Anti-epiphyte defenses in the red seaweed *Gracilaria vermiculophylla*: non-native algae are better defended than their native conspecifics

Running headline: Epiphytes on non-native *Gracilaria vermiculophylla*

Shasha Wang[1*¶], Gaoge Wang[2¶], Florian Weinberger[1], Dapeng Bian[3], Masahiro Nakaoka[4] and Mark Lenz[1]

[1] Department of Benthic Ecology, GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel, 24105 Kiel, Germany

[2] College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

[3] Xunshan Group Co., Ltd, Rongcheng 264316, China

[4] Akkeshi Marine Station, Field Science Center for Northern Biosphere, Hokkaido University, Akkeshi, Hokkaido 088-1113, Japan

[*]Corresponding author: Shasha Wang

E-mail: swang@geomar.de

Fax: +49 431 600 1671

[¶]These authors are both first authors and contributed equally to this work.

Summary

1. Epibiosis in the marine environment is a stressor that may determine invasion success in introduced species. Previous comparisons showed resistance to epibionts can be higher in non-native than in resident seaweed species, but we do not know whether it is an intrinsic trait of the non-natives or it has been acquired during the invasion process. To elucidate this question, a comparison between native and non-native populations of the same species is needed.

2. Resistance against two groups of epiphytes was assessed in living thalli and in artificial substrata coated with surface extracts, both gained from four Asian (native) and four European (non-native) populations of the red alga *Gracilaria vermiculophylla*. Two diatom species and two filamentous macroalgae were used as micro- and macro-epiphytes, and one of each type was collected in Asia, while the other came from Europe. Laboratory assays were done in both distributional ranges of *G. vermiculophylla* and in different seasons. We used *G. vermiculophylla* from four populations in each range and used a fully-crossed design with the factors a) 'Origin of *Gracilaria*', b) 'Origin of epiphytes', c) 'Season' and d) 'Solvent used for extraction'.

3. Both groups of epiphytes, regardless of their origin, attached less to living thalli and to surface extracts from non-native *G. vermiculophylla*. Fewer diatoms attached to hexane-based extracts, while fewer *Ceramium* filaments settled on extracts gained with dichloromethane.

4. *Synthesis*. Our results show for the first time that non-native individuals of a marine organism are better defended against epiphytes than native conspecifics. Furthermore, we found evidence that at least a part of the defense is based on extractable secondary metabolites. We discuss several mechanisms that could explain the increased resistance to epiphytes in non-native individuals, including the release from enemies in the non-native range, which could lead to an

increase in algal performance during the invasion process. We suggest that an enhanced defense against epiphytes after introduction is one reason for *G. vermiculophylla*'s invasion success. Our observation may also apply to other basibiont-epibiont and host-enemy systems, including plant-plant, plant-animal and animal-animal interactions, in aquatic environments and could be a key feature of bioinvasions.

Key-words: anti-fouling, biological invasions, chemical defense, Enemy Release Hypothesis, epiphytes, fouling, *Gracilaria vermiculophylla*, invasion ecology, non-native seaweeds

Introduction

Biological invasions are an important component of global change (Mack *et al.* 2000; Ricciardi 2007) and can cause severe ecological or economic problems by altering local biodiversity and affecting the services of ecosystems (Pimentel, Zuniga & Morrison 2005; Williams & Smith 2007; Vilà *et al.* 2011; Newton *et al.* 2013; Paini *et al.* 2016). Therefore, bioinvasions in the aquatic and terrestrial realm are receiving attention by scientists, authorities and environmental managers worldwide (Torchin & Mitchell 2004; Olenin *et al.* 2014). This growing awareness is needful, since the number of successful invasions is rising rapidly. In the marine environment, for instance, it increases exponentially and about 20% of the invasions are due to the spread of macroalgae (Schaffelke, Smith & Hewitt 2006).

To manage invasive species and to alleviate their negative effects, it is necessary to understand the mechanisms that determine the success or failure of invasions: Why do certain species invade new habitats successfully while others fail? Which factors allow the establishment and spread of introduced species? Currently, several hypotheses suggest explanatory concepts for these questions. Among them, the Enemy Release Hypothesis (ERH) is one of the most well-known (Keane & Crawley 2002). It states that when species are introduced into new habitats they are confronted with an abiotic and biotic environment that can be substantially different from the one they adapted to over evolutionary time scales. If co-evolved antagonists are absent in the new habitat and resident predators or parasites cannot recognise the newly introduced species as a resource, a release of the introduced species results from enemy control (Vermeij *et al.* 2009; Cacabelos *et al.* 2010). Furthermore, the Evolution of Increased Competitive Ability Hypothesis (EICA), which is an extension of the ERH, argues that non-native species that are released from their native enemies do not need to defend at all or, at least, less than in their home range. This

release would allow them to invest more energy into growth, reproduction or into tolerating environmental stress (Hierro, Maron & Callaway 2005; Joshi & Vrieling 2005; Lenz *et al.* 2011), what, in turn, should make them competitively superior to native species (Blossey & Nötzold 1995; Müller-Schärer, Schaffner & Steinger 2004). Finally, the "Novel Weapons"-hypothesis by Callaway and Ridenour (2004) suggests that non-native species should be less susceptible to resident enemies than native ones, because they possess biochemical defense mechanisms to which native antagonists have not adapted to so far.

Most if not all marine organisms are prone to colonization by sessile life forms (epibionts), including bacteria, protists, microalgae, macroalgae and invertebrates. This phenomenon is known as epibiosis and it can severely impair the performance of the host organisms (basibionts) (Wahl 2008; Thomsen *et al.* 2012). It has been reported that epibionts can affect the growth and survival of macroalgal hosts by a) limiting the uptake of oxygen, carbon dioxide and nutrients through the thallus surface, b) reducing the amount of light available for photosynthesis, c) physically inhibiting sporulation, d) decreasing thallus flexibility, and/or e) increasing the palatability of the thallus (Wahl, Hay & Enderlein 1997; Hemmi *et al.* 2005). Macroalgae therefore need physical or chemical defenses to minimize colonization of their surfaces.

All the hypotheses described above should also hold true for host-epibiont interactions, but have so far been widely neglected as a factor determining invasion success. So far, studies that tried to elucidate the relevance of host-enemy interactions for biological invasions mainly focused on non-native spermatophytes and their antagonists as well as their competitors in terrestrial systems (Keane & Crawley 2002; Verhoeven *et al.* 2009), while less research has been done on marine species (Wikström *et al.* 2006). Furthermore, most studies on non-native species - resident enemy interactions in marine systems considered herbivory (Wikström *et al.* 2006;

Forslund, Wikström & Pavia 2010; Engelen *et al.* 2011; Hammann *et al.* 2013), while little is known about the interactions between non-native basibionts and resident epibionts (Strong, Maggs & Johnson 2009; Baer & Stengel 2014). A comparison between the non-native brown alga *Fucus evanescens* and the native *Fucus vesiculosus* in Swedish waters revealed that resistance to epibiosis was higher in the non-native species (Wikström & Kautsky 2004; Wikström & Pavia 2004). However, we lack knowledge whether this difference is based on species-specific traits or whether resistance to epibiosis can be gained during the invasion process. This could, for instance, be due to the release from other enemies that would allow the allocation of energy to defenses against epibionts.

To elucidate whether non-native seaweed species are better defended against epibionts than their native conspecifics, we compared the susceptibility to epiphytism by microalgae and macroalgae between native and non-native populations of the red macroalga *Gracilaria vermiculophylla*. This perennial seaweed originates from the Northwest Pacific but during the last four decades it invaded many coastal habitats in the eastern Pacific (Bellorin, Oliveira & Oliveira 2004), the eastern Atlantic (Rueness 2005) and the western Atlantic (Freshwater *et al.* 2006; Thomsen *et al.* 2006) and the Mediterranean Sea (Sfriso *et al.* 2012). *Gracilaria vermiculophylla* has proven to be a particularly suitable marine model organism for the testing of theoretical concepts that predict the causes or consequences of biological invasions. It has, for example, been demonstrated that non-native populations of this species have a much lower genetic diversity than native populations (Kim, Weinberger & Boo 2010), while, nonetheless, the former proved to be more resistant towards herbivory (Weinberger *et al.* 2008; Rempt *et al.* 2012; Hammann *et al.* 2013; Hammann *et al.* 2016) and heat stress (Hammann 2014). These findings shed light on

potential reasons for the invasion success of *G. vermiculophylla* and stimulated curiosity about how this seaweed interacts with epibionts in its non-native range.

Putative differences in the resistance against micro-epiphytes between native and non-native populations of *G. vermiculophylla* have been studied for bacteria (Saha *et al.* 2016), while we are not aware of studies that were done with eukaryote micro-epiphytes and macro-epiphytes. We conducted common garden experiments, i.e. individuals of *G. vermiculophylla* from different geographic ranges were transplanted into a common environment, with *G. vermiculophylla* from Asia and Europe and with micro- and macro-epiphytes coming from the macroalga's native and non-native range.

Materials and methods

Collection of *Gracilaria vermiculophylla* and of epiphytes

Algal individuals were collected from four native and four non-native populations (Table 1). Sampling was conducted three times: from May to June and again from August to September 2014 as well as from June to July 2015. Laboratory experiments were conducted in June (summer) and in October (autumn) 2014 at the Helmholtz Centre for Ocean Research at Kiel, Germany, and in September (autumn) 2014 at the Xunshan Group Co., Ltd, Rongcheng, China, as well as in July (summer) 2015 at the Akkeshi Marine Station, Akkeshi, Japan (Table 2). Prior to experiments, living algal specimens from all sampling sites were transferred to the respective laboratory and kept under laboratory conditions for at least one week to allow them to recover from the transport (see Appendix S1 in Supporting Information for details).

Two pennate diatom species of the genus *Stauroneis* were isolated from individuals of *G. vermiculophylla* that were collected in Rongcheng and in the Kiel Fjord, respectively. Individuals of *Ceramium tenerrimum* were collected in Rongcheng, while specimens of *Ceramium virgatum* stem from Kiel Fjord (see Appendix S2 for details).

Algal surface extraction

Before the extraction procedure, adherent water was removed from the algal thalli in a centrifuge (Eppendorf 5810 R) with 200 rpm for 30 s. Surface associated metabolites were extracted from 80 g algal wet mass using the ‘dipping technique’ (de Nys, Dworjanyn & Steinberg 1998; Nylund *et al.* 2007), by dipping the algal individuals into a stirred mixture of dichloromethane (DCM) and hexane 1:4 (v/v) for 5 s. This procedure was benign and was chosen after different mixtures of solvents and dipping times were tested with regard to their effects on the survival of

epidermal cells which was verified with Evan's blue (Figure S1). This was done to make sure that any damaging of cell walls, which could have led to the leaching of non-surface compounds, was avoided. The resulting solution was immediately filtered through a paper filter (Macherey Nagel, 185 mm in diameter) to remove particles and the solvents were then evaporated under vacuum at 30 °C. The residue was re-dissolved in hexane to exclude non-polar compounds and this step was repeated until the hexane appeared colourless. The residue that remained after this first extraction step was then re-dissolved in DCM to extract existing polar components. Finally, 4 ml of both extracts were collected and stored at -20 °C.

Extracted surface area

To identify the extracted surface area, the relationship between algal surface area and algal wet weight was determined. Ten algal fragments, taken haphazardly across all populations, were carefully dried with paper and then scanned and weighed. The imaging software Image J (National Institute of Health, Bethesda, Maryland, USA) was used to analyze the surface area of each fragment. The algal thallus was viewed as a cylinder, so the projection area = thallus diameter × thallus length. The surface would then be = π × thallus diameter × thallus length = π × projection area. We identified the average surface area per g algal material across all ten fragments as 46.06 ± 2.8 $cm^2\ g^{-1}$ (mean ± SD). The total extracted surface area was 80 g * 46.06 ± 2.8 $cm^2\ g^{-1}$ = 3684.8 ± 224 cm^2.

Defense capacity against diatoms in living *Gracilaria vermiculophylla* thalli

We combined living thalli of *G. vermiculophylla* from both ranges (native and non-native) with diatoms from Kiel as well as Rongcheng, respectively. For each basibiont-epibiont combination, six fragments of 2 cm were cut from six randomly chosen algal individuals (n = 6) and were

placed separately into the wells of a 6-well plate (Standard, Sarstedt AG & Co.) together with 3 ml of a homogenized diatom suspension per well. The covered plate was then incubated for 3 h. A pilot study was done with different incubation periods (3 h, 5 h and 6 h), but diatom attachment did not increase any further after 3 h (Table S1). During incubation the plates were placed on a rotary shaker (100 rpm) that served to gently homogenize the suspension. Afterwards, each algal fragment was rinsed with 3 ml of sterile seawater. Algal pieces were then transferred to tubes containing 50 ml of sterile seawater and all attached diatoms were extricated by shaking the tube with a vortex shaker for 3 min. The shaken-off diatoms were collected on polycarbonate filters (0.2 µm pore size, 25 mm in diameter), which were then inspected under a fluorescence microscope and photographed. The photos were later used to assess the number of diatoms per algal fragment. The tested algal area was determined by scanning the fragments afterwards and quantifying their surface area with Image J.

Defense capacity against *Ceramium* sp. in living *Gracilaria vermiculophylla* thalli

The majority of studies about the attachment of epiphytes or epizoans to hosts focused on colonization by microscopic propagules and spores, which are the mobile stages of the otherwise sessile epibionts. However, vegetative thalli of *Ceramium* sp. can directly attach or penetrate into the host by the formation of hapteria (Leonardi *et al.* 2006; Lion *et al.* 2006; Michetti *et al.* 2016). We therefore conducted assays with *C. tenerrimum* and *C. virgatum* with filaments of these algae and organized them in the same way as the diatom trials described in the previous paragraph. For this, ten algal individuals per population of *G. vermiculophylla* were used (i.e. n = 10 per basibiont-epibiont combination). From each of these ten replicates we cut a fragment of 2 cm, while a *Ceramium* filament of the same length was then bound to *G. vermiculophylla* using colored paper clips. This was done to shorten the distance between the fragments and by this to

increase the likelihood of attachment. These pairs were put into Petri dishes containing 30 ml of the modified culture medium of Provasoli's enriched seawater (PES) (Bold & Wynne 1978). The covered Petri dishes were incubated for two weeks and attachment rates were quantified after this time.

Chemical defense capacity against diatoms in *Gracilaria vermiculophylla* surface extracts

These assays were organized in the same way as the ones with living thalli. For the assays with extracts we used an extract concentration that was five times higher than the natural surface concentration. This was done to compensate for the possible degradation and incomplete extraction of active compounds. In one cylindrical well of a 96-well plate (flat bottom, Greiner bio-one), 100 µl of both, DCM and hexane, cover a total surface area of 94 mm^2. Thus, 5.1 µl of surface extracts and 94.9 µl of pure solvent were then pipetted into each well to cover the aspired wall area. Wells loaded with pure DCM and hexane were later used as controls. Solvents were then evaporated overnight in a freeze-dryer. After this, 100 µl of the homogenized diatom suspension were transferred into the wells. Four wells of each experimental group received extracts and diatoms, while four wells received extracts only to check for extract background fluorescence. Control wells were treated in the same way. Afterwards, the covered 96-well plate was incubated for 3 h and then each well was rinsed with 200 µl of sterile seawater. Finally, fluorescence intensity per well was measured and the number of diatoms per well was calculated from fluorescence intensity by using the linear function that was established in a pilot study (Appendix S3, Figure S2).

Chemical defense capacity against *Ceramium* sp. in *Gracilaria vermiculophylla* surface extracts

These assays were organized in the same way as the ones with living thalli. In a 6-well plate, 120 µl of solvent can cover the total surface area of the bottom of one well. A paper filter (Carl Roth, 3.5 cm in diameter) was put into each well to avoid erosion by solvents. For applying a fivefold natural surface concentration, 52 µl of surface extracts and 68 µl of pure solvent were then pipetted into each well, while we had five wells per population. Some wells received pure DCM or hexane and served as controls. The solvent was then evaporated overnight in a freeze-dryer. After that, 5 ml of PES medium and ten *Ceramium* filaments (1 cm) were transferred to each well. The covered 6-well plate was then incubated for two weeks. Afterwards, the proportion of *Ceramium* filaments that attached to the paper filter was quantified.

Statistical analyses

All statistical and graphical analyses were done using the free statistical computing software R (R Development Core Team 2014). We used mixed effect-modelling to analyze the data from our multifactorial experimental approach. In case of the assays with living thalli, our experimental design included three fixed factors: 1) 'Origin' with the levels 'Native' and 'Non-native' (i.e. origin of *Gracilaria*), 2) 'Diatom' / '*Ceramium*' with the levels 'China' and 'Germany' (i.e. origin of epiphytes), and 3) 'Season' with the levels 'Summer' and 'Autumn' (i.e. the time of the experiment). In the assays with surface extracts we had one more fixed factor: 'Solvent' with the levels 'DCM' and 'hexane'. In all analyses, the algal sampling sites were included as a random factor, while the two types of epiphytes (diatoms/*Ceramium*) were analyzed separately. To achieve homogeneity of variances and normality of errors, data from the assays with diatoms and living thalli were square root transformed and data from the assays with diatoms and surface extracts were log-transformed. However, homogeneity of variances could not be achieved for all factors. We therefore included weights for 'Season' and 'Diatom' to

account for the differences in the variance structures between their factor levels in the modelling. For this we used the varIdent function of the nlme package in R (Zuur *et al.* 2009). Test assumptions were checked graphically with residual plots (Zuur, Ieno & Elphick 2010). In addition, a mixed effect-modelling, with the factors 'Origin', 'Diatom' / '*Ceramium*', 'Season' and 'Material'(i.e. thalli and surface extracts), was used to analyze the data from the two assays with diatoms and from the two assays with *Ceramium* sp., respectively, in a common approach (see results in Table S2, S3). To achieve homogeneity of variances and normality of errors, data from the two assays with diatoms were square root transformed.

Results

Defense capacity against diatoms in living *Gracilaria vermiculophylla* thalli

After 3 h of exposure to colonization by diatoms, a three-way-interaction among the factors 'Diatom', 'Origin' and 'Season' was observed: Fewer diatoms from Rongcheng attached to non-native than to native *G. vermiculophylla* in autumn and this difference was less pronounced in Summer and less observed with diatoms from Kiel in both seasons (Fig. 1, Table 3). For both diatom species fewer cells (by 60% less) attached to non-native than to native *G. vermiculophylla* individuals (Fig. 1, Table 3) and only 4% of the unexplained variation was found to be covered by the random factor 'Site'. Furthermore, for both diatom species, settlement rates were on average by 66% lower in summer than in autumn. Averaged across the two seasons, diatoms from Kiel settled by 21% less often on *G. vermiculophylla* thalli than their congeners from Rongcheng (Fig. 1, Table 3).

Defense capacity against *Ceramium* sp. in living *Gracilaria vermiculophylla* thalli

After two weeks of colonization by *Ceramium* filaments there was an interaction between '*Ceramium*' and 'Origin' (Fig. 2, Table 4). *Ceramium virgatum* (from Kiel, Germany) attached less to non-native than to native *G. vermiculophylla*, while this difference was less pronounced in *C. tenerrimum* (from Rongcheng, China). An interaction between '*Ceramium*' and 'Season' also emerged since attachment rates of *C. tenerrimum* differed between autumn and summer, while this was not the case for *C. virgatum* (Fig. 2, Table 4). Filaments of both *Ceramium* species attached, on average, by 33% less often to non-native than to native *G. vermiculophylla* specimens (Fig. 2, Table 4). In this case, 37% of the unexplained variation was covered by 'Site'. Furthermore, attachment rates, averaged across both *Ceramium* species, were by 10% lower in

autumn than in summer, while they, when averaged across both seasons, did not differ between the two *Ceramium* species (Fig. 2, Table 4).

Chemical defense capacity against diatoms in *Gracilaria vermiculophylla* surface extracts

The results from this assay are generally in accordance with those of the diatom trials with living thalli. After exposing the surface extracts to diatom settlement for 3 h, an interaction among the factors 'Solvent', 'Diatom' and 'Season' was observed: Attachment rates of diatoms from Kiel on surfaces coated with DCM-based extracts were lower in summer than in autumn, but no such difference was observed on surfaces coated with hexane-based extracts or with diatoms from Rongcheng on any coated surfaces (Table 5). Additionally, fewer diatoms attached (by 9% less) to surfaces coated with extracts from non-native than from native *G. vermiculophylla* (Figs 3 and 4, Table 5) and only 2% of the unexplained variation was covered by 'Site'. In general, diatom settlement rates were again by 22% lower in summer than in autumn (Figs 3 and 4, Table 5). Interestingly, different from the assays with living thalli, diatoms from Kiel settled two times more often than diatoms from Rongcheng (Fig. 3, Table 5). Moreover, we found fewer diatoms (by 4% less) attached to surfaces covered with non-polar compounds (extracted with hexane) than to those coated with polar compounds (extracted with DCM) (Fig. 4, Table 5).

Chemical defense capacity against *Ceramium* sp. in *Gracilaria vermiculophylla* surface extracts

In autumn we found, after two weeks of exposing surface extracts to colonization by *Ceramium* filaments that fewer (by 13% less) *Ceramium* filaments attached to surfaces coated with moderately polar compounds than on such covered with non-polar compounds. This was not the case in summer and this difference led to an interaction between 'Solvent' and 'Season' (Fig. 6,

Table 6). In addition, there was an interaction between '*Ceramium*' and 'Season': Attachment rates of *C. virgatum* were lower in autumn than in summer, while this difference was less pronounced in *C. tenerrimum* (Fig. 5, Table 6). Furthermore, fewer *Ceramium* filaments (by 10% less) attached to surfaces coated with extracts gained from non-native than to surfaces with extracts from native *G. vermiculophylla* (Figs 5 and 6, Table 6) and only 0.5% of the unexplained variation was covered by 'Site'. In general, *Ceramium* filaments attached 22% less often in autumn than in summer (Figs 5 and 6, Table 6), but attachment rates never differed between the two *Ceramium* species (Fig. 5, Table 6).

Discussion

For this intra-specific comparison, we sampled individuals of *G. vermiculophylla* at different sampling locations in either the native or the non-native range of the species. Within the respective ranges, the different sampling sites were located in nearly the same biogeographical region according to the Marine Ecoregions of the World (MEOW) system suggested by Spalding *et al*. (2007). The sites in the native range were located in the Cold Temperate Northwest Pacific/Yellow Sea (China) and in Northeastern Honshu (Japan) as well as in the Warm Temperate Northwest Pacific/Central Kuroshio Current (Japan), which is adjacent to Northeastern Honshu. The sites in the non-native range were located in the Northern European Sea/Baltic & North Sea (Germany) and in the Celtic Sea as well as the Lusitanian/South European Atlantic Shelf (France), which is adjacent to the Celtic Sea. Furthermore, we took care that the distances between the various sampling sites in the native as well as in the non-native range were similar, in order to have the same degree of between-site variability within the ranges. We therefore assumed that the within-range variability, which could be attributed to potential differences in the diversity and composition of the resident flora and fauna as well as to climate conditions, would be low. This assumption was confirmed by the low amount of unexplained variation (0.5% to 4%) that was actually covered by the random factor 'Site' in most of our statistical modellings. This was true for diatom attachment rates on both living thalli and on extracts and for *Ceramium* attachment to extracts. In contrast to this, *Ceramium* attachment rates on living thalli varied considerably (37% of the unexplained variation) among sites within both ranges and we cannot plausibly explain this deviation from the otherwise consistent picture.

In our study we used two types of epiphytes to test for inter-population differences in *G. vermiculophylla* with regard to its susceptibility to epiphytes: Diatoms as a common type of

376 micro-epiphytes and *Ceramium* filaments as a macro-epiphyte. We found that both of them,
377 independent of their actual origin, attached by 60% and 33%, respectively, less to the living thalli
378 of European *G. vermiculophylla* than to those of Asian conspecifics. In general, such a difference
379 could either due to the fact that non-native *G. vermiculophylla* individuals are better defended
380 and therefore attract fewer/repel more epiphytes or it could be due to lower settlement rates of
381 native colonizers on the non-native macroalga. However, our experimental design excluded the
382 latter option, since we exposed non-native macroalgae to epiphytes from the native as well as
383 from the non-native range and both combinations showed the same trend. This finding indicates
384 that non-native *G. vermiculophylla* are better defended against epiphytes than those that stem
385 from the native range.

386 In macroalgae, resistance to epibiosis can be mediated by a) the thallus surface structure
387 (Schumacher *et al.* 2007; Chapman *et al.* 2014), b) surface associated bacterial communities that
388 repel epibionts (Boyd, Adams & Burgess 1999; Dobretsov, Dahms & Qian 2006), and c)
389 surface-bound secondary metabolites that have anti-epibiont activities (Nylund *et al.* 2007; Saha
390 *et al.* 2011; Thabard *et al.* 2011). The question is now whether one or more of these mechanisms
391 changed with regard to their mode of action or with regard to their efficiency during the invasion
392 process. Such a change could, *inter alia*, be caused by a directional selection of genotypes that
393 exhibit a low susceptibility to epibionts during transport or after release into the new habitat.
394 However, we cannot think of a scenario during these phases that would specifically select for
395 resistance to epibiosis. Furthermore, since the non-native gene pool is a reduced subset of the
396 gene pool in the donor region, it is possible, although presumably not very likely, that by chance
397 an epibiosis-resistant genotype was highly frequent among the introduced individuals. Finally, a
398 change in the quality of anti-epiphyte defenses could be attributed to increased energy resources,

399 which are a consequence of the release from abiotic and biotic pressures in the new environment
400 (Joshi & Vrieling 2005). Under such conditions, non-native seaweeds may reduce specific
401 defenses they developed against enemies in their native range and shift energy resources towards
402 more general anti-enemy defenses. An observation made at our study site in the native range
403 hints at the potential relevance of the last mechanism: In Rongcheng, China, an amphipod
404 species, *Caprella* sp., is the main grazer of *G. vermiculophylla* in many habitats and it can
405 consume substantial parts of this local alga during summer (S. Wang, pers. obs.). In Kiel,
406 Germany, so far no herbivore makes use of this alga to such an extent and it seems that the
407 grazing pressure on *G. vermiculophylla* is generally lower than in Rongcheng (Hammann *et al.*
408 2013). However, we do not have information whether the picture is the same in the other non-
409 native habitats that we sampled in Europe. If *G. vermiculophylla* is mostly ungrazed in coastal
410 habitats in Europe, this could have allowed the non-native *G. vermiculophylla* to allocate a larger
411 part of their energy budget to anti-epibiont defenses and this possibly caused their lower
412 attractiveness for colonizers.

413 An important aspect of our study was to identify properties of *G. vermiculophylla* that mediate
414 its anti-epiphyte defenses. For this we compared epiphyte attachment rates on living thalli to
415 those on extract coated surfaces. Here we observed that the general trend in epiphyte attachment
416 was the same for living thalli and extract coated substrata. However, the effect size, i.e. the
417 difference in the susceptibility to epiphytes in native and non-native *G. vermiculophylla*, was
418 consistently smaller in the latter assays: Fewer diatoms (on average 9% for extracts, 60% for
419 living thalli) and fewer *Ceramium* filaments (on average 10% for extracts, 33% for living thalli)
420 attached to substrata that were covered with extracts from European *G. vermiculophylla* than to
421 those with extracts from Asian specimens. This, first of all, confirms that resistance to epiphytes

422 in *G. vermiculophylla* has, at least partly, a chemical component. If the lower susceptibility in
423 non-native *G. vermiculophylla* is due to this chemical component, it could either be based on an
424 increased synthesis of active compounds (Forslund, Wikström & Pavia 2010) or due to the
425 presence of some chemical compounds that are novel to resident enemies in these individuals
426 (Enge *et al.* 2012). Overall, extracts exhibited a lower inhibitory activity against diatom and
427 *Ceramium* settlement than living algae. This difference could be due to the fact that active
428 metabolites were insufficiently captured by the extraction process or degraded after extraction.
429 Alternatively, other non-chemical components such as surface properties - which were of course
430 excluded in the assays with extracts - could also have contributed to the overall deterrence.
431 Finally, the compounds which were responsible for the anti-epiphyte activity we observed in
432 living *G. vermiculophylla* may not only have stemmed from the thallus surface but also from the
433 inside of algal cells. This reason could have been relevant since some epibionts, including
434 species of the genus *Ceramium*, penetrate into algal thalli and therefore also get in contact with
435 their interior (e.g. Leonardi *et al.* 2006). We have no data that could elucidate which of the three
436 scenarios was responsible for the picture we observed. However, since we used only two
437 solvents for the extractions (i.e. hexane and DCM) that cover a limited part of the polarity
438 spectrum, it is at least likely that we missed relevant compounds and thereby underestimated the
439 potential of chemical defenses in *G. vermiculophylla*.

440 So far, no tests have been made to investigate whether the surface texture, microtopography or
441 consistency of *G. vermiculophylla* thalli mediates a defense against epibionts. Such effects are
442 known from *Saccharina* species (Chapman *et al.* 2014; da Gama, Plouguerné & Pereira 2014)
443 that belong to the brown macroalgae and possess an outer cell wall with a mucilage consisting of

444 alginic acid with traces of sulphated fucoidan that could, theoretically, act as a low-adhesion,
445 gelatinous covering.

446 Whatever the mechanism is, a low susceptibility to epiphytes in non-native populations of *G.*
447 *vermiculophylla* can, at least partly, explain the invasion success of the species. It has been
448 proposed that marine algal invaders have more effective anti-epibiont defenses than comparable
449 resident species, e.g. in its non-native habitats in northern Europe the brown alga *Fucus*
450 *evanescens* is known to get less colonized by filamentous algae and sessile invertebrates than its
451 native congener *Fucus vesiculosus* (Wikström & Pavia 2004). When their surface is free of
452 epibionts, macroalgae can take up more oxygen, carbon dioxide and nutrients. Furthermore, they
453 receive more light for photosynthesis and are less prone to dislodgement caused by
454 biomechanical drag. Furthermore, they may be less attractive to grazers (Wahl, Hay & Enderlein
455 1997). Therefore, algae, which are free of epibionts or show low degrees of epiphyte or epizoan
456 cover, should have more energy available for reproduction and growth, as well as for tolerating
457 adverse environmental conditions – what in turn should increase their potential to establish and
458 spread in new environments.

459 The Enemy Release Hypothesis proposes that non-indigenous species are commonly released
460 from biotic pressures, e.g. grazing, in their recipient habitat, because they leave their co-evolved
461 antagonists behind while, at the same time, resident enemies fail to recognize the new species as
462 a food source (Keane & Crawley 2002). In this context, Hammann *et al*. (2013) found that the
463 periwinkle species *Littorina brevicula,* which lives in the native range of *G. vermiculophylla,*
464 consumes more of this seaweed, regardless from which distributional range the algal material
465 stems, than *Littorina littorea*, which is from its non-native range. This finding is presumably due
466 to the fact that *L. brevicula* coevolved with *G. vermiculophylla* and can make better use of it as a

food source. A somewhat comparable preference of native over non-native *G. vermiculophylla* was also shown by the diatoms we used in the assays with living thalli: diatoms from Rongcheng generally attached in higher numbers to *G. vermiculophylla* from both distributional ranges than diatoms from Kiel. This finding may also be due to the fact that these diatoms recognize *G. vermiculophylla* more readily as a suitable settlement substratum than diatoms from the non-native range. Surprisingly, we observed the opposite picture when we tested the surface extracts. This mismatch suggests that, besides a chemical defense, other properties of the thallus surface, such as its structure or the presence of biofilms, play a role in the anti-diatom defense in living thalli. However, we did not find a difference between the attachment rates exhibited by the two *Ceramium* species for both substrata. However, it is not clear why this epiphyte did not show a preference. It is possible that *C. virgatum* – with regard to *G. vermiculophylla* as a settlement substratum - generally has the same settlement capacity as *C. tenerrimum*. The absence of a difference is somewhat surprising, because the way the *Ceramium* filaments attach to algal surfaces constitutes a very intimate connection of the two organisms. It is most often characterized as an infection of the basibiont, because it is mediated by the formation of hapteria that first attach to the thalli of the host and then penetrate into its tissue (Lion *et al.* 2006). Lion *et al*. (2006) found that after wounding *Gracilaria chilensis* released oxylipins, which suppressed the development of hapteria in *Ceramium rubrum*. This fact indicates that co-evolution occurs between *Ceramium* species and their hosts and hence a difference in settlement rates between *C. tenerrimum* and *C. virgatum* would be likely.

Previous studies have suggested that season (Culioli *et al.* 2002; Hellio *et al.* 2004) can influence the capacity of a seaweed to defend itself against epibionts. In accordance with this finding, we found differences in epiphyte settlement rates between summer and autumn of the same year of

which we assume that they are attributed to differences in the anti-epiphyte activity of *G. vermiculophylla*. Fewer diatoms attached to both living fragments and extract-coated surfaces in summer, while fewer *Ceramium* filaments attached to those substrates in autumn. Such inter-seasonal differences in anti-epiphyte defenses presumably correlate with natural fluctuations in the overall propagule abundance in the colonizer pool (Steinberg & Vanaltena 1992; Amade & Lemée 1998; Wahl *et al.* 2010; Rickert *et al.* 2015), which means that marine macroalgae can adjust their anti-epibiont activities to quantitative or qualitative changes in colonization pressure. In both regions where we collected algae, diatoms are more abundant from April to June than from August to October (Trimonis, Vaikutiene & Gulbinskas 2010; Wang *et al.* 2014), while *Ceramium* is more abundant during the latter time span (Weinberger *et al.* 2014; S. Wang, pers. obs.). However, we collected our data only during the course of one year and we therefore do not have robust evidence for seasonality in the defense capacity of *G. vermiculophylla*. To establish such a pattern, assays would need to be repeated over several years.

A further interesting observation that we made was that fewer diatoms attached to surfaces covered with non-polar compounds than to those coated with polar compounds, while the opposite was true for *Ceramium* filaments. This indicates that the defenses against these two epiphytes are mediated by compounds that differ in polarity. A similar observation has been reported earlier: surface compounds extracted with a mixture of hexane and DCM from *Caulerpa filiformis* significantly inhibited spore settlement of *Polysiphonia* sp., while more polar compounds, which were extracted with DCM from surfaces of the same species, inhibited settlement and germling development of gametes of *Ulva australis* (Nylund *et al.* 2007).

Our study is the second biogeographical comparison of defense capacities against epibionts between native and non-native populations of *G. vermiculophylla* (Saha *et al.* 2016), which is

513 now invasive in many coastal areas worldwide. However, it gives the first evidence that the
514 capacity to defend against epibionts is higher in non-native individuals than in native –
515 regardless of whether the epibionts originate from the native of the non-native range of *G.*
516 *vermiculophylla*. Our findings therefore seemingly contradict the observations made by Saha *et*
517 *al*. (2016), who focused on seaweed-bacteria interactions and showed that non-native *G.*
518 *vermiculophylla* are better defended against bacterial epibionts from the non-native range but, at
519 the same time, have reduced their capacity to defend themselves against epibionts from their
520 home range. The contradiction may be due to the use of different micro-epibionts. Bacteria are
521 the first colonizers of bare substrata in the marine environment (Wahl 1989) and can regulate the
522 production of bioactive compounds, motility, and biofilm formation by Quorum Sensing (QS),
523 which is a density-dependent cell-cell signaling communication among bacteria (da Gama,
524 Plouguerné & Pereira 2014). Furthermore, it is known that bacterial biofilm formation can
525 mediate further colonization by eukaryote micro- and macro-epibionts. The differences between
526 epibacteria and other epibionts could have led to the evolution of different defense strategies
527 against them in seaweeds. Unlike compounds that function against eukaryote micro- and macro-
528 epibionts through growth inhibition or lethality, most antimicrobial settlement and attachment
529 defenses impact the behavior of bacteria, such as swarming (Rasmussen & Givskov 2006).

530 Even though our study focused on macrophyte-epiphyte interactions in the marine environment,
531 our findings should be applicable to all basibiont-epibiont interactions in aquatic systems,
532 including plant-plant, plant-animal and animal-animal combinations, since epibionts are
533 widespread and most of them are generalists (Wahl & Mark 1999). Additionally, our findings
534 may also be applicable to host-herbivore interactions in aquatic systems, since, similar to
535 epibionts, many herbivores are generalists and an increased chemical resistance to herbivory has

already been documented in non-native plants and seaweeds (Forslund, Wikström & Pavia 2010).

We conclude that the lower susceptibility to epiphytes that we observed in non-native *G. vermiculophylla* cannot be explained by a lower epibiont pressure experienced by the non-native individuals, but is due to an elevated resistance to epibiosis that, at least partly, is linked to an enhanced chemical defense capacity. Our study therefore provides the first evidence of an increased resistance to epibiosis in introduced populations of a widely distributed marine species. This change in its performance during the invasion process may be critical for the invasion success of the macroalga.

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Author contributions

M.L., F.W. and S.W. conceived and designed the experiments. S.W. performed the experiments and collected algal material. G.W., D.B. and M.N. contributed to algal collection and the labs in China and Japan for bioassays. M.L. and S.W. performed statistical analyses. S.W. wrote the manuscript, and M.L., F.W. and S.W. contributed to revisions.

Data accessibility

The authors confirm that all data underlying the findings are fully available without restriction. Data are available at https://doi.pangaea.de/10.1594/PANGAEA.865230 (Wang *et al*. 2016). R scripts: uploaded as online supporting information.

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Table 1. Sampling information of the four native and four non-native populations of *Gracilaria vermiculophylla*.

Origin	Collection site	Geographic Coordinate	1st sampling	2nd sampling	3rd sampling	Salinity	Attachment status	Morphology	Epibionts (incomplete information)
Native	Rongcheng, China, Yellow Sea	37°9'4.29"N, 122°33'35.60"E	21.05.2014	23.09.2014	06.07.2015	28-33	Holdfast	Brown, relatively more branches	Diatoms, *Ceramium tenerrimum*, Ulva sp., *Polysiphonia* sp., *Folliculina* sp.
	Qingdao, China, Yellow Sea	36°3'0.6"N, 120°20'59.1"E	21.05.2014	21.09.2014	06.07.2015	28-33	Holdfast	Brown, relatively more branches	Diatoms, *Ceramium tenerrimum*, Ulva sp., *Cladophora* sp.
	Akkeshi, Japan, Northeastern Honshu	43°1'25.80"N, 144°52'47.20"E	01.05.2014	19.08.2014	16.07.2015	29-33	Holdfast	Brown, relatively more branches	Diatoms, *Ceramium kondoi*, *Circeis spirillum*
	Tokyo, Japan, Central Kuroshio Current	35°19'25.72"N, 139°38'8.30"E	01.06.2014	02.08.2014	10.07.2015	29-33	Holdfast	Brown, relatively more branches	Diatoms, Ulva sp.

Non-native	Kiel, Germany, Baltic Sea	54°23'57.03"N, 10°12'53.12"E	02.06.2014	12.08.2014	12.07.2015	15-20	Free-floating	dark brown, relatively fewer branches	Diatoms, *Ceramium tenuicorne*, Ulva sp., *Pilayella* sp., *Amphibalanus improvises*, *Mytilus edulis*
	Nordstrand, Germany, North Sea	54°29'10.0"N 8°48'44.8"E	25.05.2014	04.09.2014	08.07.2015	30-32	Free-floating	dark brown, relatively fewer branches	Diatoms, Ulva sp. *Chaetomorpha* sp., *Cladophora* sp., *Amphibalanus improvises*, *Mytilus edulis*
	Belon, France, South European Atlantic Shelf	47°49'35.80"N, 3°40'20.50"W	18.05.2014	08.09.2014		2-33	Free-floating	dark brown, relatively fewer branches	Diatoms
	Pouldouran, France, Celtic Seas	48°45'55.90"N, 3°12'1.40"W	18.05.2014	08.09.2014	18.06.2015	2-33	Free-floating	dark brown, relatively fewer branches	Diatoms

816 Holdfast: thalli attached by a holdfast to the substratum; Free-floating: thalli not attached by a holdfast;

817 **Table 2.** Overview of the locations and timing over the attachment assays with *Gracilaria*
818 *vermiculophylla* and different epiphytes.

Epiphytes	**Assays after 1st sampling**	**Assays after 2nd sampling**	**Assays after 3rd sampling**
Diatoms from Rongcheng	Kiel, 11.06.2014	Kiel, 24.10.2014	Akkeshi, 20.07.2015
Diatoms from Kiel	Kiel, 11.06.2014	Kiel, 24.10.2014	
***Ceramium tenerrimum* from Rongcheng**	Rongcheng, 30.09.2014	Rongcheng, 30.09.2014	
***Ceramium virgatum* from Kiel**	Kiel, 10.06.2014	Kiel, 23.10.2014	

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826 **Table 3.** Influence of season, origin of *Gracilaria vermiculophylla* and origin of diatoms on the
827 attachment rates of diatoms on living thalli.

Source of variation	numDF	denDF	F - value	p - value
Season	1	220	283.691	<.0001
Origin	1	6	138.724	<.0001
Diatom	1	220	24.636	<.0001
Diatom:Origin	1	220	4.429	0.0365
Diatom:Season	1	220	2.047	0.1540
Origin:Season	1	220	40.930	<.0001
Diatom:Origin:Season	1	220	11.439	0.0009

828 Results from mixed-effect modelling.

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838 **Table 4.** Influence of season, origin of *Gracilaria vermiculophylla* and origin of *Ceramium* on
839 filament attachment rates on living thalli.

Source of variation	numDF	denDF	F - value	p - value
Season	1	20	8.4325	0.0088
Origin	1	6	30.5134	0.0015
Ceramium	1	20	2.1081	0.1620
***Ceramium*:Origin**	1	20	13.1757	0.0017
***Ceramium*:Season**	1	20	75.8923	<.0001
Origin:Season	1	18	2.2500	0.1510
***Ceramium*:Origin:Season**	1	18	0.5625	0.4629

840 Results from mixed effect-modelling.

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Table 5. Influence of season, solvent, origin of *Gracilaria vermiculophylla* and origin of diatoms on the attachment rates of diatoms on surface extracts.

Source of variation	numDF	denDF	F - value	p - value
Season	1	238	283.0	<.0001
Solvent	1	238	17.4	<.0001
Origin	1	6	11.8	0.0139
Diatom	1	238	1772.5	<.0001
Solvent:Season	1	238	21.9	<.0001
Diatom:Season	1	238	648.9	<.0001
Solvent:Diatom	1	238	0.2	0.6686
Solvent:Origin	1	238	1.3	0.2479
Diatom:Origin	1	238	0.1	0.7242
Origin:Season	1	238	0.0	0.9599
Solvent:Diatom:Season	1	238	6.0	0.0154
Solvent:Diatom:Origin	1	234	0.1	0.8000
Solvent:Origin:Season	1	234	0.7	0.4059
Diatom:Origin:Season	1	234	0.3	0.6021
Solvent:Diatom:Origin:Season	1	234	0.0	0.9073

Results from mixed effect-modelling.

Table 6. Influence of season, solvent, origin of *Gracilaria vermiculophylla* and origin of *Ceramium* on filament attachment rates on surface extracts.

Source of variation	numDF	denDF	F - value	p - value
Season	1	307	124.418	<.0001
Solvent	1	307	11.090	0.0010
Origin	1	6	27.095	0.0020
Ceramium	1	307	0.126	0.7224
Solvent:Season	1	307	9.835	0.0019
***Ceramium*:Season**	1	307	20.782	<.0001
Solvent:*Ceramium*	1	298	2.308	0.1297
Solvent:Origin	1	298	0.126	0.7224
***Ceramium*:Origin**	1	298	0.051	0.8211
Origin:Season	1	298	0.026	0.8717
Solvent:*Ceramium*:Origin	1	298	0.001	0.9742
Solvent:*Ceramium*:Season	1	298	3.395	0.0664
Solvent:Origin:Season	1	298	0.235	0.6281
***Ceramium*:Origin:Season**	1	298	0.026	0.8717
Solvent:*Ceramium*:Origin:Season	1	298	2.718	0.1003

Results from mixed effect-modelling.

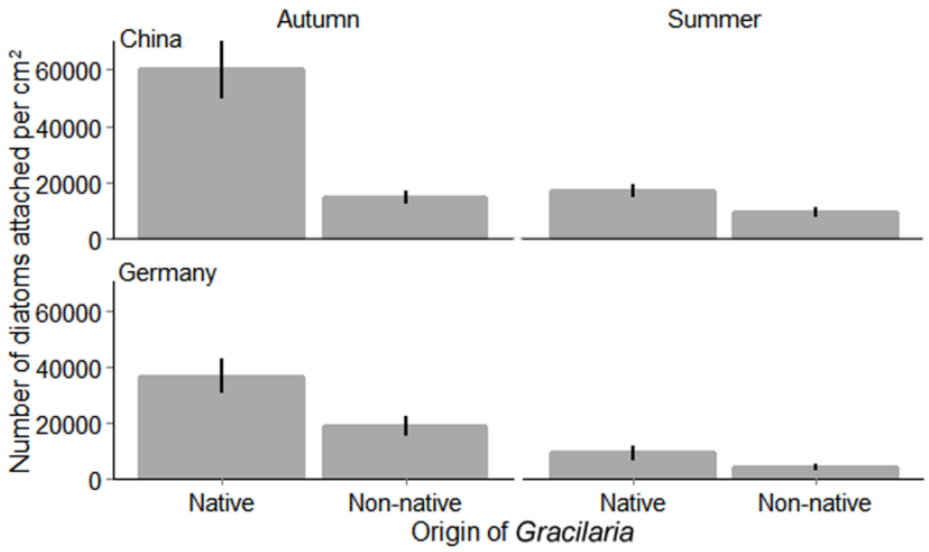
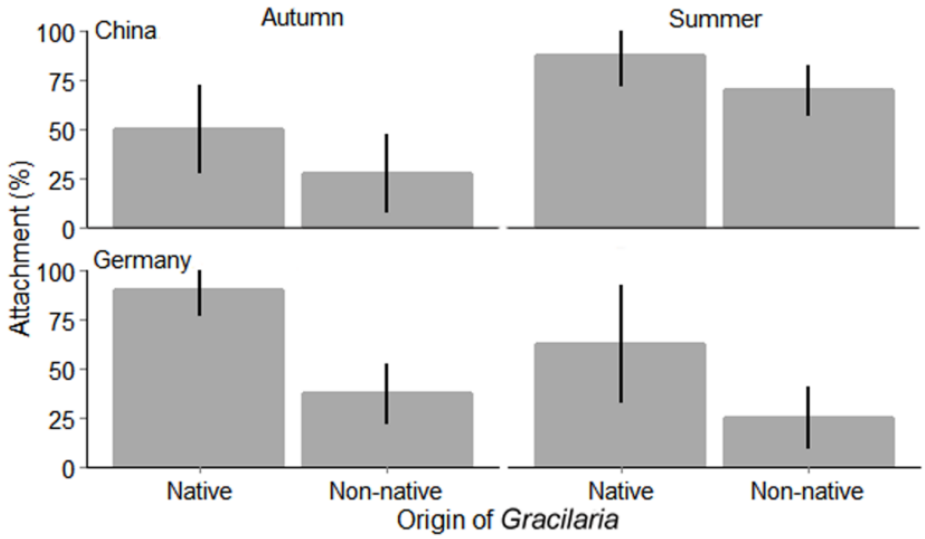


Fig. 1. Colonization of living thalli of native and non-native *Gracilaria vermiculophylla* by diatoms from both origins. Assays were run in summer and in autumn 2014 and in summer 2015. Means and 95% CIs. n =24 to 48 in each group.



874

875 **Fig. 2.** Colonization of living thalli of native and non-native *Gracilaria vermiculophylla* by
876 *Ceramium* from both origins, *C. virgatum* from Germany and *C. tenerrimum* from China. Assays
877 were run in summer and in autumn 2014. Means and 95% CIs. n = 40 in each group.

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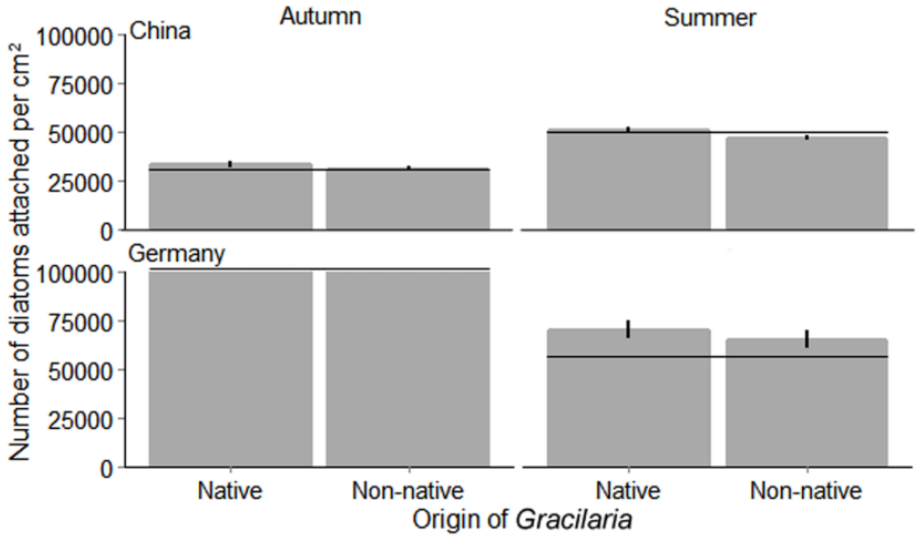

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887 **Fig. 3.** Colonization of surface extracts from native and non-native *Gracilaria vermiculophylla*
888 by diatoms from both origins. Assays were run in summer and in autumn 2014. Means and 95%
889 CIs. n = 32 in each group. The horizontal lines indicate mean colonization rate on controls, which
890 were without extracts (n = 8).

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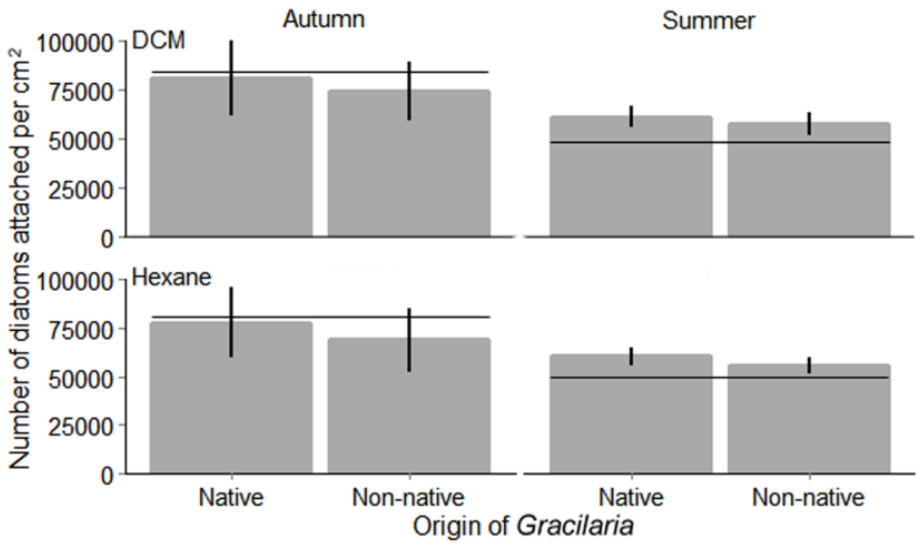

Fig. 4. Colonization of DCM and hexane surface extracts from native and non-native *Gracilaria vermiculophylla* by diatoms from both origins. Assays were run in summer and in autumn 2014. Means and 95% CIs. n = 32 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 8).

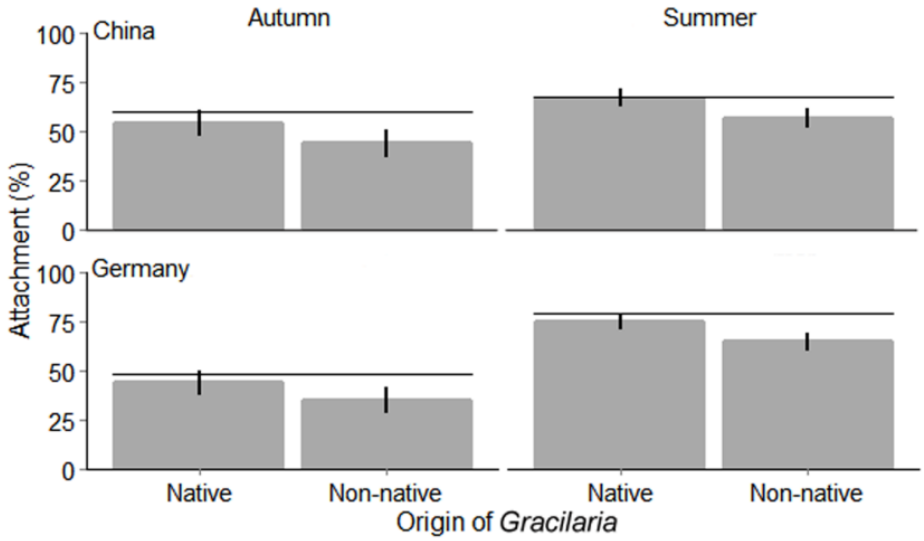

Fig. 5. Colonization of surface extracts from native and non-native *Gracilaria vermiculophylla* by *Ceramium* from both origins, *C. virgatum* from Germany and *C. tenerrimum* from China. Assays were run in summer and in autumn 2014. Means and 95% CIs. n = 40 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 10).

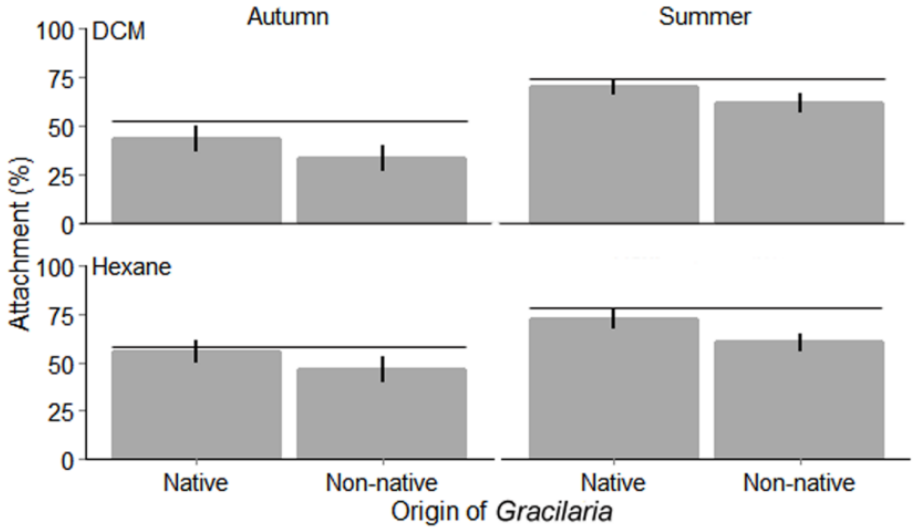

Fig. 6. Colonization of DCM and hexane surface extracts from native and non-native *Gracilaria vermiculophylla* by *Ceramium* from both origins, *C. virgatum* from Germany and *C. tenerrimum* from China. Assays were run in summer and in autumn 2014. Means and 95% CIs. n = 40 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 10).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Transport and cultivation of *Gracilaria vermiculophylla*.

Appendix S2. Collection, isolation, transport and cultivation of epiphytes.

Appendix S3. Establishing the relationship between diatom density and fluorescence intensity.

Table S1. Change of fluorescence intensities of attached diatoms with concentration of diatoms in suspensions after different incubation periods.

Table S2. Influence of season, material (extracts vs. thalli), origin of *Gracilaria vermiculophylla* and origin of diatoms on the attachment rates of diatoms on *G. vermiculophylla*.

Table S3. Influence of season, material (extracts vs. thalli), origin of *Gracilaria vermiculophylla* and origin of *Ceramium* on filament attachment rates on *G. vermiculophylla*.

Figure S1. Determination of solvents and dipping times for surface extraction of *Gracilaria vermiculophylla*. (a) Healthy algal cells. The alga was extracted by: (b) Methanol-hexane mixture 1:9 (v/v) for 5 s. (c) Methanol-hexane mixture 1:19 (v/v) for 5 s. (d) Propanol-hexane mixture 1:9 (v/v) for 5 s. (e) Propanol-hexane mixture 1:19 (v/v) for 5 s. (f) dichloromethane (DCM)-hexane mixture 1:3 (v/v) for 5 s. (g) DCM-hexane mixture 1:4 (v/v) for 10 s. (h) DCM-hexane mixture 1:4 (v/v) for 7 s. (i) DCM-hexane mixture 1:4 (v/v) for 5 s. Scale bars: 10 μm.

Figure S2. The relationship between fluorescence intensity and diatom density. (a) diatom from Rongcheng, China. (b) diatom from Kiel, Germany.