

In situ common garden assays demonstrate increased defense against natural fouling in non-native populations of the red seaweed *Gracilaria vermiculophylla*

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Abstract The susceptibility of native and non-native populations of the red alga *Gracilaria vermiculophylla* to fouling was compared in common garden experiments. Native and non-native algae were enclosed into dialysis membrane tubes, and the tubes were exposed to natural fouling. Fouling on the outside of the tubes was mediated by chemical compounds excreted by *G. vermiculophylla* that diffused through the membranes. Fouling pressure was significantly higher in the Kiel Fjord (non-native range) than in Akkeshi Bay (native range), but, at both sites, tubes containing non-native *G. vermiculophylla* were less fouled than those with native conspecifics. This is the first in situ evidence that susceptibility to fouling differs between native and non-native populations of an aquatic organism. The technique of enclosing organisms into dialysis tubes represents a simple, efficient

and accurate way to test chemical antifouling defenses and could possibly be applied to other organisms.

Introduction

Human-mediated introductions of marine non-native species constitute considerable threats to coastal ecosystems and related economies, such as aquaculture and fisheries, by affecting species diversity, ecosystem functions and services (Lubchenco et al. 1991; Schaffelke et al. 2006; Vitousek et al. 1996; Williams and Smith 2007). To manage such problems, it is necessary to understand the mechanisms that allow non-native species to succeed in new environments (Cacabelos et al. 2010; Johnson and Chapman 2007; Vermeij et al. 2009).

Several hypotheses have been proposed that could explain which mechanisms promote bioinvasions. One of the most widely discussed ones is the enemy release hypothesis (ERH) (Keane and Crawley 2002). This concept states that non-native species have an advantage over native species because they escape their natural coevolved enemies of their

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native range and are not recognized by enemies, such as predators or parasites, in the new range. The evolution of increased competitive ability hypothesis (EICA) (Blossey and Nötzold 1995), an amendment of ERH, suggests that non-native species could, for the aforementioned reasons, invest more energy into growth rather than warding-off predators and parasites. This would make them more competitive than native species. Both concepts, ERH and EICA, emphasize that the successful invasions by non-native species may depend on the release from coevolved enemies. In contrast, the novel weapons hypothesis (NWH) (Callaway and Ridenour 2004) suggests that non-native species are less sensitive to enemies in the invaded habitat. This is because they possess defenses which are highly efficient since the resident enemies are not adapted to them.

Most theories that seek to identify drivers of invasion success in plants or animals have been tested in terrestrial environments (Colautti et al. 2004; Keane and Crawley 2002), while less tests have been conducted in the marine realm (Wikström et al. 2006). Among the tested examples, the majority of cases are terrestrial plant–herbivore and marine seaweed–herbivore systems (Lake and Leishman 2004; Parker et al. 2006; Stastny et al. 2005; Vermeij et al. 2009). In contrast, much less is known about the effect of foulers on the invasion success of aquatic macrophytes (but see Strong et al. 2009; Svensson et al. 2013), despite the fact that epibiosis can severely impair the performance of host organisms (basibionts) (Wahl 2008). For instance, a biofilm can alter the chemical conditions at the host interface through its metabolic activities (Thevanathan et al. 2000) and even insulate the host surface from the vital resource light (Costeron et al. 1987). Filamentous epiphytes may increase drag, decrease flexibility (Hemmi et al. 2005) and compete with the host organisms for light and nutrients (Buschmann and Gómez 1993; Honkanen and Jormalainen 2005; Wahl 1989). Some epizoans weaken the host surface and thus enhance the success of predation by crushing predators (Bach et al. 2006; Buschbaum et al. 2007). Therefore, the host organisms need to develop efficient physical or chemical defenses to minimize colonization of their body surfaces. As a consequence, ecological theories such as ERH, EICA, or NWH may be valid for aquatic host–epibiont systems and the present study aimed at testing this in situ with a non-native red alga, *Gracilaria vermiculophylla* (Ohmi) Papenfuss.

Gracilaria vermiculophylla, originating from the cold and warm temperate Northwest Pacific Ocean (Tseng and Xia 1999), has invaded many coastal habitats in the northern hemisphere from the eastern Pacific (Bellorin et al. 2004; Saunders 2009) to the mid-western Atlantic (Freshwater et al. 2006; Thomsen et al. 2006) and the eastern Atlantic (Guillemin et al. 2008; Sfriso et al. 2010; Weinberger et al. 2008). It has been suggested that the biotic and abiotic conditions in the recipient area may be critical for the

establishment and spread of introduced marine organisms (Nyberg and Wallentinus 2009; Streftaris et al. 2005). Correspondingly, numerous studies have revealed that various intrinsic traits, such as the reproductive versatility (Krueger-Hadfield et al. 2016) and the ability to tolerate low light conditions, extreme temperatures (Hammann et al. 2016b), starvation (Nyberg and Wallentinus 2009), salinity (Nejrup and Pedersen 2012; Weinberger et al. 2008), ultraviolet radiation (Roleda et al. 2012) and grazers (Hammann et al. 2013) are important factors that may affect the invasion success in *G. vermiculophylla*. Moreover, previous studies showed that chemical defenses that protect *G. vermiculophylla* from generalist herbivores might also allow the alga to invade new habitats (Hammann et al. 2016a; Rempt et al. 2012). Further, a recent study that compared the susceptibility of native and non-native populations of *G. vermiculophylla* under identical conditions in laboratory bioassays demonstrated that non-native populations of this seaweed were better defended against two tested foulers than native conspecifics (Wang et al. 2016). This suggested for the first time that epibionts may compromise the performance of non-native seaweeds in their new environments to such an extent that more resistant individuals can have a selective advantage, similar as predicted by the NWH for resistance to grazing (Callaway and Ridenour 2004). However, in nature, seaweeds are usually not exposed to single foulers, but to communities of various epibionts that are very diverse and highly dynamic and it is an open question whether non-native algal individuals would be also more resistant toward such assemblages than native individuals. Therefore, the present study focused on testing whether native and non-native populations of *G. vermiculophylla* differ in their susceptibility to natural fouling in situ.

For this study, four native *G. vermiculophylla* populations were sampled in East Asia in two adjacent ecoprovinces (following the concept suggested by Spalding et al. (2007)): the cold temperate Northwest Pacific [ecozones: Yellow Sea (China) and Northeastern Honshu (Japan)] and the warm temperate Northwest Pacific [ecozone: Central Kuroshio Current (Japan)]. Furthermore, algal individuals from three non-native populations were sampled within the Northern European Seas ecoprovince [ecozones: Baltic Sea (Germany), North Sea (Germany) and Celtic Seas (France)]. In addition, one non-native population from outside Europe was included, which is located in the cold temperate Northeast Pacific (ecoprovince: Oregon, Washington, Vancouver coast and shelf). The objective was to expose individuals from all populations to the same natural fouling and to compare their resistance. Thus far, no biogeographic comparisons ever tested directly whether susceptibility to natural fouling differs among aquatic organisms originating from different ecological zones or even ecoprovinces. This is because the methodological challenge is considerable: To directly expose organisms originating from different

populations to the same natural fouling pressure, they need to be released into the same environment, which would be unethical. To overcome this difficulty, algal specimens of different origin were in the present study individually enclosed into dialysis tubes and in this form exposed in the sea. In addition, the intensity of natural fouling pressure in native and non-native habitats was also compared.

Materials and methods

Experiment 1: Monitoring of fouling pressure on *Gracilaria vermiculophylla* and on artificial substrata in native and non-native habitats

To compare the total abundance of foulers and the composition of fouling communities on *G. vermiculophylla* between native and non-native populations of the alga, exposure trials were conducted in the Kiel Fjord (54°19'48.5"N 10°8'58.8"E), Germany (non-native range of *G. vermiculophylla*) and in Ailian Bay (37°10'22.4"N 122°34'38.5"E), Rongcheng, China (native range of *G. vermiculophylla*), respectively, from May to July 2014. Each month 15 intact individuals of *G. vermiculophylla* were collected manually from the shallow subtidal in both sites. During transport from the collection sites to the respective nearby laboratory, algal individuals were kept separately in 3 l plastic bags, which were placed in cooler boxes.

Prior to the trials, all visible fouling organisms were removed from the surface of *G. vermiculophylla* with a soft brush that did not damage the host. During the cleaning, the algae remained submersed in seawater to prevent desiccation. Five grams of each algal individual was put into a standardized net bag made of polypropylene with a mesh size that allowed foulers (such as juvenile invertebrates) to enter the bags (Novanet Kunststoff, maximum length: 20 cm, maximum width: 10 cm, mesh width: 9 mm). In order to assess the fouling pressure on non-living surfaces in the respective study area, 15 PVC panels (10 × 10 cm)

roughened with 60 grit sandpaper were exposed at the same site as the algae and retrieved monthly during the time of the trials.

For exposure, PVC panels and net bags filled with algal individuals were paired one to one and each pair was tied to a single rope with a distance of 5 cm between plate and bag. A stone was tied to each PVC panel to stabilize it in the water column and to ensure that it remained vertically orientated. Thereafter, each pair was deployed at a depth of 0.5 m. Fouling organisms that established on the panels as well as on the living algae during the course of 1 month were fixed in a 4% formalin–seawater solution, and their abundance and composition were then identified and quantified using a stereomicroscope. Coverage by fouling species on the panels and on *G. vermiculophylla* was assessed as percentage ranging from zero to 100%. Foulers were identified to the lowest possible taxonomic level, but due to the small size of many recruits, taxonomic resolution was often restricted to the class.

Experiment 2: Susceptibility to in situ fouling in native and non-native *Gracilaria vermiculophylla*

Individuals of *G. vermiculophylla* were collected at two occasions between June and August 2015 at eight sites located in five different countries within the native and the non-native range of the species (Table 1). All sampling areas were shallow bays and estuaries. Although the algal material was collected at eight different sites, the common garden field experiments were performed at only two locations: At the institute's pier of GEOMAR, Kiel Fjord, Germany, in June and August and at the pier of the Akkeshi Marine Station, Akkeshi Bay, Japan, in July 2015 (due to technical and financial constraints, experiments 1 and 2 could not be conducted at the same location in the native range). To compare susceptibility to in situ fouling between native and non-native *G. vermiculophylla* populations, living algal specimens from all sampling sites were transferred to both Kiel and Akkeshi. For transport, algal individuals were

Table 1 Geographic locations of the sampling sites and timing of sampling events for native and non-native populations of *Gracilaria vermiculophylla*

Origin	Collection site	Geographic coordinate	First sampling	Second sampling
Native	Rongcheng, China, Yellow Sea	37°9'4.29"N, 122°33'35.60"E	05.06.2015	16.08.2015
	Qingdao, China, Yellow Sea	36°3'0.6"N, 120°20'59.1"E	08.06.2015	18.08.2015
	Akkeshi, Japan, Northeastern Honshu	43°1'25.80"N, 144°52'47.20"E	12.06.2015	13.08.2015
	Tokyo, Japan, Central Kuroshio Current	35°19'25.72"N, 139°38'8.30"E	11.06.2015	08.08.2015
Non-native	Kiel, Germany, Baltic Sea	54°21'9.7"N, 10°8'34.2"E	19.06.2015	19.08.2015
	Nordstrand, Germany, North Sea	54°29'10.0"N 8°48'44.8"E	18.06.2015	19.08.2015
	Pouldouran, France, Celtic Seas	48°45'57.30"N, 3°12'2.50"W	16.06.2015	22.08.2015
	Port Moody, Canada, Vancouver Coast	49°16'47.99"N, 122°51'6.08"W	–	30.07.2015

individually packed in plastic bags with seawater-moistened paper tissue, and these bags were then placed in cooling containers. The length of transport, even between Kiel and Akkeshi, never exceeded 5 days. In June 2015, for equipping the field experiments in Kiel (conducted in June) and Akkeshi (conducted in July), specimens from all populations were transferred to Kiel first. Then, half of the individuals from all populations outside Japan were transported from there to Akkeshi by air cargo. After their arrival, the algae were carefully inspected, but no loss was observed due to transportation stress. Populations inside Japan were also sampled in June 2015, and the collected algal individuals were transported to Akkeshi directly. For the experiments conducted in Kiel in August 2015, which were not repeated in Japan, algal individuals from all sampling sites were transferred to the laboratory in Kiel directly. In all experiments, algal specimens were acclimatized to the locally prevailing abiotic conditions (water temperature and salinity) for at least 1 week prior to exposure in the field. In Kiel, this took place in a climate room with constant water temperature (15 °C) and light (30 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in a 12-h light–dark cycle. The algal material was kept in permanently aerated 14 l aquaria filled with seawater from the Kiel Fjord (salinity 15–18), which was completely renewed by an automatic seawater flow-through every 3.5 h (flow-through rate 50 ml/min). Seaweeds sampled under high salinity conditions (e.g. in Rongcheng and Qingdao) were acclimatized to the salinity conditions in Kiel Fjord over the course of several days by decreasing salinity by two units per day over a period of 1 week. In Akkeshi, algae were kept individually in separate beakers with aeration, which were placed in a climate room at 18 °C and with light conditions of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12-h light–dark cycle. The seawater used for algal cultivation was obtained from the nearby Akkeshi Bay (salinity 30–33) and was exchanged daily. Seaweeds from low salinity environments (Baltic Sea and Pouldouran estuary) were slowly adapted to the condition in Akkeshi Bay by increasing salinity by two units per day over a period of 1 week.

Algae from all populations (replication $n = 5$ per population), as well as a control group of five algal mimics, i.e. bundles of cut black plastic cable ties in a size similar as the algal individuals, were exposed to natural colonization by epibionts. To prevent the release of spores, fragments or microorganisms associated with *G. vermiculophylla* into the water column, algal individuals (5 g each) and algal mimics were put into dialysis membrane tubes (Spectra/Por® 6 membrane, MWCO: 1 kD), which are manufactured from natural cellulose reconstituted from cotton linters. The dialysis membrane allows small molecules with a molecular weight of less than approximately 1 kD to pass, but it holds back the much larger *G. vermiculophylla* spores and microorganisms as well as bigger molecules like proteins. Each of these tubes was then fixed at a water depth of 0.5 m to a vertically

orientated rope, while a small stone was tied to the end of each rope to ensure vertical orientation and to stabilize it in the water column. A distance of 1 m was kept between the single ropes. Pretests had indicated that neither dialysis tubes nor *G. vermiculophylla* show signs of degradation when they are exposed under such conditions for 4 weeks. Exposure time during experiment 2 was 14 days in the Kiel Fjord and 18 days in Akkeshi Bay, and again, no signs of algal stress like change in pigmentation or necrosis were observed. After exposure, the dialysis membrane tubes were retrieved from the water and unfolded for inspection under a stereomicroscope. Settlement of fouling organisms on the lateral area was quantified by estimating percent cover for diatoms and by counting the number of individuals in case of solitary foulers. These data were obtained from three circular plastic frames (\varnothing 1 cm) per tube, which were placed randomly on the unfolded membranes. Furthermore, the total abundance of fouler species was estimated as percent cover within one randomly placed frame (6 × 6 cm) that was placed on each membrane.

Statistical analyses

Compositions of fouling communities (experiment 1) were statistically compared by one-factorial analysis of similarity (ANOSIM) and by non-metric multidimensional scaling ordination (nMDS) using PRIMER 6. Prior to the analyses, data points were excluded in case the fouling abundance of a sample was zero and in case the average abundance of one fouler across all samples was smaller than 0.5%. All further statistical and graphical analyses were done using the free statistical computing software R. Mixed-effect modeling was used for analyzing the total fouling coverage (experiment 2). The modeling included two fixed factors: (1) ‘*Gracilaria* origin’ with the levels ‘native’ and ‘non-native’ and (2) ‘exposure site’ with the levels ‘Japan’ and ‘Germany.’ Furthermore, the various sampling sites of *G. vermiculophylla* were included as a random factor nested in ‘*Gracilaria* origin.’ The abundances of the single fouler species were analyzed in separate approaches with one-way designs with the factor ‘*Gracilaria* origin’ with the levels ‘native’ and ‘non-native’ (experiment 2). For this, we used either generalized linear models with Poisson family or the nonparametric Kruskal–Wallis rank sum test. The total abundance of foulers (experiment 1) was compared between (a) the experimental sites (Germany vs. China) and (b) the substrata (*G. vermiculophylla* vs. PVC panels) with the nonparametric Mann–Whitney *U* test. The *t* test was used to compare the total fouling coverage on dialysis tubes containing mock substrates in both exposure sites. Homogeneity of variances was checked graphically on the base of boxplots or residual plots, while normality of errors was verified by histograms of the residuals and by Shapiro–Wilk’s *W* test.

Results

Experiment 1: Monitoring of fouling organisms on *Gracilaria vermiculophylla* and artificial substrata

Overall, in the Kiel Fjord, six different taxonomic classes were identified on both living (*G. vermiculophylla* thalli) and non-living (PVC panels) substrata. In Rongcheng, four classes were identified on *G. vermiculophylla* individuals, while seven classes were recorded on PVC panels (Fig. 1). Also, the taxonomic groups observed on PVC panels and on *G. vermiculophylla* in the same site differed considerably (Fig. 1). In both Kiel and Rongcheng, one of the two most abundant taxa on *G. vermiculophylla* was Ciliates. The most abundant group on *G. vermiculophylla* in Rongcheng were red algal epiphytes (Florideophyceae, primarily of the genera *Ceramium* and *Polysiphonia*), while the second most

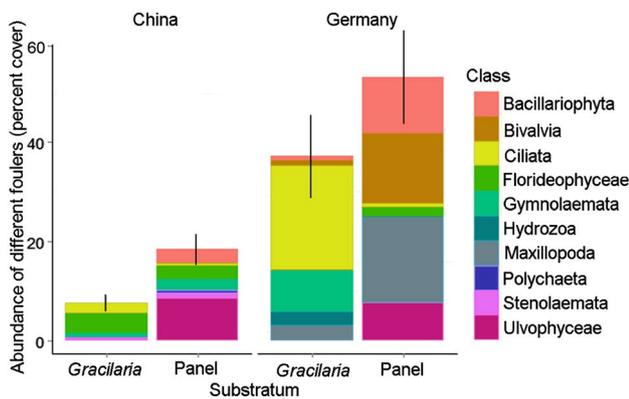
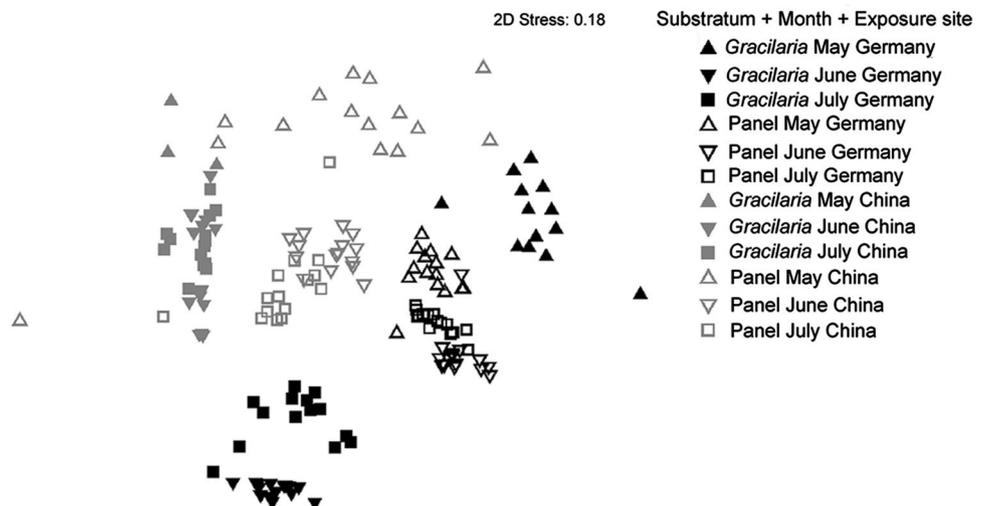


Fig. 1 Composition of fouling communities that established on *Gracilaria* individuals and on PVC panels at sites in the native (China) and non-native (Germany) range of *Gracilaria vermiculophylla* averaged over the three trials in May, June and July 2014. Means and 95% CIs ($n = 45$)

Fig. 2 Similarity between fouling communities that established on *Gracilaria* individuals and on PVC panels at sites in the native (China) and non-native (Germany) range of *Gracilaria vermiculophylla* in May, June and July 2014 ($n = 15$) determined by multidimensional scaling



abundant group on *G. vermiculophylla* in Kiel were Bryozoans of the class Gymnolaemata. In contrast, PVC panels in Rongcheng were dominated by green algal epiphytes (primarily of the genus *Ulva*) and diatoms. These groups were also abundant on PVC panels in Kiel, but the most abundant groups on PVC panels in Kiel were bivalves of the genus *Mytilus* and barnacles of the genus *Amphibalanus*—two groups that were completely absent in Rongcheng.

Interestingly, averaged across both substrata, the total abundance of foulers was higher in Kiel than in Rongcheng (Fig. 1, $W = 1172$, $P = 8.17e-13$, Mann–Whitney– U test). The total abundance of foulers was generally lower on *G. vermiculophylla* individuals than on PVC panels, and this was the case at both study sites (Fig. 1, $W = 2798$, $P = 0.001$, Mann–Whitney U test). In Kiel, this difference was 16% (*Gracilaria* $37 \pm 25\%$ and PVC panels $53 \pm 30\%$, mean \pm SD), and Bacillariophyta, Bivalvia and Maxillopoda were consistently less abundant on *Gracilaria* than on panels. In Rongcheng, the difference was about 10% (*Gracilaria* $8 \pm 5\%$ and PVC panels $18 \pm 14\%$), and Ciliata and Florideophyceae were consistently more abundant, while Bryozoans of the class Gymnolaemata were consistently less abundant on *Gracilaria* than on panels.

Across both exposure sites and all months, the compositions of fouling communities on *G. vermiculophylla* and on PVC panels (factor ‘Substratum’) were significantly different, but there was a certain overlap as indicated by ANOSIM ($R = 0.312$, $P = 0.001$, Fig. 2). Across both substrata and all months, the composition of fouling communities in Kiel differed significantly from that in Rongcheng, and there was also a certain overlap (ANOSIM: $R = 0.538$, $P = 0.001$, Fig. 2). Across both substrata and exposure sites, the composition of fouling communities was not very different among months (ANOSIM: $R = 0.239$, $P = 0.001$, Fig. 2). In Germany, however, the composition of fouling communities on *G. vermiculophylla* in May was very different from that in

June and July, and the same was true for fouling communities on PVC panels in China (Fig. 2).

Experiment 2: Susceptibility to in situ fouling in native and non-native *Gracilaria vermiculophylla*

Overall, compared to Akkeshi Bay, the composition of fouling communities on dialysis tubes exposed in the Kiel Fjord (Fig. 3) was more similar to that observed on PVC panels than to that observed on *G. vermiculophylla* exposed at the same site in experiment 1 (Fig. 1). It consisted of diatoms, bivalves of the genus *Mytilus*, barnacles of the genus *Amphibalanus*, green algae of the genus *Ulva* and red algae of the genus *Ceramium*. This contrasted with the site in Japan, where bivalves, barnacles and *Ulva* were absent, but sessile polychaetes were observed in addition to diatoms and red algae of the genus *Ceramium* (Fig. 3).

The mean abundance of foulers on dialysis tubes containing native or non-native *Gracilaria* or control mock substrates was always higher in Germany than in Japan (Fig. 3). This difference was not statistically significant when only tubes containing mock substrates were considered (*t* test, $P = 0.2852$), which was due in part to limited numbers of replication of such tubes ($n = 5$ in Japan and $n = 10$ in Germany). However, dialysis tubes containing *G. vermiculophylla* were significantly more fouled in Germany than in Japan (Fig. 3; Table 2). Further, all fouling experiments revealed that dialysis membrane tubes that contained algal individuals from non-native populations of *G. vermiculophylla* were less fouled than those filled with algae from native populations. The size of the difference was, on average, 6%, and it was statistically significant (Fig. 3; Table 2); less than 0.001% of the unexplained variation was found to be actually covered by the random factor ‘site.’ No

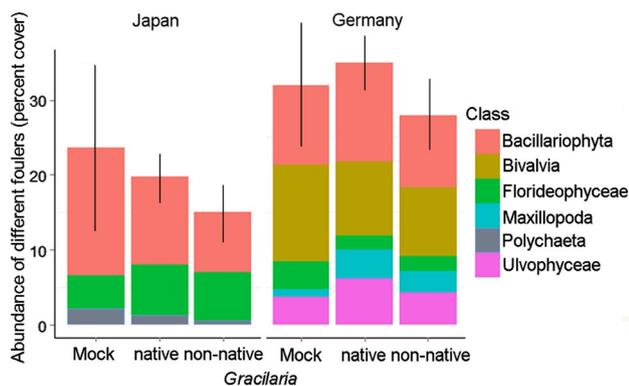


Fig. 3 Composition of fouling communities that colonized dialysis tubes filled with mock, native and non-native *Gracilaria vermiculophylla*. Tubes were exposed to natural fouling once in Japan (Akkeshi Bay) and twice in Germany (Kiel Fjord) in June to August 2015. Means and 95% CIs ($n = 5-40$)

Table 2 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) and the site of exposure (native/Japan vs. non-native/Germany) on natural fouling rates on dialysis membrane tubes containing living algal individuals

Source of variation	numDF	denDF	F value	p value
<i>Gracilaria</i> origin	1	6	9.7881	0.0204
Exposure site	1	100	38.3475	<0.0001
Origin: site	1	100	0.3064	0.5811

Results from mixed-effect modeling. *numDF* degrees of freedom in the numerator, *denDF* degrees of freedom in the denominator

interaction was detected between ‘*Gracilaria* origin’ and ‘exposure site’ (Table 2).

In June, five fouling species (*Mytilus edulis*, *Amphibalanus improvisus*, *Ulva* sp., *Ceramium tenuicorne* and diatoms) were found on the dialysis tubes that were exposed in the Kiel Fjord (Fig. 4). With the exception of *C. tenuicorne*, the differences in fouling rates between tubes with native and non-native *G. vermiculophylla* were statistically significant: *M. edulis*, *A. improvisus*, *Ulva* sp. and diatoms settled by 56, 73, 15 and 8%, respectively, less on dialysis tubes with non-native individuals than on those with native conspecifics (Table 3). In August, four fouling species (*A. improvisus*, *Ulva* sp., *C. tenuicorne* and diatoms) were present on the dialysis tubes in the Kiel Fjord (Fig. 5). All of them settled significantly less on tubes with non-native *G. vermiculophylla* individuals: *A. improvisus* by 59%; *Ulva* sp. by 58%; *C. tenuicorne* by 52%; and diatoms by 12% (Table 3).

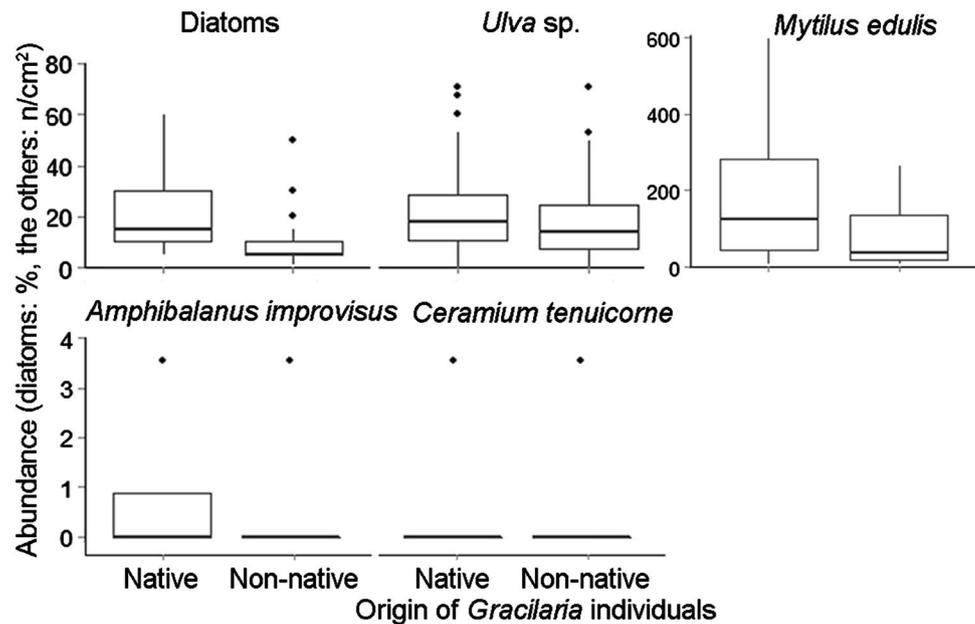
In Akkeshi, three fouling species (*Circeis spirillum*, *Ceramium kondoi* and diatoms) were recorded on the dialysis membrane tubes filled with *G. vermiculophylla* (Fig. 6). Diatoms and *C. kondoi* settled significantly less (by 3 and 18%) on tubes containing non-native *G. vermiculophylla*, while the difference for *C. spirillum* (50% less on non-native *G. vermiculophylla*) was marginally significant (Table 4).

Discussion

Differences in fouling pressure between locations in *Gracilaria*’s native and non-native range

The monitoring of field fouling pressure revealed that both substrata—living *G. vermiculophylla* individuals and PVC panels—were more heavily fouled in the Kiel Fjord, Germany, (non-native range of *G. vermiculophylla*) than in Ailalian Bay, Rongcheng, China (native range). Also in the field fouling experiments with dialysis tubes containing *G. vermiculophylla*, less fouling was observed in the native range (Akkeshi Bay, Japan) than in the non-native range (Kiel Fjord, Germany). Thus, both sets of experiments provided similar results, although the native study sites were located

Fig. 4 Abundances of different foulers that colonized dialysis tubes filled with individuals of *Gracilaria vermiculophylla* from either native or the non-native populations of the alga. Tubes were exposed to natural fouling in Germany (Kiel Fjord) in June 2015. The abundance of diatoms was determined as % of substrate surface covered, and the abundance of other groups was determined by counting. Boxplots show medians, interquartiles and outliers ($n = 15\text{--}20$)



in different ecozones. This suggests that, at least during summer, *G. vermiculophylla* faces a more severe fouling pressure in its new environment in the Kiel Fjord.

There are various possible explanations for the higher fouling pressure in the Kiel Fjord. First of all, it could be that the system there is generally more productive than the two Asian sites, due to higher nutrient concentrations in the eutrophic environment of the Western Baltic. A higher primary and secondary productivity would also mean that fouling organisms and their propagules occur in higher abundances and fouling rates should therefore be higher in eutrophic than in oligotrophic systems (Korpinen et al. 2007). However, nutrient concentration data available for the Kiel Fjord, Ailian Bay and Akkeshi Bay do not support the assumption that eutrophication is generally higher in the Kiel Fjord (suppl. Table S1). Much rather, the fact that the Kiel Fjord is a semi-enclosed environment without significant wave action and tides, while Rongcheng Bay and Akkeshi Bay are sea areas with heavy wave action and pronounced tidal amplitudes and turbulent stress, could explain the difference in fouling rates. Crimaldi et al. (2002) showed that turbulent stress events influence larval settlement success, and Koehl et al. (2013) found that wave action induced by ship wakes can reduce settlement rates by fouling organisms. It should be noted that the monitorings conducted to quantify the fouling pressure only considered one location in the non-native range of *G. vermiculophylla*. Therefore, it is uncertain whether sites in the non-native range are generally subject to more fouling pressure than sites in the native range of *G. vermiculophylla*. In any case, the finding in this study certainly contradicts and, therefore, falsifies the predictions of the ERH and EICA (Blossey and Nötzold 1995;

Keane and Crawley 2002), which both suggest that introduced species should experience a reduction in the diversity and the abundance of coevolved enemies in their new range compared to their native range. Indeed, most marine fouling organisms are relatively unspecific with respect to host choice (Wahl and Mark 1999), and in this light, the probability of release from specialized fouling organisms during invasions of aquatic organisms appears as relatively low.

***Gracilaria's* defense against fouling organisms is, at least partly, based on small molecules**

A second observation while monitoring the fouling pressure in the field was that foulers at both testing sites were generally more abundant on PVC panels than on *G. vermiculophylla* individuals and only a few groups of foulers (e.g. Ciliates) were more abundant on the alga. Although there are many differences, such as shape, between the two substrata, this finding suggests the presence of a physical and/or chemical antifouling defense in *G. vermiculophylla* against most foulers. A similar picture was reported by Rickert et al. (2015), who observed higher densities of the barnacle *A. improvisus* on PVC panels compared to the thalli of two nearby *Fucus* species. In an experimental approach, the authors could identify surface-bound metabolites as the reason for the deterrent effect that *Fucus* showed against the settlement of *A. improvisus*. Evidence of chemical defenses against algal macrofoulers has also been reported for *Gracilaria chilensis*, a species that is phylogenetically relatively closely related to *G. vermiculophylla* (Lion et al. 2006). Moreover, previous studies revealed that extractable

Table 3 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) on natural fouling rates on dialysis membrane tubes filled with individuals of the alga exposed to natural fouling in Germany (Kiel Fjord) in June and August 2015

Month	Fouler	Test	df	SS	MS	Chi squared	Deviance residuals	DF residuals	Deviance	F value	p value
June	<i>Mytilus edulis</i>	GLM Poisson	1				2245.9	103	15.115		2.2e-16
	<i>Amphibalanus improvisus</i>	Kruskal–Wallis rank sum test	1			5.9353					0.01484
	<i>Ulva</i> sp.	GLM Poisson	1				12.393	103	1331.6		0.000431
	<i>Ceramium tenuicorne</i>	Kruskal–Wallis rank sum test	1			2.8974					0.0887
	Diatoms	one-way ANOVA	num df = 1, den df = 103	9.94	9.937					17.2	
August	<i>Amphibalanus improvisus</i>	Kruskal–Wallis rank sum test	1			17.567					0.0277
	<i>Ulva</i> sp.	GLM Poisson	1				715.39	118	1787.2		2.2e-16
	<i>Ceramium tenuicorne</i>	GLM Poisson	1				55.273	118	524.07		1.05e-10
	Diatoms	Welch-adjusted one-way ANOVA	num df = 1, den df = 100.136						16.4181		0.0001

df degrees of freedom, SS sums of squares, MS mean squares

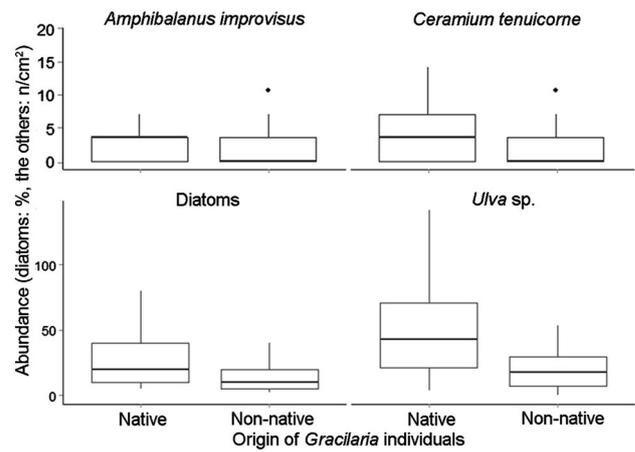


Fig. 5 Abundances of different foulers that colonized dialysis tubes filled with individuals of *Gracilaria vermiculophylla* from either native or the non-native populations of the alga. Tubes were exposed to natural fouling in Germany (Kiel Fjord) in August 2015. The abundance of diatoms was determined as % of substrate surface covered, and the abundance of other groups was determined by counting. Box-plots show medians, interquartiles and outliers ($n = 20$)

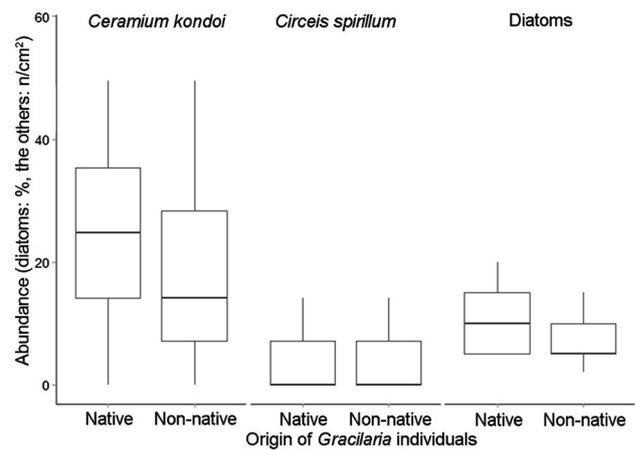


Fig. 6 Abundances of different foulers that colonized dialysis tubes filled with individuals of *Gracilaria vermiculophylla* from either native or the non-native populations of the alga. Tubes were exposed to natural fouling in Japan (Akkeshi Bay) in July 2015. The abundance of diatoms was determined as % of substrate surface covered, and the abundance of other groups was determined by counting. Box-plots show medians, interquartiles and outliers ($n = 15-20$)

surface-bound metabolites from *G. vermiculophylla* mediate the defenses of *G. vermiculophylla* against epibacteria (Saha et al. 2016), diatoms and *Ceramium* filaments (Wang et al. 2016). In contrast, physical antifouling defense strategies, such as epithallus sloughing and gelatinous or microstructured surfaces—that have been reported from some macroalgae (da Gama et al. 2014; Yamamoto et al. 2013)—were so far not observed in *G. vermiculophylla* by us or others. Nonetheless, given that

Table 4 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) on natural fouling rates on dialysis membrane tubes fouled with individuals of the alga exposed to natural fouling in Japan (Akkeshi Bay) in July 2015

Fouler	Test	df	Chi squared	Deviance residuals	DF residuals	Deviance	p value
<i>Circeis spirillum</i>	Kruskal–Wallis rank sum test	1	3.4495				0.0633
<i>Ceramium kondoi</i>	GLM Poisson	1		24.016	103	968.16	9.56e–04
Diatoms	Kruskal–Wallis rank sum test	1	9.963				0.0016

df degrees of freedom

the absence of evidence is no evidence of the absence, it cannot be excluded with certainty that physical defense mechanisms contributed to the difference in fouling rates that was observed between *G. vermiculophylla* thalli and non-living surfaces. However, the inclusion of *G. vermiculophylla* into dialysis tubes in experiment 2 not only prevented algal spores or associated microorganisms from entering the water column, but it also excluded any impact of physical antifouling defense mechanisms of *G. vermiculophylla* on foulers. The approach, thus, permitted us to evaluate the extent to which algal excretion of metabolites affects fouling organisms. Interestingly, the fouling communities on dialysis tubes containing *G. vermiculophylla* were more similar to fouling communities on PVC panels than to fouling communities that developed directly on the surface of the algae, and this could suggest that relevant physical defense mechanisms against foulers exist in *G. vermiculophylla*. However, algal specimens that originated from different populations affected the fouling communities on dialysis tubes in different ways. This not only indicates that chemical defenses exist, but it also strongly suggests that the capacity for such defenses varies among populations. Using dialysis membranes that contained living algae instead of artificial substrata coated with surface extracts as in our previous study (Wang et al. 2016) allowed us to assess the capacity of *G. vermiculophylla* for chemical antifouling defenses in a more accurate way. This is because extracted metabolites are usually subject to oxidation and other degradation processes, while living algae warrant for a relatively constant release of undegraded compounds. No signs that the algae suffered from being kept in tubes, such as a change in color or necrosis, were observed during the field fouling experiment.

Defense strength rather than avoidance by colonizers was responsible for the observed inter-population differences in fouling rates

The fouling experiments with living *G. vermiculophylla* thalli enclosed in dialysis membranes revealed that individuals from non-native populations of the alga were generally less susceptible to fouling than native conspecifics. This

was the case in both test areas. Therefore, since non-native algal individuals were exposed to natural fouling in both the native and the non-native range of *G. vermiculophylla*, we can exclude that a lower preference of native colonizers for the non-native macroalga was the sole reason for the observed picture. Thus, it is likely that there are differences in the composition and/or concentration of bioactive metabolites that were released from the surface of *G. vermiculophylla* and that those leached through the dialysis membranes. This result is consistent with the findings of a previous study (Wang et al. 2016), in which epiphytes originating from both distribution ranges of *G. vermiculophylla* exhibited less readiness to settle on surface extracts of non-native specimens than on surface extracts of native individuals.

However, it cannot be explained with certainty why the antifouling defense capacity of *G. vermiculophylla* changed during the invasion process. The simplest explanation is that fouling-resistant genotypes were selected during the invasion process, i.e. during transport and establishment in the new habitat, and that their frequency is, therefore, higher in non-native than in native populations. This scenario would be in agreement with theories that predict a selection of an increased defensive capacity during biological invasions, such as the NWH. In its non-native distribution range, *G. vermiculophylla* is mostly found in extremely sheltered lagoons and estuaries (Weinberger et al. 2008; Krueger-Hadfield et al. 2016). In contrast, populations in the native range—including Qingdao and Rongcheng—are often located on more wave-exposed rocky shores. As outlined above, wave exposure generally reduces fouling pressure, and in this light, the selection of an increased antifouling defense may have facilitated the settlement and spread of *G. vermiculophylla* in non-native environments with particularly high fouling pressure, such as the Kiel Fjord.

Alternatively, the increased antifouling capacity of non-native *G. vermiculophylla* could result from an adaptation to other biotic pressures, such as grazing. Non-native *G. vermiculophylla* populations are more strongly defended against herbivores than native populations (Hammann et al. 2013) and compounds that deter consumers are sometimes also active against epibionts. For example, in *Asparagopsis*

armata bromoform was shown to deter not only mesograzers, but also microsettlers (Paul et al. 2006a, b). Likewise, the defense of *G. vermiculophylla* against herbivores is at least partially due to a capacity for production of oxylipins from arachidonic acid after wounding (Hammann et al. 2016a) and those compounds were shown to also deter algal epibionts of Gracilarioids (Lion et al. 2006). Non-native populations of *G. vermiculophylla* were already shown to have a stronger capacity for production of oxylipins than native populations (Hammann et al. 2016a). However, oxylipin production requires activation by heavy wounding (Rempt et al. 2012), and although certain foulers of Gracilarioids (i.e. *Ceramium* species) can cause tissue wounding when they anchor themselves in the host (Leonardi et al. 2006), such wounding was excluded in this experiment: Direct, immediate contact of host and foulers was prevented by the dialysis tubes. Therefore, other compounds than oxylipins must be responsible for the deterrent effects observed in this study. Nevertheless, the increased capacity for production of those unidentified defense compounds in non-native populations of *G. vermiculophylla* could also provide additional defense strength against other biological enemies that are not foulers.

A third explanation could be that a release from other and more severe biotic pressures (e.g. grazing) in the new environment of the alga led to a reallocation of resources into antifouling defense (Blossey and Nötzold 1995). In Akkeshi, Japan, local populations of *G. vermiculophylla* were heavily grazed by the amphipod species *Caprella scaura* and *Ampithoe lacertosa* during summer 2015 and in Rongcheng, China, another *Caprella* species was also intensively feeding on *G. vermiculophylla* during summer and autumn 2014 (Wang pers. obs.). In contrast to this, in Kiel, Germany, the overall grazing pressure on *G. vermiculophylla* appears as generally lower than in Asia (Hammann et al. 2013). If this reduced feeding pressure generally applies to non-native habitats, then *G. vermiculophylla* in these habitats could possibly allocate more resources to defenses against generalist foulers than native populations (Blossey and Nötzold 1995). Since it has been reported that non-native *G. vermiculophylla* populations are more defended against herbivores than native populations (Hammann et al. 2013, 2016a), this scenario is not very likely.

Furthermore, it is possible that the more intense fouling pressure in the non-native habitat stimulated the higher antifouling defenses in *G. vermiculophylla*. Such a demand-driven antifouling defense regulation has been suggested by Saha and Wahl (2013) and Rickert et al. (2016). Saha and Wahl (2013) found that the anti-settlement activity of *F. vesiculosus* at two geographically distinct locations in Germany (Gelting and Poel) varied temporally, reaching a peak in summer/autumn, which was in phase with the density of bacterial cells in the plankton. Rickert et al. (2016) showed that in individuals of the two *Fucus* species *F. vesiculosus*

and *F. serratus*, which inhabit adjacent habitats in the Western Baltic Sea, the strength of a chemical defense against microfouling varied seasonally and that fluctuations in the defense level tend to match with fluctuations in microfouling pressure. However, such demand-driven defenses against fouling in *G. vermiculophylla* would require a highly sensitive sensing system, as direct contact between host and foulers was excluded in the experiments by the dialysis tubes.

The influence of scattering over a larger geographic scale in sampling sites on the within-range variability in antifouling defense

For the intra-specific comparison presented here, individuals of *G. vermiculophylla* were sampled from four different ecozones with the environmental conditions so different in the Baltic Sea, North Sea and Eastern Pacific within the non-native range of the species. However, antifouling defense primarily differed between ranges, while within-range variability of antifouling defense was low: Less than 0.001% of the unexplained variation went back to the random factor 'site.' Thus, non-native populations in Europe and at Port Moody (E Pacific) showed a similar antifouling defense capacity even though they probably were the result of two independent introduction events. This suggests that the observed picture could generally apply to non-native populations of *G. vermiculophylla*. More comparative studies considering non-native populations of *G. vermiculophylla* in other parts of the world are needed to confirm this assumption.

In conclusion, the fouling experiments with living algae described here give the first in situ evidence that individuals of *G. vermiculophylla* from non-native populations are generally less susceptible to natural fouling than native conspecifics. This is true regardless of whether the non-native algal individuals are exposed in the native or in the non-native range of *G. vermiculophylla*. This indicates that the observed difference goes back to the defense properties of the algae and not to the fact that the foulers present were not able to recognize the algae as a suitable settlement substratum. Since all surface properties of the alga were excluded by enclosing them in membrane tubes, it is clear that the antifouling properties were mediated by chemical compounds, which were released by the algae and were able to pass through the membrane. However, so far there is no information about which chemical compounds are involved in this. Finally, this is the first study in which living algae enclosed in dialysis membrane tubes were exposed in the field to assess natural fouling rates. This technique represents a more simple, efficient and accurate way to test chemical antifouling defenses in seaweeds than using artificial substrata coated with extracts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Data accessibility All experimental data underlying this publication are available from the PANGAEA repository (doi: <https://doi.pangaea.de/10.1594/PANGAEA.865280>).

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