von ihrem Ursprungsgebiet – deutlich schlechter als alle invasiven Populationen. Das lässt vermuten, dass Hitzeschockresistenz

bereits in einem sehr frühen Stadium der Invasion unter Selektion war. Die Resistenz gegenüber UV-C-Strahlung unterschied sich nicht signifikant zwischen nativen und invasiven Populationen. In den Mangel- und Kupferstressexperimenten wurden an den zwei Versuchsstandorten unterschiedliche Ergebnisse erzielt, was darauf schließen lässt, dass Akklimatisierungseffekte eine Rolle gespielt haben könnten.

Beim Testen von Invasionstheorien könnte es deshalb von großer Wichtigkeit sein 1) invasive und native Populationen einer Art zu untersuchen, dabei 2) verschiedene Populationen zu verwenden, die einen möglichst großen Teil des jeweiligen Gebietes abdecken und 3) die Experimente sowohl im nativen als auch im neu besiedelten Gebiet der invasiven Art durchzuführen.

In der Ostsee könnte *G. vermiculophylla* die native Braunalge *Fucus vesiculosus* – die ein wichtiges Habitat für zahlreiche Arten darstellt – sowohl durch direkte Konkurrenz um Ressourcen bedrohen als auch dadurch, dass sie einen Lebensraum für Mesograzer bietet, die jedoch bevorzugt *F. vesiculosus* fressen. Über ein Jahr hinweg wurden Mesokosmen-Experimente in der Kieler Förde durchgeführt um den direkten und den indirekten Effekt von *G. vermiculophylla* auf *F. vesiculosus* zu untersuchen. War sie in hohen Dichten anwesend, verringerte *G. vermiculophylla* sowohl das Wachstum von adultem *F. vesiculosus* als auch Überleben von *Fucus*-Keimlingen. Mit *G. vermiculophylla* assoziierte Grazer hatten ebenfalls negative Auswirkungen auf adulten *F. vesiculosus*. Meine Ergebnisse weisen darauf hin, dass *G. vermiculophylla* *F. vesiculosus* sowohl durch direkte Konkurrenz um Ressourcen beeinflusst als auch dadurch, dass sie die native Art einem höheren Fraßdruck aussetzt.

1. Introduction

Williams and Smith (2007) define an introduced seaweed (or invader) as “a species belonging to the Phyla Charophyta, Chlorophyta, Ochrophyta (formerly Phaeophyta) or Rhodophyta that has been introduced beyond its native range through human activities and has become successfully established in the new locale”. Introduced species are commonly considered to be invasive when they incur or are likely to incur negative ecological or economic impacts (Boudouresque & Verlaque 2002). This definition, however, is difficult because in many cases studies about the impacts of introduced species in their new ranges are lacking and the attribution of a negative effect is a very subjective interpretation (Inderjit et al. 2006).

In my work I will thus use the terms “non-native”, “introduced”, “invader”, “invasive”, “non-indigenous species (NIS)” or “alien species” as descriptions for species that were transported to a new habitat through human activities and have established and spread in their introduced range, but without necessarily inducing any negative impacts.

Seaweeds are frequent marine invaders (Schaffelke et al. 2006). Their share in total marine NIS is roughly 10 – 40%, with estimated absolute numbers of 300 species (Williams & Smith 2007). In some regions, non-native seaweeds comprise about 5% of the total flora (Ribera & Boudouresque 1995). Main vectors for seaweed invaders are hull fouling and shellfish farming, but in 40% of the known algal introductions the precise mode of introduction remains unidentified (Williams & Smith 2007). Especially red corticated macrophytes are commonly introduced via shellfish aquaculture. Introductions via ballast water only play a minor role for seaweed introductions, although they are generally very common for marine species (Carlton & Geller 1993, Ruiz et al. 1997). Fishing gear is likely to be a more important vector for secondary introductions, after a seaweed species has already established itself in a new environment (Williams & Smith 2007). Movements of pleasure crafts and other small boats could also contribute to the dispersal of non-native seaweeds within the introduced range (Inderjit et al. 2006).

Although rates of invasion have never been higher than today with thousands of marine species being transported between geographic regions, there are still large research gaps in invasion ecology (Johnson & Chapman 2007).

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In the following, I will introduce my study species, the macroalga *Gracilaria vermiculophylla*, and report about its invasion history, especially on the German Baltic coast, and its suitability as a model organism to investigate seaweed invasions (section 1.1). Subsequently, I will pose the question why some species do become invasive while others do not and summarize the main hypotheses to answer this question (section 1.2). In this context I will focus on two important subjects: The role of abiotic stressors (section 1.2.1) and the role of herbivory (section 1.2.2) for the invasion success of plants in general and seaweeds in particular. Then I will give a brief overview of the ecological impacts of seaweed invasions in the invaded habitats, while concentrating on *G. vermiculophylla* and its suspected effects on the Baltic Sea communities (section 1.3). Finally I will pose my research questions (section 1.4).

1.1 *Gracilaria vermiculophylla* as an example of an invasive seaweed

The red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss has a dark brown, cylindrical thallus up to 100 cm in length and 5 mm in diameter and is irregularly branched ((Bellorin et al. 2004) and see Fig. 1). It was originally described from Akkeshi bay, Hokkaido, Japan (Ohmi 1956, Papenfuss 1967, Terada & Yamamoto 2002). Its native distribution range is in the cold and warm temperate Northwest Pacific, ranging from the Northern Sea of Japan to the East China Sea and the Central Kuroshio Current (Tseng & Xia 1999).



Fig. 1: *G. vermiculophylla*, Kiel Fjord. The seaweeds usually grow unattached and are often found drifting in shallow waters or beached (left). They are dark brown, when desiccated almost black, and their thallus is cylindrical and irregularly branched (right).

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Ten years ago, there were first records of this species from areas outside its native range and authors suspect that *G. vermiculophylla* has been invading new habitats throughout the northern hemisphere for almost two decades now: In Europe, the species was first discovered on the Brittany coast in 1996 and later in the vicinity of oyster farms near Roscoff, France (Mollet et al. 1998, Rueness 2005). Bellorin et al. (2004) provide the first formal record for *G. vermiculophylla* from the Pacific coast of North America (Baja California, Mexico), but an unidentified species of *Gracilaria* that was detected in California is likely the first discovery (Goff et al. 1994, Rueness 2005). Saunders (2009) was able to show that the species is also occurring on the Northern parts of the Pacific Coast (British Columbia, Canada). *G. vermiculophylla* has spread to the North American Atlantic coast as well and was reported from Virginia (Thomsen et al. 2006) and North Carolina (Freshwater et al. 2006). Today the seaweed has furthermore established itself in the Mediterranean Sea (Sfriso et al. 2010), on the Atlantic coast of Morocco (Guillemin et al. 2008) and in Northern European Seas, including the Baltic Sea (Schories & Selig 2006, Nyberg 2007, Thomsen et al. 2007a, Thomsen et al. 2007b, Weinberger et al. 2008).

The Baltic Sea is – similar to other European brackish water seas – subject to intense invasion of alien species (Paavola et al. 2005). During the last few decades invasion rates of species into the Baltic Sea increased (Leppäkoski et al. 2002) and today more than 110 aquatic non-indigenous species are known from this sea (Schories & Selig 2006). One reason for this might be that native species richness reaches a minimum at intermediate salinities, while NIS are well adapted to these salinity levels already in their native range and can thrive in brackish waters (Paavola et al. 2005).

G. vermiculophylla is one of the relatively few macroalgal invaders in the Baltic Sea (Baltic Sea Alien Species Database 2007) and was detected for the first time at Kiel in 2005 ((Schories & Selig 2006), see Fig. 2). A survey of the German Baltic Sea showed that the species was only established in Kiel Fjord, Germany, in 2006 and 2007 and even a mass occurrence with ground coverage of 50 – 100% was observed in parts of the fjord. East of the Kiel Bight only single drifting individuals were found. In 2008, *G. vermiculophylla* underwent a marked decline in the Kiel Fjord. In 10 locations in the Kiel fjord *G. vermiculophylla* virtually disappeared from 2007 to 2008. The trend already changed in 2009, and the populations started to recover. In the following three years there were no big

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changes in comparison to 2009 and populations remained relatively stable, but at a low level (coverage max. 10%). Today, *G. vermiculophylla* in the Kiel Fjord seems to have recovered from the massive breakdown in 2007/2008 and ground coverages of up to 50% were again observed in 2013 (see Fig. 3A and B).

In contrast with the decline in the Kiel Fjord newly established populations were detected in 2008 at Sehlendorf (40 km eastward of Kiel) and at Heiligenhafen (70 km eastward of Kiel) and these were virtually healthy (see Fig. 2 and 3C). The population in Sehlendorf is located in Sehlendorfer Strandsee, a shallow and muddy lagoon with relatively low salinity (8 PSU). Early in 2008 the population in the lagoon – unattached individuals, partly covered by mud - was detected and it clearly increased in biomass until autumn 2008 (although the overall ground coverage did still not exceed 1 % at that time). In Heiligenhafen, *G. vermiculophylla* was detected in autumn 2008, firmly anchored in the sandy sediment. The populations in Heiligenhafen and Sehlendorf continued to grow and in some parts of these areas a ground coverage of up to 25% could be observed in 2011. The population in Heiligenhafen was not visited in 2012, but in 2013 it had declined markedly (Fig. 3C). In 2009, *G. vermiculophylla* was found in the lagoon of Großenbroderfähre and this is still the easternmost population established on the German Baltic coast (see Fig. 2). Up to now, this population remained very stable at a low level (see Fig. 3D). In 2013, *G. vermiculophylla* was discovered on the island of Fehmarn. Also in 2013, *G. vermiculophylla* was for the first time found north of the Kiel Fjord (in Eckernförde and Flensburg Bight) (see Fig. 2). These findings correspond well with genetic analyses indicating that *G. vermiculophylla* reached the German Baltic Sea through the Kiel Kanal (Stacy Krueger-Hadfield, Nicole Kollars and Erik Sotka, personal communication) and from this starting point spread both to the northwest and to the east (see Fig. 2).

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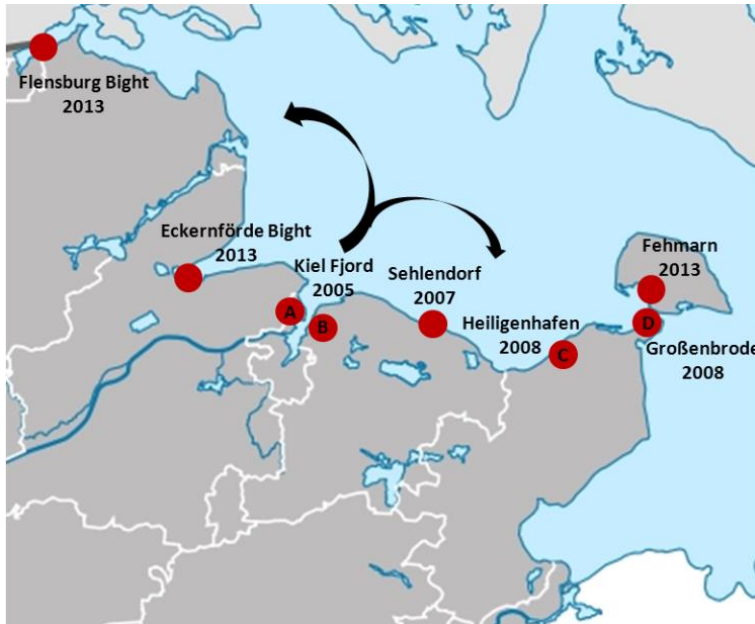


Fig. 2: Distribution and spread of *G. vermiculophylla* on the German Baltic Sea coast. Red dots mark selected places where the seaweed has been found. Letters A – D indicate exemplary populations that underwent an interesting dynamic throughout the years, which is illustrated in Fig. 3.

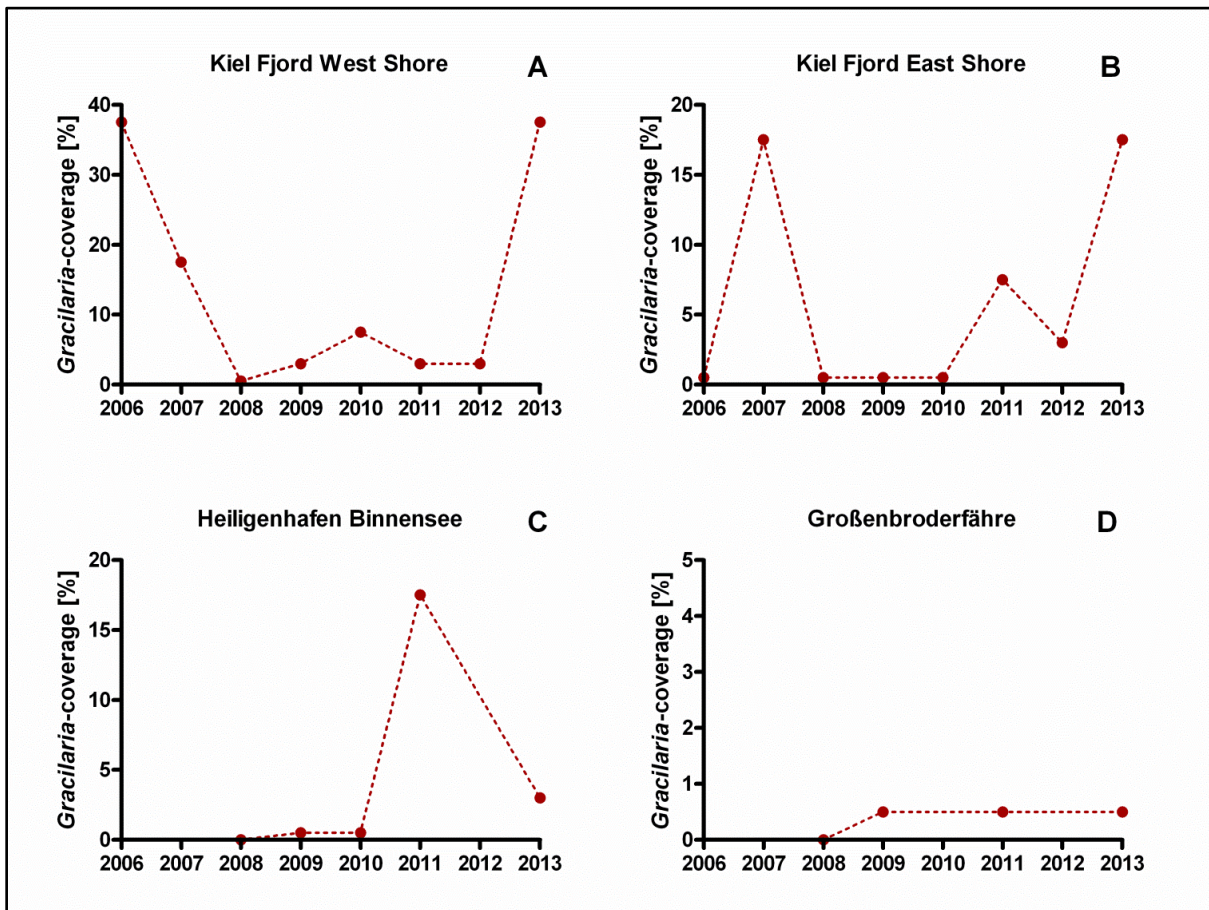


Fig. 3: Development of *G. vermiculophylla* on the German Baltic Sea coast. Population dynamics at four selected locations on the German Baltic Sea coast. Geographical positions can be found in Fig. 2.

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The invasion process resulted in considerable loss of genetic diversity in non-native populations of *G. vermiculophylla* (Saunders 2009, Kim et al. 2010). COX1 sequences of *G. vermiculophylla* individuals from the Canadian Pacific coast were identical with sequences from Portugal and Rhode Island except for one single base substitution in one individual from Canada. Moreover, they differed from COX1 data in GenBank for *G. vermiculophylla* from Japan by only one single substitution over 510 base pairs (Saunders 2009). Based on analysis of the COX1 gene from 312 individuals of *G. vermiculophylla* collected in 37 native and 32 introduced locations, Kim et al. (2010) suggest that all invasive populations of *G. vermiculophylla* in the east and west Atlantic and in the east Pacific do have a common donor region in the East Sea/Sea of Japan. New analyses refine this suggestion: The areas of origin for the invasive populations of *G. vermiculophylla* are likely the Pacific coastlines of northern Japan and Hokkaido island (Stacy Krueger-Hadfield, Nicole Kollars and Erik Sotka, personal communication).

G. vermiculophylla is a suitable model organism to study hypotheses in invasion ecology. It has established populations in various habitats on different coasts around the world and its large tolerance towards environmental stress (Yokoya et al. 1999, Thomsen & McGlathery 2007, Weinberger et al. 2008, Nyberg & Wallentinus 2009) makes it suitable for common garden-like experiments that require long-distance transportation under suboptimal conditions.

1.2 Why do species become invasive?

One of the main questions in invasion biology is: Why do some species become invasive while others do not (Boudouresque & Verlaque 2002, Johnson & Chapman 2007)? A variety of hypotheses has been proposed to answer this question (e.g. reviewed in (Hierro et al. 2005, Inderjit et al. 2006) and see below). There are mainly two different approaches to explain why a species becomes a successful invader: The reasons for invasion success could either be assigned directly to physiological traits of the invader (e.g. ability to survive the introducing process, life-history strategy, high rate of reproduction, capacity to overcome abiotic factors, see section 1.2.1) or they could be attributed to the invasibility of the new habitat (Valentine et al. 2007). Valentine et al. (2007) were able to show that life history

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traits are not necessarily useful to predict invasion, although they could find some characteristics that many invasive seaweeds do have in common, like a capacity for rapid growth and effective mechanisms for short and long-distance dispersal. Resistance to herbivory is often predicted to be an especially important trait (e.g. (Nyberg & Wallentinus 2005), but also see section 1.2.2). With regard to the invasibility of the new habitat, Johnson and Chapman (2007) state the importance of disturbances or resource availability in the invaded range. Also the ability of the recipient environment to prevent or facilitate survival and establishment of new species could play a major role (Streftaris et al. 2005).

Most theories predicting invasion success of species were first postulated for terrestrial plants and later transferred to seaweeds (Inderjit et al. 2006), but sound tests for the adaptability of these hypotheses to seaweed invasions are scarce.

There are other major gaps in the investigation of biological invasions: In most studies, traits of invaders have been compared with those of (related) native species in the introduced range, providing only limited information. The comparison of invasive and source populations of the *same* species would be a better method to distinguish between traits that are species-specific and traits that are only present in an invasive ecotype (Hierro et al. 2005). Furthermore, the majority of studies on non-native plants has been performed exclusively in the introduced range and knowledge about the abundance, interactions and impacts of exotic species in their native range is surprisingly low. The idea that an invader is more abundant in its introduced as compared to its native range is therefore often merely an assumption (Hierro et al. 2005). To close this gap, Hierro et al. (2005) stipulate the importance of studying invasive species both in their native and their introduced range, preferably in common garden studies that use plants from several populations wide across each range. Also a study of Williams et al. (2008) demonstrates that the detection of trait differences may require measuring invaders' responses to more than one environment, as phenotypic plasticity may account for observed local differences as well.

In my thesis I will therefore not only elucidate possible reasons for the invasion success of *G. vermiculophylla* and thus add to the general understanding of seaweed invasions, but I also will present results of experiments conducted both in the native and the invasive range with several populations of this invader.

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Local mass appearances and ecological dominance of *G. vermiculophylla* have repeatedly been observed in areas outside of its native range (Freshwater et al. 2006, Thomsen et al. 2006, Thomsen et al. 2007a, Pinon-Gimate et al. 2008, Weinberger et al. 2008). To my knowledge, mass appearances have not been reported for native populations of *G. vermiculophylla*. This discrepancy could result from many possible reasons. A high tolerance towards environmental stressors could facilitate both the introduction and spread of this species as well as its performance and ecological dominance in the invaded areas (see section 1.2.1 and paper I). Also, more intense control by native than by non-native feeding enemies could explain the invasion success of the seaweed, as *G. vermiculophylla* from non-native populations seems to be less affected by herbivores than native seaweeds from the same environment (see section 1.2.2 and papers II and III).

1.2.1 Resistance toward abiotic stress (paper I)

A wide tolerance to environmental stressors is often hypothesized to promote the invasion success of species (Williamson & Fitter 1996, Boudouresque & Verlaque 2002). Anthropogenic transportation into new areas may go along with particularly severe stress conditions. This could be especially relevant for aquatic organisms, as their life cycle usually lacks a dormant distribution stage that is comparable to seeds.

Van Kleunen et al. (2010) conducted a meta-analysis of 117 field or experimental-studies that have investigated trait differences of invasive and non-invasive terrestrial organisms. They were able to show that invasive species were overall performing better than non-native species with regard to e.g. physiology, growth rate and general fitness. Nyberg and Wallentinus (2005) evaluated characteristics of 113 marine macroalgae introduced in Europe to detect species traits that might allow predictions of invasion success. Their expectation was that successful invaders should possess higher tolerances for dehydration to survive transportation in e.g. fishnets, on deck or as packing material. They also supposed tolerance ranges for salinity and temperature to be higher in invasives. However, tolerance towards none of the three environmental factors differed between native and non-native seaweeds. The authors attributed these results to the lack of data. Indeed, studies that compare stress tolerances of marine organisms are rare and most of the available data is not about

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seaweeds, but about mussels or oysters (Bayne 2002, Braby & Somero 2006, Krasso et al. 2008, Schneider 2008, Zardi et al. 2008) or other invertebrates (Lenz et al. 2011).

Several authors report that *G. vermiculophylla* is highly tolerant with regard to various environmental stressors: It grows in a wide range of salinities (Weinberger et al. 2008), it is able to survive desiccated and in darkness (Nyberg & Wallentinus 2005, Thomsen & McGlathery 2007), and tolerates both high and low nutrient levels (Thomsen & McGlathery 2007).

Studies about *G. vermiculophylla* in its native range are rare. However, individuals from a population in Japan seem to tolerate a broad range of environmental conditions, like high variations of temperature (5–30°C) and salinity (5–60‰) (Yokoya et al. 1999).

There are more stressors that could be relevant for invasion success of *G. vermiculophylla* and had not been thoroughly investigated so far – neither for native nor for non-native populations. Human-mediated transport vectors might be related with extreme temperatures; therefore tolerance towards heat stress could be a relevant trait. So called stress proteins or heat shock proteins are important mechanisms to deal with heat stress and other abiotic stressors (Lewis et al. 1999). The fast induction of stress protein expression or the possession of higher constitutive levels of stress proteins could be an important mechanism for a successful invader. The ability to cope with metal pollution – e.g. through the possession of effective antioxidant mechanisms (Halliwell & Gutteridge 1984, Foyer et al. 1997) – might be relevant for introduced species as well, as new species are often introduced into harbors, estuaries or other coastal areas that might be heavily polluted with toxic trace metals like copper. A capacity to resist UV-C radiation may be relevant for marine invasive organisms as well, as UV-C radiation treatment of ballast water is often applied (Sutherland et al. 2001, Kurtela & Komadina 2010, Liu et al. 2011). Also, organisms' capacities to cope with DNA damage are often studied by a UV-C treatment (Caldwell et al. 2007).

1.2.2 Resistance toward feeding stress (papers II and III)

Herbivores may affect plant invasions in an important way and plant-herbivore-interactions are used to explain both success (as predicted by the Enemy Release Hypothesis) and failure of plant invasions (Biotic Resistance Hypothesis).

The “natural enemies hypothesis” or “enemy release hypothesis” (ERH) has first been articulated by Darwin already in 1859 and has later been amplified and modified by others (Williams 1954, Elton 1958, Gillett 1962, Keane & Crawley 2002). Introduced plants are supposed to become highly abundant, as co-evolved specialist enemies that were present in the native range are lacking in the new range. It posits that release from specialist natural enemies (herbivores and pathogens) enables exotics to become abundant in their new range. On the other hand, the “biotic resistance hypothesis” (Elton 1958) states that native species (not only herbivores, but also pathogens and competitors) can prevent non-native plants from invading new communities, thus providing an explanation for a plants’ failure of establishment.

Both hypotheses are based on a common assumption: While specialists are predicted to control plants in their native range, generalist herbivores are supposed to provide biotic resistance in the new range (Strong et al. 1984). This differentiation between impacts of specialist enemies on the one and generalist enemies on the other hand is not entirely applicable to marine systems. Significantly more marine herbivores are characterized by a low degree of host specialization and thus introduced seaweeds are often not released from grazers (Hay & Steinberg 1992).

Consequently, another hypothesis should be mentioned in this context: If plants are not released from their natural enemies in their new environment, they might possess mechanisms that make them resistant towards herbivory and therefore contribute to their invasion success. The presence of generalist enemies in the invaded range might therefore result in a selection of defensive traits in introduced plants (Blossey & Nötzold 1995, Callaway & Ridenour 2004). This could include the development of high levels of phytochemicals which native grazers (but also competitors and pathogens) did not encounter before (“novel weapons hypothesis”, (Callaway & Ridenour 2004, Cappuccino & Carpenter 2005, Cappuccino & Arnason 2006)).

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Several studies conducted in the marine environment were able to show that grazers from the introduced range prefer native over non-native seaweeds (Weinberger et al. 2008, Monteiro et al. 2009, Cacabelos et al. 2010, Engelen et al. 2011, Nejrup et al. 2012). However, these studies only examined the effect of herbivores on non-native seaweeds in the introduced range and lack information about seaweed-herbivore-interactions in the native range. If the enemy release hypothesis is true, herbivores should reduce seaweed growth in the native range. In the invaded range, though, those seaweeds should suffer only little herbivore damage. Studies that compared grazer-herbivore-interactions in both ranges are scarce (Maron & Vilà 2001), yet would be crucial to detect the reasons for invasion success of the seaweeds (Hierro et al. 2005). A study by Wikström et al. (2006) revealed that *Fucus evanescens* is less exposed to herbivory in its non-native range in Sweden than in its native range in Iceland. Grazers are probably deterred by an increased chemical defense in invasive populations.

The resistance of *G. vermiculophylla* to mesograzers likewise results at least in part from a high content in phytochemicals (Nylund et al. 2011, Rempt et al. 2012). Feeding enemies of *G. vermiculophylla* have been shown to be deterred by arachidonic acid derived hydroxyeicosatetraenoic acids and by prostaglandins that are produced by the seaweed after mechanical wounding (Nylund et al. 2011, Rempt et al. 2012).

1.3 Consequences of invasion: *G. vermiculophylla* in the Baltic Sea (paper IV)

In 2007, the ecological effects of only 17 of the known 277 introduced seaweeds had been studied (Williams & Smith 2007). Furthermore, if there is information about impacts of seaweed invaders, it often originates from field surveys or observational studies, while experimental studies about the impacts of invasive algae on their recipient ecosystems are relatively scarce (Schaffelke & Hewitt 2007), but increasing in number. Williams and Smith (2007) conducted the first systematic summary of reported ecological effects of seaweed invaders on the native communities. The majority of the studies showed negative effects, especially on native seaweeds, but also on community structure. Space monopolization is an effect which is often observed and in many cases is followed by a decline in native species' abundance (reviewed in (Schaffelke & Hewitt 2007)). However, there are also studies

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indicating that introduced seaweeds do not have any impacts on the invaded communities – or even affect them positively (Williams & Smith 2007).

G. vermiculophylla has been included into a list of the most invasive seaweeds in Europe (Nyberg 2007), as it does not only spread rapidly, but also tends to change the flora and fauna of invaded habitats (Nyberg 2007). Also in the Western Baltic Sea, *G. vermiculophylla* seems to be an established part of the flora that continues to spread and can reach – at least locally – markedly high densities (see section 1.1). Two recent studies show that *G. vermiculophylla* can have a detrimental effect on a species that is native to the Baltic Sea, namely the seagrass *Zostera marina* (Martínez-Lüscher & Holmer 2010, Höffle et al. 2011). I conducted the first study to assess the ecological impact of *G. vermiculophylla* on other macroalgae (see paper IV). Nyberg (2007) had predicted that the invasive seaweed would compete with the native brown alga *Fucus vesiculosus* and observations from the Kiel Fjord indicated that the native seaweed may indeed have declined in habitats where the invader has spread (Weinberger et al. 2008). This is a reason for concern as *F. vesiculosus* is an important habitat-forming species in the Western Baltic Sea and its populations have been on the decline – most likely due to eutrophication – for several decades (Torn et al. 2006).

In the Baltic Sea, herbivores preferably utilize *G. vermiculophylla* as refuge (Weinberger et al. 2008, Thomsen 2010), but nevertheless choose to feed on *F. vesiculosus* rather than *G. vermiculophylla* (Weinberger et al. 2008). This phenomenon whereby plants provide a habitat for a shared consumer, thereby increasing feeding pressure on another plant species is termed refuge-mediated apparent competition (Orrock et al. 2010).

G. vermiculophylla may thus pose two additional threats to *F. vesiculosus*. It may reduce the growth of adult individuals and interfere with the settlement of germlings by overgrowing *Fucus* habitats and it might furthermore provide a habitat for grazers that feed on adjacent *F. vesiculosus*.

1.4 Research questions

To find out why *G. vermiculophylla* is such a successful invader, I conducted a series of experiments in which the seaweed was exposed to abiotic stressors and to herbivory. These experiments were conducted both in the native and in the invaded range of the seaweed by comparing the performance of seaweeds from 8 invasive populations and 6 native populations. Given the fact that *G. vermiculophylla* has successfully established itself in the German Baltic Sea, I furthermore assessed the impact on a habitat forming native seaweed to predict possible ecological consequences of the invader's spread. The results of these experiments have been compiled in four papers and are presented in the following (section 3).

What is the role of abiotic stress for the invasion success of G. vermiculophylla? (paper I)

I investigated the role of an elevated resistance towards different abiotic stressors (heat shock, long-term depletion, copper exposure, UV-C-treatment) for the invasion success of *G. vermiculophylla*. To identify basic cellular mechanisms underlying stress resistance in this species, analyses of heat shock protein expression, H₂O₂ decomposition potential and haloperoxidase expression potential supplemented this study.

How does defense against grazers influence the invasion success of G. vermiculophylla? (papers II and III)

I examined if the invasion success of *G. vermiculophylla* can be attributed to release from natural enemies and / or to an elevated production of phytochemicals that deter possible grazers. Feeding assays were conducted with grazers originating from both the native and the invaded range of the seaweed.

To understand possible mechanisms that influence seaweed-herbivore-interactions I also monitored the presence and concentration of phytochemicals among 12 different populations of *G. vermiculophylla*, using a metabolomic profiling approach (see paper III). This work was performed in collaboration with Martin Rempt and Prof. Dr. Georg Pohnert from the Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University, Jena.

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Furthermore, metabolites of *G. vermiculophylla* and selected arachidonic acid derived metabolites were tested for their bioactivity towards grazers from the native and the invaded range.

How did a selection towards resistance to multiple stressors take place? Can stress resistance be linked with invasion history? (papers I – III and general discussion)

These questions will be discussed briefly in the single manuscripts. In the general discussion (section 4) results of those manuscripts will be merged and I will attempt an assessment of the relevance of resistance towards the various stressors (both abiotic and biotic) and try to explain why *G. vermiculophylla* was able to develop multiple stress resistances. Based on that I will draw conclusions regarding the invasion history of the seaweed.

*What are the ecological consequences of *G. vermiculophylla* spreading in the Baltic Sea? (paper IV)*

The purpose of my study was to investigate experimentally whether *F. vesiculosus* was affected by the presence of *G. vermiculophylla* or by mesograzers associated with it. As life stages can be influenced differently by invaders, I focused not only on the effect of the invader on the growth of adult *F. vesiculosus*, but also examined the effect upon the survival of germlings.

2. Methods

2.1 Sampling of *G. vermiculophylla* from native and invasive populations

For all stress-related experiments (papers I – III), six populations of *G. vermiculophylla* originating from the native range in South Korea and China and eight populations from the invaded range in Germany, Denmark, France and Mexico were used (see Table 1). The identity of all studied populations was confirmed by DNA barcoding of the cytochrome c oxidase I subunit (COX1), either by (Kim et al. 2010) or by me, using the methodology described in (Kim et al. 2010) (Table 1). In total, I produced two sequences each for populations from Heiligenhafen, Mandö, Nibe, Nordstrand, and Nyborg, four sequences for populations from Horsens and Mexico and eight sequences for populations from Donghae and Odo-ri. For results see section 4.3.

G. vermiculophylla individuals for the feeding experiment and for the heat shock stress experiment were collected during sampling trips in spring 2010, while specimens employed in the copper-stress, the long-term stress and the UV-C-stress experiments were sampled together in autumn 2010. This utilization of individuals originating from the same populations allows for a general comparison of the single experiments.

Table 1. *G. vermiculophylla*. Geographic location and taxonomic reference (GenBank accession numbers are given for new cox1 gene sequences generated in the framework of the present studies) of native and non-native populations.

	Population/abbreviation (sea area)	Geographic coordinates	Taxonomic reference
Native	Donghae/Dong (East Sea, South Korea)	37°29'27.65"N, 129°08'30.42"E	JQ736332
	Odo-ri/Odo (East Sea, South Korea)	36°09'52.40"N, 129°24'00.07"E	JQ736333
	Jindo/Jin (Yellow Sea, South Korea)	34°25'16.98"N, 126°20'48.21"E	Kim et al. (2010)
	Gyeokpo/Gye (Yellow Sea, South Korea)	35°37'05.54"N, 126°28'01.70"E	Kim et al. (2010)
	Daecheon/Dae (Yellow Sea, South Korea)	36°19'23.07"N, 126°30'07.81"E	Kim et al. (2010)
	Qingdao/Qing (Yellow Sea, China)	36°03'18.51"N, 120°21'57.01"E	Kim et al. (2010)
Non-native	Heiligenhafen/HHafen (Baltic Sea, Germany)	54°22'48.14"N, 10°58'58.94"E	JQ736335
	Nyborg/Ny (Baltic Sea, Denmark)	55°17'54.23"N, 10°49'07.35"E	JQ736339
	Horsens/Hor (Baltic Sea, Denmark)	55°51'35.02"N, 9°52'35.76"E	KC731475
	Nibe/Nibe (Limfjord, Denmark)	56°59'03.34"N, 9°37'48.89"E	JQ736338
	Mandö/Man (Wadden Sea, Denmark)	55°18'35.59"N, 8°38'34.74"E	JQ736337
	Nordstrand/Nord (Wadden Sea, Germany)	54°31'48.01"N, 8°52'18.88"E	JQ736336
	St.-Pol-de-Leon/St.-Pol (Brittany, France)	48°40'41.09"N, 3°58'08.04"W	Kim et al. (2010)
	Ensenada/Ens (Baja California, Mexico)	31°46'28.67"N, 116°36'40.92"W	JQ736334

2.2 Comparison of populations' resistance to biotic and abiotic stress by MDS

An analysis was conducted to find a general pattern of populations' responses to various stressors. Survival rates of *G. vermiculophylla* (heat shock, copper stress, UV-stress, long-term stress), and consumption rates (feeding experiment) and relative deterrence reactions of two snail species were compiled and a mean value for every population with regard to each stressor was calculated. Populations from Horsens, Denmark, could not be included into this analysis because they were not tested for their deterrent effect towards snails. Data were analyzed with the Primer6&PERMANOVA+ software package (PRIMER-E Ltd., Plymouth, U.K.), using the Bray-Curtis distance measure and visualized by non-metric multidimensional scaling (MDS). Information contained in a distance matrix is displayed as points in a low-dimensional space in a way that relative distances between all points are in the same rank order as the relative dissimilarities of the samples (Clarke & Warwick 2001). Samples that are very similar (here: with regard to stress resistance) are represented by points that are close together. The results are presented and discussed in section 4.

3. List of papers and contribution of authors

1. Hammann M, Wang G, Boo SM, Aguilar Rosas LE, Weinberger F: Selection of increased heat shock resistance happened early during the invasion history of the seaweed *Gracilaria vermiculophylla*. Submitted to PLoS ONE.

MH, FW designed the experiments. GW, SMB, LEAR enabled the sampling of *G. vermiculophylla* from non-European populations. GW provided the facilities to conduct experiments in the native range of *G. vermiculophylla* in Qingdao, China. MH conducted the experiments and laboratory work. MH analyzed the data, MH wrote the paper. GW, SMB, LEAR, FW commented on and made corrections to manuscript drafts.

2. Hammann M, Wang G, Rickert E, Boo SM, Weinberger F (2013): Invasion success of the seaweed *Gracilaria vermiculophylla* correlates with low palatability. *Marine Ecology Progress Series* 486: 93-103

MH, FW designed the experiments. GW, SMB enabled the sampling of *G. vermiculophylla* from non-European populations. GW provided the facilities to conduct experiments in the native range of *G. vermiculophylla* in Qingdao, China. MH, ER conducted the experiments and laboratory work. MH analyzed the data, MH wrote the paper. GW, SMB, FW commented on and made corrections to manuscript drafts.

3. Hammann M, Rempt M, Pohnert G, Wang G, Boo SM, Weinberger F: Metabolomic profiling reveals a higher potential for wound activated chemical defense in non-native than in native populations of a seaweed. Manuscript draft.

MH, FW designed the experiments. GW, SMB enabled the sampling of *G. vermiculophylla* from non-European populations. GW provided the facilities to conduct experiments in the native range of *G. vermiculophylla* in Qingdao, China. MH, MR conducted the experiments and laboratory work. MH, FW, MR, GP analyzed the data, FW, MH wrote the paper. GW, SMB, MH, FW commented on and made corrections to manuscript drafts.

4. Hammann M, Buchholz B, Karez R, Weinberger F (2013): Direct and indirect effects of *Gracilaria vermiculophylla* on native *Fucus vesiculosus*. *Aquatic Invasions* 8 (2): 121-132

FW, MH, BB, RK designed the experiments. MH, BB, FW conducted the experiments and laboratory work. MH analyzed the data, MH wrote the paper. FW, RK commented on and made corrections to manuscript drafts.

The first three out of seven experiments conducted in the framework of this study represent the content of my diploma thesis.

Paper I

Running head: Resistance against abiotic stress in *G. vermiculophylla*

Selection of increased heat shock resistance occurred early during the invasion history of the seaweed *Gracilaria vermiculophylla*

Mareike Hammann^{1*}, Gaoge Wang², Sung Min Boo³, Luis E. Aguilar-Rosas⁴, Florian Weinberger¹

- (1) Helmholtz Centre for Ocean Research (GEOMAR), Düsternbrooker Weg 20, 24105 Kiel, Germany
(2) College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, 266003 Qingdao, China
(3) Department of Biology, Chungnam National University, Daejeon 305-764, Korea
(4) Instituto de Investigaciones Oceanológicas, UABC, Km 103 Carretera Tijuana-Ensenada, México

(*). Corresponding author, email: mhammann@geomar.de

Abstract

Most macroalgae and other aquatic organisms feature life cycles without a stage of dormancy that is comparable to seeds. During anthropogenic transportation they are therefore probably more exposed to non-natural stresses than plant invaders. Nonetheless, the role of stress resistance in invasion processes of aquatic organisms has rarely been studied in a rigorous manner. We compared the responses of native and invasive populations of the seaweed *Gracilaria vermiculophylla* to different abiotic stressors in common garden-type experiments. Specimens from six native populations in East Asia and from eight invasive populations in Europe and on the Mexican Pacific coast were maintained together under identical conditions and three short-term stress experiments (with the stressors heat shock, copper toxicity and UV-C radiation) and one long-term depletion stress experiment were conducted with them. All experiments were carried out twice - one time in the native range in Qingdao, China and one time in the invaded range in Kiel, Germany - to rule out local acclimation effects. After heat shock, the invasive populations survived significantly better than the native populations in both testing sites. The resistance towards heat shock was clearly correlated with an increased expression of heat shock protein 70 (HSP70) and a decreased expression of haloperoxidase, suggesting that increased HSP70 expression and reduced allocation into haloperoxidase expression was selected during the invasion history. This selection probably happened early, as the same combination of traits was detected in very different realms within the invasive range. In contrast with heat shock, the resistance towards UV-C radiation – although clearly and reproducibly different among populations - was not significantly different among native and invasive populations. Different results were obtained in the two testing sites with respect to long term depletion stress and copper toxicity resistance, suggesting that acclimation may possibly affect those capacities in *G. vermiculophylla*.

KEY WORDS: Biological invasion · invasive seaweeds · abiotic stress · *Gracilaria vermiculophylla* · HSP70 · Haloperoxidase · copper toxicity · UV-C radiation

Introduction

Tolerance against environmental stress is an important determinant of the successful invasion of species [1]. This has been confirmed in several common garden-like studies that compared stress-related traits in native and non-native terrestrial organisms: Through meta-analysis of 117 field- or experimental-garden studies, van Kleunen et al. [2] demonstrated that invasive species generally have higher values of performance-related traits compared to non-invasive species. On the other hand, there are only few comparable studies of marine organisms, and most of them are on mussels or oysters [3-7]. Lenz et al. [8] showed in a globally replicated study that non-native marine invertebrates are more tolerant towards environmental stress than taxonomically related native species in the same environment.

However, comparisons among species may only provide limited information. Their evolutionary separation usually happened during thousands of years and traits that evolved during invasion processes may not be distinguished from other traits. A more rigorous way to identify mechanisms that enable an organism to successfully invade new areas is the comparison of invasive and source populations of the same species [9]. Such comparisons can distinguish between traits that are species-specific and traits that are only present in an invasive ecotype, and they should preferably be conducted both in the introduced and in the native range, as phenotypic plasticity may also account for local differences [10]. However, information from such common garden-type experiments is largely missing for aquatic invasive organisms.

The red macroalga *G. vermiculophylla* (Ohmi) Papenfuss is considered to be one of the most invasive seaweeds in the northern hemisphere [11]. Its native area ranges from the East Sea/Sea of Japan to the East China Sea, following the Central Kuroshio Current. During the last two decades a rapid expansion of its distribution area has been observed: *G. vermiculophylla* has spread to the cold and warm temperate North American West and East coasts [12-15], as well as to the Mediterranean Sea [16], the Atlantic coast of Morocco [17] and Northern European Seas [18,19]. As a result of this invasion process, there has been a considerable loss of genetic diversity in non-native populations of *G. vermiculophylla*, whose common genetic origin appears to lie in a donor region within the East Sea/Sea of Japan [20]. This bottleneck effect did not weaken the capacity of *G. vermiculophylla* for ecological dominance: Local mass appearances have been repeatedly reported from non-native, [14,18,21,22], but so far not from native populations.

A relatively high tolerance of *G. vermiculophylla* towards several environmental stressors possibly facilitates the establishment and spread of this species as well as its performance and ecological dominance in the invaded areas. For example, *G. vermiculophylla* from Shikoku Island, Japan, is able to tolerate high variations of salinity (5–60‰) [23] and *G. vermiculophylla* from the German Baltic Sea tolerates even salinities close to 0 ‰ for many weeks [19].

However, the very first stages prior to establishment may be particularly critical for the invasion success of aquatic organisms: their life cycle usually lacks a dormant distribution stage that is comparable to seeds, but anthropogenic transportation into new areas may go along with particularly severe stress conditions. Also with this respect *G. vermiculophylla* appears as relatively well primed: Specimens from an invasive population in Sweden were shown to be capable of surviving up to six months in darkness without water – a trait that could be very

important during transportation [24]. To cope with such stress, seaweeds do not only need to endure emersion and desiccation, but also a long time of starvation, as they are not able to absorb nutrients. For example, long periods of darkness could be endured by using the storage carbohydrate floridean starch [25].

G. vermiculophylla is also able to tolerate relatively strong variations of temperature (5–30°C; [23]). Tolerance to heat stress could play an important role in the course of invasion processes, as human-mediated transportations may be related with extreme temperatures that seaweeds usually do not experience in their natural environment [26,27]. Heat leads to a denaturation or aggregation of cellular proteins and specific chaperons such as heat shock proteins (HSPs) prevent proteins from such degradation and play a primary role in protecting cells from injuries. Their induction by environmental stress provides a central tolerance mechanism in many species [28] and also in seaweeds [29]. Chaperons are not only induced by heat, but also by many other environmental stressors, including exposure to trace metals or organic pollutants, changes in osmolarity, hypoxia, anoxia and exposure to UV radiation [30]. HSP70 is the best studied of the HSPs, as it is highly conserved and easy to detect due to its specific reactions [30,31]. Thus, invasive species or populations could possess a higher tolerance towards temperature stress if they either induce HSP expression faster than native species or populations or if they possess higher constitutive levels of HSP. There are only few studies that compared temperature tolerance between native and invasive species and the results seem to be species specific [32].

Metal pollutions are very common in many coastal areas worldwide. The ability to cope with metal pollution might be relevant for introduced species, as a lot of introductions take place related with anthropogenic actions. Hull-fouling and aquaculture are the most significant vectors for seaweed invaders [33]. New species are thus often found in harbors, estuaries or other coastal areas that may be heavily polluted. Together with mercury, copper is one of the most toxic trace metals to marine algae [34]. Excess copper has various effects on seaweeds. For instance, it inhibits photosynthesis, reduces pigment concentration and it may lead to chlorosis and restricted growth [35]. In part, the toxicity of copper results from its amplifying effect upon the cellular production of reactive oxygen species (ROS), e.g. hydrogen peroxide (H₂O₂), which results in the establishment of an oxidative stress condition if scavenging capacities of the antioxidant defenses are insufficient [36,37]. ROS damage membrane lipids, proteins and other cellular compounds either directly or through acceleration of lipid peroxidation and may thus result in programmed cell death, a self-destruction mechanism also known as apoptosis [38,39].

An important vector for marine invasive organisms is ship ballast water. According to a convention of the International Maritime Organisation (IMO) ballast water should be either exchanged or treated [40] and besides of thermal and chemical operations UV-C radiation treatment of ballast water is often applied [41-44]. Thus, a capacity to resist to UV-C radiation may be relevant for marine invasive organisms, although UV-C does not reach the earth's surface under natural conditions, as it is blocked by the stratosphere. High doses of UV-B or UV-C primarily result in DNA photodegradation and UV-C treatment has often been used to study responses to DNA damage [45]. UV-C radiation may further cause DNA fragmentation [38] and oxidative stress, subsequently disrupting the function of organelles, chloroplasts and mitochondria [38] and leading to apoptosis ([38,46]. The production of ROS also plays an important role during UV-induced programmed cell death in *Arabidopsis thaliana* [47].

Efficient antioxidative mechanisms could be important for the survival of various invasion-related stresses, as increased intracellular concentrations of ROS may result from several different environmental impacts. ROS are usually rapidly buffered by the cellular antioxidant system, which includes various antioxidant enzymes and water- and lipid-soluble antioxidant compounds [39,48]. Haloperoxidases that mediate the oxidation of halides by hydrogen peroxide are a main component of the antioxidative systems in gracilarioids [49] as well as in other red and brown algae [50].

The questions we ask in our study are the following: (1) is *G. vermiculophylla* as a whole species extremely stress tolerant already in its area of origin, or (2) were just single pre-adapted populations of this species able to invade new areas? Or are maybe (3) only the non-native populations particularly tolerant towards stress because of selection processes during the invasion process? Moreover, is (4) stress tolerance a general trait in invasive populations that protects against several kinds of stressors or is it specific for certain stressors, that might then be considered as selectors of successful invaders?

To answer these questions, *G. vermiculophylla* individuals from six native and eight invasive populations were tested in several subsequent experiments for their tolerance towards four different abiotic stresses: (A) heat shock, (B) depletion (= long-term desiccation in combination with darkness), (C) copper toxicity stress, and (D) UV-C radiation stress. All these experiments were run twice, one time in Kiel, Germany and one time in Qingdao, China, in order to find out whether the observed stress tolerance was really a trait of a respective population or rather only due to local adaptation [10]. These experiments were supplemented with analyses of HSP70 expression, H₂O₂ decomposition potential and haloperoxidase expression, in order to identify basic cellular mechanisms that determine relevant stress resistance traits during the invasion process of *G. vermiculophylla*.

Material and Methods

Collection, transport and cultivation of *Gracilaria vermiculophylla*

Six native and eight invasive populations of *G. vermiculophylla* were sampled for the experiments. The native populations were obtained from locations along the Korean peninsula and the Shandong province, China, while the invasive populations originated from the Danish-German peninsula of Jutland and Schleswig-Holstein, the west of France and the Pacific coast of Mexico (Table 1). The samples were collected between February 10th and April 22nd 2010 and between September 17th and October 12th 2010.

The identity of all *G. vermiculophylla* populations utilized in this study was confirmed by DNA barcoding as previously described [20,51]. Native individuals were easily distinguishable, as they all grew attached to hard substrata, *G. vermiculophylla* from invasive populations, however, often grew unattached. Therefore, the possibility cannot be ruled out that some “individuals” used in the experiment were in fact clones, despite the fact that both native and invasive seaweeds were collected from extended areas of >1000 m², in order to avoid risk of taking clonal samples.

Table 1: Geographic location of native and invasive populations of *G. vermiculophylla*.

	Population/Abbreviation (Coast)	Geographic coordinates
a) native	Donghae/Dong (East Sea/KR)	37°29'27.65"N, 129°08'30.42"E
	Odo-ri/Odo (East Sea/KR)	36°09'52.40"N, 129°24'00.07"E
	Jindo/Jin (Yellow Sea/KR)	34°25'16.98"N, 126°20'48.21"E
	Gyeokpo/Gye (Yellow Sea/KR)	35°37'05.54"N, 126°28'01.70"E
	Daecheon/Dae (Yellow Sea/KR)	36°19'23.07"N, 126°30'07.81"E
	Qingdao/Qing (Yellow Sea/CN)	36°03'18.51"N, 120°21'57.01"E
b) invasive	Heiligenhafen/HHafen (Baltic Sea/D)	54°22'48.14"N, 10°58'58.94"E
	Nyborg/Ny (Baltic Sea/DK)	55°17'54.23"N, 10°49'07.35"E
	Horsens/Hor (Baltic Sea/DK)	55°51'35.02"N, 9°52'35.76"E
	Nibe/Nibe (Limfjord/DK)	56°59'03.34"N, 9°37'48.89"E
	Mandö/Man (Wadden Sea/DK)	55°18'35.59"N, 8°38'34.74"E
	Nordstrand/Nord (Wadden Sea/D)	54°31'48.01"N, 8°52'18.88"E
	St.-Pol-de-Leon/St.-Pol (Brittany/F)	48°40'41.09"N, 3°58'08.04"W
	Ensenada/Ens (Baja California/MX)	31°46'28.67"N, 116°36'40.92"W

Live specimens from all *G. vermiculophylla* populations were transferred to both Qingdao (China) and Kiel (Germany) for the experiments. The algae were transported in cooling containers while wrapped in lab paper soaked with seawater [24]. To minimize stress, transportation periods never lasted longer than five consecutive days. Logistic circumstances did not always allow for a direct transfer between collection site and experimental site. Specimens from native populations could be brought to Kiel and Qingdao directly, while those from invasive populations were brought to Kiel first. There they were given at least two weeks of time under non-stressful conditions to allow them to regenerate before one half of them were transferred to Qingdao. Transportation stress was not observed to result in losses. All algae were given a regeneration period of at least one week under non-stressful conditions at the experimental site before experiments were carried out [24].

At the Qingdao site, *G. vermiculophylla* was maintained in separate Erlenmeyer flasks under identical conditions (room temperature (approx. 20°C), indirect sunlight ($\text{PAR}_{\text{max}} 20 \mu\text{mol m}^{-2} \text{s}^{-1}$), no aeration). Yellow Sea water, which was exchanged daily, served as the medium. At the Kiel site, the specimens were cultivated indoors in separate basins filled with Baltic Sea water from the Kiel Fjord as a medium. The conditions were identical for each basin (water temperature 15 to 20 °C, $\text{PAR} 30 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h d⁻¹, aeration). In order to maintain an identical water composition in all the tanks, the water was constantly exchanged between them and a central storage container. To prevent an exchange of algal propagules between tanks with samples of different origin, the water was UV sterilized at the exit of the central unit before being redistributed into the tanks. At the Qingdao site, preparations included gradual acclimatization of the specimens sampled at the low salinity conditions of the Baltic Sea to fully marine conditions over the course of several days. In analogy, seaweeds from fully marine conditions were gradually accustomed to a low salinity environment (15 ± 2 PSU) before using them in experiments at the Kiel site.

Experimental set-ups

In all experiments ten *G. vermiculophylla* individuals from each population were used and all four experiments were conducted in a similar manner: *G. vermiculophylla* individuals were exposed to a single stress factor for a certain period of time. To assess relevant stress levels preliminary experiments were conducted with single individuals from one native and one invasive population. The aim was to apply stress levels that were (1) likely to induce a detectable effect, (2) not lethal to all of the seaweeds and (3) ecologically relevant.

The **copper exposure** was started on the 22nd of October at Qingdao and the 9th of December 2010 at Kiel. *G. vermiculophylla* individuals were kept in single Petri dishes in seawater enriched with CuSO₄ (6.6 µM Cu) under in other respects non-stressful conditions (water temperature 15 - 20°C, low light) for 14 days, when their survival status was evaluated. The chosen copper concentration is about 2.4 times above mean concentrations in some highly polluted estuaries and about 2100 times above the average natural content in sea water [52]. This stress level was the lowest that induced visible damage to *G. vermiculophylla* in a preliminary experiment.

Heat shock experiments were conducted between the 27th of April and the 9th of May 2010 in Qingdao and between the 11th and the 23rd of June 2010 in Kiel. Beakers with Yellow Sea water or Baltic Sea water, respectively, were heated in a water bath and *G. vermiculophylla* individuals (0.25g each) were incubated in the beakers for a period of three hours at 40.5°C ± 0.5. This stress level was chosen because water temperatures of up to 40°C have been observed in environments that potentially harbor *G. vermiculophylla* [53] and because visible effects were observed in a preliminary experiment at this condition. After heat exposure all specimens were transferred into separate 500 ml flasks and cultured under non-stressful conditions (water temperature 15 - 20°C, low light) for 13 days before their survival status was evaluated.

A modified protocol by Nyberg and Wallentinus [24] was used to study **depletion stress** effects. Pieces of *G. vermiculophylla* thalli (0.1 g each) were placed individually into Ziploc (SC Johnson, Racine, Wi., U.S.A.) freezer bags and incubated at 4°C and in darkness for a period of five months as described by Nyberg & Wallentinus. The experiment in Qingdao lasted from 30th of April to 5th of October 2010 and the algal pieces were wrapped into lab paper soaked in seawater. The experiment in Kiel lasted from 13th of January to 18th of June 2010 and lab paper was not used.

For the **UV-treatment** the seaweed individuals were exposed to UV-C-radiation (UV-lamp: 254 nm, ~ 7000 mW m⁻² nm⁻¹, distance to seaweeds 2 cm) in single open Petri dishes (water depth 1 cm) for three hours. The experiments took place between the 21st of October and the 4th of November 2010 in Qingdao and between the 8th of December and the 22nd of December 2010 in Kiel. All algae were subsequently incubated for 14 d under the same non-stressful conditions as in the heat shock experiment before their survival status was evaluated.

At the end of each experiment survival was determined by separating living and dead algal tissue of each individual. In depletion experiments the surviving part of the seaweed was weighed and survival [%] was calculated based on the initial biomass. In other experiments the percentage of living tissue of each alga was estimated on a scale from 0 to 100 % (in steps of 5 %) and pictures of all the specimens were taken to be able to reconsider the decisions.

Protein expression analysis after heat shock was only conducted at Kiel. One *G. vermiculophylla* individual from every population was split into two parts. One part was directly frozen at -80°C , while the other part was heat-shocked for three hours as described above and immediately frozen at -80°C . For protein extraction, the samples were ground in liquid nitrogen. 0.3 g of each algal sample were incubated on a shaker with 1ml extraction buffer (0.1 M Tris-HCl, 2 mM EDTA, 10 mM MgCl_2 , 10 mM NaHCO_3 , 1 mM Dithiothreitol, 10% Glycerin, 1% Triton X-100) for one hour. Afterwards the samples were centrifuged for 2 min at 13000 rpm at 4°C and the supernatant was kept for further analysis. The protein concentration of the supernatant was determined photometrically [54], using staining reagent by Bio-Rad Laboratories (Munich, Germany).

For analysis of HSP70 expression, a volume containing 20 μg protein was applied to a precast gel (Mini-Protean TGX, Bio-Rad, Munich, Germany) and proteins were fractionated by approximate size by sodium dodecyl sulfate polyacrylamide gel electrophoresis [55]. In a next step, the Western Blotting technique was applied to confer the proteins from the gel to a suitable membrane [56]. The membranes were incubated with a polyclonal primary antibody solution (host: rabbit; dilution 1: 3000; AS08371, Agrisera AB, Vännäs, Sweden) with reactivity toward six different isoforms of cytoplasmic HSP70 from *Arabidopsis thaliana* overnight at 4°C . Afterwards the membranes were washed in a TBS-T buffer solution [148.9 mM NaCl, 76.8 mM Tris-HCl, 0.05 % Polysorbate 20, pH 7.3] and an alkaline phosphatase-linked secondary anti-rabbit IgG antibody (host: goat, dilution 1:5000; Santa Cruz Biotechnology, Santa Cruz) that catalyzes the following colorimetric reaction was applied. Membranes were stained with a nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl-phosphate-solution (NBT/BCIP Ready-To-Use tablets; Roche, Mannheim).

For analysis of haloperoxidase expression a volume containing 45 μg protein was applied to a precast gel (Mini-Protean TGX, Bio-Rad, Munich, Germany). Proteins were then separated by polyacrylamide gel electrophoresis under non-denaturing conditions and haloperoxidases were stained for 30 min with o-dianisidine in the presence of KI as has been previously described for other *Gracilaria*-species [49].

Western blotting membranes were photographed and electrophoresis gels were scanned. The size and intensity of bands was then analyzed, using the image processing program FIJI [57].

H_2O_2 decomposition potential. To assess the capacity to decompose externally applied H_2O_2 individuals of each *G. vermiculophylla* population ($n=3$, 0.5 g each) were incubated at Kiel at non-stressful conditions (water temperature 15 - 20°C , low light) on a shaker in Petri dishes containing 30 ml of seawater. Seawater controls containing no seaweed were also run. H_2O_2 (final concentration: 250 μM) was added from a stock solution and after 15 min remaining H_2O_2 contents of the solutions were quantified. To this end ferricyanide-catalyzed luminol-dependent luminescence after oxidation by H_2O_2 was quantified with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) [58].

Statistical analysis

Prior to statistical tests, data on survival were either rank-transformed (survival after heat shock, copper stress and haloperoxidase isoform 3) or ArcSin-transformed (survival after UV- and depletion stress) to achieve homogeneity of variances and normality. Homogeneity of variances could not be achieved in all cases (Levine's test, $p < 0.05$) and in those cases results were taken as statistically significant if the probability of error was less than 1% [59]. Survival data were analyzed by three factorial, nested ANOVA with the factors location (= experimental site; Qingdao or Kiel), region (native or invasive) and population (nested in region). Tukey's post-hoc test was conducted to detect differences among populations. Differences in HSP70 expression and haloperoxidase expression after heat shock were analyzed by two factorial ANOVA with the factors region (native or invasive) and treatment (either heat shocked or control). To detect differences between native and invasive *G. vermiculophylla* individuals with regard to H₂O₂ degradation capacity a t-test was conducted. A linear regression was fitted between survival rates of *G. vermiculophylla* populations used at the two locations (Qingdao and Kiel), in order to find out whether patterns in survival correlate with each other. Linear regression was also used to detect correlations among survival after heat shock and presence of HSP70. Transformations and statistical tests were conducted using the STATISTICA 8 software package (StatSoft, Inc., Tulsa/USA), while regressions were fitted using the GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla/USA).

Results

Heat shock. Overall, native and invasive specimens of *G. vermiculophylla* clearly differed in their survival rates after heat shock ($p < 0.001$; see Tab. 2 and Fig. 2). While > 60% of the tissue of the invasives survived the treatment in both testing sites, only 7% of the native tissue endured the stress. A linear regression between the survival rates of *G. vermiculophylla* populations in both experiments showed a significant correlation ($p < 0.001$, $r^2 = 0.72$; see Fig. 1A), indicating that populations in both experiments reacted to the heat shock in a similar manner. Nonetheless, nested 3-way-ANOVA revealed that the place where the experiment was conducted influenced the survival rates (significant interaction between the factors "location" and "region"; see Tab. 2): Survival of native specimens did not differ between the two experiments, while survival of invasive specimens was better in Qingdao as compared to Kiel (Fig. 2). Also single populations of *G. vermiculophylla* reacted differently with regard to survival after heat shock (see Tab. 2 and Fig. 3). No differences were observed within the native populations, while invasive populations clustered in two groups: *G. vermiculophylla* from brackish water populations in the Baltic Sea and Limfjord (Heiligenhafen, Horsens, Nyborg, Nibe) performed significantly ($p < 0.01$) or non-significantly better than population from fully marine conditions in Mexico and Europe.

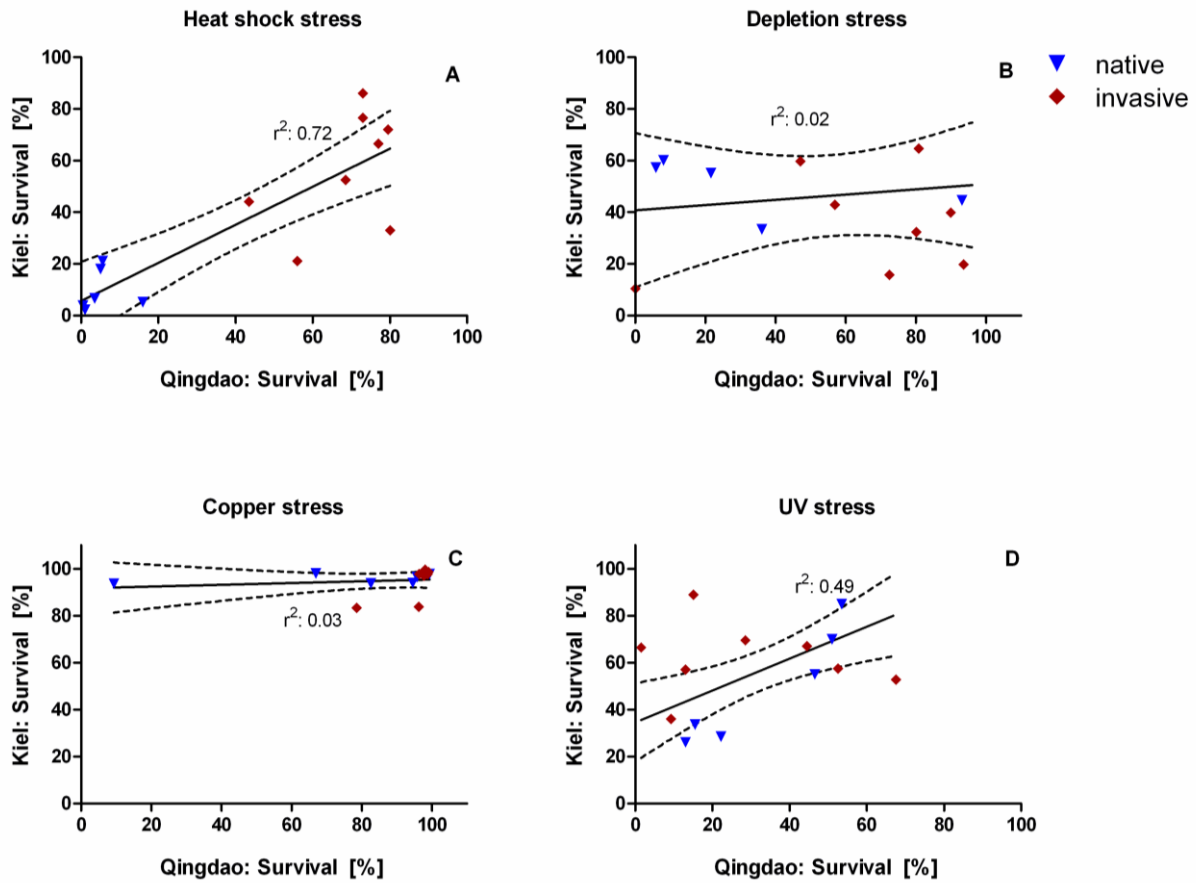


Figure 1: Influence of experimental location. Linear regression correlating the survival of *G. vermiculophylla* in experiments conducted in Qingdao, China and Kiel, Germany. (A) Heat shock stress, (B) Depletion stress, (C) Copper stress, and (D) UV stress. Dotted lines indicate the 95 % CI.

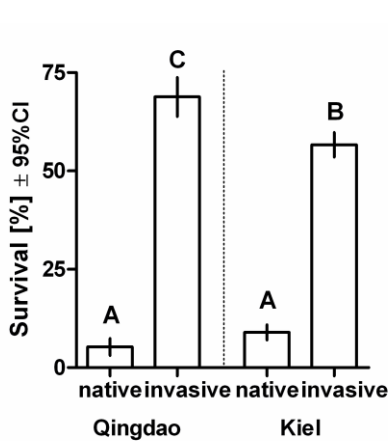


Figure 2: Heat shock experiment. Average survival of *G. vermiculophylla* from native and invaded regions after heat shock in experiments conducted in two locations in Qingdao, China and Kiel, Germany. $n_{\text{native}} = 60$, $n_{\text{invasive}} = 80$. Different letters indicate groups that were significantly different in a Tukey-test ($p < 0.01$).

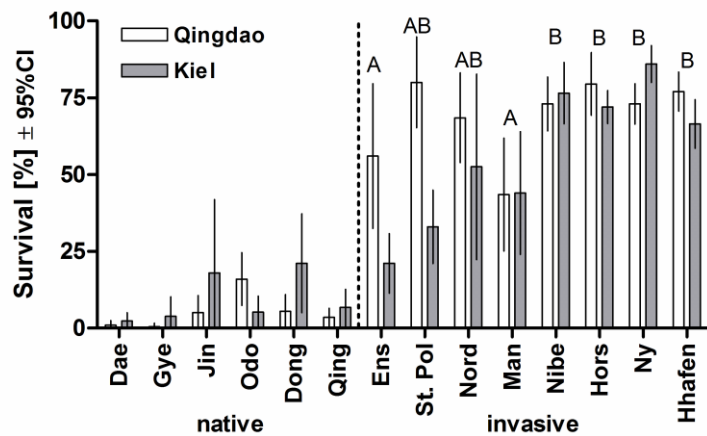


Figure 3: Heat shock experiment. Survival of different *G. vermiculophylla* populations after heat shock. White bars show survival in experiments conducted in Qingdao, China while grey bars show survival in Kiel, Germany. Different letters indicate populations that were significantly different in a Tukey-test ($p < 0.01$) either within the native or within the invaded region. $n = 10$.

Table 2: Heat shock experiment. Influence of the origin of *G. vermiculophylla* populations and the experimental location (either Qingdao, China or Kiel, Germany) on survival of *G. vermiculophylla* in a nested design ANOVA with the factor "population" nested within the factor "region" (either native or invasive).

	SS	DF	MS	F	p
Population (region)	153460	12	12788	7.84	<0.001
Location	2588	1	2588	1.59	0.21
Region	981825	1	981825	601.86	<0.001
Location x Region	21228	1	21228	13.01	<0.001
Error	412722	253	1631		

Depletion stress. Interestingly, *G. vermiculophylla* from the non-native region survived depletion stress better (65% vs. 44%) at Qingdao, while the opposite case was true for the experiment conducted in Kiel (natives 61% vs. invasives 36%; Fig. 4). This was reflected in a significant interactive effect of location and region, while the overall differences among specimens from different regions or between experiments conducted at different locations turned out to be insignificant (see Tab. 3). However, single populations reacted differently with regard to survival after depletion stress ($p < 0.001$; see Tab. 3 and Fig. 5). In the invasive group specimens originating from Donghae (South Korea) survived significantly better than individuals from all other native populations. Within the invasives, the population from Ensenada (Mexico) showed a worse survival than all other seaweeds except those from Mandö (Denmark). *G. vermiculophylla* individuals from Ensenada were also the only group that died completely in one of the experiments, while in all the other populations at least a small part of the algal tissue survived. Survival in the two subsequent experiments was not significantly correlated ($p = 0.64$, $r^2 = 0.02$; see Fig. 1B).

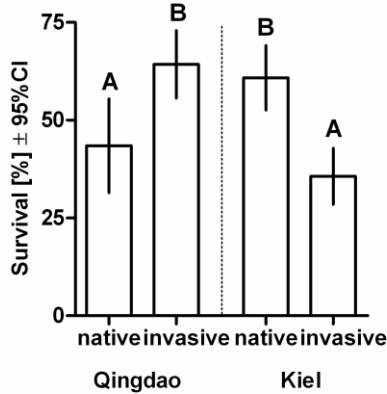


Figure 4: Depletion stress experiment. Average survival of *G. vermiculophylla* from native and invaded regions after depletion stress in experiments conducted in two locations in Qingdao, China and Kiel, Germany. $n_{\text{native}} = 60$, $n_{\text{invasive}} = 80$. Different letters indicate groups that were significantly different in a Tukey-test ($p < 0.01$).

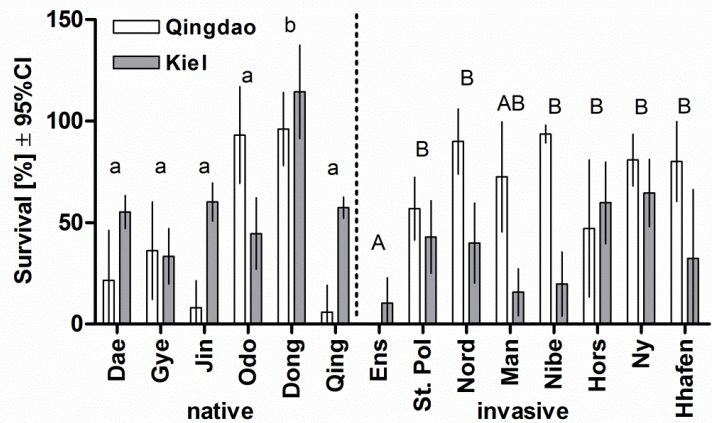


Figure 5: Depletion stress experiment. Survival of different *G. vermiculophylla* populations after depletion stress. White bars show survival in experiments conducted in Qingdao, China while grey bars show survival in Kiel, Germany. Different letters indicate populations that were significantly different in a Tukey-test ($p < 0.01$) either within the native or within the invaded region. $n = 10$.

Table 3: Depletion stress experiment. Influence of the origin of *G. vermiculophylla* populations and the experimental location (either Qingdao, China or Kiel, Germany) on survival of *G. vermiculophylla* in a nested design ANOVA with the factor "population" nested within the factor "region" (either native or invasive).

	SS	DF	MS	F	p
Population (region)	4.09	12	0.34	14.29	<0.001
Location	0.07	1	0.07	2.84	0.09
Region	0.01	1	0.01	0.45	0.50
Location x Region	0.97	1	0.97	40.57	<0.001
Error	6.11	257	0.02		

Copper stress. For the copper treatments, it did not make a difference whether the experiment was conducted in Qingdao or in Kiel. In contrast, the source region of specimens influenced the reaction during copper exposure ($p < 0.001$; see Tab. 4 and Fig. 6) and overall the invasives showed higher survival rates in presence of the stressor. However, this difference was mostly due to the poor survival of one single native population (Odo, South Korea) in the experiment in Qingdao, which lowered the overall survival rates of the native specimens in this experiment by almost 13%. Correspondingly, survival of the Odo population during copper exposure differed significantly from the responses of the two best performing populations, Gyeokpo and Donghae (both South Korea), which survived better ($p < 0.001$; see Tab. 4 and Fig. 7). Within the invasive populations Ensenada (Mexico) survived less than all other populations except of Nordstrand (Germany). Survival in the two subsequent experiments was not significantly correlated ($p = 0.54$, $r^2 = 0.03$; see Fig. 1C).

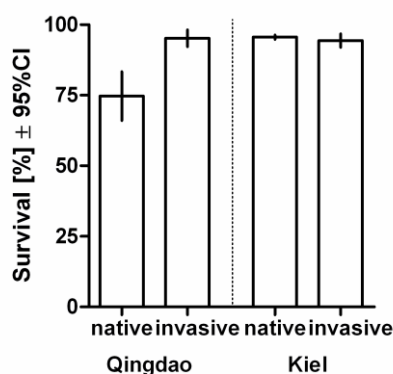


Figure 6: Copper stress experiment. Average survival of *G. vermiculophylla* from native and invaded regions after copper stress in experiments conducted in two locations (Qingdao, China and Kiel, Germany). $n_{\text{native}} = 60$, $n_{\text{invasive}} = 80$.

Table 4: Copper stress experiment. Influence of the origin of *G. vermiculophylla* populations and the experimental location (either Qingdao, China or Kiel, Germany) on survival of *G. vermiculophylla* in a nested design ANOVA with the factor "population" nested within the factor "region" (either native or invasive).

	SS	DF	MS	F	p
Population (region)	594132	12	49511	13.797	<0.001
Location	6285	1	6285	1.751	0.19
Region	121590	1	121590	33.884	<0.001
Location x Region	6859	1	6859	1.911	0.17
Error		258			

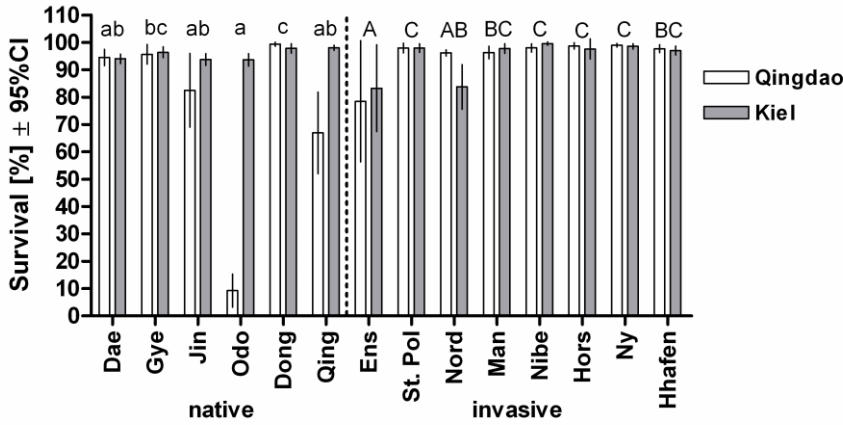


Figure 7: Copper stress experiment. Survival of different *G. vermiculophylla* populations after copper treatment. White bars show survival in experiments conducted in Qingdao, China while grey bars show survival in Kiel, Germany. Different letters indicate populations that were significantly different in a Tukey-test ($p < 0.01$) either within the native or within the invaded region. $n = 10$.

UV-stress. In the UV-treatment native and invasive specimens exhibited overall no significantly different responses ($p = 0.06$; see Tab. 5 and Fig. 8). However, there was a significant difference between the experiments conducted in China and in Germany ($p < 0.001$; see Tab. 5 and Fig. 8), with overall higher survival rates in Germany. On average, invasive specimens performed in Kiel better than native specimens, but this was not statistically significant. Both within native and within invasive populations significantly different capacities to tolerate UV-C radiation were detected ($p < 0.001$; see Tab. 5 and Fig 9). Particularly resistant were the populations in Donghae, Qingdao and Nibe, and their resistance to UV-C was in a similar way exhibited in Germany and in China, so that the data observed in both locations correlated significantly ($p < 0.01$, $r^2 = 0.49$; see Fig. 1D).

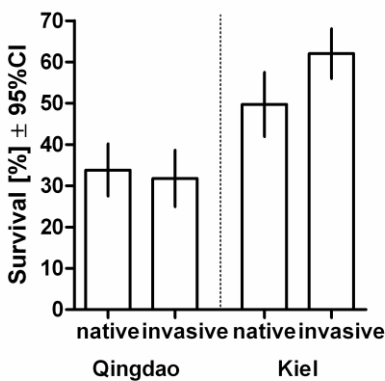


Figure 8: UV stress experiment. Average survival of *G. vermiculophylla* from native and invaded regions after UV stress in experiments conducted in two locations (Qingdao, China and Kiel, Germany). $n_{\text{native}} = 60$, $n_{\text{invasive}} = 80$.

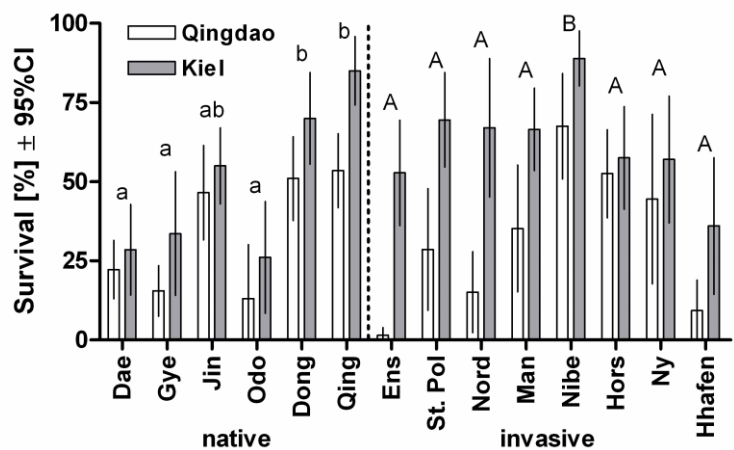


Figure 9: UV stress experiment. Survival of different *G. vermiculophylla* populations after UV stress. White bars show survival in experiments conducted in Qingdao, China while grey bars show survival in Kiel, Germany. Different letters indicate populations that were significantly different in a Tukey-test ($p < 0.01$) either within the native or within the invaded region. $n = 10$.

Table 5: UV stress experiment. Influence of the origin of *G. vermiculophylla* populations and the experimental location (either Qingdao, China or Kiel, Germany) on survival of *G. vermiculophylla* in a nested design ANOVA with the factor "population" nested within the factor "region" (either native or invasive).

	SS	DF	MS	F	p
Population (region)	14.16	12	1.18	14.03	<0.001
Location	5.97	1	5.97	71.04	<0.001
Region	0.30	1	0.30	3.62	0.06
Location x Region	0.33	1	0.33	3.88	0.049
Error		263			

Protein expression and H₂O₂ degradation potential. Western blotting with an antibody reactive against HSP70 from *Arabidopsis thaliana* resulted in the formation of one distinct band (Fig. 11) representing a protein of approximately 70 kDa, which suggested that the antibody was also reactive without crossreactions toward HSP70 from *G. vermiculophylla*. Expression of HSP70 was significantly higher in invasive as compared to native individuals of *G. vermiculophylla* ($p < 0.001$, see Fig. 10 and 11 and Tab. 6). The difference between heat-shocked and non-heat-shocked individuals was nearly significant ($p = 0.052$) with a higher expression of HSP70 in the heat-shocked individuals. The average survival per *G. vermiculophylla* population after heat shock (see experiment above) correlated significantly ($p < 0.01$) with the expression of HSP70 after heat-shock (see Fig. 12).

Table 6: Expression of HSP70. Influence of the origin of *G. vermiculophylla* populations and the treatment level (either heat shocked or control) on expression of HSP70 in *G. vermiculophylla* in a two factorial ANOVA.

	SS	DF	MS	F	p
Region	1.24E+09	1	1.24E+09	33.13	<0.001
Treatment	1.55E+08	1	1.55E+08	4.14	0.05
Region * Treatment	5.31E+06	1	5.31E+06	0.14	0.71
Error	8.97E+08	24	3.74E+07		

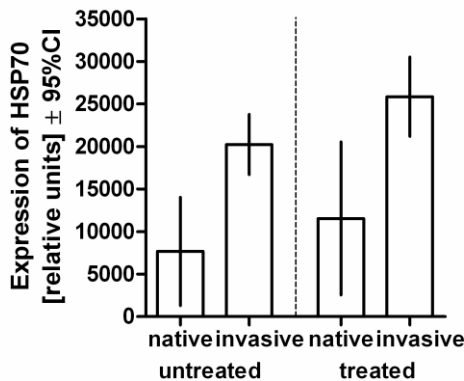


Figure 10: Expression of HSP70. Average expression of HSP70 in *G. vermiculophylla* from native and invaded regions either after heat shock (right) or in unstressed control individuals (left). $n_{\text{native}} = 6$, $n_{\text{invasive}} = 8$.

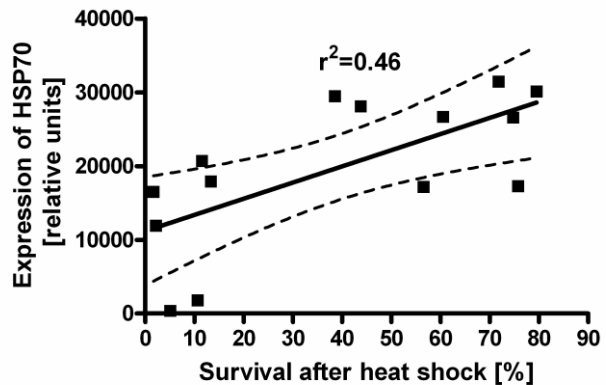


Figure 12: Heat shock treatment and expression of HSP70. Linear regression correlating the survival of *G. vermiculophylla* after heat shock with expression of HSP70. Dotted lines indicate the 95 % CI.



Figure 11: Expression of HSP70. Expression of HSP70 in single individuals of different *G. vermiculophylla* populations either after heat shock (treated) or in unstressed control individuals (not treated).

In-gel staining of haloperoxidase after electrophoresis under non-denaturing conditions resulted in the formation of three distinct bands that represent different isoforms of haloperoxidase (Fig. 13). The first band was only visible in single individuals of *G. vermiculophylla* and was therefore not evaluated statistically. The expression of the second isoform of haloperoxidase (see band 2 in Fig. 13) was significantly higher in native as compared to invasive individuals of *G. vermiculophylla* ($p < 0.001$, see Fig. 14 and Tab. 7a). Heat shocked individuals of *G. vermiculophylla* expressed more of this isoform than control individuals ($p < 0.01$, see Fig. 13, 14 and Tab. 7a). The third isoform (see band 3 in Fig. 13) was in tendency down regulated in heat-stressed specimens of *G. vermiculophylla*, however, this trend was not significant. Also, no significant difference was detected between native and invasive individuals of the seaweed (see Fig. 14 and Tab. 7b).

Similar as the expression of haloperoxidase isoform 2, the capacity of non-heat-shocked individuals to decompose externally applied H_2O_2 was higher in native as compared to invasive *G. vermiculophylla*, but this difference was statistically not significant ($t = 1.62$, $p = 0.12$; Fig. 15).

Table 7: Expression of haloperoxidase. Influence of the origin of *G. vermiculophylla* populations and the treatment level (either heat shocked or control) on expression of two different isoforms of haloperoxidase (corresponding to the bands 2 (a) and 3 (b) in Fig. 13) in *G. vermiculophylla* in a two factorial ANOVA.

a)	SS	DF	MS	F	p
Region	1.78E+13	1	1.78E+13	21.30	<0.001
Treatment	7.76E+12	1	7.76E+12	9.30	<0.01
Region * Treatment	2.62E+12	1	2.62E+12	3.14	0.09
Error	2.00E+13	24	8.34E+11		

b)	SS	DF	MS	F	p
Region	42.15	1	42.15	0.71	0.41
Treatment	150.67	1	150.67	2.53	0.12
Region * Treatment	9.67	1	9.67	0.16	0.69
Error	1428.04	24	59.50		

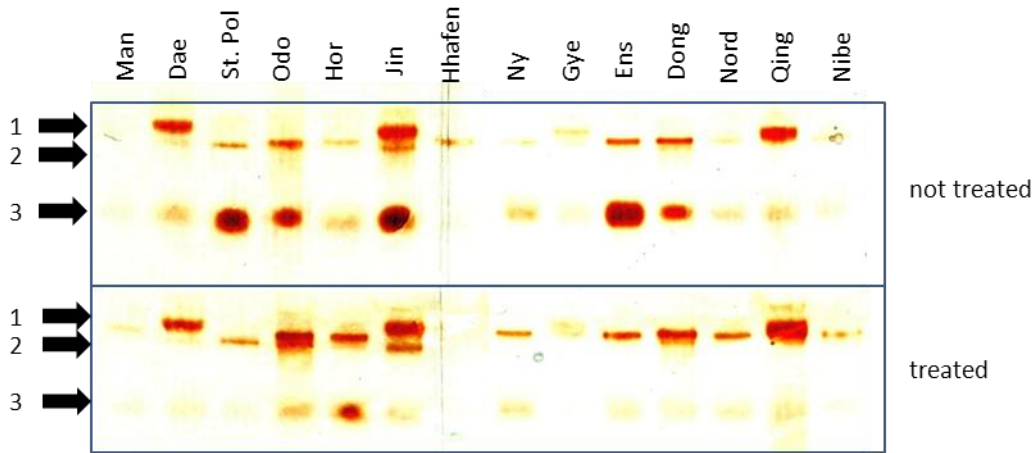


Figure 13: Haloperoxidase expression. Expression of three different isoforms of haloperoxidase in individuals of different *G. vermiculophylla* populations either after heat shock (treated) or in unstressed control individuals (not treated).

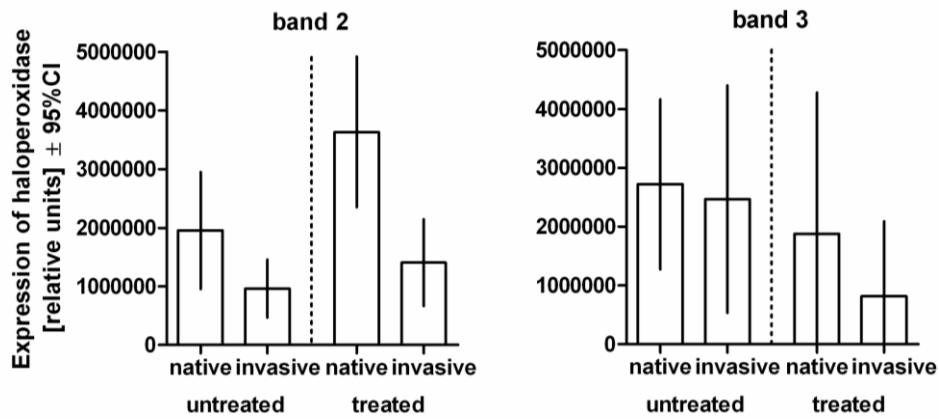


Figure 14: Haloperoxidase expression. Average expression of two different isoforms of haloperoxidase (corresponding to the bands 2 (left) and 3 (right) in Fig. 13) in *G. vermiculophylla* from native and invaded regions either after heat shock or in unstressed control individuals. $n_{\text{native}} = 6$, $n_{\text{invasive}} = 8$.

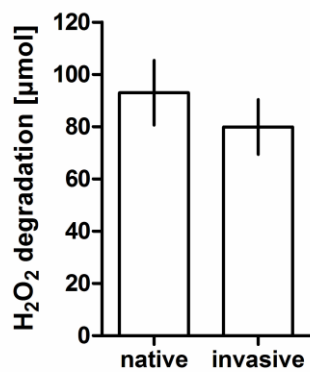


Figure 15: H₂O₂ degradation. Average degradation of H₂O₂ in *G. vermiculophylla* from native and invaded regions after heat shock. No significant differences were detected in a t-test. $n_{\text{native}} = 10$, $n_{\text{invasive}} = 20$.

Discussion

On the basis of our results the investigated 14 populations of *G. vermiculophylla* clearly differ with respect to stress resistance. However, different stresses are apparently tolerated at different degrees by each population and a significant difference in stress resistance between the two groups of native and invasive populations was only found in two out of four experiments (heat shock and copper stress). In two out of four experiments (heat shock and UV-stress) responses of the seaweeds correlated between the experiments conducted in Kiel and Qingdao, indicating that effects were mainly caused by the respective stressor. In the two other experiments (copper and depletion stress) the results did not correlate between the two experimental locations. This could indicate that local adaptation played a role, but other factors may also have influenced the results (see below).

The survival after heat shock was significantly higher in all invasive as compared to native populations in both Qingdao and Kiel. This would lead to the assumption that this strong resistance does exist in invasive populations, whereas it did not exist in native populations. Therefore the resistance to heat shock must have evolved in the course of the invasion process - either during the transport or in the new area.

The expression of HSP70 also differs between native and invasive specimens of *G. vermiculophylla*. As we find a significant correlation between expression of HSP70 and heat shock response we conclude that HSPs could at least in part be responsible for the heat shock reaction of *G. vermiculophylla*. Another fact is especially interesting: HSP70 expression is not only more elevated in heat shocked invasive individuals, but the difference also exists in non-stressed native and invasive individuals. The expression of high levels of HSP70 thus seems to be a constitutive trait in invasive individuals of *G. vermiculophylla*. The maximum sea water temperatures experienced in nature by most of the tested native populations of *G. vermiculophylla* are higher than those experienced by the invaded populations, so that acclimatization to natural temperature conditions in the new areas seems to be an unlikely explanation for the high heat stress tolerance of the invasive populations. Furthermore it seems relatively unlikely that the elevated tolerance to heat stress has evolved independently in several areas worldwide. The possession of elevated levels of HSP70 could rather be a trait that was useful to endure stress experienced in an early phase of invasion, probably during transportation.

With regard to depletion stress the response patterns of *G. vermiculophylla* differed between the experiments conducted in Kiel and Qingdao: In the native range we observed a better survival of invasive individuals, while the opposite was true for the experiment conducted in Kiel. There are mainly two possible reasons for our results: Either different conditions in the laboratories in Qingdao and Kiel or an influence of transportation to the respective experimental places. In both locations, *G. vermiculophylla* was stored in fridges at identical conditions. The only differences were in the water that was used to wetten the algae prior to their exposition in the fridge (Baltic Sea water at Kiel; Yellow Sea water at Qingdao) and in the presence (Qingdao) or absence (Kiel) of lab paper during the incubation. The presence of wetted lab paper in the Qingdao-experiment could have influenced the humidity surrounding the seaweeds or growth conditions for associated microorganisms. Another explanation for the differences could be that those seaweeds that endured the longer transportation to the experimental places (the invasives for the experiments

carried out in Qingdao and the natives for the experiments carried out in Kiel) developed a higher resistance towards depletion stress through acclimation and therefore performed better in the following depletion stress experiment. As two experimental runs brought different results it would be necessary to repeat the experiment - preferably in all the locations of origin of the seaweeds included in the trial. Effects of local acclimation were also detected in other common-garden experiments [10].

The differences in *G. vermiculophylla*'s reaction to copper stress remain relatively small. The only exception is the population from Odo in the experiment conducted at Qingdao. Remains of the specimens used in this experiment were kept in the lab under identical conditions as in the experiment, but in seawater without copper addition. They remained healthy and did not show any signs of stress, so that the copper seems causative for the degradation of the Odo population. The definite reason for the poor performance of the population from Odo in the experiment remains unknown, but a possible explanation could be that individuals of this population had encountered metal pollution before they were sampled and now their threshold of resistance was exceeded. Apart from that, the concentration of copper was obviously too low to cause clear effects in the other populations, despite of the results of our preliminary experiments and of other authors' findings. For example, growth rates and photosynthesis of *Gracilariopsis lemaeiformis* declined by > 70% when cultured in medium with copper addition for 96 hours at concentrations similar to those that we employed in our experiments (5 and 10 μM Cu) [60]. Also the growth rates of *Gracilaria tenuistipitata* were generally reduced when the seaweed was exposed to copper (0.16 – 3.15 μM Cu). In this light it seems that both native and invasive populations of *G. vermiculophylla* are relatively tolerant towards copper pollution, which could hint at the possession of effective antioxidant or detoxification [61] mechanisms and which could make *G. vermiculophylla* an invader with a generally higher probability of success than other gracilarioids. Indeed, *G. vermiculophylla* seemingly contains more haloperoxidase than other *Gracilaria*-species that have been studied (compare Fig. 13 and Fig. 9 in [62]).

However, while native populations performed less or equally well as invasive populations with respect to copper stress and UV-C radiation (which also often requires effective mechanisms to reduce harmful ROS [38]) native individuals of *G. vermiculophylla* expressed significantly higher levels of isoform 2 of haloperoxidase and by trend decomposed more externally applied H_2O_2 than invasive individuals. The expression of haloperoxidase isoform 2 was also higher after the individuals experienced heat shock, suggesting that the enzyme is employed in heat shock stress management. Thus, while the invasive individuals of *G. vermiculophylla* primarily expressed higher levels of HSP70 after heat shock, native individuals of the seaweed primarily employed haloperoxidase to deal with temperature stress. However, given the bad performance of native specimens in the heat shock experiment, the expression of higher levels of HSP70 seems to be the more effective way to cope with this stressor. Invasive populations thus apparently evolved not only toward an increased expression of HSP70, but also toward reduced allocation of resources into the less efficient haloperoxidase expression.

Conclusion

Resistance towards abiotic stress seemingly played an important role during the invasion history of *G. vermiculophylla*, as a selection of heat shock resistance clearly took place. In its area of origin, the seaweed is not generally stress tolerant, but there are marked differences with regard to different populations and different stressors. Correspondingly, the studied stress tolerance traits of *G. vermiculophylla* seem not to protect against multiple stressors. For example, HSP70 expression only correlated with heat shock resistance, and not with survival of metal pollution or UV-C radiation – despite several studies that attribute this chaperon also a function in the resistance against the two latter stresses [29].

It becomes apparent from the general presence of heat shock resistance among invasive populations in Europe and W Mexico that single populations or even individuals within a specific donor population of *G. vermiculophylla* may have been selected early during the invasion history of this species because of their pre-adaptation to heat shock. A selective introduction of specific pre-adapted genotypes was also supposed to be the key to invasion success for the South African ragwort *Senecio inaequidens* [63].

However, high tolerance toward heat shock is apparently not the only trait that was relevant for the invasion success of *G. vermiculophylla* as herbivory-resistant genotypes were also selected [51]. Thus the low palatability to herbivores in the invaded range [19,64] may have been another mechanism facilitating the spread of this seaweed.

Higher levels of HSP70 expression in invasive relative to native species were also found in the few available studies with other aquatic organisms: The invasive tunicate *Diplosoma listerianum* showed higher HSP70 levels than the native *Distaplia occidentalis* [32]. Greater induction of HSP70 and higher HSP70 induction temperatures were detected in the invasive mussel *Mytilus galloprovincialis* as compared to its native congener *Mytilus trossulus* [65,66]. The invasive brown alga *Undaria pinnatifida* from Asia expressed more HSP70 than native seaweeds in the invaded habitat, but showed similar magnitudes in expression as Japanese populations of *U. pinnatifida* did [67]. On the other hand, we are only aware of few examples of elevated chaperon expression in non-native species or populations of terrestrial invasive organisms [68,69], despite the fact that invasive terrestrial organisms have been much more investigated than invasive aquatic organisms. In this light heat shock protein expression may possibly be a more relevant determinant of invasion success in aquatic organisms than in terrestrial organisms.

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Paper II



Invasion success of the seaweed *Gracilaria vermiculophylla* correlates with low palatability

Mareike Hammann^{1,*}, Gaoge Wang², Esther Rickert¹, Sung Min Boo³,
Florian Weinberger¹

¹Helmholtz Centre for Ocean Research (GEOMAR), Düsternbrooker Weg 20, 24105 Kiel, Germany
²College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, 266003 Qingdao, China
³Department of Biology, Chungnam National University, Daejeon 305-764, Korea

ABSTRACT: Differences with respect to anti-herbivore defense were investigated in invasive and native populations of the seaweed *Gracilaria vermiculophylla*. Specimens from 6 native populations in East Asia and from 8 populations invasive in Europe and the Mexican Pacific coast were maintained under identical conditions and offered to herbivorous snails from both the native range (*Littorina brevicula*) and Europe (*L. littorea*) in no-choice feeding assays. *L. brevicula* consumed in total significantly larger amounts of *G. vermiculophylla* tissue than did *L. littorea*. Further, both snail species least consumed the seaweed specimens originating from either non-native populations or from populations native to the Korean East Sea/Sea of Japan. The Korean East Sea/Sea of Japan had previously been identified as putative donor region of all the invasive populations of *G. vermiculophylla*. Thus, populations in the donor region as well as non-native populations in different invaded realms feature an increased capacity to resist feeding pressure. Differences in nutrient content did not account for the observed patterns of consumption, as palatability and carbon to nitrogen (C:N) ratio were not significantly correlated. Thus, mechanical or chemical defenses or the content of feeding cues influenced the behavior of the snails. We suggest that low palatability contributed to the invasion success of the species.

KEY WORDS: Biological invasion · Invasive seaweeds · Enemy release hypothesis · Novel weapons hypothesis · Herbivory · *Gracilaria vermiculophylla*

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INTRODUCTION

For terrestrial plants and also for seaweeds (Nyberg & Wallentinus 2005), grazing defense is thought to be an important biological trait that determines invasiveness. High levels of novel phytochemicals (so called 'novel weapons') that native plants, grazers, or pathogens have not previously encountered have often been predicted to favor introduced plants in new environments (Callaway & Ridenour 2004, Cappuccino & Carpenter 2005, Cappuccino & Arnason 2006, Forslund et al. 2010). Correspondingly, pres-

ence of generalist enemies in new environments might result in a selection of defensive traits in introduced plants (Blossey & Notzold 1995, Callaway & Ridenour 2004). In contrast, the 'enemy release' hypothesis (Keane & Crawley 2002) attributes the success of introduced plants to a lack of specialist enemies in the invaders' new ranges. Under such conditions, invasive plants might lose their protection against specialist herbivores, allowing a shift towards higher protection against generalist herbivores (Joshi & Vrieling 2005). However, in contrast to terrestrial plants, introduced seaweeds are often not

*Email: mhammann@geomar.de

released from grazers, as significantly more herbivores in the sea than on land are generalists (Hay & Steinberg 1992).

Several studies have quantified grazing on invasive seaweed species in their new range, and in most cases, grazers from the introduced range preferred native over non-native seaweeds (Weinberger et al. 2008, Monteiro et al. 2009, Cacabelos et al. 2010, Engelen et al. 2011, Nejrup et al. 2012). This low feeding pressure could explain the invasiveness of non-native algae. However, all those studies were conducted only in the invader's introduced range. Hierro et al. (2005) state the importance of studying exotic species not only in their introduced ranges, as there is a gap in knowledge about the ecology of these species in their native habitats. For testing herbivory-related invasion hypotheses, comparisons of the effects of grazers in the native and the introduced range are therefore crucial. Wikström et al. (2006) were the first to compare native and non-native populations of a seaweed species with regard to feeding pressure and grazing defense, instead of comparing invasive and co-occurring native species. Their study revealed that the rockweed *Fucus evanescens* is less exposed to herbivory in its non-native range in Sweden than in its native range in Iceland, probably due to an increased chemical defense in invasive populations. Both Iceland and Sweden belong to the same Northern European coastal ecoprovince, and the inventories of seaweed-consuming species that are present in these regions show considerable overlap (Spalding et al. 2007). Introduction into new ecoprovinces or even realms might confront the invader with more drastic changes in interspecific interactions. Our aim was therefore to expand the comparison of invasive and non-invasive macroalgal populations over a larger geographical scale, in order to cover environments with more divergent species inventories.

The red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss was chosen as a model organism, as its large tolerance towards environmental stress (Yokoya et al. 1999, Thomsen & McGlathery 2007, Weinberger et al. 2008, Nyberg & Wallentinus 2009) makes it suitable for common garden-like experiments that require long-distance transportation under suboptimal conditions. The native distribution range of the species is in the cold and warm temperate Northwest Pacific ecoprovinces, ranging from the Northern Sea of Japan to the East China Sea and the Central Kuroshio Current. During the last 2 decades, *G. vermiculophylla* has not only spread to the cold (Saunders 2009) and warm (Bellorin et al. 2004) tem-

perate North American west coasts, but also to most ecoprovinces of the temperate Northern Atlantic realm: cold (Saunders 2009) and warm (Freshwater et al. 2006) temperate North American east coasts, Mediterranean Sea (Sfriso et al. 2010), Lusitanian coasts (Guillemin et al. 2008), and Northern European seas, including the Baltic Sea (Schories & Selig 2006, Nyberg 2007, Thomsen et al. 2007a,b, Weinberger et al. 2008). *G. vermiculophylla* spreads rapidly, tends to change the flora and fauna of invaded habitats, and has been included among the most invasive seaweeds in Europe (Nyberg 2007). The invasion process has resulted in a considerable loss of genetic diversity in non-native populations of *G. vermiculophylla*, all of which seem to have a common genetic origin in a donor region within the Korean East Sea/Sea of Japan (Kim et al. 2010).

Local mass appearances and ecological dominance of *Gracilaria vermiculophylla* have repeatedly been observed in areas outside of its native range (Freshwater et al. 2006, Thomsen et al. 2006, 2007a, Pinon-Gimate et al. 2008, Weinberger et al. 2008). To our knowledge, mass appearances have not been reported for native populations of *G. vermiculophylla*. This discrepancy could result from many possible reasons. A more stringent control by native than by non-native feeding enemies is one of them, as *G. vermiculophylla* from non-native populations seems to be less affected by herbivores than native seaweeds from the same environment (Weinberger et al. 2008, Nejrup et al. 2012).

Unlike terrestrial plants, many algae only have a low proportion of structural material that could act as a morphological defense against grazers. Therefore they rely on other defense mechanisms, such as the use of chemical feeding deterrents (Hay & Steinberg 1992). Also, *Gracilaria vermiculophylla* is capable of defending itself from herbivory by mesograzers: if tissue is damaged—either by artificial mechanical wounding or by grazing—the alga produces oxylipins (Nylund et al. 2011, Rempt et al. 2012). These compounds deter potential grazers of *G. vermiculophylla*, such as the omnivorous isopod *Idotea balthica* (Nylund et al. 2011) or the herbivorous sea snail *Echinolittorina peruviana* (Rempt et al. 2012). Wound-activated oxylipins are also molecular messengers that mediate induced defenses of gracilarioids (Weinberger et al. 2011).

The consumption of *Gracilaria vermiculophylla* by certain mesograzers may therefore be reduced due to chemical defenses of the alga. On the other hand, herbivores depend on the ingestion of plant-derived nitrogen and are therefore assumed to prefer plants

with a high proportion of nitrogen, thus with a low carbon to nitrogen (C:N) ratio (Mattson 1980). In accordance with this, *Littorina littorea* consumed and retained more food in nutrient-enriched environments and gained a higher biomass (Diaz et al. 2012). Likewise, feeding preferences of the snail *L. sitkeana* among different seaweeds were primarily determined by the C:N ratio (Van Alstyne et al. 2009), and the amphipod *Gammarus locusta* actively chose high-quality nutrient-rich food as well (Kraufvelin et al. 2006).

The main hypotheses tested in the present study were the following: (1) *Gracilaria vermiculophylla* is less palatable to grazers from the invaded range as compared to grazers from the native range because invader and grazers in the new habitat do not share a co-evolutionary history (as predicted by the enemy release hypothesis), (2) *G. vermiculophylla* originating from non-native populations is in general less palatable than *G. vermiculophylla* from native populations because it has evolved resistance against herbivores during the invasion process (as predicted by the novel weapons hypothesis), and (3) a disposition to relatively low palatability of *G. vermiculophylla* populations from the Korean East Sea/Sea of Japan has facilitated the invasion success of specimens from this donor region (through selection of preadapted genotypes).

To test these hypotheses, *Gracilaria vermiculophylla* from 6 native and 8 invasive populations was fed in no-choice feeding experiments of common-garden type to (1) the snail *Littorina brevicula* Philippi, which has a similar distribution range in East Asia as *G. vermiculophylla* (Son & Hong 1998,

Kim et al. 2003), and (2) *L. littorea* L., a common grazer in most of the regions invaded by *G. vermiculophylla* in the North Atlantic realm. *L. littorea* is able to feed on a variety of macroalgae (Watson & Norton 1985, Diaz et al. 2012), and its Asian conspecific is a generalist herbivore as well (Molis et al. 2008). Feeding experiments with *L. brevicula* were conducted in Qingdao, China, and feeding assays with *L. littorea* were carried out in Kiel, Germany. C:N ratios of *G. vermiculophylla* specimens offered in feeding assays were analyzed in order to investigate whether differences in nutrient content explained differences in palatability. The combination of several native and non-native seaweed populations with congeneric grazers of either the native or the introduced range in a common garden was a new approach in the frame of testing herbivory-related invasion theories in marine ecology. Furthermore, it also ensured that the detected effects were not only due to local adaptation (Williams et al. 2008).

MATERIALS AND METHODS

Collection, transport, and cultivation of *Gracilaria vermiculophylla*

Between 10 February and 22 April 2010, 6 native populations of *Gracilaria vermiculophylla* were sampled along the Korean peninsula and in Shandong Province, China, and 8 non-native populations were sampled around the Danish-German peninsula of Jutland and Schleswig-Holstein, in the west of France, and on the Pacific coast of Mexico (Table 1).

Table 1. *Gracilaria vermiculophylla*. Geographic location and taxonomic reference (GenBank accession numbers are given for new *cox1* gene sequences generated in the framework of the present study) of native and non-native populations

	Population/abbreviation (sea area)	Geographic coordinates	Taxonomic reference
Native	Donghae/Dong (East Sea, South Korea)	37° 29' 27.65" N, 129° 08' 30.42" E	JQ736332
	Odo-ri/Odo (East Sea, South Korea)	36° 09' 52.40" N, 129° 24' 00.07" E	JQ736333
	Jindo/Jin (Yellow Sea, South Korea)	34° 25' 16.98" N, 126° 20' 48.21" E	Kim et al. (2010)
	Gyeokpo/Gye (Yellow Sea, South Korea)	35° 37' 05.54" N, 126° 28' 01.70" E	Kim et al. (2010)
	Daecheon/Dae (Yellow Sea, South Korea)	36° 19' 23.07" N, 126° 30' 07.81" E	Kim et al. (2010)
	Qingdao/Qing (Yellow Sea, China)	36° 03' 18.51" N, 120° 21' 57.01" E	Kim et al. (2010)
Non-native	Heiligenhafen/Hhafen (Baltic Sea, Germany)	54° 22' 48.14" N, 10° 58' 58.94" E	JQ736335
	Nyborg/Ny (Baltic Sea, Denmark)	55° 17' 54.23" N, 10° 49' 07.35" E	JQ736339
	Horsens/Hor (Baltic Sea, Denmark)	55° 51' 35.02" N, 9° 52' 35.76" E	KC731475
	Nibe/Nibe (Limfjord, Denmark)	56° 59' 03.34" N, 9° 37' 48.89" E	JQ736338
	Mandö/Man (Wadden Sea, Denmark)	55° 18' 35.59" N, 8° 38' 34.74" E	JQ736337
	Nordstrand/Nord (Wadden Sea, Germany)	54° 31' 48.01" N, 8° 52' 18.88" E	JQ736336
	St.-Pol-de-Leon/St.-Pol (Brittany, France)	48° 40' 41.09" N, 3° 58' 08.04" W	Kim et al. (2010)
	Ensenada/Ens (Baja California, Mexico)	31° 46' 28.67" N, 116° 36' 40.92" W	JQ736334

The identity of all studied populations was confirmed by DNA barcoding, either by Kim et al. (2010) or by us, using the methodology described in Kim et al. (2010) (Table 1). *G. vermiculophylla* individuals from native populations were easily distinguishable, as each plant was growing attached to hard substratum. However, *G. vermiculophylla* from non-native populations was often growing unattached, and so it cannot be ruled out that some of the used 'individuals' were in fact clonal, although both native and non-native plants were sampled in extended areas of >1000 m² to avoid taking clonal samples.

For the feeding experiments with *Littorina brevicula* and *L. littorea*, specimens from all *Gracilaria vermiculophylla* populations were transferred alive to both Qingdao (China) and Kiel (Germany), respectively. They were transported wrapped into seawater-wetted lab paper and in cooling containers (Nyberg & Wallentinus 2009). In order to exert as little stress as possible, transportation periods never exceeded 5 d, and for logistic reasons the transportation between collection site and experimental site could not always be direct. Specimens originating from native populations were transported directly to Qingdao and Kiel, while specimens originating from non-native populations were brought to Kiel first. After a regeneration period where they were kept under non-stressful conditions for at least 2 wk, one half was transported to Qingdao, while the other half remained in Kiel. Losses due to transportation stress were not observed. After transportation and prior to feeding assays, all specimens were kept under non-stressful conditions for at least 1 wk before conducting the feeding assays to allow for regeneration. In Qingdao, they were for this purpose cultivated in separate Erlenmeyer flasks at identical conditions (room temperature, i.e. approx. 20°C; indirect sunlight, i.e. maximum photosynthetically active radiation [PAR_{max}] of 20 μmol m⁻² s⁻¹; no aeration). The medium (Yellow Sea water) was exchanged daily. At Kiel, the cultivation for regeneration took place in separate indoor tanks under identical conditions (water temperature: 15 to 20°C; PAR: 30 μmol m⁻² s⁻¹ for 16 h d⁻¹; aeration). The medium was Baltic Sea water from the Kiel Fjord. It was constantly exchanged between all tanks and a central storage container, in order to maintain an identical composition in all tanks. At the exit of the central container and prior to redistribution into the tanks, the water was UV-sterilized, in order to prevent exchange of algal propagules among tanks containing material of different origin. For experiments conducted in Qingdao, seaweeds that were sampled at low salinity condi-

tions (Baltic Sea) were slowly (stepwise over several days) acclimatized to fully marine conditions, while seaweeds from fully marine conditions were slowly adapted to low salinity conditions (salinity: 15 ± 2) before using them for experiments in Kiel.

Collection of *Littorina brevicula* and *L. littorea*

Three days before the feeding experiment started, *Littorina brevicula* individuals were collected on the Yellow Sea coast in Qingdao (36° 03' 18.51" N, 120° 21' 57.01" E) at a site where *Gracilaria vermiculophylla* was present. Snails were maintained in the laboratory in an aquarium containing 5 l of Yellow Sea water (salinity: 33), which was exchanged twice every day. During this period they were allowed to graze on green algae (*Ulva* sp.). The diameter of the snails was measured prior to the experiment. Snails with a diameter between 7 and 9 mm were most common in the habitat at the time of sampling (April) and were therefore used for experiments.

Littorina littorea individuals were collected on the shore of the Kiel Fjord (54° 21.965' N, 10° 8.908' E) at a site where *Gracilaria vermiculophylla* was present. They were kept in a flow-through aquarium containing 5 l of Baltic Sea water (salinity: 15 ± 2) prior to the bioassay. During this period of 3 d, they were allowed to graze on green algae (*Ulva* sp.). For this experiment, snails with a diameter between 8 and 12 mm were used.

Feeding assays

The experiments with *Littorina brevicula* were conducted in April 2010 and experiments with *L. littorea* in May 2010. For the no-choice feeding assays, 10 algal individuals per *Gracilaria vermiculophylla* population were used. Each of the 10 replicates was cut in 2 pieces of 0.1 ± 0.005 g (blotted wet weight): One was exposed to snails and the second one was used as control of autogenic changes during the course of the experiment. At Qingdao, all algal pieces were incubated in Petri dishes (diameter: 9 cm) containing water from the Yellow Sea (salinity: 33), which was exchanged every day. The experiment lasted for 7 d, and 4 snails were used in each treatment. At Kiel, the experimental setup was identical, with 3 exceptions: the water originated from the Kiel Fjord (salinity: 15 ± 2), and only 3 snails were used per Petri dish, as they were larger than those at Qingdao. Also, *L. littorea* individuals were allowed to

consume the algae for 12 d, as the feeding rates were generally lower than for *L. brevicula*. Experiments were run in a constant temperature room (16°C) in Kiel. In Qingdao, those facilities were not available, but temperatures were in the same range (15 to 20°C).

At the end of each experiment, algae exposed and unexposed to snails were weighed again and net growth was determined. To get comparable growth data for both experiments, the daily weight change of *Gracilaria vermiculophylla* was determined and related to the respective soft body dry weight (DW) of the snails. Soft body DW was ascertained from shell diameters of the 2 snail species as follows. The diameter of snails of different sizes was measured (*Littorina brevicula*: n = 46, diameter: 4 to 10 mm; *L. littorea*: n = 61, diameter: 6 to 15 mm). The soft body was removed from the shell and dried at 50°C until weight constancy. Regressions were then calculated between shell diameters and soft body DW of both species, so that the soft body DW of the snails in each Petri dish could be assessed.

C:N analysis

Remains of every *Gracilaria vermiculophylla* individual used in the feeding assays were conserved in silica gel for C:N analysis. From both series of experiments, 5 randomly selected replicates per population were analyzed. The dried algal pieces were ground in a mixer mill (Retsch), and C:N ratios were determined in a Euro Vector EA 3000 Elemental Analyzer (Eurovector).

Statistical analysis

Prior to statistical tests, consumption data and C:N ratios were Box-Cox-transformed to achieve homogeneity of variances and normality. Homogeneity of variances could not be achieved for all factors (Levine's test, $p < 0.05$), and in these cases, results were taken in the following tests as statistically significant if the probability of error was $< 1\%$ (Underwood 1997), while other results were taken as statistically significant at a p-level of 5%. Differences in consumption rates of either *Littorina brevicula* or *L. littorea* were analyzed by nested-design ANOVA, with the factor 'population' nested within the factor 'region' (native or non-native). Differences among single populations were detected with Tukey's post hoc test. A linear regression was fitted between con-

sumption rates of *L. brevicula* and *L. littorea*, in order to find out if both snail species preferably fed on the same populations of *Gracilaria vermiculophylla*. Linear regression was also used to detect correlations among C:N ratios and feeding patterns. Box-Cox transformations and statistical tests were conducted using the STATISTICA 8 software package (StatSoft), while regressions were fitted using GraphPad Prism 4 software.

RESULTS

Both *Littorina brevicula* and *L. littorea* found *Gracilaria vermiculophylla* individuals originating from native populations more palatable, and consumed > 3 times as much of them compared to specimens originating from invasive populations ($p < 0.001$; Fig. 1, Table 2).

Interestingly, there was a high variability within the native populations, and the origin of native specimens influenced consumption rates of *Littorina brevicula* significantly ($p < 0.001$; Fig. 2A, Table 2). Seaweeds from Jindo (South Korea) were consumed more than all other native populations, except for those from Qingdao (China) and Gyeokpo (South Korea). Populations from Daechon, Odo, and Donghae (South Korea) were consumed significantly less than the seaweed individuals from Jindo, and had an average (\pm SE) biomass loss of 14.7 ± 2.9 mg d⁻¹ (g DW *L. brevicula*)⁻¹. Variability within the invasive

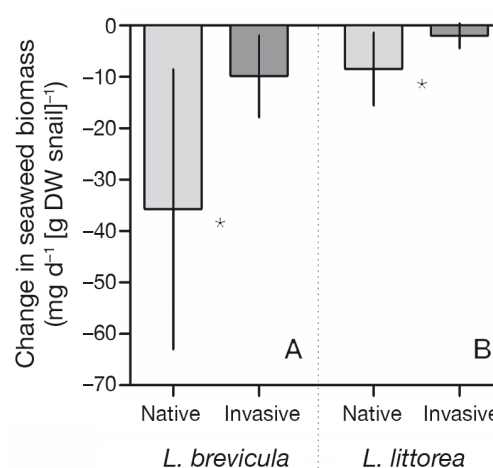


Fig. 1. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Average biomass loss of *G. vermiculophylla* from native and invasive populations caused by consumption by (A) *L. brevicula* and (B) *L. littorea* in feeding experiments. *Native and invasive populations significantly different in a nested 2-way ANOVA (see factor 'region' in Table 2; $p < 0.001$). n = 7 to 10. Whiskers: $\pm 95\%$ CI

Table 2. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Influence of the origin of *G. vermiculophylla* populations on the biomass consumed by *L. brevicula* and *L. littorea* in feeding experiments, using a nested-design ANOVA with the factor 'population' nested within the factor 'region' (either native or invasive)

	SS	df	MS	F	p
<i>L. brevicula</i>					
Population (Region)	4.50×10^{10}	12	3.75×10^9	4.41	<0.001
Region	3.02×10^{10}	1	3.02×10^{10}	35.56	<0.001
Error	1.04×10^{11}	122	8.50×10^8		
<i>L. littorea</i>					
Population (Region)	3.34×10^8	12	2.78×10^7	4.34	<0.001
Region	1.37×10^8	1	1.37×10^8	21.46	<0.001
Error	7.24×10^8	113	6.41×10^6		

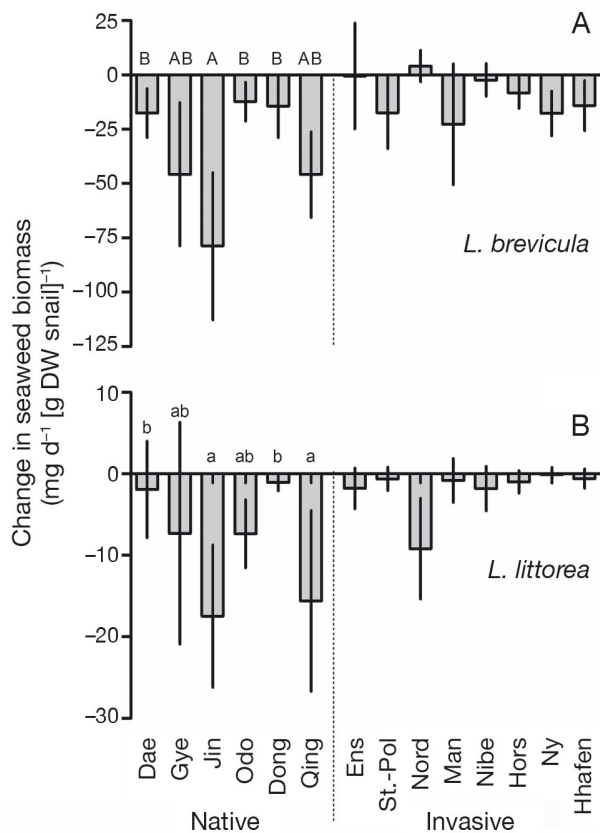


Fig. 2. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Consumption of biomass of different *G. vermiculophylla* populations by (A) *L. brevicula* and (B) *L. littorea* in feeding experiments. Different letters indicate populations that were significantly different in a Tukey test ($p < 0.01$) either within the native or within the non-native region. $n = 7$ to 10 . Whiskers: $\pm 95\%$ CI. See Table 1 for population abbreviations

populations was lower than within native populations, and Tukey's post hoc test did not detect any significant differences between them (see Fig. 2A).

Average (\pm SE) biomass loss of invasive populations was $10.2 \pm 2.5 \text{ mg d}^{-1}$ ($\text{g DW } L. brevicula$) $^{-1}$, and thus similar to populations from Daecheon, Odo, and Donghae.

Consumption of *Littorina littorea* also varied significantly within native populations, but not within invasive populations ($p < 0.001$; Fig. 2B, Table 2). Specimens from Jindo (South Korea) and Qingdao (China) were consumed significantly more than algae from other native populations, with the exception of those from Gyeokpo (South Korea) and Odo (South Korea). Material from Dae-

cheon and Donghae (South Korea; biomass loss [\pm SE]: 1.9 ± 2.4 and $1.1 \pm 0.4 \text{ mg d}^{-1}$ [$\text{g DW } L. littorea$] $^{-1}$, respectively) was consumed significantly less than specimens from Jindo and Qingdao, and showed consumption rates in a similar range as *Gracilaria vermiculophylla* from invasive populations (average [\pm SE] biomass loss: $2.0 \pm 0.5 \text{ mg d}^{-1}$ [$\text{g DW } L. littorea$] $^{-1}$).

Littorina brevicula and *L. littorea* tended to find the same populations of *Gracilaria vermiculophylla* most palatable (Fig. 3). Two of the native populations (Jindo from South Korea, and Qingdao from China) were consumed most by both herbivores, while the consumption of 2 other native populations (Daecheon and Donghae, South Korea) was similar to that of invasive populations. Seaweeds from Gyeokpo (South Korea) were in both experiments consumed at an intermediate range. Seaweeds from Odo (South Korea), however, differed between experiments in

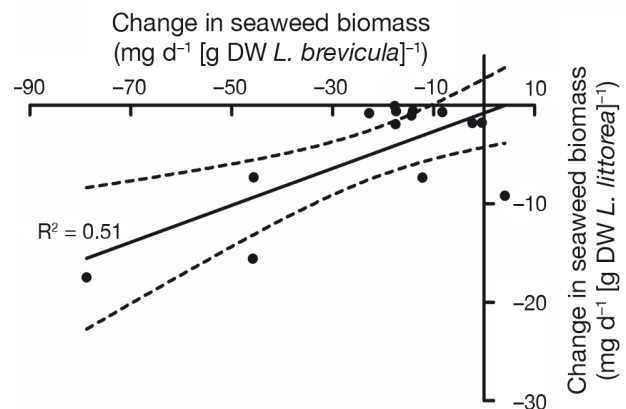


Fig. 3. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Linear regression correlating the biomass of *G. vermiculophylla* per population consumed by *L. brevicula* and *L. littorea*. Dotted lines: 95% CI

the native and the invaded range— while they were not much consumed by *L. brevicula*, the consumption by *L. littorea* was at an intermediate level.

However, the total amounts consumed were significantly higher for *Littorina brevicula*, which fed more than 5-fold the amount of *Gracilaria vermiculophylla* compared to *L. littorea* (Fig. 1). The difference becomes even more distinct when the combinations of snails and seaweeds existing in nature (*L. brevicula* and *G. vermiculophylla* from Qingdao and *L. littorea* and *G. vermiculophylla* from the German Baltic coast) are compared— *L. brevicula* consumed 90-fold the amount consumed by *L. littorea*.

At the end of both experiments, the C:N ratios differed between native and invasive populations of *Gracilaria vermiculophylla* ($p < 0.001$; Fig. 4, Table 3), with slightly higher relative nitrogen contents in the invasive species. Significant differences in C:N ratios were also detected among single populations (experiment in Qingdao: $p < 0.001$; Fig. 5A, Table 3; experiment in Kiel: $p < 0.01$; Fig. 5B, Table 3). However, there was no obvious distribution pattern among native or invasive algae, and the variability was high.

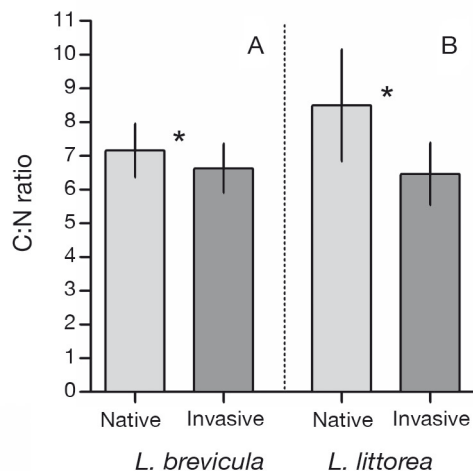


Fig. 4. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Means of C:N ratios for native and invasive *G. vermiculophylla* populations consumed by (A) *L. brevicula* and (B) *L. littorea*. *Native and invasive populations significantly different in a nested 2-way ANOVA (see factor 'region' in Table 3; $p < 0.001$). $n = 5$. Whiskers: $\pm 95\%$ CI

Table 3. *Gracilaria vermiculophylla*. Influence of the origin of *G. vermiculophylla* populations on C:N ratios of the seaweed in experiments in Qingdao and Kiel, using a nested-design ANOVA with the factor 'population' nested within the factor 'region' (either native or invasive)

	SS	df	MS	F	p
Qingdao					
Population (Region)	0.018	12	0.002	8.81	<0.001
Region	0.002	1	0.002	13.60	<0.001
Error	0.009	55	0.0002		
Kiel					
Population (Region)	0.021	11	0.002	2.80	<0.01
Region	0.020	1	0.020	28.1	<0.001
Error	0.034	49	0.0007		

We detected no significant correlations between C:N ratios and biomass consumption by *Littorina brevicula* or *L. littorea* (Fig. 6).

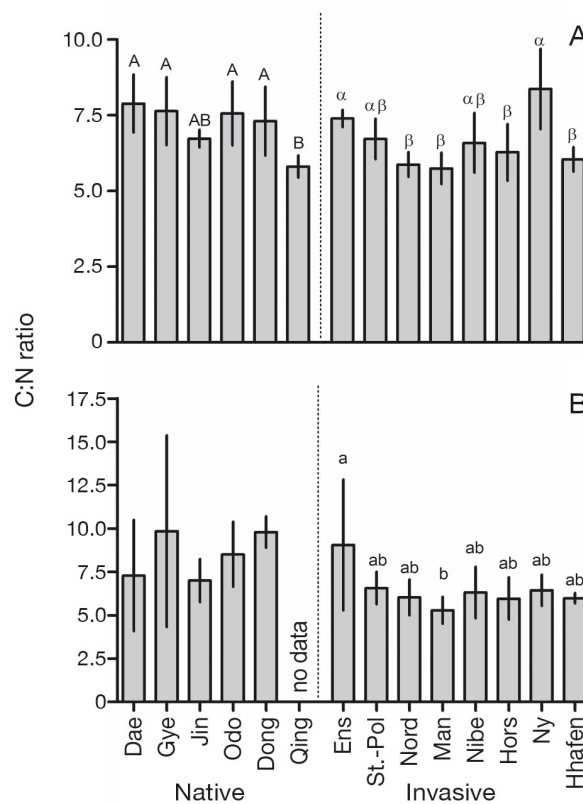


Fig. 5. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Averages of C:N ratios for single *G. vermiculophylla* populations consumed by (A) *L. brevicula* and (B) *L. littorea*. Different letters indicate populations that were significantly different in a Tukey test ($p < 0.01$) either within the native or within the non-native region. $n = 5$. Whiskers: 95% CI. See Table 1 for population abbreviations

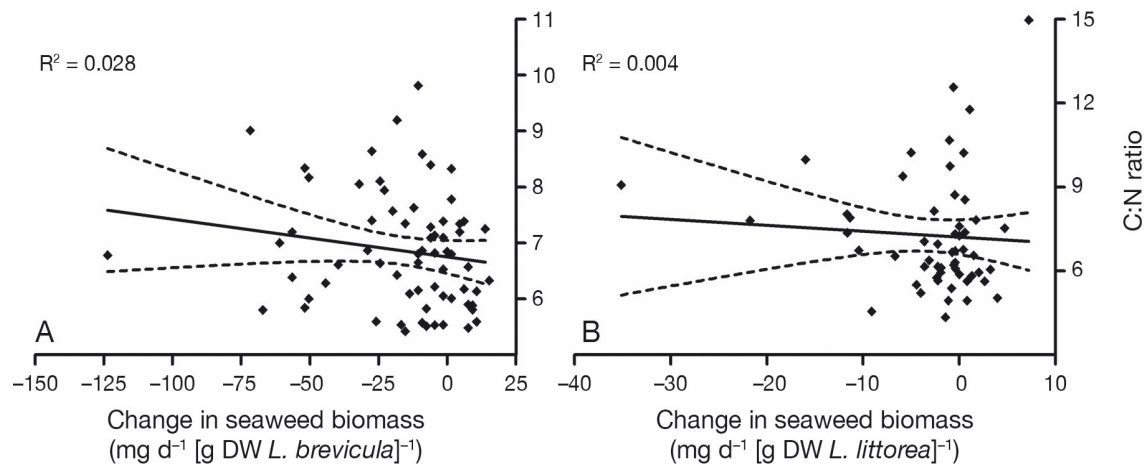


Fig. 6. *Gracilaria vermiculophylla*, *Littorina brevicula*, and *L. littorea*. Linear regression of C:N ratios of *G. vermiculophylla* biomass and biomass consumed by (A) *L. brevicula* and (B) *L. littorea*. Dotted lines indicate 95% CI

DISCUSSION

The employment of both native and non-native populations of *Gracilaria vermiculophylla* in combination with congeneric generalist herbivores from the native range (*Littorina brevicula*) and the invaded range (*L. littorea*) allows us to answer several questions: Are non-native populations of the seaweed in general more sensitive to grazing than native populations? Do feeding rates differ among single populations? Do *L. brevicula* and *L. littorea* consume similar amounts of the seaweed or is *G. vermiculophylla* less palatable to the grazer in the new range? The comparison between the consumption rates of the 2 snail species has to be looked at with some caution, as conditions at the experimental sites were similar, but not identical. It is possible that *L. brevicula* has in general higher consumption rates than *L. littorea* or that it coped better with the laboratory conditions. However, the behavior of all snails seemed normal, and losses during the experimental period were not observed. Light and temperature regimes were in a similar range, but the salinity differed, as we used the salinity conditions that were present at the respective sampling sites of the snails to avoid stress. Nevertheless, the differences are quite remarkable: *L. brevicula* from the native range consumed in total significantly higher amounts (\pm SE) of algal tissue ($21.4 \pm 2.8 \text{ mg d}^{-1} \text{ g}^{-1}$ when averaged over all algal populations) than the phylogenetically related *L. littorea* from the non-native range ($4.3 \pm 0.8 \text{ mg d}^{-1} \text{ g}^{-1}$). The ratio was 90-fold when naturally occurring matches of seaweed and herbivore in Qingdao (China) and in the German Baltic were compared. At

Qingdao, *G. vermiculophylla* inhabits the upper eulittoral close to the supralittoral fringe. *L. brevicula* is the main character species of this habitat (Morton 1990), and the alga is frequently subject to consumption by the snail (authors' pers. obs.). In the atidal Baltic Sea at Kiel and Heiligenhafen, both *L. littorea* and *G. vermiculophylla* inhabit the upper sublittoral, and the snail is 1 of the 2 main feeding antagonists, together with the isopod *Idotea baltica* (Weinberger et al. 2008). However, both consumers preferably feed on the native brown seaweed *Fucus vesiculosus* rather than on *G. vermiculophylla* when both are offered in 2-way-choice experiments (Weinberger et al. 2008). Nejrup & Pedersen (2010) examined *in situ* grazing losses of non-native *G. vermiculophylla* in Denmark over an entire growth season, but did not find significant losses due to grazing. The feeding pressure on native populations of *G. vermiculophylla* in the field has not been determined yet. However, on the Chinese and South Korean coasts, *L. brevicula* can occur in markedly high numbers, while densities of *L. littorea* on the German and Danish coasts seem to be lower (authors' pers. obs.). At the same time, our experiments indicate that the grazer *L. littorea* from the invaded range is less attracted or more deterred by the metabolic composition of *G. vermiculophylla* than the snail from the native range.

It was most obvious that both snail species preferred to feed on the native seaweeds. Both *Littorina littorea* and *L. brevicula* consumed more than 3 times larger amounts of native than of invasive *Gracilaria vermiculophylla*. Apparently, *G. vermiculophylla* originating from the invaded range was less palatable. Consequently, the question should be asked whether

differences in C:N ratios could be responsible for the observed differences in consumption of the algal material. Similarly to the differences in consumption, C:N ratios also differed significantly among native and invasive algal specimens. Slightly higher C:N ratios were detected in native individuals. However, snails of the genus *Littorina* have been attracted to food with low rather than high C:N ratios (Van Alstyne et al. 2009). Moreover, there were no significant correlations between C:N ratios and biomass consumption by *L. brevicula* or *L. littorea*. The C:N contents of the seaweeds can therefore not explain the observed feeding patterns. In this light, it seems more likely that the snails were influenced by specific deterrents or feeding cues. *G. vermiculophylla* is able to defend itself from feeding by up-regulation of chemical compounds. After wounding, the alga produces—among other substances—arachidonic acid derivatives, which were found to deter mesograzers such as the omnivorous isopod *Idotea baltica* (Nyland et al. 2011) or the herbivorous sea snail *Echinolittorina peruviana* (Rempt et al. 2012). It seems likely that these or other substances also act as feeding deterrents against *L. brevicula* and *L. littorea*. In a study with the invasive red alga *Bonnemaisonia hamifera*, Enge et al. (2012) were able to show that low consumption in the new range of the seaweed can be directly attributed to a specific chemical defense against native generalists that did not encounter this type of chemical defense before. They conclude that novel chemical weapons against naive herbivores might provide a mechanistic explanation for algal invasion success. The lower palatability of non-native populations of *G. vermiculophylla* could similarly be due to an increased level of chemical defenses. This low palatability to one of the main grazers in invaded habitats could partly explain the invasion success of *G. vermiculophylla*. However, numerous other traits also cannot be excluded as potential factors of invasion success, as *G. vermiculophylla* can survive and spread under a wide range of abiotic conditions and is also able to survive extensive periods of desiccation and burial (Yokoya et al. 1999, Nyberg 2007, Thomsen & McGlathery 2007, Weinberger et al. 2008, Nyberg & Wallentinus 2009, Nejrup & Pedersen 2012).

The variation of palatability within native populations of *Gracilaria vermiculophylla* is high, but overall *Littorina brevicula* and *L. littorea* found the same populations most palatable. The consumption of 2 native populations (Daechon and Donghae, South Korea) by both snails was similar to that of invasive populations, while seaweeds from Odo

(South Korea) were of low palatability to *L. brevicula* only. Of all the native populations included in the present study, the Donghae population is genetically most similar to invasive populations (Kim et al. 2010), and genetic data hint at the Korean East Sea/Sea of Japan being the donor region for all invasive *G. vermiculophylla* populations (Kim et al. 2010). In this light, it is interesting that both populations from the Korean East Sea/Sea of Japan (Donghae and Odo) included in the present study were also among those native populations that have been fed on least. Possibly, low palatability is more widely spread among populations in the Korean East Sea/Sea of Japan; such pre-adapted genotypes were introduced into new habitats, and in their new environment they could settle and spread, as they were not controlled by grazers. Such a selective introduction of specific pre-adapted genotypes was also assumed for the South African ragwort *Senecio inaequidens* (Bossdorf et al. 2008).

CONCLUSIONS

Both *Littorina brevicula* and *L. littorea* consumed significantly higher amounts of native than of invasive *Gracilaria vermiculophylla*. The low palatability to *L. littorea* could thus be one reason for the invasion success of the seaweed. Furthermore, the results of our study show that it can be of great importance to compare more than only one native and one non-native population of a species to understand the mechanisms underlying an invasion process. *G. vermiculophylla* populations originating from different ecoprovinces exhibit a high variability, especially within the native populations. A simple comparison of populations from the Baltic Sea and Qingdao might have led to the assumption that native populations are in general more palatable than non-native populations. However, consumption rates of *G. vermiculophylla* from some Korean populations were just as low as those of invasive populations. Therefore, we conclude that the invasion success of this species might have been facilitated by the introduction of herbivory-resistant genotypes.

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Paper III

Metabolomic profiling reveals a higher potential for wound activated chemical defense in non-native than in native populations of the seaweed *Gracilaria vermiculophylla*

Mareike Hammann^{1*}, Martin Rempt², Georg Pohnert², Gaoe Wang³, Sung Min Boo⁴, Florian Weinberger¹

(1) Helmholtz Centre for Ocean Research (GEOMAR), Düsternbrooker Weg 20, 24105 Kiel, Germany

(2) Institute for Inorganic and Analytical Chemistry, Instrumental Analytics/Bioorganic Analytics, Friedrich Schiller University, Lessingstraße 8 D-07743 Jena, Germany

(3) College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, 266003 Qingdao, China

(4) Department of Biology, Chungnam National University, Daejeon 305-764, Korea

(*) Corresponding author, email: mhammann@geomar.de

Abstract

The capacity for wound activated production of oxylipins was compared among 118 specimens of the red macroalga *Gracilaria vermiculophylla* that originated from five native and seven non-native populations. Despite their previously demonstrated lower genetic diversity non-native populations exhibited a higher variability of metabolomic profiles than native populations. Further, profiles of non-native populations from Europe and the Mexican Pacific coast were more similar among each other than to any native populations from Asia. This difference was in large part due to an increased presence of arachidonic acid derived oxylipins, including prostaglandin E₂ and prostaglandin A₂, 15-keto prostaglandin E₂ and hydroxylated and dihydroxylated eicosatetraenoic acids, after wounding. Several of these compounds deter herbivores or are regulators of induced defenses. Two of these compounds, prostaglandin A₂ and dihydroxylated eicosatetraenoic acid, had a deterring effect on snails of the genus *Littorina* when tested in bioassays. Also oxylipin extracts of *G. vermiculophylla* deterred these snails, and the deterring effect was higher for extracts from non-native populations of the seaweed. Non-native populations of *G. vermiculophylla* appear thus as better adapted to challenge by herbivores and other biological enemies that cause local tissue or cell disruption.

KEY WORDS: Fatty acid · *Gracilaria vermiculophylla* · Metabolomics · Oxylipin · Prostaglandin · Seaweed invasion

Introduction

Ecological hypotheses predict that natural enemies such as herbivores can influence the dynamics of biological invasions (Elton 1958, Maron & Vilà 2001, Keane & Crawley 2002). It is also assumed that strongly defended organisms will be more successful invaders when the pressure of biological enemies in a new environment is high (Callaway & Ridenour 2004, Forslund et al. 2010) and that genetic adaptation of defense-related traits may result from differences in feeding pressure among native and non-native environments (Blossey & Nötzold 1995, Callaway & Ridenour 2004). A large body of experimental work has examined the validity of these concepts for terrestrial plants (Levine et al. 2004), while marine organisms remain nearly unexplored. Significantly more herbivores are generalists in the sea than on land (Hay & Steinberg 1992). This probably affects the defense ecology of seaweeds, reduces the probability of enemy release during macroalgal invasions (Forslund et al. 2010), and might favour selection for defensive traits (Callaway & Ridenour 2004). We therefore examined whether defense-related traits of native and non-native populations of the red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss are divergent.

During the last two decades, *G. vermiculophylla* has spread from its native distribution range in East Asia to the North American West and East coasts, as well as to Europe, and this process resulted in considerable loss of genetic diversity in non-native populations (Kim et al. 2010). The species continues to invade new environments (Weinberger et al. 2008, Saunders 2009, Sfriso et al. 2010) and has been included into a list of the most invasive seaweeds in Europe (Nyberg 2007). *G. vermiculophylla* originating from invasive populations often appears as relatively insensitive toward herbivory, as compared to native species from the same environment (Weinberger et al. 2008, Nejrup & Pedersen 2010).

This was confirmed in common-garden type experiments that directly compared the palatability of *G. vermiculophylla* specimens originating from native and non-native populations toward two herbivorous periwinkles, *Littorina brevicula* Philippi from the native range of *G. vermiculophylla* in East Asia (Son & Hong 1998, Kim et al. 2003), and *L. littorea* L., a common grazer in most of the regions invaded by the seaweed in the North Atlantic realm (Hammann et al. 2013).

The resistance of *G. vermiculophylla* to mesograzers results at least in part from a high content in oxylipins, which are predominantly produced after mechanical wounding (Nylund et al. 2011, Rempt et al. 2012). Arachidonic acid derived hydroxyeicosatetraenoic acids (HETE), as well as prostaglandins (PG), are most prominent among the wound activated metabolites (Fig. 1) and have repeatedly been shown to deter feeding enemies of *G. vermiculophylla*, such as the isopod *Idotea balthica* (PGA₂ (Nylund et al. 2011)) or the

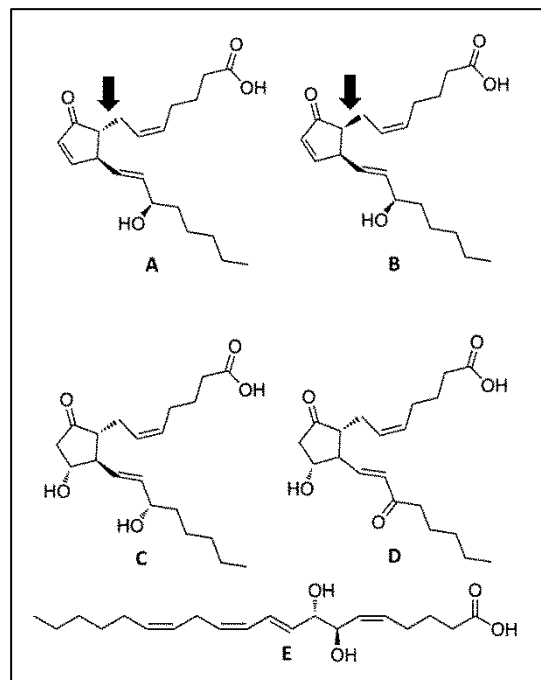


Fig. 1: Chemical structures. Structures of the metabolites used for bioassays with *L. brevicula* and *L. littorea*. (A) PGA₂, (B) 8-iso-PGA₂, (C) PGE₂, (D) 15-keto-PGE₂, and (E) 7,8-di-HETE. The difference between PGA₂ and 8-iso-PGA₂ is in the steric conformation only (arrows).

herbivorous sea snail *Echinolittorina peruviana* (7,8-di-HETE (Rempt et al. 2012)). Also, cases of lethal intoxication of humans with PGE₂ after consumption of *G. vermiculophylla* have been recorded in the past (Noguchi et al. 1994). Wound activated oxylipins thus target a relatively wide array of potential enemies and are also molecular messengers that mediate induced defenses of gracilarioids (Weinberger et al. 2011).

We therefore monitored the presence and concentration of such compounds among 12 different populations of *G. vermiculophylla*, using a metabolomic profiling approach. Furthermore, extracts of *G. vermiculophylla* containing oxylipins were tested for their bioactivity towards *L. littorea* and *L. brevicula*. Additionally, selected arachidonic acid derived metabolites that proved to differ in concentration between native and invasive populations of *G. vermiculophylla* were tested for their reactivity towards the snails as well.

Methods

Between the 10th of February and the 22nd of April 2010 five native populations were sampled along the Korean peninsula and in Shandong Province, China, and seven non-native populations were sampled around the Danish-German peninsula of Jutland and Schleswig-Holstein, in the west of France and on the Pacific coast of Mexico (Table 1). The identity of all studied populations with *Gracilaria vermiculophylla* (Ohmi) Papenfuss was confirmed by DNA barcoding, either by others or by us, using the methodology described in Kim et al. (2010) (Table 1). All specimens were transferred alive in cooling containers to Kiel/Germany, with the exception of those from Qingdao and Jindo, which were transferred to Qingdao/China. The algae had to be cultivated in the laboratory prior to wound activation, in order to allow for regeneration from transportation stress and because it was impossible to sample and wound activate all specimens on the same day. At Kiel they were cultivated in separate indoor tanks at identical conditions (water temperature 15 to 20°C, PAR 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h d⁻¹, aeration). The medium (salinity: 30 PSU) was Baltic Sea water from the Kiel Fjord with addition of 13 g l⁻¹ Instant Ocean sea salt. It was constantly exchanged between all tanks and a central storage container, in order to maintain an identical medium composition in all tanks. The medium was UV sterilized at the exit of the central container and prior to redistribution into the tanks, in order to prevent exchange of algal propagules among tanks containing material of different origin. At Qingdao cultivation of all specimens was conducted in separate Erlenmeyer flasks at identical conditions (water temperature 20°, indirect sunlight, without aeration). The medium (Yellow Sea water) was daily exchanged.

Wound activation of all specimens was conducted between the 6th and the 27th of April 2010. For wound activation 0.5 g of each algal specimen was mortared and transferred into a 15 ml Falcon tube. After an incubation period of 30 min the material was shock frozen in liquid nitrogen and lyophilized. For extraction of oxylipins the dry biomass was grinded and 10 mL of Methanol (MeOH) were added. After an incubation period of 45 min on a shaker extract and residue were separated by filtration. MeOH was evaporated *in vacuo* and the extract was stored at -20°C. Prior to ultra-performance liquid chromatography (UPLC) with electrospray ionization mass spectrometric (ESI-MS) detection the extract was redissolved in 200 μl MeOH which was then directly transferred to inserts of 1.5 mL sample vials.

An Acquity UPLC (Waters, Eschborn/Germany) equipped with a 50-mm Acquity UPLC BEH C18 column (2.1mm, 1.7 μm) at a column temperature of 30°C was used for separation of the metabolites. The injection volume was 1 μL . The LC mobile phases were A = 0.1% formic acid and 2% acetonitrile in water and B = 0.1% formic acid in acetonitrile (all UPLC/MS grade; Biosolve, Valkenswaard/The Netherlands). The LC gradient with a flow rate of 0.6 mL min^{-1} was the following: 0 % B at 0 min; 50 % B at 0.5 min; 100 % B at 5 min; 100 % B at 5.5 min; 0% B at 6 min. Post column, a diode array detector (DAD) was installed and the outlet of the DAD was coupled to a Q-TOF micro-MS (Waters) operated with an ESI source in negative mode with a scan rate of one scan per second, an interscan delay of 0.1 s, and a scan range from 100 to 1000 Da. The ESI parameters were the following: collision energy 5 V, capillary voltage 2700 V, sample cone 35 V, cone gas 35 $\text{L N}_2 \text{h}^{-1}$, and desolvation gas 700 $\text{L N}_2 \text{h}^{-1}$.

Raw MS data were used to automatically generate a list of intensities of mass-retention time pairs (markers) using the normalized and mean centered peak area, calculated with Apex Track peak detection (implemented in the MarkerLynx V4.1 software supplied by Waters). For data collection, retention time windows of 0.2 min and m/z windows of 1 Da were allowed as tolerance, and peaks within this window were considered to be identical. The so prepared markers were used for further multivariate statistics analysis. Ion intensities of distinct m/z mass peaks occurring at distinct retention times were integrated and listed for all samples.

Table 1 Geographic location, taxonomic reference and sample numbers analyzed of native and non-native populations of *G. vermiculophylla*, as well as within-population dispersion of metabolomic profiles. Taxonomic reference papers confirm the identity of studied populations with *G. vermiculophylla* based on DNA barcoding. Within-population multivariate dispersion is expressed as the mean Bray-Curtis distance \pm SE. Distances marked by different letters were significantly different in a permutational test for homogeneity of multivariate dispersions ($p < 0.05$).

Population	Geographic coordinates	Taxonomic reference	Samples	Multivariate dispersion
Native				
Donghae (East Sea/KR)	37°29'27.65"N, 129°08'30.42"E	Hammann et al. 2013	10	34,0 \pm 1,0 ^{CEFG}
Odo (East Sea/KR)	36°09'52.40"N, 129°24'00.07"E	Hammann et al. 2013	10	36,1 \pm 2,7 ^{ABCDEFGH}
Jindo (Yellow Sea/KR)	34°25'16.98"N, 126°20'48.21"E	Kim et al. 2010	10	38,8 \pm 2,8 ^{ABCDEF}
Gyeokpo (Yellow Sea/KR)	35°37'05.54"N, 126°28'01.70"E	Kim et al. 2010	10	32,4 \pm 1,2 ^{FGH}
Qingdao (Yellow Sea/CN)	36°03'18.51"N, 120°21'57.01"E	Kim et al. 2010	9	30,0 \pm 1,2 ^{GH}
Mean of native populations			5	34,3\pm3,0
Non-native				
Heiligenhafen (Baltic Sea/D)	54°22'48.14"N, 10°58'58.94"E	Hammann et al. 2013	9	38,2 \pm 1,9 ^{ABCDEF}
Nyborg (Baltic Sea/DK)	55°17'54.23"N, 10°49'07.35"E	Hammann et al. 2013	10	39,6 \pm 1,1 ^{ABC}
Nibe (Limfjord/DK)	56°59'03.34"N, 9°37'48.89"E	Hammann et al. 2013	10	37,0 \pm 0,8 ^{BCDE}
Mandö (Wadden Sea/DK)	55°18'35.59"N, 8°38'34.74"E	Hammann et al. 2013	10	41,3 \pm 1,1 ^{ABC}
Nordstrand (Wadden Sea/D)	54°31'48.01"N, 8°52'18.88"E	Hammann et al. 2013	10	36,4 \pm 1,2 ^{BCDEF}
St.-Pol-de-Leon (Brittany/F)	48°40'41.09"N, 3°58'08.04"W	Kim et al. 2010	10	39,2 \pm 1,8 ^{ABCDE}
Ensenada (Baja California/MX)	31°46'28.67"N, 116°36'40.92"W	Hammann et al. 2013	10	39,5 \pm 1,6 ^{ABCDE}
Mean of non-native populations			7	38,8\pm1,5

Each algal specimen was wound activated, extracted and analyzed in duplicate. Duplicate samples were randomized and blank runs were regularly performed in order to exclude potential contamination. Mass peaks corresponding to arachidonic acid, 8-HETE, 7,8-di-HETE, PGE₂, 15-keto-PGE₂ or PGA₂ were identified by comparison with mass spectra and retention times of standard compounds (Nylund et al. 2011, Rempt et al. 2012) and quality control samples were regularly run with PGE₂, 15-keto PGE₂, PGA₂ and 7,8-di-HETE to verify for stability of the analytic procedure. Further mass peaks representing arachidonic acid derivatives were identified by comparison with their increased degree of stable isotope labeling after incubation with 2,2-[²H₂]-arachidonic acid (Rempt & Pohnert 2010). Mass peak areas representing different fragments of identical sample components were cumulated prior to data analysis. Mass peaks occurring in less than 5 % of all analyzed samples were eliminated. The mass peak areas obtained for both duplicates were averaged, in order to reduce within-sample variability. Data of 236 LC/MS runs from the duplicate samples were thus combined to 118 data sets from the different sites (as indicated in Table 1). The resulting data matrix of ion intensities of 933 mass retention time pairs in 118 samples was range-transformed and analyzed with the Primer6&PERMANOVA+ software package (PRIMER-E Ltd., Plymouth, U.K.), using the Bray-Curtis distance measure.

Bioassays with *L. brevicula* were conducted in Qingdao, China, and bioassays with *L. littorea* were carried out in Kiel, Germany. *L. brevicula* individuals were collected three days before the deterrence experiment started on the Yellow Sea coast in Qingdao (36°03'18.51"N, 120°21'57.01"E) at a site where *G. vermiculophylla* was present. Before and during the experimental period they were kept in the laboratory in an aquarium containing 5 l of Yellow Sea water, which was exchanged twice every day. They were allowed to graze on green algae (*Ulva* spec.) and were only taken out of their aquarium to conduct the bioassays. Snails between 7 and 9 mm in diameter were most common in the habitat at that time of the year and were therefore used for experiments. *L. littorea* individuals were collected on the shore of the Kiel Fjord (N54°21.965', E10°8.908') at a site where *G. vermiculophylla* was present. They were kept in a flow-through aquarium containing 5 l of Baltic Sea water until the bioassays were finished. During this period they were allowed to graze on green algae (*Ulva* spec.). The snails were only removed from their aquarium to carry out the bioassays, for which snails with a diameter between 8 and 12 mm were used.

For the bioassays, *G. vermiculophylla* extracts (200 µL) were pipetted into a glass Petri dish and covered with hot seawater (5 mL, ca. 60°C) containing 2% agar. By gentle shaking, extracts were distributed homogenously in the water-agar-mixture. After cooling, this resulted in a layer of jellified seawater that contained the extract at approximate natural concentrations. Ten replicates for each *G. vermiculophylla* population were prepared. Solvent controls were made accordingly way with pure methanol (200 µL). To test for deterrence, either 15 individuals of *L. brevicula* or 15 individuals of *L. littorea* were placed upon agar containing algal extracts. After 7 min the Petri dishes were turned upside down, and the individuals that remained attached to the agar and the individuals that fell down were counted (Rempt et al. 2012). For each of the snail species bioassays were run on four subsequent days until *G. vermiculophylla* extracts from all populations had been tested. One and the same snail individual was never employed in the bioassays twice on the same day. Every day, 10 to 30 solvent controls were run in the same manner. Numbers of either *L. brevicula* or *L. littorea* attached or unattached to the solvent

controls were summed up for all tests. The approximate deterrence of snails by the agar containing extract relative to agar containing no extract was calculated as odds ratio (Fisher & Van Belle 1993):

$$\text{Relative deterrence} = \frac{\frac{(\text{Sum of snails on extract,unattached})+0.5}{(\text{Sum of snails on extract,attached})+0.5}}{\frac{(\text{Sum of snails on solvent control,unattached})+0.5}{(\text{Sum of snails on solvent control,attached})+0.5}}$$

A relative deterrence of 1 indicates that the same number of snails attached to the agar in the treatment compared to the solvent control, meaning that there is neither a preference for the treatment nor for the control. At values <1, snails were attracted by *G. vermiculophylla* extract, while values >1 indicate that the snails were deterred by this extract. For statistical analysis, data on relative deterrence were transformed to logarithm. Within single treatments the relative deterrence was tested for significance using the Mantel-Haenszel-procedure: If the 95 % confidence interval around the geometric mean of a given odds ratio excludes 1 then this odds ratio must be regarded as significant ($p < 0.05$, Mantel-Haenszel-test) (Fisher & Van Belle 1993).

Differences in deterrence of either *L. brevicula* or *L. littorea* by *G. vermiculophylla* extracts were analyzed by nested-design ANOVA, with the factor ‘population’ nested within the factor ‘origin’ (native or invasive). Tukey’s post hoc tests were conducted to detect differences among single populations. A full factorial ANOVA with the factors ‘species’ and ‘concentration’ was conducted to find differences between the snail species when exposed to two concentrations of pure compounds. Tukey’s post hoc tests were run to discover interactive effects of the two factors. Statistical tests were conducted using the STATISTICA 8 software package (Stat-Soft).

To test the oxylipins PGA_2 , 8-iso- PGA_2 , PGE_2 , 15-keto- PGE_2 , and 7,8-di-HETE (Fig. 1) pure compounds were dissolved in methanol (for PGA_2 , 8-iso- PGA_2 , PGE_2 , 15-keto- PGE_2) or acetonitrile (for 7,8-di-HETE) and incorporated from these stock solutions into the agar as described above for extracts. Concentrations of $1 \mu\text{g g}^{-1}$ and $10 \mu\text{g g}^{-1}$ agar gel were used, corresponding with the approximate range of natural concentrations (Nylund et al. 2011, Rempt et al. 2012). A mix of these compounds in the ratio 1:1:1:1:0.5 – corresponding with the approximate natural concentration ratio – was also tested in the approximate range of natural concentrations ($4.5 \mu\text{g}$ and $45 \mu\text{g g}^{-1}$ agar gel). Solvent controls were run with corresponding concentrations of methanol or acetonitrile. Data for pure compounds were treated and analyzed as described for *G. vermiculophylla* extracts.

Results and discussion

Permutational analysis of multivariate dispersion (PERMDISP, (Anderson 2006)) detected significant differences among populations ($F = 3.93$, $p < 0.002$, Table 1), and the mean multivariate dispersion was significantly lower within native than within non-native populations ($t = 2.763$, $p = 0.05$). Thus, the relatively low genetic diversity within non-native populations of *G. vermiculophylla* (Kim et al. 2010) contrasts with relatively high metabolomic diversity.

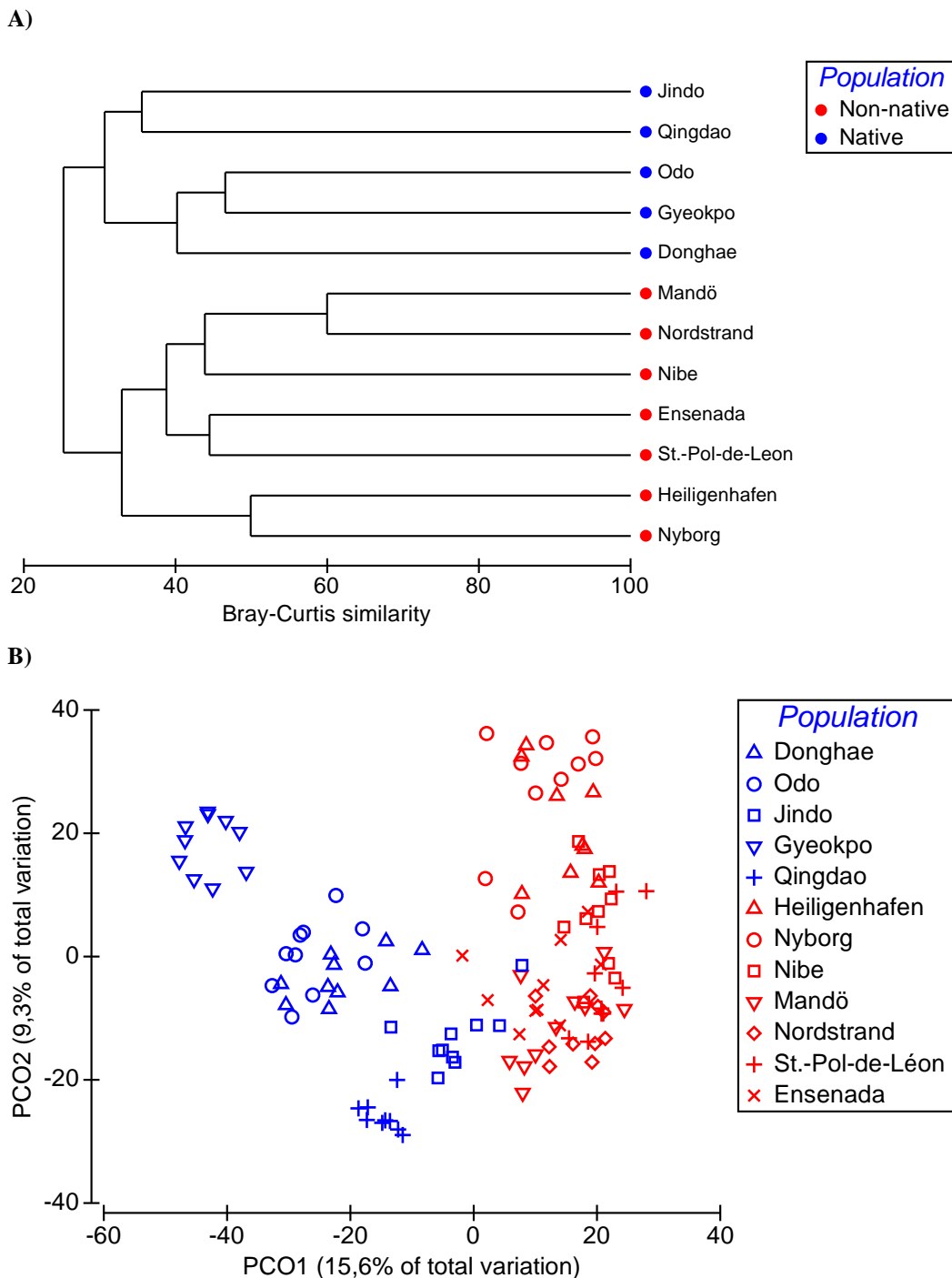


Fig. 2 Variability of the metabolic profiles of native and non-native populations of *G. vermiculophylla*, derived from the Bray-Curtis similarities of range-transformed mean ion intensity peak areas of 933 components. (A) Hierarchical agglomerative cluster plot (group average) of mean metabolic profiles. (B) Distribution of profiles of 118 specimens originating from native (blue) and non-native (red) populations among the first two principal axes of covariance, which together explain 23.9 % of the total variation among all samples.

A nested permutational multivariate analysis of variance (PERMANOVA, (Anderson 2001)) detected significant differences between native and non-native populations (Pseudo-F = 27.78, $p < 0.0001$), as well as between all non-native ($t \geq 1.4999$, $p \leq 0.0042$) and between all native ($t \geq 2.5653$, $p \leq 0.0001$) populations. Native and non-native populations formed two distinct groups exhibiting relatively low similarity of metabolomic profiles (25.2 %, Fig. 2a). A relatively high similarity of 60.0 % was detected among the two non-native populations of Nordstrand and

Mandö, corresponding with their relatively close geographical distance. However, geographic distance alone was not sufficient to explain the full pattern of similarities. For example, the three Wadden Sea and Limfjord populations were more similar to the Mexican population of Ensenada (more than 9000 km of air distance) than to the two Baltic Sea populations (less than 350 km of air distance) or to any of the native populations (less than 8000 km of air distance, Fig. 2A). Principal coordinates analysis confirmed that important differences among native and non-native populations exist, since an axis mainly separating among the metabolic profiles of non-native and native specimens of *G. vermiculophylla* was detected as the first principal coordinate (Fig. 2B).

Pairwise comparison of mass peak areas (Mann-Whitney-U-test, $n_{\text{native}}=5$, $n_{\text{non-native}}=7$, $p < 0.05$) detected 57 components that were significantly more concentrated in non-native than in native populations. Of them, 19 (33.3 %) were derived from arachidonic acid. In contrast, none of the 65 components that were significantly more concentrated in native populations was an eicosatetraenoid. PGA_2 , PGE_2 , 15-keto- PGE_2 , 7,8-di-HETE and 8-HETE were among the components that were most significantly more concentrated in non-native specimens ($p = 0.02$), as well as two further unidentified arachidonic acid derived metabolites with m/z 335 and m/z 388 (Fig. 3). These seven components were also among the 25 that were identified through canonical analysis of principal coordinates (Anderson & Willis 2003) as the most relevant for separation of native and non-native populations. Of 33 different components derived from arachidonic acid that were detected in our study, 19 were significantly and 13 were non-significantly more concentrated in non-native than in native populations of *G. vermiculophylla* (Wann-Whitney-U-test, $p < 0.05$). This pattern was usually also detected when single populations were compared (Fig. 4), although exceptions occurred occasionally (e.g., 7,8-di-HETE in samples from Ensenada and from native populations was similarly concentrated (Fig. 4E) and 8-HETE did not differ significantly between specimens from Qingdao and those from non-native populations (Fig. 4H)). Interestingly, arachidonic acid derivatives differed much more among native and non-native populations than arachidonic acid itself (Fig. 3). This could suggest that it is less the availability of arachidonic acid and more the activity or expression of enzymes required for its transformation (e.g., lipoxygenases or cyclooxygenases) that differs.

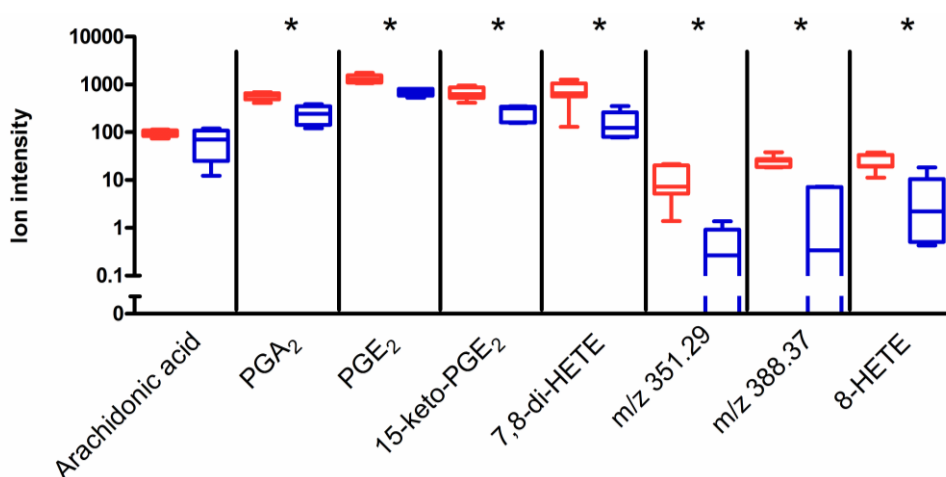


Fig. 3 Ion intensities (median \pm quartiles of population means) of arachidonic acid and seven derivatives in specimens of *G. vermiculophylla* originating from non-native (red, $n = 7$) and native (blue, $n = 5$) populations. Asterisks indicate components that differed significantly among native and non-native populations (Mann-Whitney-U-test, $p < 0.05$).

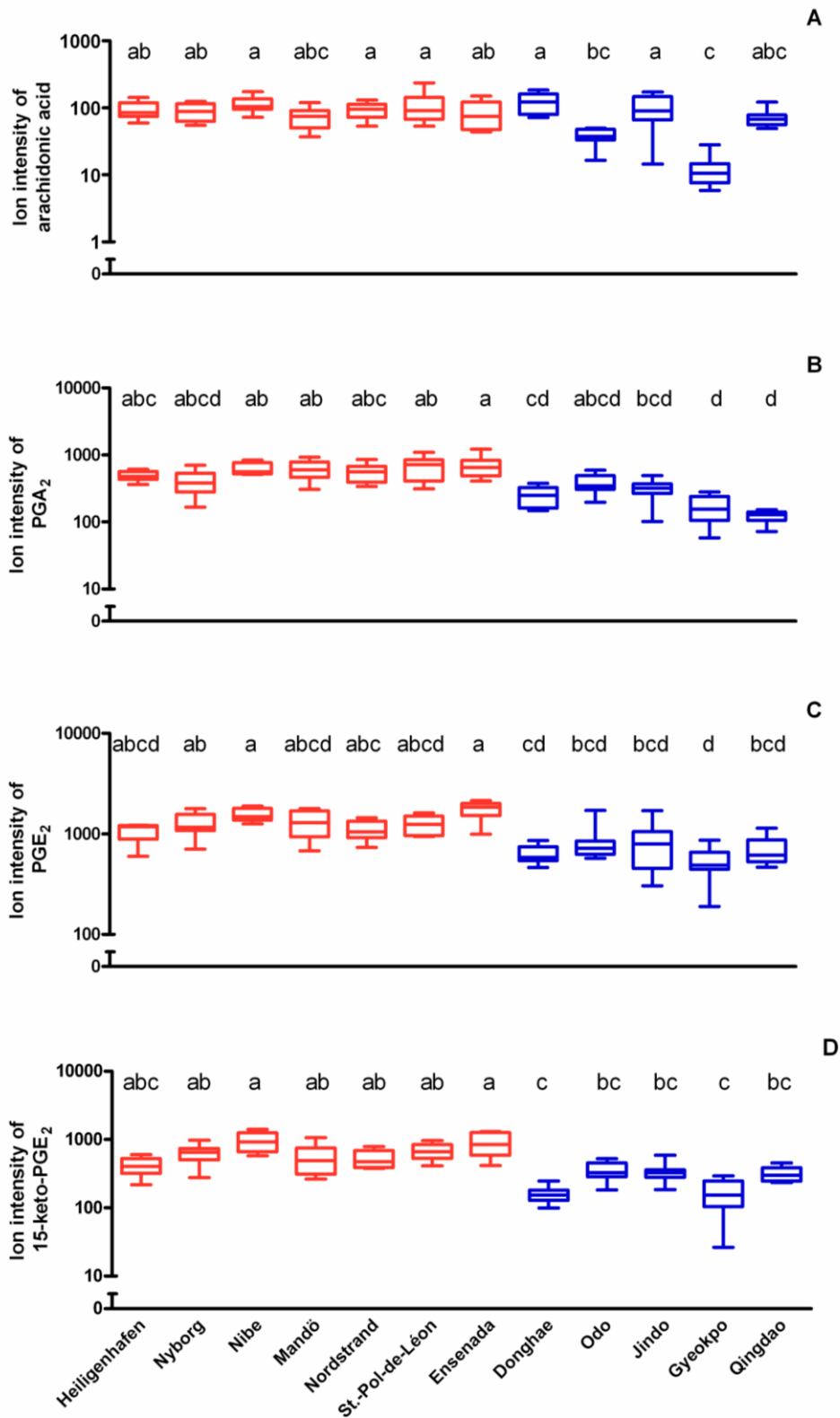


Fig. 4: Ion intensities (median \pm quartiles) of A) arachidonic acid, B) PGA_2 , C) PGE_2 , D) 15-keto- PGE_2 , E) 7,8-di-HETE, F) a component derived from arachidonic acid with m/z 355.29, G) a component derived from arachidonic acid with m/z 388.37, H) 8-HETE in specimens of *G. vermiculophylla* originating from seven non-native (red) and five native (blue) populations.

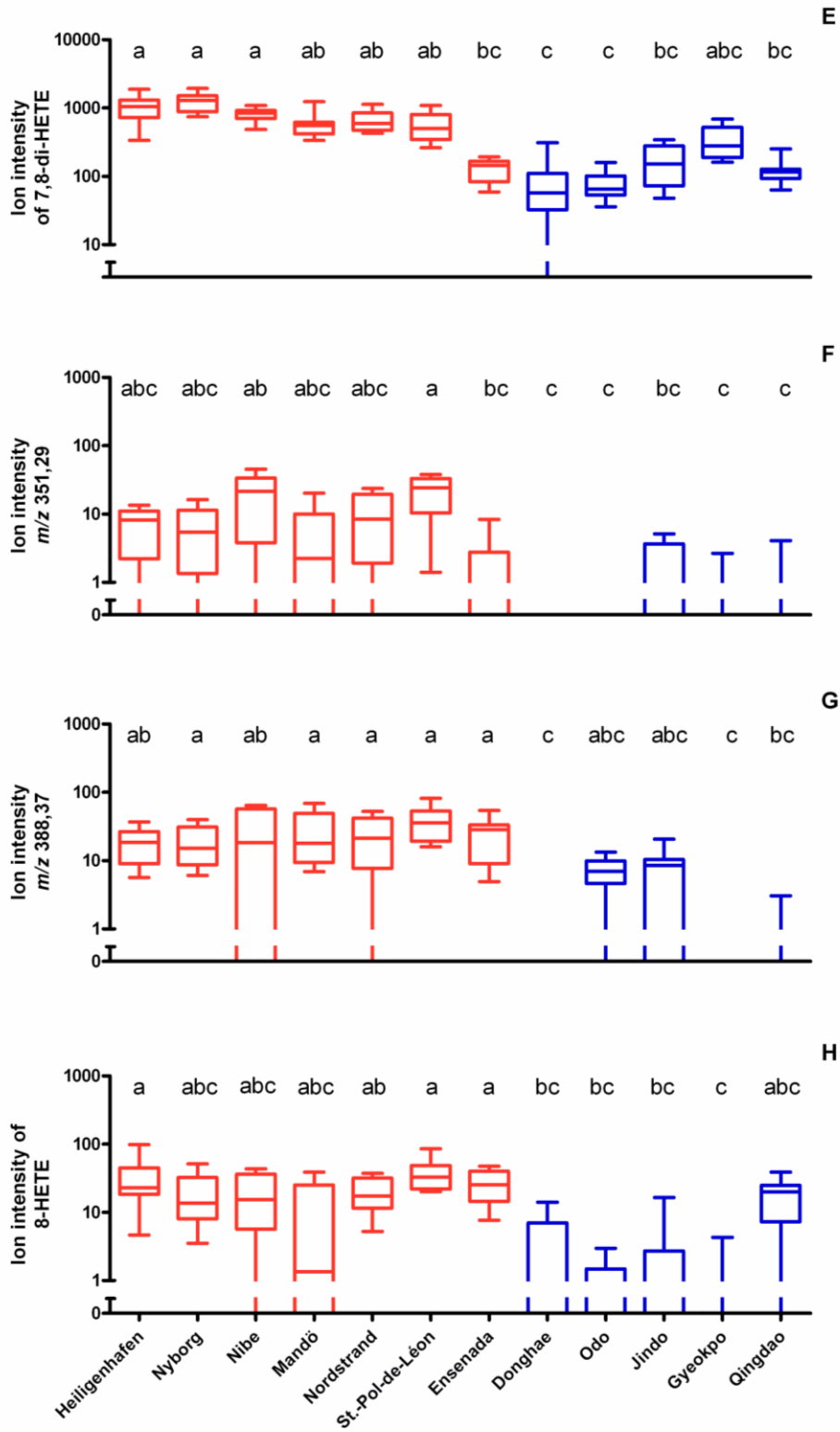


Fig. 4 continued

Table 2: *Gracilaria vermiculophylla* extracts. Influence of the origin of *G. vermiculophylla* populations on the deterrence of *L. brevicula* (A) and *L. littorea* (B) in bioassays, using a nested-design ANOVA with the factor ‘population’ nested within the factor ‘origin’ (either native or invasive).

A)	SS	DF	MS	F	p
Population (origin)	4.30	10	0.43	3.76	<0.001
Origin	3.13	1	3.13	27.35	<0.001
Error	12.24	107	0.11		

B)	SS	DF	MS	F	p
Population (origin)	6.02	10	0.60	4.21	<0.001
Origin	2.28	1	2.28	15.92	<0.001
Error	15.30	107	0.14		

Both *L. brevicula* and *L. littorea* were deterred by extracts of *G. vermiculophylla*, but *L. littorea* was generally less deterred than *L. brevicula*. For both snail species, the deterring effect of extract from non-native *G. vermiculophylla* specimens was higher than the deterrence by native populations’ extracts ($p < 0.001$, see Table 2 and Fig. 5). Variability between populations was relatively low, but significant differences were nonetheless found ($p < 0.001$, see Table 2 and Fig. 6). *L. brevicula* showed a similar distaste for extracts from all native populations, but was more deterred by *G. vermiculophylla* extract originating from the non-native population in Heiligenhafen (German Baltic Sea) than by extracts originating from Nibe, Mandö (both from Denmark), St.-Pol-de-Léon (France) and Ensenada (Mexico) (see Fig. 6A). *L. littorea* on the other hand did not discriminate between non-native populations, but was more deterred by extracts from Odo (South Korea) than by all other native populations (see Fig. 6B).

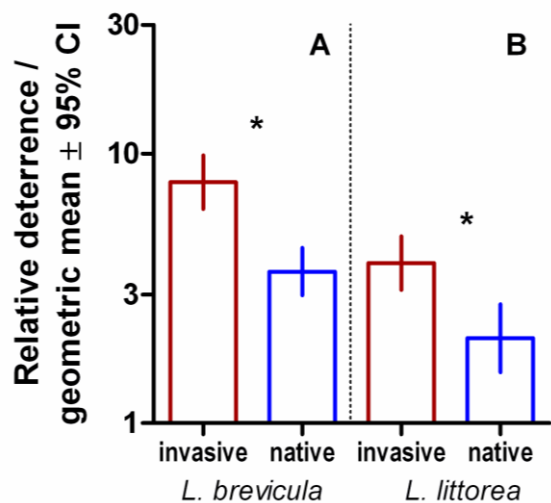


Fig. 5: *Gracilaria vermiculophylla* extracts. Average deterrence of (A) *L. brevicula* and (B) *L. littorea* by extracts of *G. vermiculophylla* from and invasive (red) and native (blue) populations. *Native and invasive populations significantly different in a nested 2-way ANOVA (see factor ‘origin’ in Table 2; $p < 0.001$). $n = 10$.

Four metabolites that are particularly prominent in *G. vermiculophylla* (PGA₂, PGE₂, 15-keto-PGE₂, and 7,8-di-HETE, Fig. 3) were chosen to investigate the bioactivity of arachidonic acid derived compounds towards snails. For the chemical structures of these metabolites see Fig. 1. A mix of these compounds was tested as well to investigate if effects of the single metabolites interact with each other. Overall, the two snail species reacted differently to the pure metabolites and to different concentrations of those compounds. For example, *L. brevicula* was only deterred by the higher concentration (10 $\mu\text{g g}^{-1}$) of PGA₂ and did not react to the lower concentration (1 $\mu\text{g g}^{-1}$), while *L. littorea* was already deterred when this substance was applied at 1 $\mu\text{g g}^{-1}$ ($p < 0.05$, see Table 3A and Fig. 7A). Because PGA₂ exhibited a clear deterrent effect toward both snails we also tested their reactions towards 8-iso-PGA₂, a stereoisomer of PGA₂ (Fig. 1).

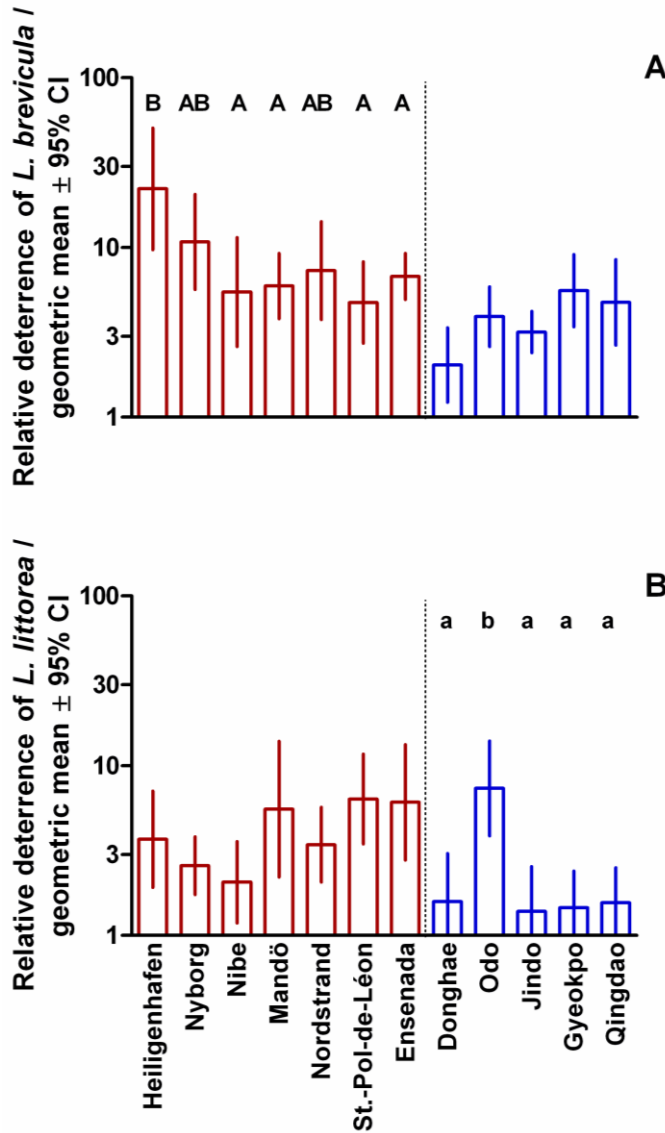


Fig. 6: *Gracilaria vermiculophylla* extracts. Average deterrence of (A) *L. brevicula* and (B) *L. littorea* by extracts of *G. vermiculophylla* from different populations. Invasive populations are presented as red bars, native populations as blue bars.

Different letters indicate populations that were significantly different in a Tukey test ($p < 0.05$) either within the native or within the non-native region. $n = 10$

The goal was to find out if isomery influences the deterring effect of those compounds. Interestingly, 8-iso-PGA₂ exhibited much less deterrence than PGA₂ (Fig. 7). *L. littorea* showed no significant reaction to 8-iso-PGA₂, while *L. brevicula* was only significantly deterred by it at the lower concentration that was tested (Fig. 7B). However, two-way-ANOVA detected a significant difference in the responses of both species ($p < 0.05$, Table 3B) and a significant deterrence of *L. brevicula* was detected, when the data obtained at both concentrations were analyzed together (Mantel-Haenszel-test, $p < 0.05$). Both species also responded differently to PGE₂, irrespective of the concentration ($p < 0.001$, Table 3C). The Mantel-

Haenszel-test did not detect any significant effects when single concentrations were tested (Fig. 7C), but a significant attracting effect of PGE₂ upon *L. littorea* was detected when data obtained at both tested concentrations were analyzed together ($p < 0.05$). In contrast, *L. brevicula* did not exhibit a significant response to PGE₂. The snails' reactions towards 15-keto-PGE₂ were dependent on the concentration ($p < 0.05$, Table 3D and Fig. 7D). Lower concentrations were for both snails in tendency more deterrent than higher concentrations, and *L. littorea* was in tendency even attracted and not deterred at the higher concentration. The snails' reactions towards this substance were, however, relatively weak. The last substance that was tested, 7,8-di-HETE, had a significant deterrent effect towards both snail species at the lower concentration and a non-significant deterrent effect at the higher concentrations (Fig 7E). Two-way-ANOVA did not detect any differences between the two snail species or the tested concentrations (Table 3E) and both snails were significantly deterred when data obtained at both tested concentrations were analyzed together ($p < 0.05$).

In conclusion, *L. brevicula* was deterred by PGA_2 (only at high concentrations), by 7,8-di-HETE and by 8-iso- PGA_2 , while *L. littorea* was only deterred by PGA_2 (also at low concentrations) and by 7,8-di-HETE. Both snails thus respond negatively to the same substances that also deter other feeding enemies of *G. vermiculophylla* (Nylund et al. 2011, Rempt et al. 2012). However, *L. littorea* was attracted by PGE_2 and in tendency also by 15-keto- PGE_2 , indicating that responses to oxylipins may be highly species specific. This might explain the different reactions of the two snail species when they were exposed to a mix of all five compounds: *L. brevicula* was at both concentrations clearly deterred by the mix, while *L. littorea* was not affected (see Table 3F and Fig. 7F). Apparently the deterring effects of PGA_2 , 8-iso- PGA_2 , and 7,8-di-HETE upon *L. brevicula* summed up, while the attracting effect of PGE_2 neutralized the deterring effect of PGA_2 and 7,8-di-HETE in the case of *L. littorea*.

Table 3: Pure compounds. Influence of concentration of the compound and species identity on deterrence of *L. brevicula* or *L. littorea* by five different oxylipins (A-E) and a mix of these metabolites (F), using a full factorial ANOVA.

A) PGA_2	SS	DF	MS	F	p
Concentration	1.31	1	1.31	10.26	<0.01
Species	2.10	1	2.10	16.47	<0.001
Concentration x Species	0.88	1	0.88	6.93	<0.05
Error	2.55	20	0.13		
B) 8-iso-PGA_2					
Concentration	0.02	1	0.02	0.28	0.60
Species	0.31	1	0.31	5.04	<0.05
Concentration x Species	0.01	1	0.01	0.09	0.77
Error	1.23	20	0.06		
C) PGE_2					
Concentration	0.06	1	0.06	0.40	0.54
Species	1.56	1	1.56	9.94	<0.01
Concentration x Species	0.03	1	0.03	0.18	0.67
Error	3.15	20	0.16		
D) 15-keto-PGE_2					
Concentration	0.71	1	0.71	5.62	<0.05
Species	0.16	1	0.16	1.26	0.27
Concentration x Species	0.42	1	0.42	3.34	0.08
Error	2.52	20	0.13		
E) 7,8-di-HETE					
Concentration	0.29	1	0.29	2.54	0.12
Species	0.002	1	0.01	0.01	0.89
Concentration x Species	0.01	1	0.01	0.07	0.78
Error	3.62	32	0.11		
F) Mix					
Concentration	0.001	1	0.001	0.01	0.93
Species	2.10	1	2.10	14.95	<0.001
Concentration x Species	0.16	1	0.16	1.11	0.30
Error	2.81	20	0.14		

PGA₂ and 7,8-di-HETE belong to the metabolites that were higher concentrated in non-native specimens of *G. vermiculophylla* (see Fig. 2). These compounds therefore possibly contributed to the more pronounced deterrent effect of extracts of *G. vermiculophylla* from non-native populations (Fig. 5). Also, deterrence of *L. littorea* was generally lower than deterrence of *L. brevicula*, which may be due to the attracting effect of PGE₂ toward *L. brevicula*. However, we cannot exclude that other oxylipin or non-oxylipin metabolites also added to the overall deterrence of *G. vermiculophylla* extracts, as we did not test all those compounds. Moreover, further substances that were not extracted by the chosen method could in theory also affect the overall deterrence of *G. vermiculophylla* toward *Littorina* species.

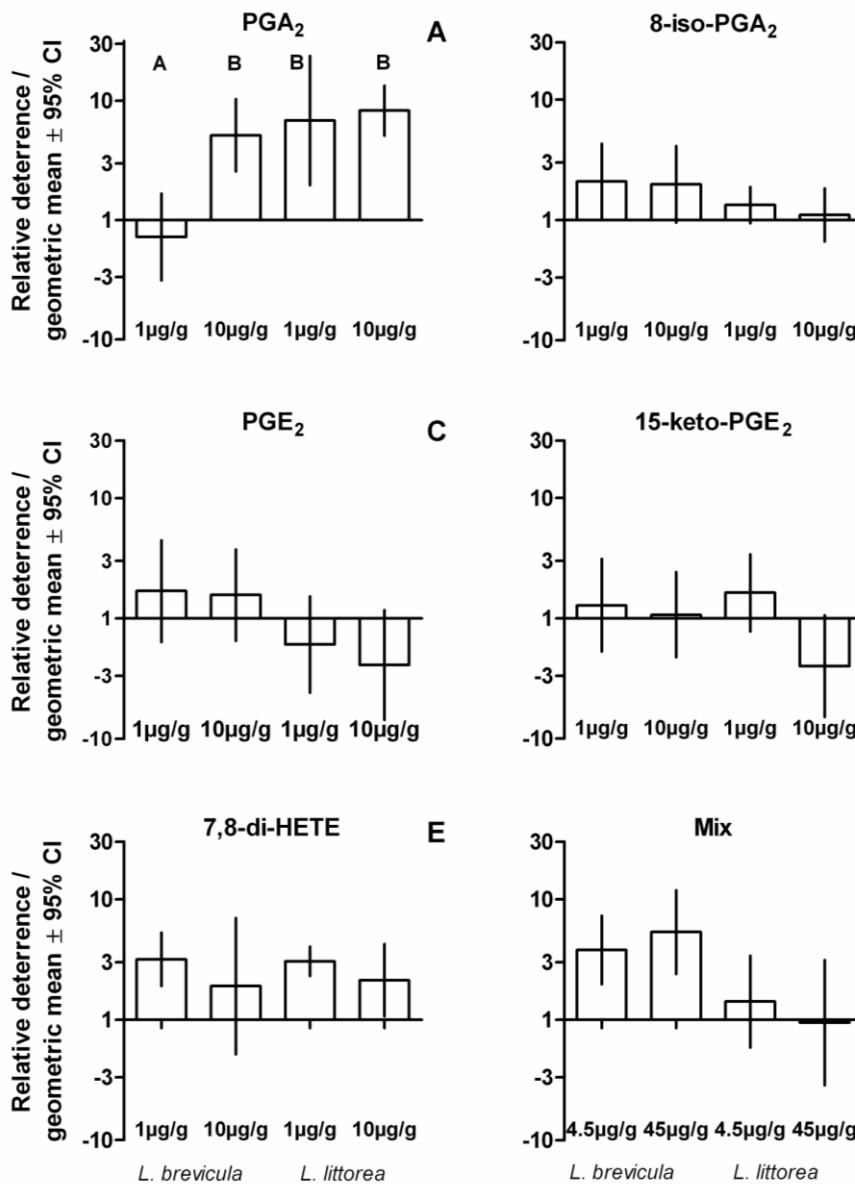


Fig. 7: Pure compounds. Average deterrence of *L. brevicula* and *L. littorea* by two concentrations of five different oxylipins (A-E) and by a mix of these metabolites (F). Different letters indicate populations that were significantly different in a Tukey test ($p < 0.05$).

Wounding and tissue disruption are inevitable consequences of herbivory and the grazing activity of mesoherbivores on *G. vermiculophylla* results in similar oxylipin profiles as mechanical wounding (Nylund et al. 2011). Non-native thalli of *G. vermiculophylla* thus appear as more defended against herbivory on the basis of oxylipins than native thalli. This suggests that resistance toward grazers may have been a necessary trait for the invasion success of *G. vermiculophylla* and that a selection for wound activated production of oxylipins has occurred, corresponding with the fact that most marine herbivores are generalists and enemy release during seaweed invasions is probably rare.

Acknowledgements

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The first three experiments presented in paper IV represent the content of my diploma thesis entitled “Impact of the invasive red alga *Gracilaria vermiculophylla* upon native *Fucus* communities”, Lüneburg, Leuphana Universität, 2008, and are therefore not part of my dissertation. Their results, however, were necessary for the meta-analysis of data and were thus included.

Paper IV

Research Article

Direct and indirect effects of *Gracilaria vermiculophylla* on native *Fucus vesiculosus*Mareike Hammann^{1*}, Björn Buchholz¹, Rolf Karez² and Florian Weinberger¹¹ Helmholtz-Zentrum für Ozeanforschung Kiel (GEOMAR), Düsternbrooker Weg 20, 24105 Kiel, Germany² Landesamt für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein, Hamburger Chaussee 25, 24220 Flintbek, GermanyE-mail: mhammann@geomar.de (MH), bbuchholz@geomar.de (BB), Rolf.Karez@llur.landsh.de (RK),fweinberger@geomar.de (FW)

*Corresponding author

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Abstract

The perennial red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss has recently been introduced to the Baltic Sea and is a potential competitor to *Fucus vesiculosus*, the most common native perennial alga in large parts of the Baltic Sea. *Gracilaria* might interfere with *Fucus* through direct competition for resources. In addition, *Gracilaria* is a favoured refuge for mesograzers, which prefer to feed on *Fucus*. Mesocosm-experiments were conducted over one year in the Kiel Fjord in order to test the direct and indirect effects of *Gracilaria* on *Fucus*. *Fucus* was incubated with *Gracilaria* at three different densities and grazers in high or low abundances. High densities of *Gracilaria* inhibited the growth of *Fucus* adults and also reduced the half-life-time of *Fucus* germlings. Associated grazers also had a negative effect on *Fucus* adults. Our results suggest that *Gracilaria* is able to influence *Fucus* in the Baltic Sea through direct competition for resources and by exposing it to higher grazer pressure.

Key words: *Fucus vesiculosus*; *Gracilaria vermiculophylla*; invasion biology; introduced species; algae; effects-community; Baltic Sea**Introduction**

Seaweeds are frequent marine invaders (Schaffelke et al. 2006). The most common effect of invasive macroalgae in their new habitat is space monopolization that results in a reduced abundance of native species (reviewed in Schaffelke and Hewitt 2007). Loss of biodiversity and effects on fish and invertebrates were also reported (e.g. Boudouresque et al. 1992; Levin et al. 2002; Relini et al. 1998). In the case of *Caulerpa*, toxic effects on other biota were documented (e.g. Paul and Fenical 1986, reviewed in Schaffelke and Hewitt). However, most information originates from field surveys or observational studies, and there are only very few experimental studies about the impacts of invasive algae on their recipient ecosystems (Schaffelke and Hewitt 2007). As a consequence, the mechanisms underlying ecological effects of invasive species are mostly unknown (Schaffelke and Hewitt 2007). Manipulative community-level field studies, combined with modeling, are

necessary to identify the impacts of introduced seaweeds on native communities (Williams and Smith 2007).

The perennial red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss (hereafter: *Gracilaria*) has invaded many coastal areas worldwide. From its native distribution in East Asia it has spread to other temperate areas of the northern hemisphere, primarily along both North American coasts (Bellorin et al. 2004; Freshwater et al. 2006; Thomsen et al. 2005) and in the East Atlantic between Morocco and the Western Baltic Sea (Guillemin et al. 2008; Nyberg 2007; Rueness 2005; Thomsen et al. 2007; Weinberger et al. 2008). Accounting for 74% of the entire algal biomass, *Gracilaria* dominated the shallow water habitat in Hog Island Bay, Virginia (Thomsen et al. 2006). Two recent studies show that *Gracilaria* is able to negatively impact metabolism and survival of *Zostera marina* by creating an anoxic layer (Martínez-Lüscher and Holmer 2010; Höffle et al. 2011). Possible ecological effects of *Gracilaria* on macroalgae have not been studied.

Gracilaria tolerates high irradiances and can grow in shallow water. Therefore, it is predicted to be a strong competitor for the native *Fucus vesiculosus* L. (Nyberg 2007, hereafter: *Fucus*). *Fucus* is one of the most important phytobenthic species on hard bottom habitat in the Baltic coastal zone as it provides a habitat for species-rich epiphytic and epibenthic communities (Torn et al. 2006). During the last few decades, populations of *Fucus* have declined over large areas in the Baltic Sea, most likely as a consequence of eutrophication (Torn et al. 2006). Nowadays, *Fucus* is mainly restricted to shallow water with depths between one and two meters in the western Baltic Sea (Torn et al. 2006). The presence of *Gracilaria* could increase the pressure on *Fucus* further, and observations from the Kiel Fjord indicate that *Fucus* may have declined in habitats where *Gracilaria* has spread (Weinberger et al. 2008). In the newly invaded habitats in the Baltic Sea, *Gracilaria* appears as drifting mats, sometimes partly buried in the sediment or entangled in other seaweeds or mussels. These drifting mats of *Gracilaria* overgrow both soft bottom and hard bottom substrates (Weinberger et al. 2008) and may interfere with the settlement of *Fucus* germlings and reduce the growth of adult *Fucus*.

Orrock et al. (2010) developed the idea of refuge-mediated apparent competition, i.e. an indirect interaction whereby plants provide a refuge for a shared consumer, subsequently increasing consumer pressure on another plant species. In the Baltic Sea, *Gracilaria* provides an attractive habitat for herbivores (Thomsen 2010), especially for isopods (*Idotea* spp.), which are often present at higher densities on *Gracilaria* than on *Fucus* when both algal species live in the same habitat (Weinberger et al. 2008). Noticeably, the common periwinkle *Littorina littorea* and the isopod *Idotea baltica* show a preference for consuming *Fucus* rather than *Gracilaria* when both algae are offered in two-way-choice experiments (Weinberger et al. 2008). Herbivores can exert a strong influence upon *Fucus* species (Dethier et al. 2005). In the Baltic Sea, grazing by isopods (*Idotea* spp.) and periwinkles (*Littorina* spp.) leads to biomass loss of *Fucus* (Engkvist et al. 2000; Råberg and Kautsky 2008; Torn et al. 2006). Thus *Gracilaria* may potentially influence the decline of *Fucus* through two different mechanisms, 1) directly through overgrowth and competition for resources, 2) indirectly as it provides a habitat for grazers that feed on adjacent *Fucus*.

The main purpose of our study was to investigate experimentally whether *Fucus* was affected by the presence of *Gracilaria* or by mesograzers associated with it. As life stages can be influenced differently by invaders, we focused not only on the effect of *Gracilaria* on the growth of adult *Fucus*, but also investigated the effect upon the survival of *Fucus* germlings.

Material and methods

To investigate the impact of *Gracilaria* upon *Fucus* two (for *Fucus* germlings) or seven (for *Fucus* adults) sequential experiments were conducted as seasonal repetitions. In these experiments *Fucus* was incubated with *Gracilaria* in mesocosms floating on the Baltic Sea surface. *Gracilaria* was applied at three different densities, in order to test whether its effects on *Fucus* were density dependent. The applied *Gracilaria* contained grazers at either high or low density, which allowed determining the influence of grazers. The effect of *Gracilaria* and/or associated grazers was measured as growth of adult *Fucus* specimens and as half-life time of *Fucus* germlings.

Set-up, stocking and incubation of mesocosms

The experiments were carried out in the Kiel Fjord in a sheltered bay (N54°21.965', E10°8.908') between March 2008 and March 2009. The mesocosms consisted of white polyethylene boxes (60 × 40 × 40 cm) and had a bottom made of gauze (mesh size: 1 mm) to allow for an exchange of water with the Baltic Sea but to inhibit migration of grazers. They were floating on the water surface and contained a water column of ca. 30 cm and a water volume of approximately 70 l.

Light and temperature data were provided through constant measurements of the research division "marine meteorology", GEOMAR.

Each mesocosm was equipped with one stone with a medium-sized adult *Fucus* - devoid of grazers - and one tile with *Fucus* germlings. The chronology of the seven subsequent experiments with adult *Fucus* (hereafter: adults' experiments), the two incubation periods of *Fucus* germlings (hereafter: juveniles' experiments) and the environmental conditions during our studies are illustrated in Figure 1.

Adult *Fucus* individuals (mean 4.6 g WW ± 0.2 SE) growing on small stones were collected from the coastline of the Kiel Fjord. They were

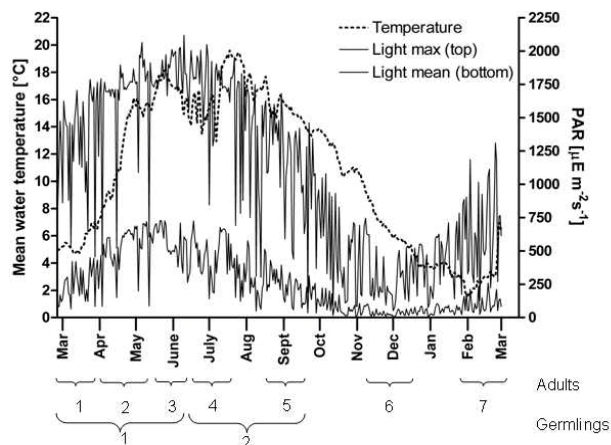
Effects of *Gracilaria* on *Fucus*

Figure 1. Development of mean water temperature in the Kiel Fjord and of mean and maximal photosynthetically active radiation (PAR) during the experiments. Also shown are the different time periods for the subsequent experiments.

replaced with newly collected specimens in the beginning of each adults' experiment. In order to obtain juveniles, tiles (15 × 15 cm) were inoculated with *Fucus* zygotes in the laboratory. One series of tiles was inoculated in January 2008 and one series in June 2008, in order to cover both reproduction periods of *Fucus* in the Baltic Sea (Berger et al. 2001). The winter germlings had an age of approximately 50d when they were transferred to the mesocosms for the first juveniles' experiment. The summer germlings were only 10 days old when the second juveniles' experiment started, but due to lower growth rates in winter the initial size of germlings was similar in both experiments (approximately 10 cells).

There were three different *Gracilaria* density treatments with ten replicates each (Figure 2): **No** *Gracilaria*, *Gracilaria* at a **low** density (5 – 10% of the 0.24 m² bottom of the mesocosm covered with *Gracilaria*, equalling 100 g of biomass FW) and *Gracilaria* at a **high** density (100 % covered, equalling 1000 g FW). These densities were considered relevant since *Gracilaria* ground coverage of up to 100% was previously observed in certain areas of the Kiel Fjord (Weinberger et al. 2008). For stocking, *Gracilaria* from drifting mats was collected together with its associated grazers with a dip net (mesh size 0.1 mm) in shallow parts of the Kiel Fjord.

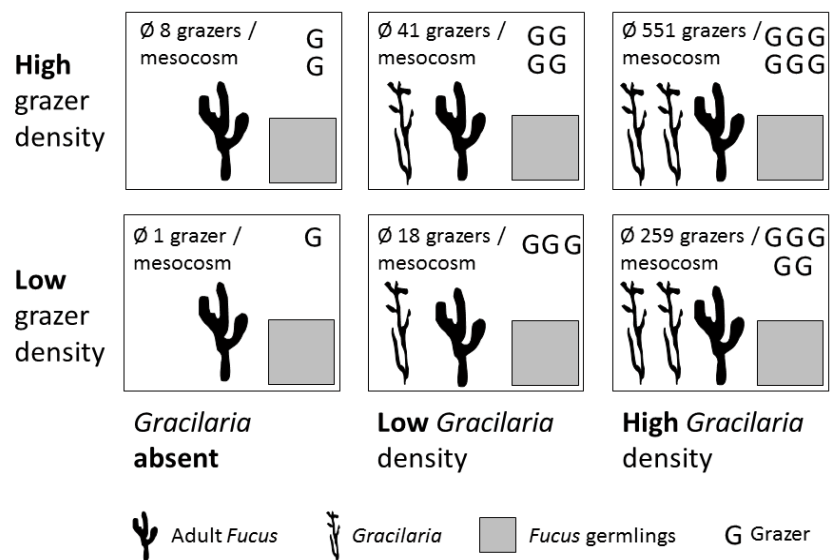
Each of the three *Gracilaria* density treatments was conducted in five mesocosms with low grazer density and in five mesocosms with high grazer density (Figure 2). In mesocosms containing no *Gracilaria* the natural density of grazers associated with *Fucus* collected in nature was regarded as high, while absence of grazers was regarded as low. The natural grazer density on *Fucus* was determined prior to each experiment by counting all grazers that were present on five *Fucus* specimens collected with a gauze net (mesh size 0.1 mm) and comparable densities of grazers were added into mesocosms without *Gracilaria*, but with high grazer density (Figure 2).

In mesocosms containing *Gracilaria* the natural density of grazers in *Gracilaria* mats was considered as high, while an artificially reduced density was considered as low. For reduction of grazer densities half of the *Gracilaria* stocking material was treated with freshwater for two hours. This procedure resulted in the removal of a part of the associated grazers and thus generated low grazer densities. Preliminary tests had confirmed the efficiency of this method. Moreover, a control experiment was conducted after each freshwater treatment, in order to ascertain that *Gracilaria* was not affected. For this purpose, six samples of freshwater treated and six samples of untreated *Gracilaria* (10 g each) – in both cases grazers had been manually removed - were incubated in single mesh bags (mesh size: 1 × 1 cm) at 15°C and under artificial light in the same aquarium, provided with sea water from the Baltic Sea. After seven days the growth of treated and untreated *Gracilaria* was determined and the effect of the two treatments was compared. No significant differences between treated and untreated *Gracilaria* were found in any of the experiments (Mann-Whitney-U-test, $p > 0.05$). Thus, the freshwater-treatment had no detectable effect upon the growth of *Gracilaria*.

Prior to the start of each experiment the initial density of grazers associated with *Gracilaria* stocking material was determined by taking samples of untreated and freshwater treated *Gracilaria* with a dip net. The samples were fixed with formaldehyde in seawater (4%) and the grazers were picked under a stereo microscope, classified and counted.

In order to reduce the impact of fouling organisms attached to the mesocosm walls, they were replaced with clean mesocosms in the

Figure 2. Experimental set-up. Three *Gracilaria vermiculophylla* densities (without, high and low) were combined with two grazer densities (low and high).



beginning of each adults' experiment. At these occasions *Gracilaria* was also removed and replaced with either 100 g or 1000 g of newly collected *Gracilaria* from the Kiel Fjord, either treated with freshwater or not, as described above. In contrast, tiles bearing *Fucus* juveniles were kept over several adults' experiments (see Figure 1), and transferred into the newly stocked mesocosms.

Sampling collection

For growth measurements, adult *Fucus* individuals were cleaned and spread under a plexiglass panel and photographed beside a benchmark in the beginning and at the end of each adults' experiment. The photos were evaluated by using a computer imaging program (Sigma Scan Pro, STATCON, Witzenhausen, Germany) which counted pixels representing the benchmark area and *Fucus*, thus allowing for calculation of the algal surface area.

Four times during each juveniles' experiment the germlings attached to tiles were counted by microscopy – in the beginning, the end and two times during the experimental run.

Animals present in the mesocosms were fixed with formaldehyde (4%) at the end of each adults' experiment. They were identified and counted under a stereo microscope. In mesocosms without *Gracilaria* and in mesocosms

with *Gracilaria* at low density all grazers were quantified, while subsamples (90 ± 36 g FW) were analyzed from mesocosms containing *Gracilaria* at high density.

We only considered potential grazers of *Fucus* for our analysis. In the Kiel fjord and adjacent waters these are the periwinkle *Littorina* spp., and the isopods *Idotea* spp., *Sphaeroma rugicauda* and *Jaera albifrons* (Sjöberg 1967; Frier 1979; Weinberger et al. 2008; hereafter: *Littorina*, *Idotea*, *Sphaeroma*, *Jaera*). *Idotea* and *Littorina* prefer to consume *Fucus* rather than *Gracilaria* in two-way-choice experiments (Weinberger et al. 2008).

Comparison of the grazer densities in single mesocosms at the beginning and at the end of adults' experiments revealed that the intended adjustment of grazer densities was largely effective. However, small isopods proved able to migrate into the mesocosms in summer and *Littorina* densities sometimes declined due to escape, but mesocosms intended to contain grazers at high and low densities generally still showed this difference at the end of each adults' experiment. However, in adults' experiment 3, 4, 5 and 7 real and intended grazer densities did not fully correspond. Thus mean values of effective grazer densities at the beginning and at the end of each experiment were calculated for each mesocosm and for each of the main grazer genera. Afterwards, the five mesocosms with

Effects of *Gracilaria* on *Fucus*

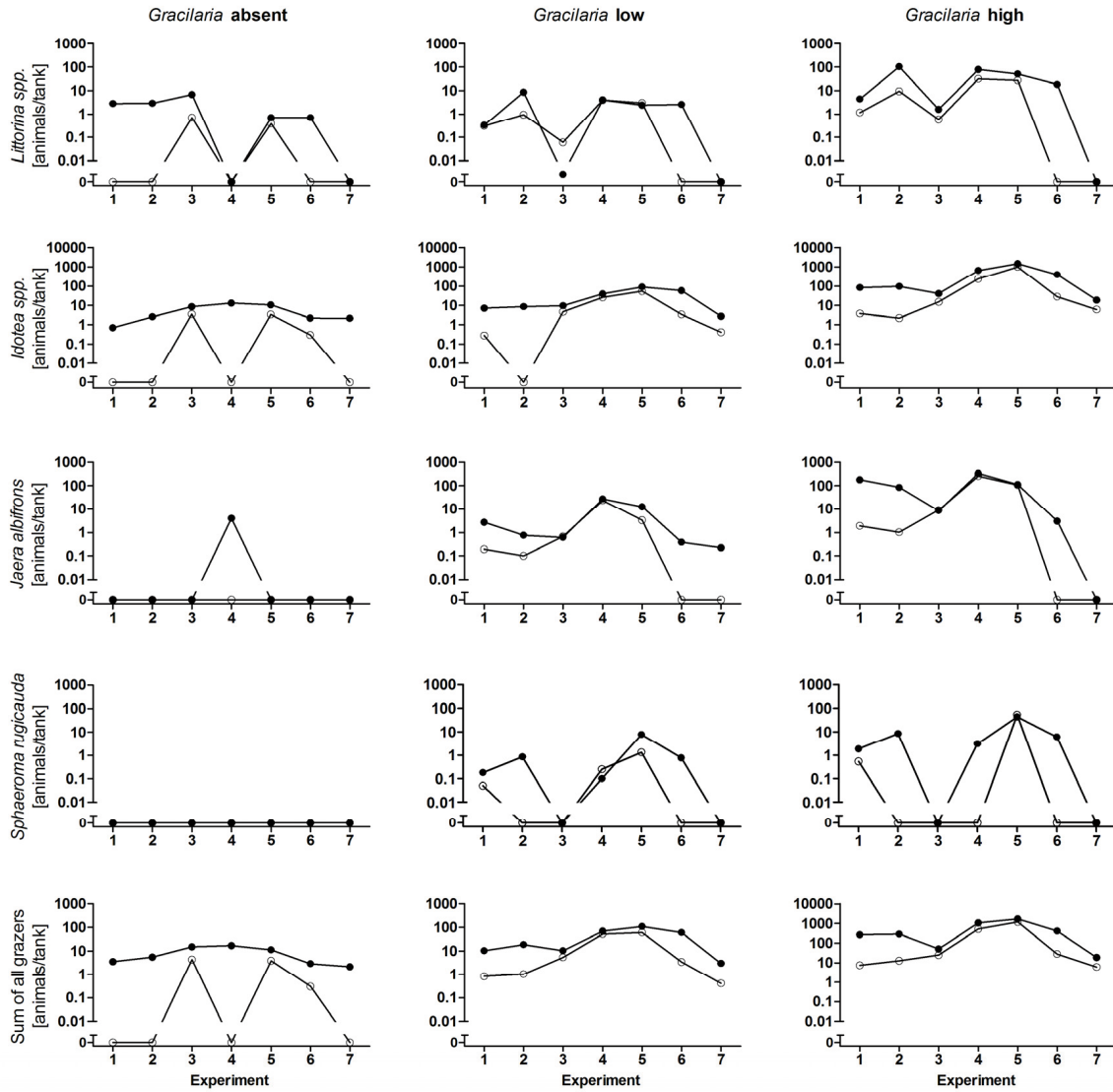


Figure 3. *Littorina* spp., *Idotea* spp., *Jaera albifrons*, and *Sphaeroma rugicauda*. Mean densities in mesocosms with high or low *Gracilaria vermiculophylla* density or without *Gracilaria* and with low (○) and high (●) grazer densities during seven single adults' experiments, n = 5.

relatively high and the five mesocosms with relatively low mean grazer density had to be chosen *post hoc* for each *Gracilaria* density. The real mean grazer densities in the two juveniles' experiments were calculated based on grazer densities determined in the beginnings and ends of adults' experiments 1 to 3 (which together covered the time window of juveniles' experiment 1, see Figure 1) and adults' experiments 4 and 5 (covering juveniles' experiment 2, see Figure 1), respectively. Real and intended grazer densities

fully corresponded in juveniles' experiment 1, while a *post hoc* distinction of mesocosms with relatively low and relatively high grazer density was necessary in juveniles' experiment 2. Figure 3 shows average grazer densities (i.e. mean values of grazer densities at the beginning and at the end of each experiment in five replicate mesocosms) in the course of the different experiments and in the different *Gracilaria* treatments for both high and low grazer treatments as assigned by us *post hoc*.

Data analysis

Computation of half-life times of *Fucus* germlings

Using the “Prism 4.03”-software (GraphPad Software, La Jolla, Ca., USA.), an exponential decay function was fitted by adaptive iteration to the densities of *Fucus* germlings that were observed at successive countings. The function used was $Y = B \times e^{(-K \times X)} + A$, with Y = germling density at a given time X , A = final germling density, B = maximal decrease of germling density, K = rate constant. In this way the half-life time Z of the germlings on each tile could be estimated, using the function $Z = 0.69/K$.

ANOVA and post-hoc tests

The effect of *Gracilaria* density, grazer density and the respective experimental period upon the half-life time of *Fucus* germlings and the growth of *Fucus* adults was tested for significant differences, using factorial ANOVA and Tukey’s post-hoc test. Prior to statistical tests, all data were rank-transformed, as this transformation best reduced heteroscedasticity. In the adults’ data set, homogeneity of variances could not be achieved for the factor “time” (Levine’s test, $p < 0.05$) and results were taken as statistically significant if the probability of type I error was less than 1% (Underwood 1997), while other results were taken as statistically significant at a p -level of 5%.

Results

Seasonal variation in environmental conditions

The environmental conditions followed a typical seasonal pattern (see Figure 1). Low temperatures of $< 5^\circ\text{C}$ were characteristic for the winter months, while peak temperatures of 20°C were reached in July and August. Light intensity was already relatively high in spring and slowly increased until June. Lowest light intensities were measured in December and January. In December and January there are only 7 h of daylight compared with 17 h in June and July.

Growth of adult *Fucus* individuals

Not surprisingly, the time when an experiment was carried out had a significant effect on the growth of *Fucus* adults ($p < 0.001$; see Table 1 and Figure 4). Growth rates were highest in May/June (experiment 3), whereas the weight of

Fucus declined in November/December (experiment 6) and moderate growth rates were achieved in spring and autumn (experiments 1, 2, 4, 5, 7). Also the presence of *Gracilaria* had a significant effect upon the growth of *Fucus* adults (Table 1 and Figure 5). The post-hoc test detected that presence of *Gracilaria* at high density resulted in significantly ($p < 0.001$) less *Fucus* growth than presence at low density or absence. Grazers alone had no significant effect on the growth of *Fucus* at the chosen alpha level ($p = 0.053$, Table 1), but ANOVA detected a significant interactive effect of *Gracilaria* density and grazer density ($p < 0.01$, see Tab. 1 and Figure 5). At low *Gracilaria* density the presence of high grazer densities significantly reduced *Fucus* growth, while grazer treatments did not affect growth at other *Gracilaria* densities. This interactive effect was particularly obvious in April/May (experiment 2; see Figure 4) and from July to December (experiments 4, 5, 6; see Figure 4).

Development of germlings

During the first experimental period from March to June, the average initial density of *Fucus* germlings on tiles was 231.1 cm^{-2} , while their average half-life was 1.40 ± 0.07 weeks (mean \pm S.E.). The average initial *Fucus* density during the second experimental period from June to September was lower than in the first period ($36.9 \text{ germlings cm}^{-2}$). The average half-life time (mean \pm S.E.: 0.45 ± 0.06 weeks) was also significantly shorter than in the first experiment ($p < 0.001$; Table 2 and Figure 6). Based on ANOVA results the factor “*Gracilaria*” also significantly influenced the survival of germlings ($p < 0.001$; Table 2). Half-life time was longest in treatments with low *Gracilaria* density and shortest in treatments with high *Gracilaria* density (Figure 7). Grazers had no significant effect upon the half-life time of *Fucus* germlings (Table 2 and Figure 7).

Discussion

The main purpose of the experiments was to test the two hypotheses (1) that *Gracilaria* affects *Fucus* directly and (2) that mesograzers associated with *Gracilaria* have an impact on *Fucus*. Both hypotheses cannot be rejected. *Gracilaria* had an influence on adult *Fucus* individuals and *Fucus* germlings. High *Gracilaria* densities

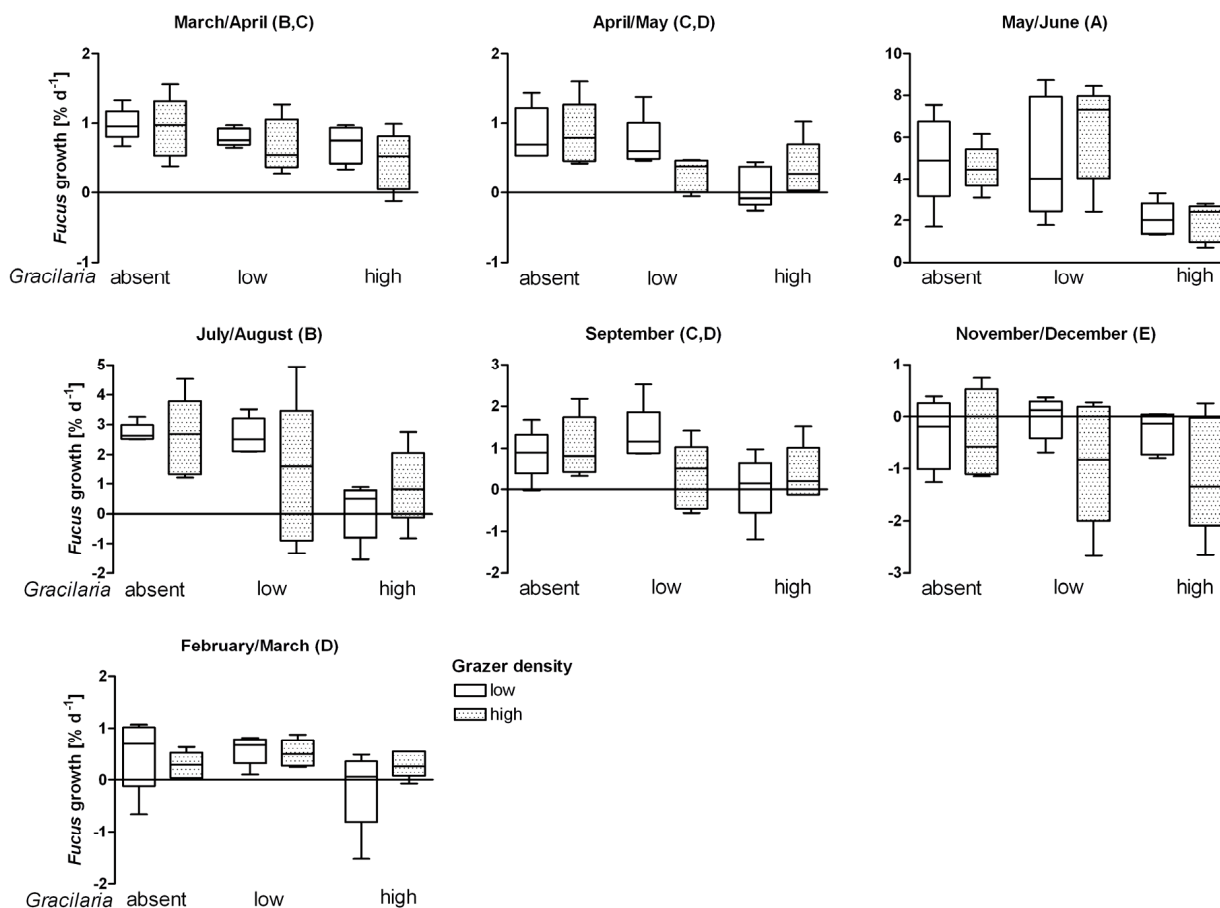
Effects of *Gracilaria* on *Fucus*

Figure 4. *Fucus vesiculosus*, adults. Growth in mesocosms with high or low *Gracilaria vermiculophylla* density or without *Gracilaria*, and with low or high grazer densities. Single results of seven successive experiments, $n = 5$. Different letters in the graph titles indicate the experiments that were significantly different in a Tukey-test ($p < 0.01$).

clearly reduced the half-life time of *Fucus* germlings and growth of *Fucus* adults as compared to low *Gracilaria* densities. Grazers only had an effect on *Fucus* adults at low *Gracilaria* densities.

Growth of adult *Fucus*

There was a significant seasonal variation in *Fucus* growth, as would be expected. Growth rates were highest in May/June when water temperature and light availability were elevated. With low water temperatures and reduced light availability in November/December, *Fucus* biomass declined.

Fucus adults grew least when *Gracilaria* was present at high density, but there was no difference in growth when *Gracilaria* was absent

or present at low density. At high density the ground coverage by *Gracilaria* was 100 %, and one can assume that such conditions result in a reduced availability of light, oxygen and nutrients for *Fucus*, despite its capacity to protrude *Gracilaria* mats due to the buoyancy provided by gas filled bladders. Also, an allelopathic effect cannot be excluded.

Grazers reduced the growth of *Fucus* as well, but the overall effect was relatively small in size (a reduction of growth by approximately 8%) and statistically not significant. It is possible that epiphytes and other fouling organisms may have interfered with the interactions of *Gracilaria*, *Fucus* and grazers. Especially in spring and early summer ephemeral algae - first *Ulothrix* spp. and later tube forming diatoms - were present on mesocosm walls and entangled in *Gracilaria* and

Table 1. Growth of *Fucus vesiculosus* adults. Effect of time when an experiment was conducted, *Gracilaria vermiculophylla* density and grazer density in a fully factorial ANOVA.

	SS	DF	MS	F	p
Time	407493	6	67915	53.08	<0.001
<i>Gracilaria</i>	65402	2	32701	25.56	<0.001
Grazer	4867	1	4867	3.80	0.053
Time* <i>Gracilaria</i>	20195	12	1683	1.32	0.21
Time*Grazer	1740	6	290	0.23	0.97
<i>Gracilaria</i> *Grazer	14116	2	7058	5.52	<0.01
Time* <i>Gracilaria</i> *Grazer	21118	12	1760	1.38	0.18
Error	211136	165	1280		

Table 2. Half-life time of *Fucus vesiculosus* germlings. Effect of time when an experiment was conducted, *Gracilaria vermiculophylla* density and grazer density in a fully factorial ANOVA.

	SS	DF	MS	F	p
Time	10837.26	1	10837.26	120.15	<0.001
<i>Gracilaria</i>	1027.03	2	513.51	5.69	<0.01
Grazer	3.60	1	3.60	0.04	0.84
Time* <i>Gracilaria</i>	129.79	2	64.90	0.72	0.49
Time*Grazer	76.18	1	76.18	0.84	0.36
<i>Gracilaria</i> *Grazer	26.75	2	13.37	0.15	0.86
Time* <i>Gracilaria</i> *Grazer	21.30	2	10.65	0.12	0.89
Error	4149.20	46	90.20		

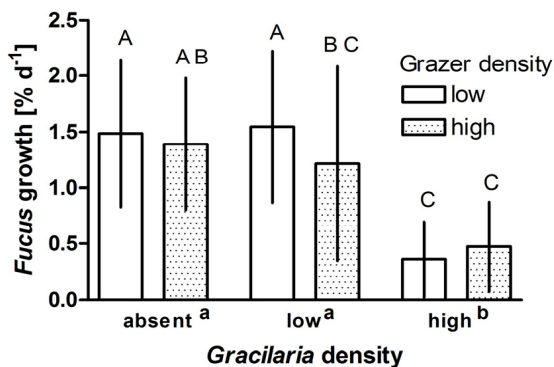


Figure 5. *Fucus vesiculosus*, adults. Growth in mesocosms with high or low *Gracilaria vermiculophylla* density or without *Gracilaria*, and with low or high grazer densities. Mean of seven successive experiments \pm 95% CI, n = 35. Different lower-case letters indicate *Gracilaria* treatments that were significantly different in a Tukey-test ($p < 0.05$), while upper-case letters indicate differences in the interactive effect of *Gracilaria* and grazers ($p < 0.05$).

on *Fucus*. These algae were also observed in spring and early summer on *Gracilaria* and on *Fucus* in nature. Several experiments have shown that many grazers (including *Idotea*) preferably feed on epiphytes and ephemeral algae, also those growing on *Fucus* (Karez et al. 2000; Orav-Kotta and Kotta 2004; Worm and Sommer 2000). Preferential consumption of ephemeric algae might thus reduce the negative effect of mesograzers upon *Fucus*.

The effect of grazers on *Fucus* differed with *Gracilaria* density. Treatments with high grazer density contained for both *Gracilaria* densities (low and high) roughly the double amount of grazers as treatments with low grazer density (see Figure 2 and Figure 3). High *Gracilaria* densities had the strongest negative impact on *Fucus* growth rates, irrespective of grazer density. Thus *Gracilaria* biomass clearly is of higher importance for *Fucus* growth than the grazers associated with it. Any effects of grazers are probably superimposed by effects of *Gracilaria* in high density. In contrast, at low *Gracilaria* density an increased density of grazers resulted in decreased *Fucus* growth. This

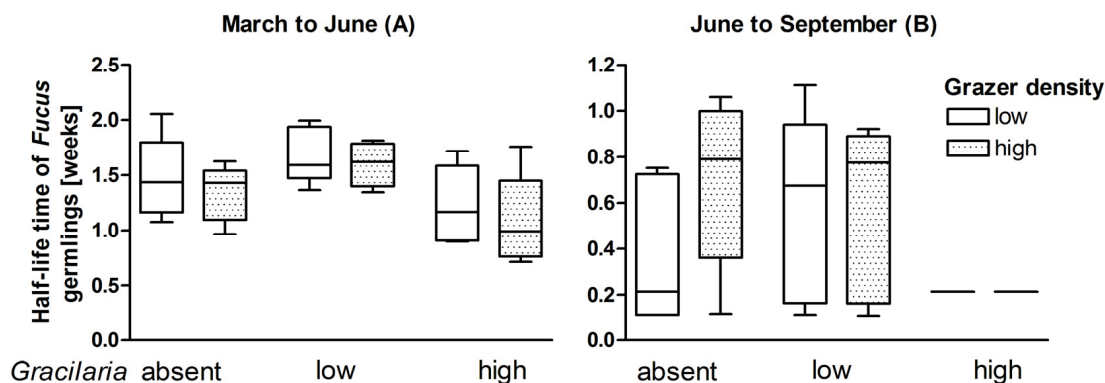


Figure 6. *Fucus vesiculosus*, germlings. Mean half-life time in mesocosms with high or low *Gracilaria vermiculophylla* density or without *Gracilaria* and with low or high grazer densities. Single results of two successive experiments, $n = 5$. Different letters in the graph title indicate that the experiments were significantly different in a Tukey-test ($p < 0.05$).

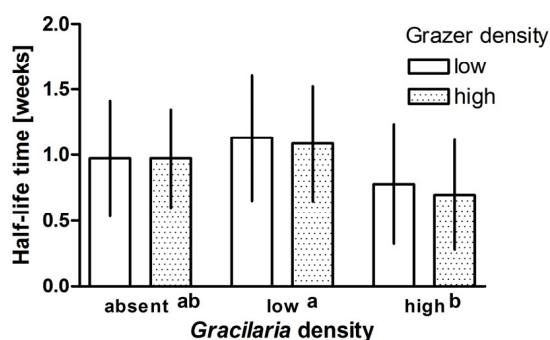


Figure 7. *Fucus vesiculosus*, germlings. Mean half-life time in mesocosms with high or low *Gracilaria vermiculophylla* density or without *Gracilaria* and with low or high grazer densities. Mean of two successive experiments \pm 95% CI, $n = 10$. Different letters indicate treatments that were significantly different in a Tukey-test ($p < 0.05$).

was not the case in absence of *Gracilaria*, where *Fucus* growth was generally high and again unaffected by grazers. *Fucus* in *Gracilaria* free environments is occasionally heavily grazed (Dethier et al. 2005; Engkvist et al. 2000), but the natural densities of grazers associated with *Fucus* during the experimental periods were too low to cause a significant decline in *Fucus* growth. Grazers thus affected growth only at low *Gracilaria* density. This indicates that the grazer population accommodated by *Gracilaria* poses an additional indirect threat to *Fucus*. However, the effect only gets visible when the direct effects of *Gracilaria* are relatively small.

Development of germlings

The early live stages of *Fucus* are generally especially vulnerable (Berger et al. 2003). In our experiments, germlings were found to have the shortest half-life time when *Gracilaria* was present at high density. This effect was especially pronounced in the second experimental period where none of the *Fucus* germlings survived at high *Gracilaria* treatments – irrespective of grazer density. Buried under thick *Gracilaria* mats, the germlings presumably suffered from a reduced availability of light and oxygen. However, both generations of *Fucus* germlings also tended to have a slightly longer half-life time in presence of *Gracilaria* at low density than in absence of *Gracilaria*, suggesting that *Gracilaria* may have both positive and negative effects upon the survival of *Fucus* germlings. In other experiments, low concentrations of *Pilayella littoralis*-exudates had a positive effect on the germination of *Fucus* zygotes (Råberg et al. 2005). This effect was attributed to the enhanced nutrient concentration in the exudates, which probably supported the germlings. Possibly, *Fucus* germlings in mesocosms with low *Gracilaria* density also profited from a slightly raised nutrient concentration, but other explanations can also not be excluded.

Ecological consequences and outlook

With some restrictions, the results of our mesocosm experiments can be compared to natural processes. The densities of both *Gracilaria*

and grazers were based on those found in nature. Also the water depth was similar to that in nature, as both species occur mainly in shallow water in the Kiel Fjord.

Growth of *Gracilaria* in the southwestern Baltic is limited by light and largely restricted to summer and water depths above 3m (Weinberger et al. 2008). With *Fucus* appearing in similar depths of water, there could be a strong competition for space and light between the two species (Nyberg 2007). Based on our studies, *Gracilaria* appears to be able to restrict growth of *Fucus* in these shallow waters. Especially in sheltered bays with little water fluctuation and where high densities of *Gracilaria* can amass, the effect of *Gracilaria* on *Fucus* could be detrimental, even though it will presumably not lead to the extinction of single populations. The further effects, however, depend very strongly upon how *Gracilaria* influences the settlement and development of *Fucus* germlings. Our experiments show that in dense *Gracilaria* mats with numerous grazers, the survival of *Fucus* germlings approaches zero. The local density of *Gracilaria* in nature therefore is a crucial factor that determines which effect the presence of *Gracilaria* actually has. Other factors such as deposited matter trapped in algal mats have also to be taken into consideration in order to estimate the overall impact of *Gracilaria* upon *Fucus*. Furthermore, it has to be held that the results of the experiments conducted cannot be applied to the entire Baltic Sea without reservations, but first and foremost to the western Baltic Sea with its special conditions of salinity, light and temperature.

Currently, *Gracilaria* appears in the German Baltic Sea mainly in its unattached form. However, the species seems to be capable of sexual reproduction at relatively low salinities (own observations). Should *Gracilaria* recruits settle on hard substrate, the competition for space with *Fucus* germlings might increase even further. Space occupation by other algae has been shown to have a strong negative effect on the settling success of spores and zygotes (Vadas et al. 1992), also those of *Fucus* ssp. (Berger et al. 2003; Råberg et al. 2005; Steen 2004; Worm and Chapman 1996). In the long run, a rise of water temperatures as a consequence of global warming might intensify the competitive situation in the Baltic Sea (Leppäkoski et al. 2002; Stachowitz et al. 2002), because *Gracilaria*, unlike *Fucus*, grows even better at higher temperatures (Yokoya et al. 1999).

Gracilaria appears to show an exceedingly strong potential of changing the habitat in which it grows. It alters the habitat architecture by forming thick mats. Thereby it works as a trap for sediment and other particles and shades other algae (Nyberg 2007).

On the other hand, *Gracilaria* in small amounts adds structural complexity to a relatively homogeneous system. It sustains a large taxonomic richness and provides a new habitat for many taxa (Nyberg 2007; Thomsen 2010). Our experiments were conducted in an environment of relatively low diversity (Rönnbäck et al. 2007), but 18 different animal taxa were nonetheless found in *Gracilaria* (data not shown).

Thomsen et al. (2012) found out that *Gracilaria comosa* had ambiguous effects in its new environment in Australia, as it inhibited the native seagrass *Halophila ovalis* itself, but facilitated most seagrass-associated invertebrates. Accordingly, the question should be asked, whether *Gracilaria*, which in fact harbours a high biodiversity, could be an adequate replacement for *Fucus* in the Western Baltic Sea if the decline of the latter continues or whether consequences of a further spread of the invader would rather be detrimental for the system. Kraufvelin and Salovius (2004) stated that substitution of *Fucus* by the filamentous green alga *Cladophora glomerata* does not immediately affect macroinvertebrate diversity negatively. Other authors also found out that following a decline of *Fucus* the majority of *I. balthica* switched to an alternate habitat (Orav-Kotta and Kotta 2004). Weinberger et al. (2008) postulated a possible functional replacement of *Fucus* by *Gracilaria* and a gain of habitat for seaweed associated organisms on soft bottom substrates. However, while animal abundances and biomass are higher in *Gracilaria* as compared to *Fucus* - probably due to the finer morphology of *Gracilaria* - epiphytic algae and sessile epiphytic animals are generally more abundant on fucoids and may lose substrates if *Fucus* is replaced by *Gracilaria*. Thereby, the habitat complexity may be reduced and energy fluxes may be affected (Kraufvelin and Salovius 2004). A decline of *Fucus* might for similar reasons be harmful to the stability of the whole community (Torn et al. 2006). Moreover, high densities of algal mats stress benthic communities directly, especially by creating oxygen deficiency (Arroyo et al. 2012; Berezina and Golubkov 2008; Norkko and Bonsdorff 1996; Norkko et al. 2000) and even night-time anoxia caused by rapid algal growth in the summer (e.g. Shen et al. 2008). In

conclusion, opportunistic and mobile taxa may benefit from algal mats consisting of *Gracilaria*, but stenoecious key species adapted to *Fucus* may be lost, and fundamental changes to community function and food web might be induced.

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5. Discussion

5.1 *G. vermiculophylla* is highly tolerant towards abiotic stress

As introduced in section 1.2, invasive *G. vermiculophylla* possesses a high tolerance towards various environmental stressors, including the ability to survive desiccation and darkness (Thomsen & McGlathery 2007, Nyberg & Wallentinus 2009), growing at both high and low nutrient levels (Thomsen & McGlathery 2007) and at wide ranges of salinities (Weinberger et al. 2008).

High resistance to depletion stress was confirmed by my results and I furthermore can add to the list of the seaweed's general traits related to stress tolerance. Differences between single populations of *G. vermiculophylla* with regard to abiotic stressors will be discussed in section 4.3. With the exception of one population (Ensenada, Mexico) in the experiment conducted in Qingdao, at least some individuals of every population were able to survive an extended period of starvation, desiccation and darkness. Some populations coped better with the stressor than others, but generally *G. vermiculophylla* might have particularly good storage mechanisms. As the ability to cope with long term survival out of water could be beneficial during extended periods of transport during the invasion process, this trait might have played a role for its invasion success. *G. vermiculophylla* can regrow its whole thallus from small fragments, so a survival of only a small part of the seaweed would be sufficient to enable its growth and spread.

G. vermiculophylla from non-native populations is able to survive short periods of extreme heat. The possession of high levels of constitutive and stress-induced HSP70 could be an important mechanism to facilitate the elevated temperature tolerance of non-native *G. vermiculophylla* individuals. This trait might have been useful to cope with stress that was experienced during early invasion, e.g. during transportation.

Moreover, the invader is very tolerant towards copper pollution, which could enable growth in anthropogenically impacted areas. Other species of *Gracilaria* suffered more from copper concentrations similar to or even lower than those employed in my study (Xia et al. 2004, Huang et al. 2010). This is in accordance with the observation that *G. vermiculophylla* seems to contain higher levels of haloperoxidase that scavenge harmful reactive oxygen species – as for example induced by metal exposure – than other gracilarioids that have been studied.

5.2 *G. vermiculophylla* is well defended towards grazers

L. littorea from the invaded range of *G. vermiculophylla* consumed significantly lower amounts of the seaweed's tissue than *L. brevicula* from the native range. This would suggest that *G. vermiculophylla* experienced a certain **enemy release**, as it is of lower palatability to grazers in the new range than to grazers in its area of origin.

Both snail species furthermore preferred to feed on the native seaweeds as compared to the invasive seaweeds. Apparently, *G. vermiculophylla* originating from the invaded range was less palatable. *G. vermiculophylla* is able to defend itself from grazing by up-regulation of chemical compounds and this ability seems to be higher in invasive populations of the seaweed. The lower palatability of non-native populations of *G. vermiculophylla* could therefore result from an increased level of chemical defenses, which would support the **novel weapons hypothesis**.

This is in accordance with the outcomes of the experiments described in paper III. For both snail species the deterring effect of *G. vermiculophylla* from non-native populations was higher than the deterrence by native populations' oxylin extracts. Furthermore, I was able to show that a variety of chemical components were significantly more concentrated in non-native than in native populations of *G. vermiculophylla*, among them many arachidonic derived substances. Two of these substances, PGA_2 and 7,8-di-HETE clearly had a deterring effect on both *L. brevicula* and *L. littorea* when tested in bioassays. The higher concentration of these metabolites in non-native individuals of *G. vermiculophylla* might thus contribute to the fact that both snail species were more deterred by *G. vermiculophylla* from non-native populations and consumed lower amounts of algal tissue.

However, some other results of the feeding experiments can't be entirely explained with our metabolomic approach. While *L. littorea* consumed significantly lower amounts of the seaweeds' tissue than *L. brevicula*, *L. littorea* was generally less deterred by oxylin extracts than *L. brevicula*. Also consumption patterns for the single populations of *G. vermiculophylla* are not always in accordance with deterrence patterns of the snails.

I was able to show that certain oxylin (here: PGE_2) do not have a deterrent effect on all grazers, but even attracted *L. littorea*. Grazers' reactions towards certain metabolic

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compounds might thus be highly species specific. Furthermore, I only tested the oxylipin fraction of the seaweeds and it is possible that other substances play a role in the defense of *G. vermiculophylla* as well. Up-regulation of other metabolites might also explain why certain populations of *G. vermiculophylla* had a stronger effect on the snails than others.

Nonetheless, non-native individuals of *G. vermiculophylla* seem to be well defended against herbivory and are consumed by one of the main mesograzers in their new habitat only in very low amounts. I thus conclude that a selection for wound activated production of oxylipins has occurred during the invasion history of *G. vermiculophylla* and that low palatability has facilitated the invasion success of the seaweed.

5.3 A selection towards resistance to multiple stressors took place

Resistance towards both biotic and abiotic stress seemingly played an important role for the invasion success of *G. vermiculophylla*. Significant differences in stress resistance between native and invasive populations were not detected for all the investigated stressors, but only for heat shock, copper stress and feeding defense related traits (palatability, chemical composition, deterrence of grazers). However, results of the copper stress experiment differed between the two experimental locations and were likely influenced by local adaptation effects and by deviant reactions of one single population. For heat shock and palatability results were reproducible when the experiments were conducted in Kiel and Qingdao, indicating that effects were little affected by acclimation. Also results of the UV-stress experiment were reproducible, but did not differ between native and invasive populations. A selection of heat shock resistance and of resistance towards feeding thus clearly took place, while other traits have either been of lower relevance for the invasion success or are rather species specific traits than traits of the invasive ecotype(s).

However, although both heat shock resistance and resistance to grazers are higher in non-native populations of *G. vermiculophylla*, there is an important difference between these traits: The strong resistance against heat shock did not exist in any of the investigated native populations but did exist in all invasive populations included in my experiments. In the case of feeding defense, the observed pattern was different: The consumption of 3 native

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populations by either both or at least one of the snail species was similar to that of invasive populations, indicating that a low palatability was already present in the native area. The general presence of heat shock resistance and low palatability among invasive populations in Europe and W Mexico indicates that single populations or even individuals within a specific donor population of *G. vermiculophylla* may have been selected early during the invasion history of this species because of their pre-adaptation to heat shock and/or feeding resistance.

How these multiple resistances could have been selected is hard to tell. Mechanisms to cope with the environmental factors are very specific for the different stressors: In case of heat stress, the possession and expression of higher levels of HSP70 is at least partly responsible for the high resistance of non-native populations. For feeding defense, the selection of individuals containing higher levels of oxylipins probably played an important role. It seems unlikely that the combined elevated tolerance to heat stress and towards grazing has evolved independently in several areas worldwide. The possession of elevated levels of HSP70 could rather be a trait that was useful to endure stress experienced in an early phase of invasion, probably during transportation. With regard to low palatability / feeding defense it seems likely that pre-adapted genotypes were introduced into new habitats, and in their new environment they could settle and spread, as they were not controlled by grazers.

When comparing the resistance of all *G. vermiculophylla* populations towards all stressors tested in the present studies, interesting patterns become visible: All non-native populations with the exception of Ensenada, Mexico, cluster together. The isolated position of the Mexican population is based on the fact that its survival is lower than that of most other non-native populations in the depletion stress experiment and in the copper exposure experiment. The population from Donghae, South Korea, clusters together with the group of non-natives. Populations from Daecheon, Jindo and Gyeokpo in South Korea cluster together with the population from Qingdao, China. Odo-ri – like Ensenada – stands for itself and differs from all the other populations. This is mostly due to its low survival rate in the copper stress experiment.

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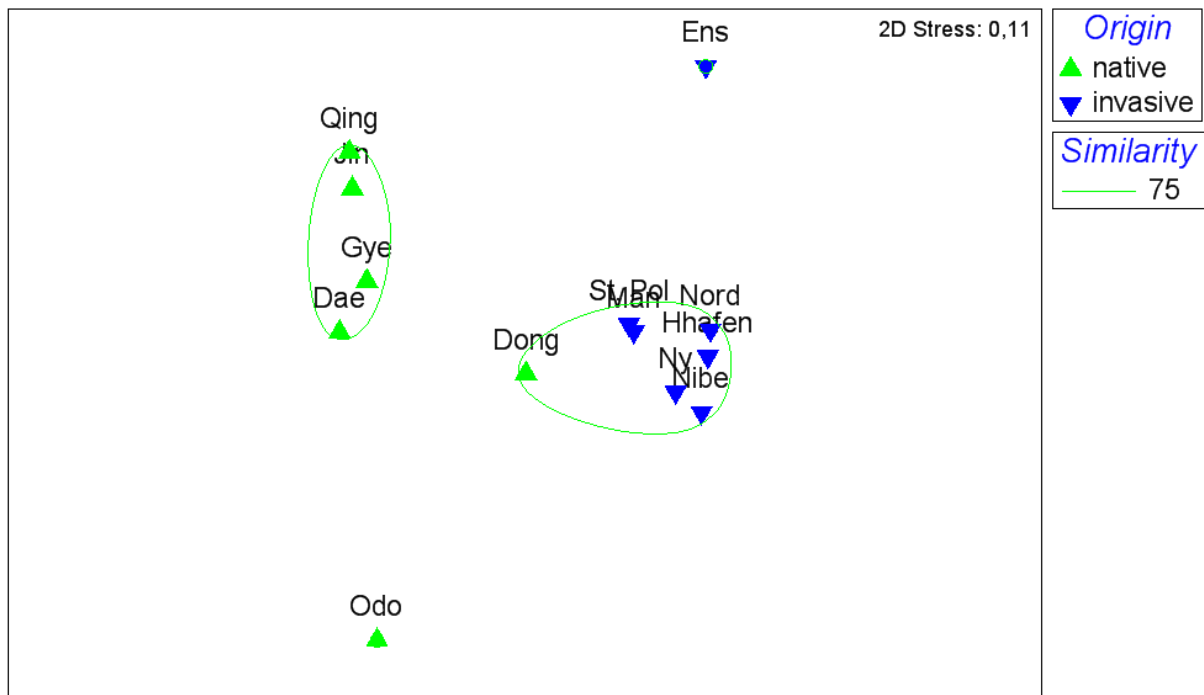


Fig. 4: Similarities between populations of *G. vermiculophylla*. MDS plot of seaweed's populations comparing their reactions towards various biotic and abiotic stressors. The green line marks populations with a similarity of $\geq 75\%$.

There is another trait that non-native populations of *G. vermiculophylla* have in common: Based on the COX1-gene, the genetic diversity in non-native populations of the seaweed has been reported to be very low (Saunders 2009, Kim et al. 2010). This is in accordance with my own genetic analyses: All non-native individuals of *G. vermiculophylla* that I investigated for my studies share the same COX1-sequence and are in this respect identical with all eight specimens from the Donghae-population. This haplotype was not detected in the population from Odo-ri, which is located in vicinity to Dongae at the Korean East Sea/Sea of Japan. Thus, my results confirm the findings by Kim et al. (2010): Of all the native populations included in the present study, the Donghae population is genetically most similar to invasive populations. In this light it is especially interesting that the population from Donghae is also the population that is most similar to the non-native cluster when comparing all stress experiments. One remarkable similarity between the population from Donghae and the non-native populations is their low palatability. Also the population from Odo-ri belonged to those that were consumed only in small amounts – at least by *L. brevicula*, suggesting that low palatability could be a general trait in this sea area.

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However, the metabolomic profile of seaweed individuals originating from the Donghae population differs from metabolomic profiles of the non-native populations (see Fig. 2 in paper III). This could be a hint that the population from Donghae is probably not the donor-population of the non-native *G. vermiculophylla* populations – despite all other similarities.

New genetic analyses refine the identification of the area of origin of the non-native populations of *G. vermiculophylla*. Donor populations for all non-native populations are likely the Pacific coastlines of northern Japan and Hokkaido islands (Stacy Krueger-Hadfield, Nicole Kollars and Erik Sotka, personal communication). Among all populations included into my study, the population from Donghae was geographically closest to the donor region, which might explain the relative – yet not complete – similarity between Donghae and the non-native populations.

It could be possible that *G. vermiculophylla* populations from this sea area are generally more stress resistant than populations from other native regions. However, studies of more populations with respect to stress resistance would be needed to come to a decision in this respect.

The fact that all non-native populations included into my study 1) have a high tolerance towards heat-stress including elevated levels of HSP70, 2) do contain high levels of oxylipins and are of low palatability to grazers and 3) are genetically at least very similar if not identical allows some assumptions about the invasion history of the seaweed. It seems very likely that all populations do have a common donor population, probably from northern Japan or Hokkaido (see above). This could be due to two different mechanisms: There could either be one population in the native range that was the origin of several introductions to different parts of the world, or *G. vermiculophylla* was introduced into one of the new habitats and – starting from that point – was transported to further areas.

Saunders (2009) suspects that the introduction of *G. vermiculophylla* to British Columbia – probably with oysters from Japan – was an event separate from introductions to California and Mexico, as the distribution of the species is highly disjunct. Another possibility would be an introduction at one site and a subsequent secondary invasion along the coast by local shipping or aquaculture measures. The suspected vector for the invasion of *G. vermiculophylla* to Europe is an introduction together with Japanese oysters to the Brittany

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coast and secondary transfers by local shipping (Rueness 2005). However, Saunders also brings up the idea that *G. vermiculophylla* made a stepping-stone passage through British Columbia to Europe as was also assumed for *Sargassum muticum*. Also Mexico could have been a possible stepping-stone, but it is difficult to assess if populations of *G. vermiculophylla* were brought to Mexico or to Europe first. *G. vermiculophylla* individuals in California (in the vicinity of the population in Mexico) and in France were both detected in the mid 90ies, so that this does not allow for further conclusions. With regard to general stress resistance, the population from Mexico differed from all other non-native populations (see Fig. 4 and discussion above). This could be a hint that populations from Mexico and from Europe were separated earlier during invasion history than the European populations. Through this isolation a differing selection could have occurred. However, larger differences between habitats – promoting divergent adaptation – might be responsible for the observed effects as well.

5.4 The consequences of *G. vermiculophylla* spreading in the Baltic Sea might be detrimental for the system – but are difficult to predict

G. vermiculophylla has now been present in the Baltic Sea for nearly ten years. During this time period it has managed to spread to several new sites and to establish persistent populations. Locally it was able to reach markedly high densities and after its decline in 2008 it seems to have recovered and ground coverages of up to 50% were again observed in Kiel Fjord in 2013. A further spread eastward into the Baltic Sea is likely, as the seaweed is able to tolerate low salinities (Weinberger et al. 2008).

The presence of *G. vermiculophylla* in high densities has detrimental impacts on both the growth of adult *F. vesiculosus* individuals and the survival of *F. vesiculosus* germlings. The main mechanism underlying this effect can be attributed to *G. vermiculophylla* covering *F. vesiculosus* under thick mats and thus reducing availability of light, oxygen and nutrients for the native seaweed. Furthermore, I was able to show that grazers associated with the invader pose an additional threat to *F. vesiculosus*.

DISCUSSION

G. vermiculophylla thus has the potential to negatively impact growth and survival of *F. vesiculosus* and by this not only harm the native seaweed itself, but also its associated community, especially epiphytic algae and sessile epiphytic animals. However, one crucial factor determining the effects of *G. vermiculophylla* is its local density (see paper IV). Based on our observations, it remains hard to tell if densities at certain sites will increase, decrease or remain stable. Some populations have been stable for years now, while others underlie strong dynamics (see section 1.1). Furthermore, I observed that *G. vermiculophylla* is no longer only occurring in its unattached form, but is also increasingly settling on hard substrate, which might intensify competition with *F. vesiculosus* germlings. Also global factors like rising water temperatures might influence the competitive situation in the Baltic Sea (Leppäkoski et al. 2002, Stachowicz et al. 2002), because *G. vermiculophylla*, unlike *F. vesiculosus*, grows even better at higher temperatures (Yokoya et al. 1999).

I can thus conclude that *G. vermiculophylla* does have the potential to interfere with the development of *F. vesiculosus* in the Baltic Sea; however, predictions about actual consequences of the invader's establishment and spread remain difficult, since they depend on many factors that are difficult to assess.

6. Conclusion

On the importance of testing multiple populations of a species in its native and its invaded range

The results of my experiments show that it can be of great importance to study 1) invasive and source populations of the same species, and to 2) not restrict the investigations to merely one invasive and one native population, but to use several populations that have been sampled widely across each range. The execution of common garden-like studies in 3) both the native and the introduced range allows for a better interpretation of stress-related traits, as results of experiments can strongly depend on the environment where they have been conducted.

A comparison between an invasive population and its putative donor population allows to exclude traits that are generally present in the species and thus to identify traits that are really specific for the invasive ecotype. In the case of *G. vermiculophylla* heat tolerance is obviously a trait that was not strongly developed in the native range, but is present in all tested invasive populations, indicating that resistance towards heat stress was under selection during the invasion process. However, if I had only investigated the seaweed in its introduced range, I could have drawn the conclusion that *G. vermiculophylla* is generally very heat resistant.

Moreover, some traits do not only differ between all native and all invasive populations, but also between single populations within each range. The outcome of my feeding experiment is a good example stressing the importance of studying more than one native and one invasive population of a species in order to be able to understand certain mechanisms underlying the invasion process. In this case, I was able to show that a low palatability is not only a trait of invasive populations of *G. vermiculophylla*, but that it is also present in some native populations. Knowing this, I can conclude that the invasion success of *G. vermiculophylla* might have been facilitated by the introduction of herbivory-resistant genotypes.

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9. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation mit dem Titel „Invasion Ecology of Marine Macroalgae: The relevance of stress resistance for the invasion success of *Gracilaria vermiculophylla* and consequences of its spread“ selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt und habe die Dissertation nach den Regeln guter wissenschaftlicher Praxis (Standard wissenschaftlichen Arbeitens nach den Empfehlungen der DFG) verfasst.

Kiel, den _____

Invasion Ecology of Marine Macroalgae

Dissertation Mareike Hammann 2014