

***Zostera marina* and epibionts: the dynamics of  
chemical defenses.**

**Dissertation**

for the Academic Degree Dr. rer. Nat. at the Faculty of Mathematics and  
Nature Sciences of the Christian-Albrechts-University Kiel, Helmholtz  
Centre for Ocean Research GEOMAR

Submitted by

**Chi Guan**

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**gez.**

**Dekan Prof. Dr. Natascha Oppelt**



*Dedicated to my love Nancy*



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## Summary

Marine ecosystems and terrestrial ecosystems differ in many aspects and one of these differences is in biofouling, which is considerably more important under water. The role of bacteria in biofouling is essential, as they precondition living and non-living surfaces for the settlement of other marine organisms. In most cases micro- and macro-epiphytes have harmful or even detrimental effects on their hosts, as they compete for resources, reduce the uptake of nutrients or solar energy and in some cases microepiphytes can even be pathogens. The bacterial density in the marine environment is rather high, with, for instance,  $0.7$  to  $2.24 \times 10^6$  cells/ml in the western Baltic Sea. However most of the marine plants, such as the habitat forming species *Zostera marina* L., remain largely free of fouling although they are constantly exposed to those microbes. Such observations suggest that *Z. marina* may be capable of an antifouling defense. It was the primary goal of this work to identify relevant defense compounds and environmental factors that regulate the dynamics of their production.

The chemical defense of *Z. marina* has been studied since the 1930s and various primary and secondary metabolites - especially phenolic compounds – have been identified as potentially defensive compounds that could defend plants from fouling. However, almost all of those compounds were isolated from the tissue of *Z. marina* and not from plant surfaces, where fouling actually occurs and the defensive effect would be required. In addition, only few studies determined minimum active concentrations of compounds that are required to deter ecologically relevant settlers and compared them to the concentrations of those compounds on host surfaces, which is important to identify ecologically relevant compounds that drive the natural antifouling defense of *Z. marina*. In this dissertation, ecologically relevant non-toxic repellents of bacterial microfoulers that are present in extracts from eelgrass surfaces were identified. In subsequent bioassay-guided fractionation steps, rosmarinic acid and two sulphated flavonoids (luteolin-7-sulphate and diosmetin /chrysoeriol-7-sulphate) were identified as bioactive components. All three compounds were dose-dependent in terms of antifouling defense, but rosmarinic acid turned out to be the most dominant and hence the most ecologically relevant of the three compounds. Concentrations of the two flavonoids and also of the known deterrent compound

zosteric acid were rather low in surface extracts but high in tissue extracts, which illustrates the importance to study surface extracts if ecologically relevant compounds are to be identified (Chapter I).

To study the responses of *Z. marina* to temperature increase with the respect of chemical antifouling defense I incubated *Z. marina* several months in the Kiel Outdoor Benthocosm facility under a simulated natural temperature regime with or without application of periodic or single heat waves (+5 °C within one week) compared to the seasonal environmental change heat waves increased the antifouling defense relatively moderately, and the increase was more obvious in the last month of incubation, when the seasonal temperature increase was at its maximum. The surface concentration of rosmarinic acid was not altered by heat wave treatments but by the seasonal environmental change. At the same time it correlated to some degree with the abundance of potential bacterial foulers, suggesting that a demand-driven regulation of antifouling defense possibly exists in *Z. marina*. Given that heat waves had no reducing effect on the deterrent capacity of *Z. Marina*, global warming in the future will possibly not decrease the resistance of eelgrass against bacterial foulers (Chapter II).

To further study the effects of seasonal changes on the antifouling defense activity of *Z. marina* in the natural Baltic Sea environment, seagrass surface extracts were collected monthly over one vegetation period. The peak of defense activity appeared in summer, when the abundance of potential bacterial foulers was also higher than in all other seasons, which provided additional evidence of a demand-driven antifouling defense strategy in *Z. marina*. Also the seasonality of rosmarinic acid surface concentrations correlated with the bacterial fouling pressure and the density of bacteria that settled on eelgrass plants, which was also observed in the benthocosm experiment described in Chapter II. Besides of rosmarinic acid, additional surface compounds must be relevant for the defense activity of eelgrass against fouling, since both measures correlated to some degree only. In addition, abiotic factors such as temperature and nitrogen supply seem to play important roles in the regulation of antifouling defenses and hence the seasonal fluctuation of this activity. However, the influence of temperature is perhaps indirect, as it drives an increased fouling pressure in summer (Chapter III).

In summary, this thesis demonstrates that a chemical defense of *Z. marina* against fouling exists and it identifies the ecologically most relevant surface compounds. Abiotic and possibly also biotic factors that influence the chemical defense and the production of rosmarinic acid by *Z. marina* were detected, which provides new insights into the dynamics of eelgrass-bacteria interactions and provides a basis for further research concerning the reaction of eelgrass to bacterial foulers.

## Zusammenfassung

Marine und terrestrische Ökosysteme unterscheiden sich in vielfacher Hinsicht und auch hinsichtlich der Bedeutung von Aufwuchs, der gewöhnlich unter Wasser viel stärker zur Entwicklung kommt. Eine wesentliche Rolle für die Entwicklung von Aufwuchs spielen Bakterien, die sowohl lebende als auch nicht-lebende Oberflächen für die Besiedlung durch andere marine Organismen konditionieren. Mikro- und Makroepiphyten wirken in den meisten Fällen eher nachteilig oder sogar schädlich auf ihre Wirtsorganismen, weil sie um Ressourcen konkurrieren, die Verfügbarkeit von Nährstoffen und Licht reduzieren und in manchen Fällen – wenn es sich um Mikroorganismen handelt - sogar als Krankheitserreger wirken können. Die Bakteriendichte in Meerwasser ist gewöhnlich relativ hoch, beispielsweise werden in der westlichen Ostsee zwischen  $0.7$  und  $2.24 \times 10^6$  Zellen/ml gefunden. Trotzdem bleiben die meisten marinen Pflanzen – beispielsweise das Seegras *Zostera marina* L. – weitgehend frei von Aufwuchs, obwohl sie ständig diesem Pool bakterieller Besiedler ausgesetzt sind. Derartige Beobachtungen legen nahe, dass *Z. marina* gegen Aufwuchsorganismen verteidigt sein könnte. Es war die Zielsetzung der vorliegenden Arbeit, relevante Abwehrstoffe und Umweltfaktoren, die ihre Dynamik regulieren, zu identifizieren.

Die chemische Verteidigung von *Z. marina* wird bereits seit den 1930er Jahren erforscht und diverse primäre und sekundäre Metabolite – insbesondere phenolische Verbindungen – wurden bereits als potenzielle Abwehrstoffe gegen Aufwuchs postuliert. Fast alle dieser Substanzen wurden jedoch aus dem Gewebe von *Z. marina* und nicht von der Pflanzenoberfläche isoliert, an der sich Aufwuchs anheftet und an der der abwehrende Effekt auf diesen Aufwuchs wirken müsste. In den vorhergehenden Untersuchungen wurde in der Regel auch weder bestimmt, welche minimalen Dosen der identifizierten Verbindungen erforderlich wären, um einen Effekt auf ökologisch relevante Besiedler hervorzurufen, noch ob diese erforderlichen Konzentrationen an der Pflanzenoberfläche erreicht werden. In der vorliegenden Arbeit wurden diese Fragen in den Fokus genommen, um ökologisch relevante Abwehrstoffe zu identifizieren, die die Grundlage der chemischen Verteidigung von *Z. marina* gegen Aufwuchsorganismen bilden. Mehrere nicht-toxische Abwehrstoffe, die gegen bakterielle Siedler wirken und an der Oberfläche von *Z.*

*marina* vorhanden sind, wurden durch Bioassay-geleitete Isolation identifiziert, dies waren Rosmarinsäure und zwei sulfatierte Flavonoide (Luteolin-7-Sulfat und Diosmetin/Chrysoeriol-7-Sulfat). Alle drei Verbindungen hemmten mikrobielle Aufwuchsorganismen dosisabhängig, aber Rosmarinsäure erwies sich als die dominanteste und daher ökologisch relevanteste der drei Verbindungen. Die Konzentrationen der zwei Flavonoide und auch die Konzentration von Zosterinsäure – einer zuvor als potenzieller Abwehrstoff identifizierten Verbindung – waren zwar in Gewebeproben hoch, aber in Oberflächenextrakten niedrig, was die Bedeutung von Oberflächenextrakten für die Isolation ökologisch relevanter Wirkstoffe gegen Aufwuchs erneut hervorhebt (Kapitel I).

Um den Einfluss von Temperaturerhöhung auf die chemische Verteidigung von *Z. marina* gegen Aufwuchs zu untersuchen wurde die Pflanze mehrere Monate in der Kieler Freiland-Benthokosmen-Anlage einem simulierten natürlichen Temperaturregime mit oder ohne periodische Hitzewellen (+5 °C über eine Woche) ausgesetzt. Verglichen mit der natürlichen jahreszeitlichen Temperaturveränderung hatten Hitzewellen nur einen begrenzten verstärkenden Einfluss auf die chemische Verteidigung gegen Aufwuchs, und diese Zunahme war im letzten Monat des Experimentes am stärksten, als der jahreszeitliche Temperaturanstieg sein Maximum erreicht hatte. Die Oberflächenkonzentration von Rosmarinsäure wurde nicht durch Hitzewellen, aber durch die jahreszeitliche Umweltveränderung beeinflusst. Zugleich korrelierte sie in gewissem Maße mit der Dichte potenzieller bakterieller Aufwuchsorganismen, was auf eine Anpassung der Verteidigungsstärke von *Z. marina* an den Besiedlungsdruck hinweisen könnte. Angesichts der Tatsache, daß Hitzewellen keinen reduzierenden, sondern eher einen verstärkenden Einfluß auf die Verteidigungsfähigkeit von *Z. marina* hatten, ist mit einer Schwächung der chemischen Verteidigung dieser Art gegen Aufwuchs durch die für die Zukunft erwartete globale Erwärmung eher nicht zu rechnen (Kapitel II).

Um den Einfluß jahreszeitlicher Veränderung im Ostseeraum auf die Verteidigung von *Z. marina* gegen Aufwuchs weiter zu untersuchen wurden über eine Vegetationsperiode monatlich Oberflächenextrakte gesammelt. Die Abwehrstärke dieser Extrakte gegen bakterielle Aufwuchsorganismen erreichte ihr Maximum im Sommer. Zugleich war auch die Dichte

potenzieller Besiedler höher war als zu allen anderen Jahreszeiten, was erneut auf eine Anpassung der Verteidigungsstärke von *Z. marina* an den Besiedlungsdruck hinzuweisen schien. Auch die Konzentration von Rosmarinsäure in Oberflächenextrakten korrelierte mit dem Besiedlungsdruck und der Dichte von Bakterien, die direkt auf den Pflanzen herrschte, was in ähnlicher Weise im Benthokosmenexperiment (siehe Kapitel II) beobachtet worden war. Zusätzlich zu Rosmarinsäure müssen noch weitere Oberflächensubstanzen bei der Verteidigung von *Z. marina* gegen Aufwuchs eine Rolle spielen, weil beide Effektgrößen nur mäßig korreliert sind. Darüber hinaus spielen nicht nur die Temperatur, sondern auch die Versorgung mit anorganischem Stickstoff offenbar wichtige Rollen bei der jahreszeitlichen Regulation der Antifoulingaktivität. Möglicherweise ist der Temperatureinfluß jedoch nicht direkt, sondern indirekt, weil die Temperatur auch den sommerlichen Besiedlungsdruck antreibt (Kapitel III).

Zusammenfassend kann gesagt werden, daß diese Dissertation die Existenz einer ökologisch relevanten chemischen Verteidigung von *Z. marina* gegen Aufwuchsorganismen nachweist und die wichtigsten Abwehrstoffe identifiziert. Nicht nur abiotische, sondern möglicherweise auch biologische Faktoren, die die chemische Verteidigung und die Produktion von Rosmarinsäure durch *Z. marina* beeinflussen wurden identifiziert, was neue Erklärungsansätze für die Dynamik der Wechselwirkungen von Bakterien und Seegrass bietet und die Basis für weitere Untersuchungen dieser Interaktionen darstellen könnte.

## **General Introduction**

A large proportion of lifeforms on Earth lives in the ocean between the sea surface and the sea floor in more than 10000 meters depth. Different lifeforms are adapted to their environment and they can also shape them and form unique ecosystems, such as coral reefs, kelp forests, seagrass meadows etc. Marine ecosystems differ from their terrestrial counterparts in many aspects, mainly due to the fundamental differences in the prevalence of air and water in these systems. Therefore, communities in marine ecosystems often show specific adaptations. For instance, microbes, plants or animals in marine environments often need to attach to substrata on the sea bottom to avoid or reduce the influence from currents. The survival of such sessile organisms largely depends on the availability of substrata, either in certain life stages or throughout the life cycle (Wahl 2009). In the marine environment, sessile organisms constantly compete for the attachment on both living and non-living surfaces, which leads to the consequence of epibiosis if the attached substratum is another organism.

### **Epibiosis**

Briefly spoken epibiosis is the concept that describes the accumulation of sessile microorganisms, plants, algae, or animals on the surface of other host organisms (Harder et al. 2008, Wahl 1989, Wahl 2009). Unlike terrestrial ecosystems, aquatic environments are uniquely characterized by features of high density and viscosity, which supports the sessile mode of live because the relative weight of aquatic organisms is relatively lower than that of their counterparts in air, due to the higher density of water. On the other hand, aquatic organisms are able to capture nutrients and energy directly from water instead of from soils, which terrestrial plants need for permanent attachment (Wahl 2009). Further, the aquatic environment is in general more favorable for the growth and settlement of microorganisms, and also the competition for substrate by sessile macroscopic live forms is relatively fiercer in the aquatic environment than on land (Rigby 1997, Thiel & Gutow 2005). It is for this reason that not only non-living, but also living surfaces are frequently used as a substratum for attachment. Such substrates are called ‘basibionts’ if they are also organisms, while settlers are called ‘epibionts’ (Wahl 1989) and “epizoans” or “epiphytes” if they are animals or algae, respectively (Wahl 1989, Wahl 2009).

Epibiosis generally goes through four stages: 1) the absorption of chemical molecules, 2) the attachment of bacteria, 3) the colonization by diatoms, fungi, and protozoans, and 4) settlement of invertebrate larvae and algae (Wahl 2009, Railkin 2004; Maki and Mitchell 2002) (Figure 1). Dissolved macromolecules (such as polysaccharides and peptides) are initially adsorbed to a newly submerged surface. This process takes place in the first seconds or minutes of epibiosis. Those compounds attract bacterial settlers and lead to the formation of primary biofilms that are composed of bacterial cells and an extracellular matrix. In the following stage more complicated, three-dimensional, diversified secondary biofilms may develop and lead to the attachment of macrofoulers, such as invertebrate larvae and algal spores (Wieczorek and Todd 1998). All the members of such an epibiotic community regulate the settlement of new members through attraction and repellence and they may also mutually affect each other's growth by chemical signals and competition (Harder 2008; Railkin 2004; Armstrong et al. 2001). Moreover, the interactions among epibionts and basibionts usually depend on the specific environmental context.

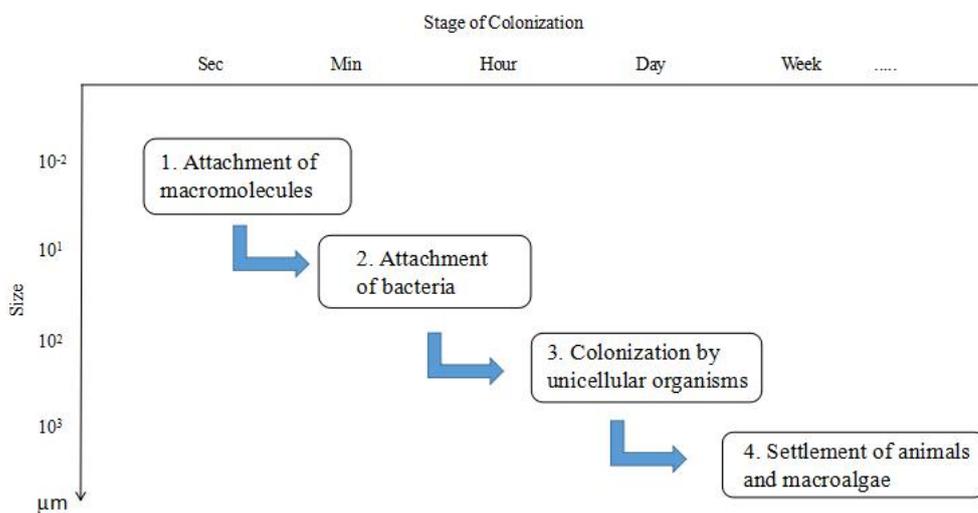


Figure 1. Sequences of colonization at surfaces in the aquatic environment.

### The consequences of epibiosis

The chemical and physical properties of the outer surface of a marine organism are the decisive

factors that influence the interaction between the basibiont and its epibionts (Wahl 2009). Exchange of gases, nutrient uptake, light absorption and/or emission or perception of chemical signals by hosts can be affected by epibionts and biological responses of hosts were triggered to cope with the unfavorable influence correspondingly, which were constantly observed in the basibionts-epibionts interaction (Harder 2008). Moreover, various processes such as heat exchange, particle adherence, surface charge and wettability may be modulated on surfaces once they are colonized (Wahl 2009). Those processes can have numerous consequences that could be both harmful and beneficial for hosts and colonizers.

*Consequences for basibionts:*

In most cases the disadvantages of epibionts on their hosts are more obvious. For instance, competition for nutrients between hosts and colonizers often occurs (Wahl 2008) and nutrients that reach the basibiont may be diminished by the epibiotic biofilms if these have similar needs. Epibiotic organisms may cause a reduction of light supply by up to 80 % to host plants, which can even lead to the mortality of host plants (Bulthuis & Woelkerling 1983). Predation can also be enhanced by epibionts, which has been described as ‘shared doom’ (Wahl & Hay 1995). On the other hand, some benefits of epibiosis for basibionts have also been observed. For example, in some symbiotic interactions nitrogenous compounds, vitamins and other secondary metabolites are provided by epibiotic diatoms or macroalgae to their basibionts (Lupton & Marshall 1984, Wahl 2008) and protective coatings by unpalatable epibionts may help hosts to escape from predation (Karez et al. 2000, Dougherty & Russell 2005, Marin & Belluga 2005, Cerrano et al. 2001) or other detrimental epibionts or pathogens (Wahl & Hay 1995; Poore & Hill 2005; Thornber 2007; Potin 2012).

*Consequences for epibionts:*

Compared to basibionts, the consequences of epibiosis on epibionts are relatively simple and more straightforward. The major advantage for epibionts is the settlement itself, which is essential for sessile animals and algal propagules. Attachment to a substrate generally protects algal propagules and animal larvae from the constant threats of strong water currents (Wahl 2009) and a capacity to

colonize living surfaces that are not accessible to all settlers provides access to a stable and exclusive environment (Oswald & Seed 1986, Riedl 1971). In addition, epibionts may possibly benefit from the deterrent effects of well defended hosts against predation and they may take advantage from the exudates or feeding habits of their hosts in terms of nutrient intake (Wahl 2009). On the other hand, epibionts have to deal with any defensive mechanisms that are exerted by their hosts and they may have to cope with severe changes of environmental variables if the host is motile and capable to move between different marine environments (Wahl 2009).

### **The role of bacteria in epibiosis**

As mentioned above, bacterial attachment starts to occur already in the first phases of epibiosis, soon after the biochemical conditioning of uncolonized surfaces (Wahl 1989, Harder 2008). The absorption of macromolecules begins in the first few seconds after the first immersion, and marine bacteria that are attracted by chemical cues may attach to the surface area through physical forces including Brownian motion, electrostatic interaction, gravity and Van-der-Waal forces (Dexter 1976, Banerjee et al. 2011). The physicochemical and biological properties of bacterial surfaces are important for this initial attachment. For instance, shapes of bacteria may determine the order of attachment of microsettlers, and rod shaped and coccoid bacteria generally settle earlier than filamentous cells (Marshall et al. 1971, Corpe 1972). Bacterial cells - along with the pre-formed macromolecular matrix system - build up the primary biofilm, which supports the colonization of uni- or multicellular eukaryotes in subsequent sequences and provides inductive cues to the larvae of animals and spores of algae (Unabia & Hadfield 1999). During the first stage of biofouling, communication among and within bacterial species is crucial for the formation and maturation of the primary bacterial biofilm. Quorum sensing (QS) is a density-dependent communication among bacterial cells which allows bacteria to respond with changes on a population-wide scale (Waters & Bassler 2005). Signaling chemicals (usually referred to as autoinducers) are produced and released for communication, and make bacterial colonies or biofilms act like a multi-cellular organism (Parsek & Greenberg, 1999). For example, in the first stage of infection, bacterial QS mediates the deactivation of virulence gene expression in pathogenic microbes within biofilms, which avoids the triggering of host defenses until a

sufficient number of pathogens has accumulated (Rice et al. 2005). Therefore, interference of the regulation can be another approach to resist biofouling.

### **Responses of the hosts**

Marine organisms are nearly continuously exposed to “fouling pressure”, the colonization attempts of potential epibionts. However, most marine plants, algae or animals remain largely free of heavy fouling in most circumstances, which suggests that basibionts have developed effective and often very specific strategies to deal with epibiosis. Those strategies are mainly based on three aspects: tolerance of epibiosis, physical (or mechanical) defense, and chemical defense (Wahl 2009). Some basibionts were reported to be capable of tolerating certain non-pathogenic microepibionts and of selectively rejecting others through targeted defenses (Schaffelke & Deane 2005). Peeling of the cuticula and/or epidermis or intensive superficial mucus production by hosts are examples of physical defenses that periodically remove all foulers from the surface (Nylund & Pavia 2005, Littler & Littler 1999), which comes along with the risk that the new ‘skin’ may again encounter settlement of numerous bacteria, fungi and diatoms (Wahl & Banaigs, 1991). In addition, macrophytes can also benefit from their epibionts in terms of defense against foulers and herbivores. For instance, chemicals released from bacterial epiphytes on the surface help their hosts to avoid heavy fouling (Borowitzka et al. 2007, Marhaeni et al. 2011) and compared to seagrass, the productive algal epiphytes attached to seagrasses are preferable food resources for the seagrass associated animals such as gastropods, amphipods and isopods (Jernakoff et al. 1996), which largely protect seagrass from grazing and in some cases from biofouling (Marhaeni et al. 2011).

Chemical defense is another approach that may be used by hosts to avoid heavy fouling. Various chemicals produced by basibionts - including terpenoids and steroids (White et al. 2008, Tsoukatou et al. 2007), fatty acids (Baze et al. 2009, Bhattarai et al. 2007), amino acids (Diers et al. 2004, Ortlepp et al. 2007), alkaloids (Diers et al. 2006, Nakamura et al. 1982, Dahlström & Elwing 2006) etc. - have already been identified as potential or demonstrated antifouling compounds in marine organisms. A number of those compounds were demonstrated to be non-toxic for foulers (See review by Fusetani 2011), as they repel or prevent the attachment of

foulers instead of killing them. There are studies indicating that plants are able to produce chemicals to inhibit QS among bacteria, which then leads to a disruption of bacterial films and repels foulers from their surface. For instance, halofuranones from the red alga *Delisea pulchra* were demonstrated to be inhibitors of quorum sensing which block the communication among bacterial cells within the epibiotic communities and thereby prevent the formation of primary bacterial biofilms (Dworjanyn et al. 2006) In fact, there is a considerable body of evidence suggesting that chemical antifouling defense by basibionts is typically not based upon the production of one extremely toxic compound that indiscriminately repels or kills all foulers. Instead, the effects of active compounds tend to be selective and specific for certain target species (Egan et al. 2000, Kubanek et al. 2003) or even induced by targeted foulers (Matz et al. 2008) or herbivores (Lankau 2007). Thus, most if not all marine organisms seemingly employ various defense mechanisms in parallel, and their combined effects keep the host free of heavy fouling in the natural marine environment.

### **The importance of surface extracts**

Most of the antifouling defense related compounds that were so far discovered have been isolated from tissue extracts and tested at tissue concentrations (Pereira & Da Gama 2008, Goecke et al. 2010, Iyapparaj et al. 2014). This approach is useful for the discovery of functional natural compounds from the marine environment for biotechnological applications, but the ecological relevance of such compounds for antifouling defense obviously depends much more on their presence at the surface of basibionts (Jennings & Steinberg 1994; Lane et al. 2009). Although some studies demonstrated the ecological importance of surface extracts coming from certain basibionts (Dworjanyn et al. 2006; Sudatti et al. 2008; Saha et al. 2011) comparable information is still largely missing for most marine organisms.

### **Regulation of antifouling defense**

Antifouling defense activity can be influenced by numerous factors. The strength of a chemical defense is usually directly related with synthesis, transport, and storage of secondary metabolites (Strauss et al. 2002, Stamp 2003, Neilson et al. 2013) and the metabolic and energy costs that can

be allocated to a given defense mechanism usually depend on other processes of life, such as growth (Cronin 2001, Mazid et al. 2011). For instance, the production of halofuranones with antifouling activity in the alga *Delisea pulchra* is significantly negatively correlated with the growth rate (Dworjanyn et al. 2006) and fucoxanthin isolated from the brown seaweed *Fucus vesiculosus* was demonstrated to be active in antifouling defense (Saha et al. 2011) in addition to its known function as an accessory pigment (Katoh et al. 1991). Likewise, another antifoulant, dimethylsulphoniopropionate (DMSP) from *F. vesiculosus*, also plays a role as osmoregulator in algae (Rudulier et al. 1996). Also the content of phenolics in seagrasses – compounds that are functional in antifouling (Iyapparaj et al. 2013, Iyapparaj et al. 2014, Mayavu et al. 2009), anti-herbivory (Grignon-Dubois et al. 2012, Steele & Valentine 2012, Vergés et al. 2011) and anti-pathogen defence (Arnold et al. 2012, Steele et al. 2005) - were correlated with CO<sub>2</sub> levels (positively) and nitrogen levels (negatively) in the surrounded environment (Mata et al. 2012). The reason for this is probably in the fact that the production of phenolic compounds in plants is an approach to store carbon and energy when nitrogen supply is limited (Planchet et al. 2015). If – as in these examples - secondary metabolites are not only relevant as defense compounds, but also in the responses to other environmental factors, then the defense activity may obviously be regulated by those other factors, for example light (Amade & Lemee 1998), temperature (Sudatti et al. 2011) or nutrient availability (Arnold et al. 2012, Buchsbaum et al. 1990) or by synergistic effects of abiotic and biotic factors (Helio et al. 2004, Marechal et al. 2004, Wahl et al. 2010).

According to the regional climate models, the mean water temperature of the Baltic Sea is predicted to increase by 3 to 5 °C in summer and by 5 to 10 °C in winter in next decades (BACC 2006). Therefore macrophytes in the Baltic Sea will be forced to deal with higher water temperatures and more frequent heatwave events (Beniston et al. 2007). Severe loss of habitat species in temperate marine environments was constantly observed during the past decades. For instance, a heatwave event lasted for 3 weeks in summer of 2003 and caused major losses of eelgrass at European coasts (Greve et al. 2003, Reusch et al. 2005). One of the reasons for large scale die-offs could be elevated pathogenic infections at high temperature conditions, a phenomenon that was demonstrated in some cases (Vergeer et al. 1995). Macrophytes are of

ecological importance due to their habitat forming role in marine ecosystems, therefore their loss may lead to serious consequences or even devastation of whole ecosystems. Therefore, a better understanding of the influence of high temperatures on the antifouling defense capacity of macrophytes such as eelgrass in the Baltic Sea is necessary to allow for the conservation of these marine ecosystems.

### **Induced chemical defense**

In addition to intrinsic and environmental factors, the antifouling defense of basibionts could also be influenced by the surrounding fouling communities. Macroalgae may perceive the invasion of certain epibionts through recognition of signaling compounds that originate from either microbes or from degradation or wounding of the basibiont (Weinberger et al. 1999, Nylund et al. 2011). Corresponding biological processes may then be activated along with the regulation of specific defense related compounds (Weinberger 2007, Egan et al. 2014). For instance, penetration of pathogens was shown to cause the breakdown of host cell walls in the red alga *Gracilaria conferta* (Weinberger et al. 2005) and the brown alga *Laminaria digitata* (Küpper et al. 2006) and the products originating from this degradation (so-called pathogen-induced molecular patterns, PIMPs) were perceived by host receptors. This recognition activated in both algal species within minutes after the perception of PIMPs an increased NADPH oxidase activity, protein kinases, the production of other defense related compounds - such as halocarbons – and an antibiotic effect on associated microorganisms (Weinberger et al. 2005, Weinberger et al. 2007). The presence of similar receptors that perceive bacterial lipopolysaccharides was recently also demonstrated for the seagrass *Thalassia testudinum* (Loucks et al. 2013). Thus, it was revealed that seagrasses contain signal transduction systems that appear to be conserved from the defense response systems of terrestrial plants. However, it is not clear whether these signalling systems and subsequent cellular responses only play a role in the defense against microbial pathogen or whether they can also target microbial epibionts in general (Loucks et al. 2013).

Compared to constitutive defenses, facultative defense strategies could be more favorable, due to their lower energy costs. Facultative defense is not constant and continuously functional, but only activated or induced when it is needed (Agrawal and Karban 1999). Most hosts have to

deal with a wide range of consumers, microbes, and pathogens and not all of them are capable to cause threats for the wellbeing of the hosts. Also for this reason most hosts probably employ strategies (i.e. induced defenses) that kill or repel enemies selectively according to demand, while beneficial or neutral organisms remain unaffected (Egan et al. 2000, Kubanek et al. 2003). Finally, demand-driven defenses also lower the risk of pathogen adaptation to the resistance mechanisms (Boyd 2006).

### **The seasonality of antifouling defense**

Not surprising if we consider the before mentioned possibilities for biotic and abiotic impact, the defense reactions of basibionts often exhibit seasonal patterns. For instance, the antifouling defense of the alga *Fucus vesiculosus* exhibited a clear seasonal pattern that corresponded with seasonal fluctuations of fouling pressure and with several abiotic factors and this seasonality was reflected in corresponding changes in the concentrations of certain defense metabolites (Hellio et al. 2004, Saha et al. 2012, Wahl et al. 2010).

### **Eelgrass**

*Zostera marina* L, also named as eelgrass, is one of the most abundant and important habitat plants in northern American and European near shore areas. It is a cold temperate species that is distributed in the northern Pacific, the northern Atlantic (even within the Arctic Circle), the Mediterranean and the Black sea (den Hartog 1970). *Z. marina* is a rhizomatous perennial herb that has shoots and long green leaves that may become up to one meter long. It is able to reproduce itself both sexually and vegetative. The plant is monoecious in term of sexual reproduction; both male and female flowers appear on the same plant. Pollination occurs after flowering and the maturation of seeds normally takes 4 or 5 weeks before they are released and sink to the bottom of the water column (de Cock 1980). Eelgrass plants may also produce new plants clonally from rhizomes and form meadow-like colonies (Phillips et al. 1983). In many near shore ecosystems, eelgrass meadows are an important component because they provide a habitat for many other species and the basis of complex food webs (Borum et al. 2004). Eelgrass populations are currently threatened by several different processes. For instance, human activities

have damaged eelgrass meadows in various ways. For example, practices in fishing and aquaculture - such as dredging and trawling – have destroyed numerous eelgrass meadows (Borum et al. 2004) and reduced light penetration depth due to eutrophication has limited the distribution of eelgrass to shallower parts of the Baltic Sea (Baden et al. 2002, Bekkby et al. 2008). Similar as other basibionts, eelgrass is also threatened by high fouling pressure. Epiphytic biofilms that are composed of micro- and macrofoulers may reduce the availability of nutrients and light, thereby hamper the efficiency of photosynthesis and metabolism in eelgrass shorten living resources (Brush & Nixon 2002). The chemical defense of eelgrass against foulers has been studied since the 1970s and various secondary compounds were isolated and identified as defense related. Among those compounds, phenolic acids such as caffeic acid and zosteric acid were among those most frequently discovered (Achamlale et al. 2009, Barrios et al. 2005, Vergeer & Develi 1997). However, all of those studies were based upon plant tissue analyses and the ecological relevance of those compounds was not demonstrated. Moreover, the analytical chemistry of plant phenolics is relatively complicated, due to their diversity and sensitivity to oxidation and degradation. In this light the analytical approaches used in some of the older studies appear as questionable.

### **Thesis outline**

When I started this dissertation project in 2012 it was known that seagrasses have a chemical antifouling defense and this defense was expected to be largely based upon phenolics (Arnold et al. 2012, Steele et al. 2005). Phenolic components isolated from *Z. marina* tissue had shown antigrazing (Lewis & Boyer 2014) and antipathogen effects (Vergeer and Develi 1997) and were expected to also affect epibionts (Todd et al. 1993). For example, Zosteric acid, a phenolic compound that was repeatedly discovered in the tissue of eelgrass (Enerstvedt et al. 2016), was proven to be an effective antifouling compound that reduces the attachment of bacteria on surfaces (Newby et al. 2006, Villa et al. 2010). However, the composition and concentration of antifouling compounds on the surface of eelgrass was still unknown. In addition, while the effect of abiotic factors such as light, temperature and nutrients on - and the seasonal patterns of - antifouling defenses had been investigated in some other aquatic plants (Saha et al. 2013, Rickert et al. 2016), such research was missing for seagrass and there was also no evidence that antifouling defense

could be driven by demand.

The aims of my research were therefore to

(A) identify those chemicals on the surface of eelgrass that are responsible for its chemical antifouling defense (Chapter I),

(B) study the regulation of relevant defense compounds and the antifouling activity in response to temperature increase (Chapter II),

(C) study the response of relevant defense compounds and antifouling activity to seasonal fluctuations (Chapter III)

A suitable surface extraction method for eelgrass leaves was developed for the isolation of relevant defense compounds of different polarity. Active extracts were generated, selected and further separated on HPLC through a bioassay guided fractionation approach. In this experiment, settlement inhibition bioassays with different bacterial foulers were used to detect repellents that were present in extracts and fractions. Purified compounds were characterized by LC-MS and NMR. Purchased chemical standards of identified compounds were then used to determine their necessary concentrations for antifouling activity. In the end, these necessary concentrations were compared to measured surface and tissue concentrations (Chapter I).

To study responses of the antifouling defense of *Z. marina* plants in response to temperature impact eelgrass was incubated in the Kiel Outdoor Benthocosm system (KOB) for four months under simulated natural conditions. In addition to a control treatment that simply simulated the seasonal temperature increase during a year without major temperature fluctuations two kinds of heat treatments were also applied in this experiment. These consisted of (1) repeated heatwave treatments that were applied in approximately monthly intervals, lasted for 1 week and peaked at a temperature that was 5 °C above the control treatment and (2) only one heatwave treatment of the same type that was applied at the end of the experimental period. Several variables (including fouling pressure, density of bacteria settled on eelgrass, antifouling defense activity etc.) were

repeatedly measured to elucidate the effects of seasonal temperature increase with or without occurrence of heatwaves on the antifouling defense activity of eelgrass (Chapter II).

To study the seasonal change of antifouling defense in eelgrass a natural stand was repeatedly sampled over a period of six months and I quantified the intensity of chemical defense in different months. Simultaneous quantification of the fouling pressure and densities of bacteria that had settled on eelgrass leaves allowed me to search for possible correlations among fouling pressure and defense. In addition various abiotic factors such as temperature, light, salinity and nutrient concentrations were also monitored and correlated with the measured responses of seagrass and microfoulers (Chapter III).

### **Study hypotheses:**

The main hypotheses of my work were these:

- 1) *Z. marina* surfaces are protected from microfouling by specific defense compounds that deter bacterial settlers.
- 2) The chemical antifouling defense activity of *Z. marina* is affected by abiotic factors and in particular by temperature.
- 3) The antifouling defense activity of *Z. marina* fluctuates seasonally.
- 4) The antifouling defense activity of *Z. marina* fluctuates with the demand.

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## **Publications and Contributions of Authors**

*Parts of this doctoral thesis have been submitted or manuscripts for publication:*

### **Chapter I. - Manuscript submitted to “Biofouling” and currently in revision.**

**Guan C.** Parrot D, Wiese J, Sönnichsen F, Saha M, Tasdemir D, Weinberger F. The role of rosmarinic acid and sulfated flavonoids in the chemical defense of the eelgrass *Zostera marina* against microfouling

*FW and CG conceived and designed the experiments. CG collected seagrass material and performed the bioassay experiments and the fractionation on HPLC. MS provided the bacterial species. DP, DT helped with fractionation on HPLC. DP, DT and FS identified compounds in LC-MS and NMR. CG and FW performed statistical analysis. CG wrote the manuscript, and FW contributed to revisions.*

### **Chapter II. - Manuscript not submitted yet.**

Guan C, Weinberger F, Saha M Heat waves accelerate the chemical defense of *Zostera marina* against microfoulers

*CG and FW conceived and designed the experiments. CG collected material and performed the experiments. MS provided bacteria for the bioassays and counted density of bacteria on PVC panels and on eelgrass. CG and FW performed the statistical analysis. CG wrote the manuscript, and FW contributed to revision*

### **Chapter III. - Manuscript not submitted yet.**

Guan C, Weinberger F, Saha M Seasonal variations in the anti-bacterial defense activity of *Zostera marina* L. correlate with seasonal variations in fouling pressure and density of microepibionts

*CG and FW conceived and designed the experiments. CG collected eelgrass materials and performed the experiments. MS provided bacteria for the bioassays CG and FW performed the statistical analysis. CG wrote the manuscript, and FW contributed to revision*

## **The role of rosmarinic acid and sulfated flavonoids in the chemical defense of the eelgrass *Zostera marina* against microfouling**

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# The role of rosmarinic acid and sulfated flavonoids in the chemical defense of the eelgrass *Zostera marina* against microfouling

## Abstract

We identified ecologically relevant defense compounds that are present on the surface of *Zostera marina* and inhibit settlement of microfoulers at natural concentration. Moderately polar eelgrass surface extracts inhibited the settlement of seven marine bacteria that originated from nonliving substrata and of one yeast. In contrast, five other bacterial strains that had been directly isolated from eelgrass surfaces were all insensitive, which suggested a selective effect of surface metabolites on the microbial communities present on eelgrass. Bioassay guided isolation of active compounds from the extracts in combination with UPLC-MS and <sup>1</sup>H-NMR spectroscopy resulted in the identification of rosmarinic acid, luteolin-7-sulfate and diosmetin-7-sulfate or its isomer chrysoeriol-7-sulfate. All three compounds are nontoxic repellents, as they did not inhibit bacterial growth but prevented bacterial settlement in a dose-dependent manner. However, only rosmarinic acid was consistently present on the surface of eelgrass at sufficiently high concentrations to control bacterial settlers.

Key words: Antifouling; Diosmetin; Luteolin; Rosmarinic acid; Seagrass-microbe-interaction; *Zostera*.

## Introduction

Marine organisms - including macrophytes - are under most conditions subject to a strong settlement pressure by micro- and macroepibionts that is comparable in intensity to that on non-living surfaces (Amstrong et al. 2001; Wahl 2009). The associations between bacterial biofilm and host could be of various types and may range from mutualistic through neutral to antagonistic relationships (Hay et al. 2004; Wahl 2008). Harmful and disadvantageous effects of microfoulers can be: 1) their inducing role encouraging settlement of macrofoulers (Dobretsov et al. 2006; 2013); 2) a reduction of light available for host photosynthesis (Rohde et al. 2008; Wahl et al. 2010) and 3) a reduction of nutrients and oxygen availability, or a detrimental effect on the pH balance on the host surface (Wahl et al. 2012). Nevertheless, under most conditions marine macrophytes are less fouled than non-living substrata (Wahl 1989; da Gama 2014), indicating the presence of a defensive mechanisms and a capacity to control attachment of undesirable microorganisms. Differences in the degree of fouling on living organisms and mimics have also been observed with seagrasses (Pinckney & Micheli 1998), flowering plants that are derived from terrestrial ancestors. During their evolution seagrasses have recolonized the marine environment (Ackerman 2007), raising the interesting question of how they adapted to marine fouling. The present investigation addresses this question, using the eelgrass *Zostera marina* as a study organism.

The eelgrass *Zostera marina* (family Zosteraceae) is widely distributed in intertidal and subtidal inshore waters of North America, Europe and Asia (Short et al. 2007). It is one of the most important habitat formers in the Baltic Sea (Rönnbäck et al. 2007). Eelgrass beds are very productive environments (Short et al. 2007; Green & Short 2003). They are nursery habitats for fish and shell fish (Duffy 2006; Fourqurean et al. 2012) and an important carbon sink (Röhr et al. 2016). Further, they form the basis of a complex food web system and of have various functions important for maintaining stable coastal ecosystems (Duarte et al, 2002; Duffy 2006; Fourqurean et al. 2012). The ecological importance of eelgrass became particularly apparent in the 1930ies and again in the 1980ies, when major outbreaks of “eelgrass wasting disease” destroyed many *Z. marina* habitats in the Atlantic (Den Hartog 1987; Short et al. 1987). The disease is caused by a

slime mold pathogen, *Labyrinthula zosterae* (Short et al. 1987) and recent work demonstrated that the infection process can be facilitated by an eelgrass associated marine yeast, *Cryptococcus fonsecae* (Weinberger et al., unpublished). Thus, biofilm components that are present on eelgrass surfaces may not only influence macrofouling, but also the disease resistance capacity of their host.

The defense mechanisms of marine macrophytes against microfoulers receive increasing attention (Scardino et al. 2011; Paradis et al. 2016; Othmani et al. 2016). Most studies suggest that metabolites produced either by the host or by certain mutualistic microorganisms that are associated with the host can inhibit antagonistic microsettlers (Egan et al. 2000; Dobretsov et al. 2005; Silva-Aciares & Riquelme 2008). For example, several seaweeds rapidly generate and excrete reactive oxygen species in response to microbial pathogens (Potin 2008; Weinberger et al. 2007). Such oxidative burst responses can build up concentrations of hydrogen peroxide and related compounds over algal surfaces that are sufficient to kill microorganisms (Potin 2008; Weinberger et al. 2007; Loucks et al. 2013). Moreover, a wide range of secondary and even primary metabolites originating from seaweeds have been demonstrated to inhibit microorganisms, either through toxic or repellent effects (Cardozo et al. 2007).

Phenolics often play important roles in the resistance of terrestrial plants against consumers and pathogens (Ranilla et al. 2010; Ghasemzadeh 2011) and similar functions have repeatedly been suggested for the phenolic metabolites that are present in seagrasses (Vergés et al. 2011; Grignon-Dubois et al. 2012), which are generally rich in phenolic metabolites (Arnold et al. 2012; Grignon-Dubois et al. 2012). For example, zosteric acid, rosmarinic acid and flavonoids and their derivatives were detected in *Zostera marina* (Quackenbush et al. 1986; Wang et al. 2012; Enerstvedt et al. 2016) and several studies demonstrated or suggested that these compounds may be responsible for antiherbivore (Lewis & Boyer 2014), antimicrobial (Vergeer & Develi 1997; Wang et al. 2012; Laabir et al. 2013) or antifouling (Mayavu et al. 2009) defenses.

However, most of the studies that so far investigated the antibiosis or antifouling effects of seaweeds and seagrasses applied total tissue extracts (Kubanek et al. 2003; Iyapparaj et al.

2014). This approach may be useful to unravel the general biotechnological potential of marine natural products, but it will not necessarily lead to the discovery of metabolites that are ecologically relevant with respect to fouling: Only compounds that are present directly at the host surface may be expected to affect settlers on this surface (Jennings & Steinberg 1994; Lane et al. 2009). During the last few years various studies have consistently shown that anti-fouling compounds are present on the surfaces of seaweeds that can inhibit or kill microfoulers and may be considered as ecologically relevant (Dworjanyn et al. 2006; Sudatti et al. 2008; Saha et al. 2011). However, comparable information is still largely missing for seagrasses.

In the present study we therefore aimed 1) to develop a method for the extraction of surface associated phenolic compounds from eelgrass leaves; 2) to investigate the general potential of such metabolites for the deterrence of microfoulers; 3) to isolate and identify bioactive metabolites through a bioassay-guided approach, and to study their specificity.

## **Material and Methods**

### ***Eelgrass material***

1 kg *Zostera marina* L. (wet weight) was sampled from Falkenstein Beach at Kiel, Germany (54°38' N / 10°17' E) in March 2013. The location belongs to the Kiel Fjord in the southwest Baltic Sea. The average water salinity at the site is around 16 ‰. The plants were transported in Ziplock bags in a cooler box to the laboratory, where they were immediately placed into an aquarium filled with natural seawater to avoid tissue damage and dehydration. The aquarium was maintained in a climate room at a temperature of 15 °C under artificial light (20-30  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) for less than one day before extraction.

### ***Solvents and reagents***

Acetonitrile, formic acid and all phenolic standard compounds were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA), while Carl Roth (Karlsruhe, Germany) supplied other solvents and reagents. LC-MS grade solvents for UPLC/MS analysis were purchased from VWR (Pennsylvania, USA).

### ***Surface extraction of eelgrass***

The ‘dipping method’ (de Nys et al. 1998) was used to prepare *Z. marina* surface extracts. Prior to extraction the tolerance of eelgrass epidermal cells to short-time immersion into different organic solvents was tested, in order to identify a condition that would extract compounds from the plant surface, but not from plant cells. Therefore, fresh plants were rinsed with artificial seawater, spin-dried in a salad spinner and dipped for different times (5s, 15s, 30s and 60s) into one of the four following solvents separately: methanol, propan-2-ol, ethyl acetate or *n*-hexane. After exposure to organic solvent, the plants were immediately rinsed again with artificial seawater and incubated for 30 min in seawater containing 0.05% of the lethal stain Evans Blue (Sigma Aldrich®, St. Louis, Missouri, USA) (Weinberger et al. 2005). Untreated plants were used as a positive control. After the staining procedure, all specimens were investigated under the microscope (magnification: 10x) for presence of stained cells. Dipping in propan-2-ol for 5 s or in *n*-hexane for 30 s proved not to damage epidermal cells, hence these solvents were selected for preparing moderately polar and unpolar surface extracts from eelgrass (Figure S1).

Prior to extraction, the plants were rinsed several times with artificial seawater, in order to remove sand, attached algae and herbivores. Only healthy green leaves were used for the extraction. All leaves were pooled, in order to obtain a sufficient quantity of extract for the subsequent fractionation steps and analyses. The leaves were spin-dried in a salad spinner, weighed and dipped into solvent as described above. Contact of cut ends of leaves with solvent was carefully avoided to avoid contamination of intracellular compounds. Crude extracts prepared in this way were immediately filtered through glass fiber-filters (0.2 µm mesh size; Carl Roth, Karlsruhe, Germany). Solvent was evaporated *in vacuo* at 30 °C and the extracts were stored in a freezer (-20°C).

Subsequently *n*-hexane extracts were redissolved in *n*-hexane, while propan-2-ol extracts were redissolved in methanol, in order to facilitate the following bioassays and fractionation steps. Methanol was selected to dissolve the propan-2-ol extracts. Due to its higher velocity, methanol evaporates better than propan-2-ol and the bioactivities of propan-2-ol extracts dissolved in

methanol or in propan-2-ol are identical (Figure S2). Thus, no bioactive compounds were lost by this change of solvent.

### ***Extracted surface volume***

The concentration of the compounds extracted from *Zostera* surfaces was related to the extracted surface volume, i.e. the volume of approximately 30  $\mu\text{m}$  thickness that forms a boundary layer over the surfaces of marine plants (Wahl et al. 2010). To determine this volume, spin-dried leaves of defined weight were spread out on a flatbed scanner and scanned together with a bench-mark. Leaf areas were then determined from the leaf projections, using the imaging software Image J (National Institute of Health, Bethesda, Maryland, USA). The mean leaf area/wet weight ratio of 5 g *Z. marina* samples that were analyzed in this way was  $78.9903 \text{ cm}^2 \text{ g}^{-1}$ . Multiplication of this value with 30  $\mu\text{m}$  leads to a surface volume of  $0.237 \text{ cm}^3 \text{ g}^{-1}$ , thus 1 g of fresh plants was on average covered by a surface volume of 0.237 ml and 100  $\mu\text{l}$  of surface volume came from 421.9 mg of *Z. marina*.

### ***Microfoulers***

Several bacterial isolates were used in bioassays to quantify the bioactivity of extracts. Eight strains that had been isolated earlier by our group from stones in the upper subtidal and lower intertidal zones of German North Sea and Baltic Sea coasts (Saha et al. 2016) were used in the bioassays. Two of these isolates phylogenetically related to the species *Vibrio cyclitrophicus* (strain ANORD3) and *Marivita litorea* (strain ANY8) were systematically applied in the bioassay-guided fractionation of deterrents from *Z. marina* extract. Six additional strains originating from the same collection were only used to test the bioactivity of purified compounds and standard compounds. These strains affiliated to *Alteromonas stellipolaris* (strain AMAN1), *Formosa agariphila* (strain AFALK3), *Loktanelia rosea* (strain AFALK1), *Staphylococcus hominis* (strain AFALK10), *Polaribacter dokdonensis* (strain ANORD1) and *Pseudoalteromonas carrageenovora* (strain AMAN4). Moreover, five bacteria were newly isolated from eelgrass plants that were collected on the 11<sup>th</sup> of August 2016 at Falkenstein beach. The isolation and identification of these bacteria by means of sequencing of the 16S rRNA gene followed Saha et al.

(2016) and DNA sequences obtained were deposited at GenBank (NCBI, Bethesda, Md.). Two of these isolates were closely related with *Pseudoalteromonas undina* NBRC 103039<sup>T</sup> (GenBank accession number AB681919). Strain B (GeneBank accession number KY440047) exhibited a similarity to the type strain of 99.71 % and strain P (GenBank accession number KY440048) showed 99.43 %). Two other isolates, strain M and strain WH were closely related with *Bacillus cereus* ATCC 14579<sup>T</sup> (GenBank accession number AE016877). The similarity to this type strain was 99.93% for strain M (GeneBank accession number KY440049) and 99.87 % for strain WH (GenBank accession number KY440050). The fifth isolate could not be identified. For bioassays, all bacteria were cultivated in liquid medium containing 25 g sea salt, 5 g peptone, and 3 g yeast extract in 1 L deionized water at 25 °C in darkness for 1-2 days before bioassays.

In addition, the yeast *Cryptococcus fonsecae* strain KF921 previously isolated from a seawater sample collected in August 1995 at the island Helgoland (North Sea, Germany) during the Victor Hensen cruise 95-21 by Dr. Karsten Schaumann (Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven, Germany) was used in bioassays. The strain was cultivated in yeast medium containing 25 g sea salt, 12 g Kobe agar, 10 g glucose, 5 g peptone, 3 g maltose and 3 g yeast extract in 1 L deionized water (pH 5.8). All the strains were maintained as cryostocks at -80°C.

### ***Bioassays***

A bioassay quantifying inhibitory effects on microbial settlement was systematically employed for the detection of bioactive compounds in *Z. marina* extracts that deter microbial settlers (bacteria or the yeast *C. fonsecae*). A second assay quantifying inhibitory effects on growth of the same microorganisms was employed in addition, in order to test whether compounds that inhibited settlement also affected growth. For both bioassays either extracts or pure compounds were added into microtiter wells (96 well plates, flat bottom, Greiner). Aliquots of each extract or compound to be tested were diluted in 100 µl of solvent and were pipetted into 8 wells. Identical amounts of pure solvent were employed into 8 separate wells as solvent controls. The solvent was then evaporated by a freeze-dryer.

### *Settlement inhibition assay*

The tendency of microorganisms to settle on the surfaces of wells impregnated with *Z. marina* surface extract was quantified following the general principles outlined in Saha et al. (2011) with minor modifications. Bacteria were cultivated on a shaker at 25°C for 24-36 hours in liquid medium (see above) and the yeast was cultivated under the same conditions for 24 h in yeast liquid medium before these microorganisms were used in the bioassay; at this time they were still in their exponential growth phase. Aliquots of the cultures (100 µl) were added into 4 of the 8 wells and 100 µl of sterile seawater were added into the remaining 4 wells as a control of extract fluorescence. After inoculation the wells were incubated for 2 h at 25°C at 100rpm. After this time unattached cells were removed by rinsing with seawater (Saha et al. 2011). Attached bacterial cells were stained with the DNA dye SYTO9 (Invitrogen, Waltham, Massachusetts, USA) as described in Saha et al. (2011) and their fluorescence intensity was measured using a Hidex Chameleon plate reader (wave length of excitation: 477-491 nm, wavelength of emission: 540 nm). Likewise, attached yeast cells were stained with the fluorescent dye Calcofluor White (Sigma Aldrich®, St. Louis, Missouri, USA) at a concentration of 0.2 mg ml<sup>-1</sup> for 10 min, and the fluorescence was measured with the same plate reader (wave length of excitation: 350nm, wave length of emission: 430nm) (Ram & Klis 2006) as a measure of relative cell abundance. Crude extracts, fractions and compounds prepared from *Z. marina* surfaces were tested at 1-fold natural concentration. In addition, standard compounds were purchased (Sigma Aldrich®, St. Louis, Missouri, USA) and tested over concentration ranges that included the natural concentration range of these compounds.

### *Growth inhibition assay*

The possible effect of *Z. marina* surface extracts on the growth of marine fouling bacteria and of *C. fonsecae* was also tested in microtiter well plates that were impregnated with *Z. marina* surface extract as described above. The wells were inoculated with 100 µl of 10 h old cultures and incubated on a shaker at 25°C at 100rpm. Light absorption at 610 nm was repeatedly measured every hour from 10 h to 24 h after inoculation, in order to monitor the relative density of suspended microbial cells. Concentration gradients ranging from 2-fold over 5-fold to 10-fold natural concentration were tested in these growth inhibition assays. The three tested concentrations

required an addition of surface extract per well and were prepared from 844 mg, 2110 mg and 4219 mg *Z. marina*, respectively. This was done because all assays were conducted in volumes of 100  $\mu$ l and a surface volume of 100  $\mu$ l was on average detected on 421.9 mg *Z. marina* (see above).

### ***Bioassay-guided fractionation***

Crude surface extracts exhibiting inhibitory activity on the settlement of *V. cyclitrophicus* (strain ANORD3) and *M. litorea* (strain ANY8) were fractionated by high pressure liquid chromatography (HPLC) on a semi-preparative LichroCart 250/10 Purospher STAR-rp 18ec column (VWR, Radnor, USA), using a Varian 940-LC with integrated photodiode array detector (PDA) and a fraction collector (Varian 940-LC, Agilent Technologies, Palo Alto, California, USA). Eluting compounds were detected at a wavelength of 280 nm. The elution consisted of a binary solvents system gradient, A (0.1% formic acid in H<sub>2</sub>O) and B (0.1% formic acid in acetonitrile): initial: 5% B, 0.1 min 10 % B, 4 min 10 % B, 50 min 50 % B, 52 min 0 % B, 54 min 0 % B; flow rate: 4.7 mL/min. The column was then washed and equilibrated for the next injection by a decrease of solvent B to 5 % at 54.2 min and maintenance of this concentration until 60 min. Six fractions (F1 to F6) were collected corresponding to the 6 main peak signals, respectively. Solvents were evaporated *in vacuo* at 30°C and the resulting fractions were dissolved in methanol before retesting for effects on bacterial settlement. Fractions exhibiting inhibitory effects against strains ANORD3 and ANY8 were additionally tested with 6 more bacteria before they were chemically characterized and identified.

### ***Characterization of inhibitory compounds***

#### *UPLC-QToF-MS analyses*

The inhibitory fractions were analysed by Acquity UPLC I-Class System coupled to Acquity UPLC PDA detector and the Xevo G2-XS QToF Mass Spectrometer (Waters<sup>®</sup>, Massachusetts, USA). The extracts and/or pure compounds were dissolved in methanol (1 mg/mL) and injected (1  $\mu$ l) into an Acquity UPLC<sup>®</sup> HSS T3 column (High Strength Silica C<sub>18</sub>, 1.8  $\mu$ m, 2.1 x 100 mm,

Waters<sup>®</sup>, operating at 60°C) was used. A binary mobile phase system (A: 0.1% formic acid in milliQ-water and B: 0,1% formic acid in acetonitrile (UPLC grade from VWR<sup>®</sup>, Pennsylvania, USA) was pumped at a flow rate of 0.8 mL/min at the following gradient: initial, 90% A; 0-13 min, 0% A linear; following by washing and reconditioning of the column. The total run was 15 min. Acquisition range of PDA detector was 190-600 nm. The MS and MS<sup>n</sup> spectra, in negative mode, were recorded during the UPLC run with the following conditions: capillary voltage: 1 kV, cone voltage: 40 V, source temperature: 150°C, cone gas flow: 50 L/h, desolvation gas flow: 1000 L/h and a collision energy ramp: 20-45 eV. Scan times were 0.1 s and acquisition range was *m/z* 50-1200. MassLynx<sup>®</sup> Software (version 4.1) was used for data acquisition. MS<sup>2</sup> spectra were used to elucidate the chemical structure and the fragmentation pattern of the purified rosmarinic acid (main compound of interest). The commercial rosmarinic acid standard was also analysed in the same manner to fully confirm the structure of the isolated rosmarinic acid from *Z. marina*. Dereplication workflow that is available in the online GNPS platform (<https://gnps.ucsd.edu/>; Wang et al. 2016) and the MassBank database (<http://www.massbank.jp/>; Horai et al. 2010) were used for the chemical identification of the phenolic compounds.

#### *NMR Analysis*

<sup>1</sup>H-NMR spectra of extracts and pure compounds (≈ 8 mg/mL) were recorded on a Bruker DRX 500 (500 MHz for <sup>1</sup>H). The residual solvent signal of methanol-*d*<sub>4</sub> was used as internal reference ( $\delta_{\text{H}}$  3.31). NMR data were processed using MestReNova version 1.3 software. The <sup>1</sup>H NMR spectra (Figure S9) of rosmarinic acid (**1**, MeOD, 500 MHz) were assigned as follows:  $\delta_{\text{H}}$  2.97-3.02 (1H, dd, *J*=14.3, 8.4 Hz, H-7' $\alpha$ ); 3.07-3.10 (1H, dd, *J*= 14.3, 4.3 Hz, H-7' $\beta$ ); 5.16-5.19 (1H, dd, *J*= 8.4, 4.3 Hz, H-8'); 6.23-6.27 (1H, d, *J*= 15.9 Hz, H-8); 6.60-6.61 (1H, dd, *J*= 8.1, 2.1 Hz, H-6'); 6.67-6.69 (1H, d, *J*= 8 Hz, H-5'); 6.76 (1H, d, *J*= 2Hz, H-2'); 6.77 (1H,d, *J*= 8.2 Hz, H-5); 6.93-6.94 (1H, dd, *J*= 8.3, 2.0 Hz, H-6); 7.03 (1H, d, *J*= 2.1 Hz, H-2); 7.52-7.55 (1H, d, *J*= 15.9 Hz, H-7)).

#### *Quantification of inhibitory compounds*

Concentration of rosmarinic acid in surface extracts of *Z. marina* was quantified by HPLC-PDA,

using the same solvent system as described above for crude extract fractionation in combination with a diode array detector. For calibration rosmarinic acid standard was injected into the system at different concentrations. Peak areas at a wavelength of 350 nm were determined and correlated with corresponding peak areas in *Z. marina* surface extracts. Sulfated flavonoids were quantified following the same principle, but corresponding non-sulfated flavonoids had to be used as standards. This approach was possible because sulfated and unsulfated luteolin show the same and very characteristic UV spectrum (Enerstvedt et al. 2016). The same is true for sulfated and unsulfated diosmetin/chrysoeriol.

### ***Statistical Analysis:***

#### *Settlement inhibition bioassays*

The strength of settlement inhibition was calculated as a log effect ratio, the logarithm of target microbe settlement in presence of extracts/compounds divided by target microbe settlement in presence of solvent controls. A settlement inhibition of 0 indicates absence of effects, while positive and negative values indicate attracting and deterring effects of extracts/compounds on microbial settlers, respectively. The one sample-t-test was used to test whether settlement inhibition by crude extracts and HPLC fractions deviated from 0 ( $p < 0.05$ ), while the two-sample-t-test was used to compare the effects obtained with different treatments ( $p < 0.05$ ). These statistical analyses were conducted using the software package Statistica 8.0 (StatSoft, Tulsa, Oklahoma, USA). To analyze the dose-dependency of anti-settlement effects of four different compounds, the logistic regression function

$$AS = AS_{\max} + (AS_{\min} - AS_{\max}) / (1 + 10^{(C - \text{Log}(IC_{50}))})$$

(with AS=activity strength, C=compound concentration,  $AS_{\max}$ =maximal activity strength,

$AS_{\min}$ =minimal activity strength and  $IC_{50}$ =compound concentration required to obtain a

halfmaximal activity strength)

was fitted to the data distributions, using the software Prism 5.0 (Graphpad, La Jolla, California, USA).

### *Anti-growth bioassays*

To analyze the growth of bacteria in presence or absence of different compounds the exponential growth equation

$$Y=Y_0*e^{(k*t)}$$

(with Y=optical density of bacterial cultures, Y<sub>0</sub>=initial optical density, t=incubation time and k=rate constant of divisions)

was fitted to the data distributions with Prism 5.0, which allowed it to also compute the doubling time as  $\ln(2)*k^{-1}$ . Differences in doubling times between solvent controls and treatments were tested for significance with the two-samples-t-test ( $p < 0.05$ ), using the software package Statistica 8.0.

## **Results**

### ***Bioactivity of crude extracts***

Propan-2-ol extracts of *Z. marina* leaves exhibited relatively strong inhibitory effects on settlement of the bacteria *V. cyclitrophicus* and *M. litorea*, as well as of the yeast *C. fonsecae* (Figure 1). In all three cases a one-sample t-test revealed that these effects were significant at  $p < 0.001$ , while low or no inhibitory effects were observed with *n*-hexane extracts (*V. cyclitrophicus*,  $p=0.2984$ ; *M. litorea*,  $p=0.3090$ ; *C. fonsecae*,  $p=0.7269$ ) (Figure 1).

The effect of propan-2-ol surface extract on the settlement of additional bacteria was tested next. Five bacteria that originated from stones were without exception significantly deterred, but this was not the case with five bacteria that were isolated from *Z. marina* (Figure 2a, one-sample-t-test,  $p < 0.05$ ,  $n=4$ ). Thus, microorganisms isolated from stones were generally inhibited by surface extracts (Figure 2b, one-sample-t-test,  $p < 0.0001$ ,  $n=7$ ), while microorganisms isolated from eelgrass were not (Figure 2b, one-sample-t-test,  $p=0.5465$ ,  $n=5$ ). Moreover, responses of both groups of bacteria also differed from each other (2-sample t-test,  $p < 0.001$ ).

In contrast to settlement activity, the growth of *V. cyclitrophicus*, *M. litorea*, and *C. fonsecae* was not inhibited by propan-2-ol extract from *Z. marina*. Instead, growth of all three microorganisms increased when such extract was present at 2-fold natural concentration (Figure S4), which was reflected in significantly decreased doubling times (Figure 3). Higher (5-fold or 10-fold natural) extract concentrations brought the growth rates back to control level.

### ***Bioassay guided fractionation of settlement inhibiting compounds***

Semi-preparative HPLC analysis of propan-2-ol surface extract of *Z. marina* resulted in a separation of at least six compounds or groups of compounds that absorb UV light (Figure 4). These compounds had retention times (min) of 28.59, 32.72, 35.68, 37.04, 37.68, and 45.55 and were collected as fractions F1 to F6, respectively.

*V. cyclitrophicus* and *M. litorea* were applied as test organisms in the following settlement bioassays that were conducted with fractions F1 to F6. Both strains were unaffected or even attracted by fractions F4, F5 and F6, but deterred by fraction F1. Fraction F2 deterred only *V. cyclitrophicus*, while fraction F3 deterred only *M. litorea* (Figure 5). When they were pooled fractions F1 to F6 exhibited on average approximately 45 % of the total bioactivity of crude extract toward *V. cyclitrophicus* and this difference was significant (Two-sample-t-test,  $p=0.0018$ ). In contrast the bioactivity of the combined 6 fractions toward *M. litorea* was not significantly different from that of the crude extract (Two-sample-t-test,  $p=0.069$ ).

Because fractions F1 to F3 exhibited repellent activity against bacterial settlers they were tested with 6 more bacterial isolates from stones. The different fractions exhibited quite variable action spectra (Figure 6A). Fraction F1 inhibited the settlement of F3 out of the 6 bacteria (*F. agariphila*, *A. stellipolaris*, *L. rosea*). In contrast, fraction F2 only inhibited settlement of *P. dokdonensis*, while fraction F3 repelled *P. dokdonensis* and *L. rosea*. The last two fractions even attracted *P. carrageenovora* (fraction F3) and *S. hominis* (fraction F2). Due to the variable responses of the different isolates only fraction F1 exhibited overall a significant inhibitory effect against all 8 bacteria that were tested (Figure 6B). Thus, fraction 1 apparently contained those compound(s) that are the most effective deterrents.

### ***Characterization of compounds***

HRMS and HRMS<sup>2</sup> data combined with <sup>1</sup>H-NMR data allowed for the identification of three main metabolites (**1** to **3**) from fractions F1 to F3, respectively (Figure 7, Table 1). Compound **1** showed an [M-H]<sup>-</sup> ion at *m/z* 359.0814, appropriate for a molecular formula C<sub>18</sub>H<sub>16</sub>O<sub>8</sub> of rosmarinic acid (Figure S7). The negative mode HRMS/MS analysis of the compound **1** yielded 6 main fragments, corresponding to *m/z* [M-H]<sup>-</sup> 197.0477 (C<sub>9</sub>H<sub>9</sub>O<sub>5</sub>); 179.0343 (C<sub>9</sub>H<sub>7</sub>O<sub>4</sub>); 161.0299 (C<sub>9</sub>H<sub>5</sub>O<sub>3</sub>); 135.0439 (C<sub>8</sub>H<sub>7</sub>O<sub>2</sub>); 123.0439 (C<sub>7</sub>H<sub>7</sub>O<sub>2</sub>) and 72.9913 (C<sub>2</sub>HO<sub>3</sub>) as depicted in Figure S7. As shown in Figure S8, this fragmentation pattern was identical to that obtained with the commercial rosmarinic acid standard. The <sup>1</sup>H-NMR data of compound **1** was in accordance with the literature (Lee et al. 2013) and it matched perfectly with those of rosmarinic acid standard (Figure S9). The UV absorption of **1** at λ<sub>max</sub> 221, 234 and 328 nm (Table 1, Figure S5), was also in agreement with that for commercial rosmarinic acid standard. All this data confirmed that F1 of *Z. marina* surface extract was pure rosmarinic acid.

Compound (**2**), luteolin-7-sulfate (λ<sub>max</sub> 223; 314 nm and *m/z* 365.0071 [M-H]<sup>-</sup>), was present in fraction 2. Its negative mode HRMS fragmentation pattern provided one main fragment at *m/z* [M-H]<sup>-</sup> 285.0371 (C<sub>15</sub>H<sub>9</sub>O<sub>6</sub>) and several minor fragments at *m/z* [M-H]<sup>-</sup> 257.0424 (C<sub>14</sub>H<sub>9</sub>O<sub>5</sub>); 241.0483 (C<sub>14</sub>H<sub>9</sub>O<sub>4</sub>) 151.0013 (C<sub>7</sub>H<sub>3</sub>O<sub>4</sub>); 133.0270 (C<sub>8</sub>H<sub>5</sub>O<sub>2</sub>); 107.0110 (C<sub>6</sub>H<sub>3</sub>O<sub>2</sub>) (Table 1, Figure S10). The minor fragments were identical to its equivalent non-sulfated parent compound (luteolin) in the MassBank database (<http://www.massbank.jp>; Horai et al. 2010). Here, the fragment ion at *m/z* 285.0371 [M-H]<sup>-</sup> - corresponding to the loss of a sulfate group (SO<sub>3</sub>) - was obtained, confirming the chemical identification of compound (**2**) in F2.

Compound (**3**) (λ<sub>max</sub> 221; 344 nm; [M-H]<sup>-</sup> ion at *m/z* 379.0225) - corresponding either to diosmetin-7-sulfate or its isomer chryseoriol-7-sulfate was present in fraction 3. It yielded main fragments by tandem mass spectrometry at *m/z* [M-H]<sup>-</sup> 299.0529 (C<sub>16</sub>H<sub>11</sub>O<sub>6</sub>); 284.0295 (C<sub>15</sub>H<sub>8</sub>O<sub>6</sub>) and two minor fragments at [M-H]<sup>-</sup> *m/z* 256.0356 (C<sub>14</sub>H<sub>8</sub>O<sub>5</sub>) and 151.0025 (C<sub>7</sub>H<sub>3</sub>O<sub>4</sub>) (Table 1, Figure S11). The negative mode mass fragmentation pattern of compound (**3**) was comparable with diosmetin and chryseoriol in the MassBank database. Also here presence of the ion fragment *m/z* 299.0529 [M-H]<sup>-</sup> (indicating SO<sub>3</sub> loss) inferred with the structural identification of compound

(3). However, it was not possible to distinguish whether compound (3) is diosmetin-7-sulfate or chrysoeriol-7-sulfate based on MS/MS analysis. Due to the very low amounts of compound (3) that yielded from the surface extract (< 1 mg) no further analytical studies were possible.

The biological activity of the identified compounds was verified in additional tests with commercially available standard compounds at different concentrations ranging from 0.005 mg/ml to 0.3 mg/ml. Rosmarinic acid (1) was tested against four bacterial strains (*V. cyclitrophicus*, *M. litorea*, *P. dokdonensis* and *F. agariphila*), which were all inhibited in a dose-dependent manner (Figure S6A). The sulfated flavonoids were not commercially available, but their unsulfated derivatives were available and used for testing. The settlement-inhibiting effect of luteolin-7-sulfate (2) prepared by us at 5-fold natural concentration was compared to the effect of purchased luteolin at a corresponding concentration of  $39.6 \mu\text{mol l}^{-1} = 14.5 \mu\text{g ml}^{-1}$ . Both compounds exhibited similar activity profile toward two different bacteria and the two-samples-t-test detected no significant differences between luteolin and luteolin-7-sulfate (*V. cyclitrophicus*,  $n=4$ ,  $p=0.5887$ ; *M. litorea*,  $n=4$ ,  $p=0.4030$ ), suggesting that luteolin and luteolin-7-sulfate exert comparable repellent activity (see Figure S3). Similar to rosmarinic acid, luteolin, as well as diosmetin, exhibited dose-dependent inhibitory effects on the settlement of four bacteria (Figures S6B, S6C). In contrast, chrysoeriol only deterred *M. litorea*, while it attracted *P. dokdonensis* and *V. cyclitrophicus* and it left *F. agariphila* unaffected in the tested concentration range (Figure 6SD).

Theoretically possible maximal activity strengths toward all four isolates could be derived from best fitting logistic dose-response functions (Figure 8A). Only in the case of chrysoeriol, two bacteria exhibited no or too weak responses in the tested concentration range. Altogether, the maximal activity strength was of comparable magnitude for rosmarinic acid and diosmetin, but consistently higher for luteolin. In contrast, required concentrations of rosmarinic acid for inducing a halfmaximal inhibition of settlement ( $\text{IC}_{50}$ ) were generally lower than those observed for luteolin and diosmetin (Figure 8B). No significant  $\text{IC}_{50}$  could be computed in two cases for chrysoeriol and in one case for luteolin.

The concentrations of all three bioactive phenolic compounds varied considerably and decreased generally in the order of rosmarinic acid (1) > diosmetin-7-sulfate or chrysoeriol-7-sulfate (3) > luteolin-7-sulfate (2), both in surface extracts and in tissue extracts (Table 2A). Surface rosmarinic acid contributed approximately 2.3 % to the total tissue content of rosmarinic acid (1), while much higher contributions to the total tissue content were detected for surface luteolin-7-sulfate (2) and surface diosmetin/ chrysoeriol-7-sulfate (3) (Table 2A).

Sulfated flavonoids can be easily subject to desulfatation, which is difficult to control under laboratory conditions (see above) and which probably also happens *in vivo*. For this reason 1-fold sulfated flavonoids that are present in eelgrass surface extracts could not only result from direct excretion, but also from degradation of excreted 2-fold sulfated flavonoids. We therefore also compared the molar concentrations of flavonoids with different degrees of sulfatation (Table 2B). Interestingly, luteolin-7,3'-disulfate and diosmetin/chrysoeriol -7,3'-disulfate were nearly absent from surface extracts, but detected in reasonable amounts in the tissue extracts (Table 2B). This discrepancy confirms that our surface extraction method really only yielded surface compounds and not tissue compounds. Further, 1-fold and 2-fold sulfated surface luteolin together contributed 4 % of the total tissue content of sulfated luteolin, while 1-fold or 2-fold sulfated surface diosmetin/chrysoeriol contributed approximately 8 times as much of the tissue content of sulfated diosmetin/chrysoeriol. This could indicate that sulfated diosmetin or chrysoeriol is more actively excreted by *Z. marina* than sulfated luteolin and rosmarinic acid.

## Discussion

Our results clearly demonstrate the existence of a chemical defense against micro-settlers on the surface of *Zostera marina* leaves. Surface extracts generated with *n*-hexane could not significantly deter microbial settlement, but middle polarity solvent extract generated with propan-2-ol inhibited the settlement of marine fouling bacteria. These extracts only contained surface compounds, since application of a lethal dye did not detect any disrupted epidermal cells after treatment with propan-2-ol and also because proportions of single compounds were not the same in surface extracts and tissue extracts. At 1-fold natural concentration propan-2-ol extracts

significantly deterred seven bacterial isolates that originated from non-living marine substrates. In contrast, five bacterial strains directly isolated from eelgrass were unaffected under the same conditions and a yeast species that is associated with *Z. marina* (Weinberger et al. unpublished) was less affected. This indicates that compounds present in propan-2-ol surface extracts have a selective effect on the microbial community associated with *Z. marina*: microorganisms that are capable to colonize eelgrass leaves tend to be less sensitive towards those compounds.

The inhibitory effect of propan-2-ol crude surface extract on microbial settlement was certainly due to repellence and not due to toxicity, since a 10-fold concentration of these extracts could not reduce microbial or yeast growth and a 2-fold concentration even caused some growth acceleration. Bioactivity-guided fractionation of the surface propan-2-ol extract further confirmed that multiple phenolic compounds are present and able to affect bacterial settlement on *Z. marina* surfaces. Based upon its light absorption characteristics and on LC-MS and <sup>1</sup>H NMR data, which were all comparable to those of a purchased standard, the most abundant compound was unambiguously identified as rosmarinic acid. Rosmarinic acid is an ester of caffeic acid, biosynthetically derived from the phenylpropanoid pathway (Petersen & Simmonds 2003). Interestingly, it occurs throughout the terrestrial and marine plant kingdom (Ravn et al. 1994). The biological roles of rosmarinic acid have been studied for years (Bulgakov et al. 2012) and include antioxidant, anti-inflammatory, antiviral, and antibacterial activities (Moon et al. 2010; Panya et al. 2010; Petersen & Simmonds 2003). For example, Wang et. al (2012) detected on average 3.13 mg g<sup>-1</sup> rosmarinic acid in dry eelgrass tissue and its nematicidal and antibacterial activities were shown in subsequent bioassays. Eelgrass tissue concentrations of rosmarinic acid in the same order of magnitude were also detected in other studies (Ravn et al. 1994; Achamlale et al. 2009; Custódio et al. 2015) and now by us. Only less than 3 % of this quantity are present in the surface volume of *Z. marina*, whereas concentrations of rosmarinic acid in the range of 15.6 and 106.8 µg ml<sup>-1</sup> were detected (Table 2). These natural surface concentrations exceed those required for an inhibition of microsettlers: The necessary concentrations for halfmaximal settlement inhibition (IC<sub>50</sub>) ranged from 5.34 to 27.2 µg ml<sup>-1</sup>. Hence rosmarinic acid is certainly relevant for the defense of eelgrass plants against various microsettlers.

Additional bioactive phenolics were also detected in *Z. marina* surface extracts. They exhibited less pronounced effects than rosmarinic acid when they were tested at natural concentration. They were very minor compounds for <sup>1</sup>H NMR analyses, but their UV and MS data were in agreement with the published data and databases for certain flavonoids, luteolin-7-sulfate, diosmetin-7-sulfate and/or chrysoeriol-7-sulfate (Enerstvedt et al. 2016). These compounds are known to be easily subject to desulfatation when they are kept in solution (Varin 1992). We observed this phenomenon through corresponding shifts in MS and elution behaviours when leaving our extracts in solvent for prolonged times at room temperature (data not shown). Such desulfatation after or during (Stowell et al. 2006) excretion perhaps explains the circumstance that 7,3'-disulfates of luteolin and diosmetin/chrysoeriol exhibited much lower concentrations than the corresponding 7-sulfates in surface extracts of *Z. marina* – despite the fact that disulfates were more or equally abundant in tissue extracts (Table 2B).

Luteolin, diosmetin and chrysoeriol have repeatedly been detected in tissues of seagrass (Buchsbaum et al. 1990; Grignon-Dubois et al. 2012; Laabir et al. 2013; Enerstvedt et al. 2016) and other plants (Alvesalo et al. 2006; Agati et al 2012; Kumar & Pandey 2013). Some of these flavonoids have been demonstrated or suggested to have functions in plant defenses and a potential use in medical applications. For instance, luteolin isolated from the seeds of *Perilla frutescens* exhibited strong antimicrobial effects against isolates of *Staphylococcus aureus* that were resistant to multiple other antibiotics (Xu & Lee 2001; Yamamoto & Ogawa, 2002). However, much less is known about the characteristics and biological activities of the sulfated derivatives of luteolin, diosmetin and chrysoeriol (but see Grignon-Dubois & Rezzonico 2012; Enerstvedt et al. 2016; Kim et al. 2016).

The fact that the commercial standards of luteolin-7-sulfate, diosmetin-7-sulfate and chrysoeriol-7-sulfate could not be purchased, complicated the quantification of these compounds, as well as the ultimate demonstration of their deterrent effect. However, luteolin and luteolin-7-sulfate exhibit the same light absorption characteristics at wave lengths  $\geq 350$  nm (Enerstvedt et al. 2016), a circumstance that allowed it to quantify luteolin-7-sulfate and to compare the bioactivities of both compounds: luteolin-7-sulfate exhibited a deterrent effect that

was not significantly different from the effect of luteolin at equal concentration.

Diosmetin-7-sulfate and chrysoeriol-7-sulfate are isomers, therefore they cannot be easily distinguished (Enerstvedt et al. 2016). Interestingly, in our bioassays diosmetin exhibited significantly higher deterrent effects than chrysoeriol, the latter even attracted some of the tested bacterial isolates. This may suggest that diosmetin-7-sulfate rather than chrysoeriol-7-sulfate is present in *Z. marina* surface extracts. However, a definite decision is currently not possible.

Irrespectively, a comparison of the natural surface concentrations of luteolin-7-sulfate (ranging from 1.6 to 5.9  $\mu\text{g ml}^{-1}$ ) and of diosmetin/ chrysoeriol-7-sulfate (ranging from 4.6 to 19.9  $\mu\text{g ml}^{-1}$ ) with the required concentrations of luteolin, diosmetin and chrysoeriol for halfmaximal deterrence of microfoulers ( $\text{IC}_{50}$ : 23.6-148.6, 12.51-132.6, and 27.3  $\mu\text{g ml}^{-1}$ , respectively) suggests that these flavonoids can only be of limited relevance for the deterrence of microfoulers. Thus, rosmarinic acid clearly appears to be the major phenolic deterrence compound on eelgrass surfaces.

However, in theory biologically active compounds may interact in additive or synergistic ways when they are present at their respective natural concentrations. Indeed, fractions of propan-2-ol crude surface extract - containing only rosmarinic acid or one of the flavonoids - did not exhibit the full deterrent effect that was observed when the complete crude extract was tested, suggesting that at least additive effects among different compounds must exist. Moreover, all six fractions containing phenolics also exhibited a weaker effect than the full crude extract when they were pooled together at their respective natural concentrations, indicating that non-phenolics with a relevant deterrent capacity toward microfoulers may also be present on eelgrass surfaces that still need to be identified. This possibility also exists because the surface extraction methods applied in our study only included moderately polar and unpolar compounds but excluded very polar compounds, due to the sensitivity of eelgrass to solvents more polar than propan-2-ol. Antifungal and antimicrobial effects of non-phenolic compounds extracted from seagrass tissue have been reported (Iyapparaj et al. 2014) and such compounds could interact with rosmarinic acid and the other phenolic compounds that we identified if they are also present on the surface of

eelgrass. Further, zosteric acid, another phenolic compound that has demonstrated antibiotic activity (Villa et al. 2010; Polo et al. 2014; Kurth et al. 2015) was detected in our crude surface extract by UPLC-MS (RT 0.84 min, m/z [M-H]<sup>-</sup> 242.9934). Its concentration was too minor to allow for bioactivity-guided isolation by semi-preparative HPLC, but an interaction of zosteric acid with rosmarinic acid and other bioactive compounds could be possible.

In conclusion, although *Z. marina* tissue contains numerous phenolic compounds only few of them are regularly present in surface extracts. Most of these phenolic compounds will not or only rarely reach concentrations that allow for a relevant ecological role as deterrents of microsettlers. Some surface phenolics apparently even attract microsettlers. Thus, eelgrass surface phenolics cannot per se be regarded as deterrents of microfoulers. The most important of the compounds relevant for antifouling defense is rosmarinic acid. We found little evidence that therelevant compounds inhibit marine microsettlers through toxic effects, although toxicity of luteolin toward bacterial human pathogens has been described (Puupponen-Pimiä et al. 2005). Much rather, those settlers are deterred through non-toxic effects. Such non-toxic repellent effects of rosmarinic acid and flavonoids have not been reported, thus, it will be interesting to elucidate the underlying mechanisms. It appears possible that their effect is related to an interference with microbial quorum sensing (Decho et al. 2010; Li & Tian 2012; Kalia 2013). Obviously the detected effects are not inclusive for all marine bacteria, insensitive microorganisms may settle on the surface of *Z. marina*, including the ‘wasting disease’ promoting yeast *C. fonsecae*, that exhibited only a limited sensitivity to surface associated compounds in our study. The concentrations of rosmarinic acid and both sulfated flavonoids are higher in eelgrass tissue than on its surface, and a relatively higher antibacterial activity of these compounds may therefore be expected toward microorganisms that can penetrate into the tissue. It will therefore be interesting to study the sensitivity of *Labyrinthula zosterae* and other eelgrass pathogens toward these compounds in the future.

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## Figure legends

Figure 1. Antisettlement activity of propan-2-ol and *n*-hexane surface extract from *Zostera marina* leaves at 1-fold natural concentration on two different bacteria and one yeast (mean $\pm$ 95% CI, n=8). Asterisks indicate activities that differed significantly from 0 (one-sample-t-test,  $p < 0.05$ ).

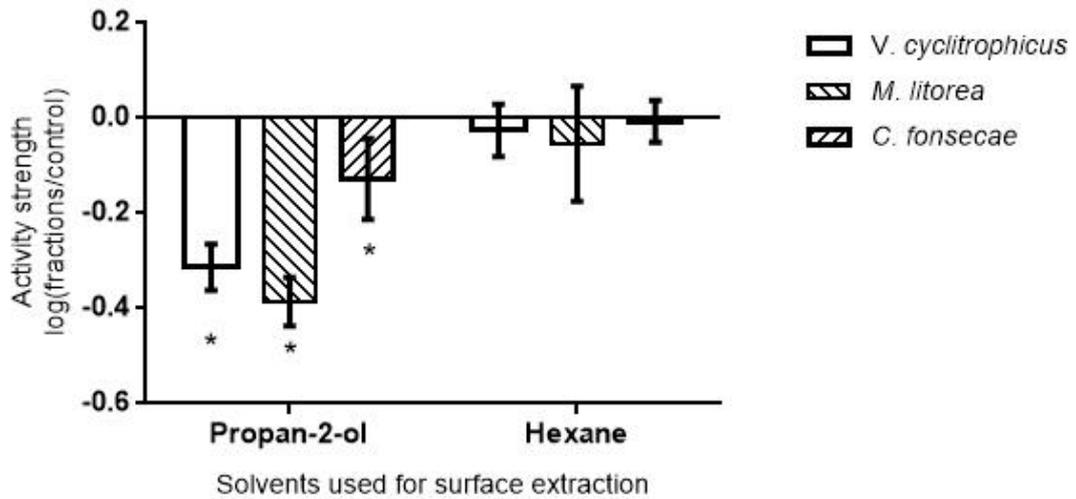


Figure 2. Antisettlement activity of propan-2-ol surface extract tested at 1-fold natural concentration on bacteria isolated from stones and plants (mean $\pm$ 95% CI). A: Activity against five individual strains of bacteria; B: Activity against grouped bacteria isolated from stones (n=7, see Figures 1 and 2a for individual responses) and bacteria isolated from plants (n=5, see Figure 2a for individual responses). Asterisks indicate activities that differed significantly from 0 (one-sample-t-test,  $p < 0.05$ ).

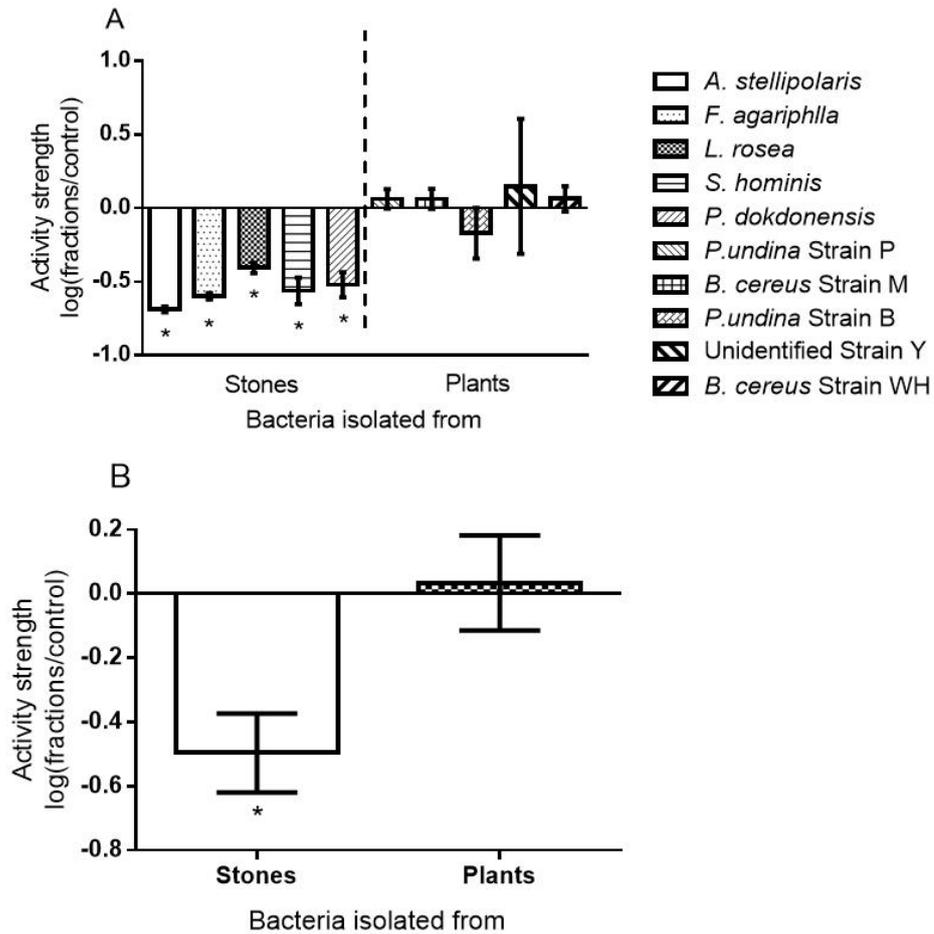


Figure 3. The inhibitory effects of propan-2-ol surface extract at 3 concentrations on the growth of A: *V. cyclitrophicus*; B: *M. litorea*; C: *C. fonsecae*, (mean $\pm$ 95%CI, n=4). 0 corresponds to the control. Asterisks indicate activities that differed significantly from the control (separated by dotted vertical line, two-sample-t-tests,  $p < 0.05$ ).

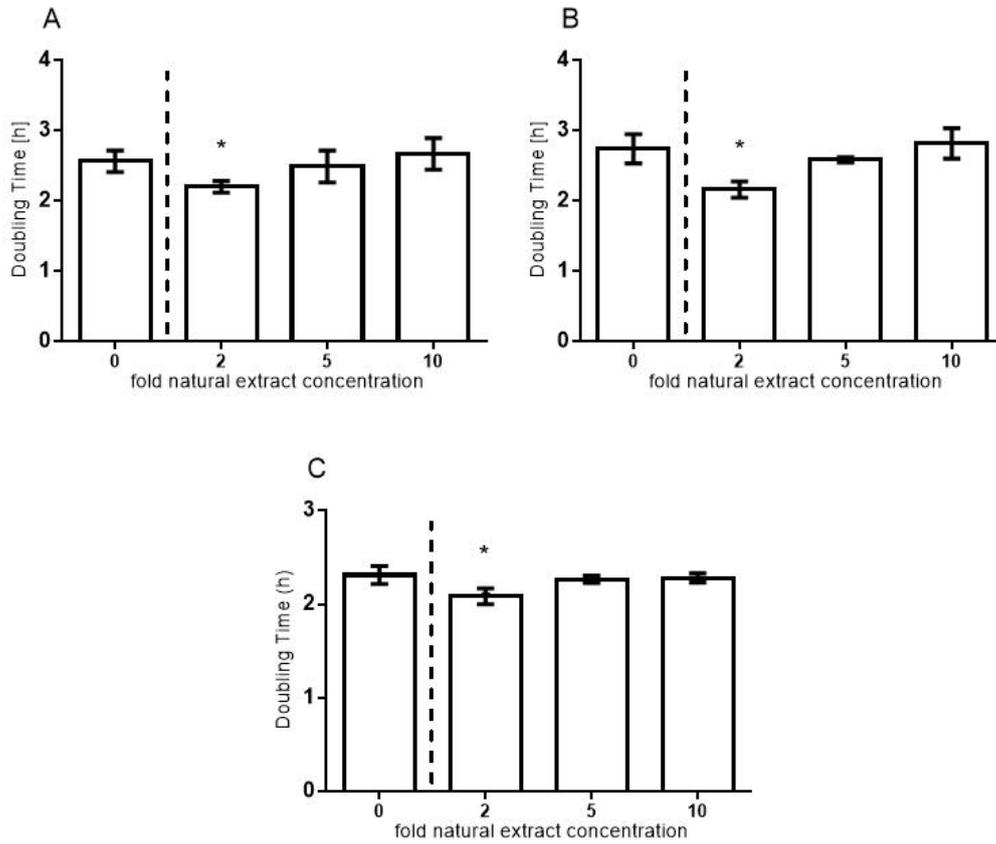


Figure 4. Fractionation of *Z. marina* surface extract prepared with propan-2-ol and redissolved in methanol, using semipreparative HPLC with PDA detection at 280 nm. Peaks 1 to 6 were collected as six different fractions as indicated.

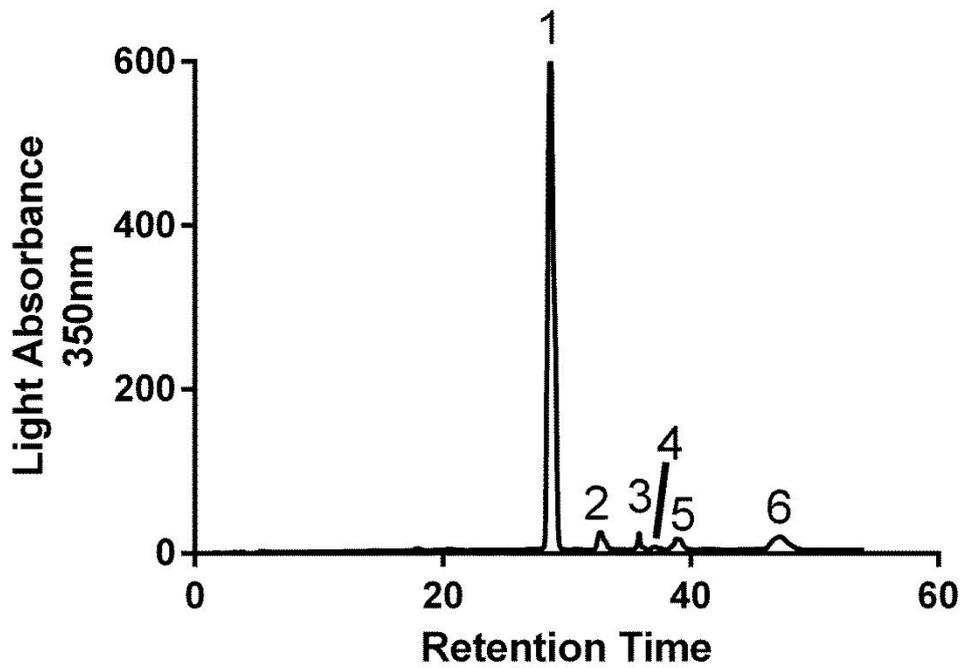


Figure 5. Antisettlement activity of six fractions prepared from propan-2-ol leaf surface extract of *Z. marina* on A: *V. cyclitrophicus*; B: *M. litorea* (mean $\pm$ 95% CI, n=4). The fractions were tested at 1-fold natural concentration. CE: Unfractionated crude extract, 6P: Fractions 1 to 6 pooled in their original concentrations. Asterisks indicate activities that differed significantly from 0 (one-sample-t-test,  $p < 0.05$ ).

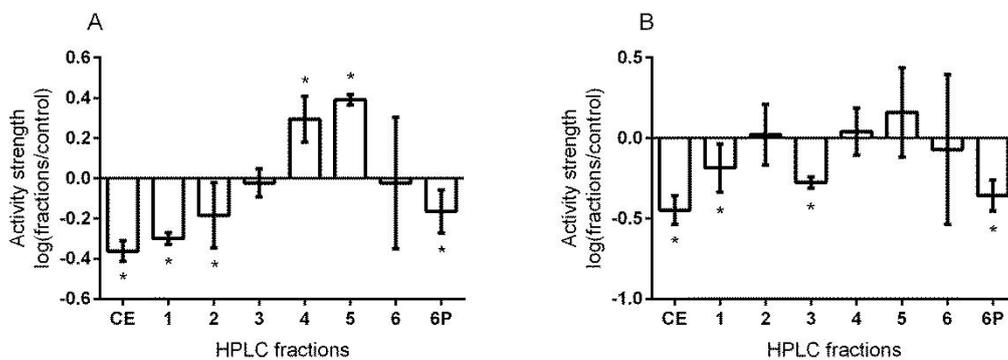


Figure 6. Antisettlement activity of 3 fractions prepared from propan-2-ol leaf surface extract of *Z. marina* toward (A) six different marine bacteria and (B) the same bacteria grouped with *V. cyclitrophicus* and *M. litorea* (compares Figure 5). Crude extract and fractions were tested at 1-fold natural concentration (Mean $\pm$ 95% CI, n=3). Measurements conducted with the same isolate in (A) and n =8 means obtained with different isolates in (B). Asterisks (\*) indicate activities that were significantly different from 0 (one-sample-t-test,  $p < 0.05$ ).

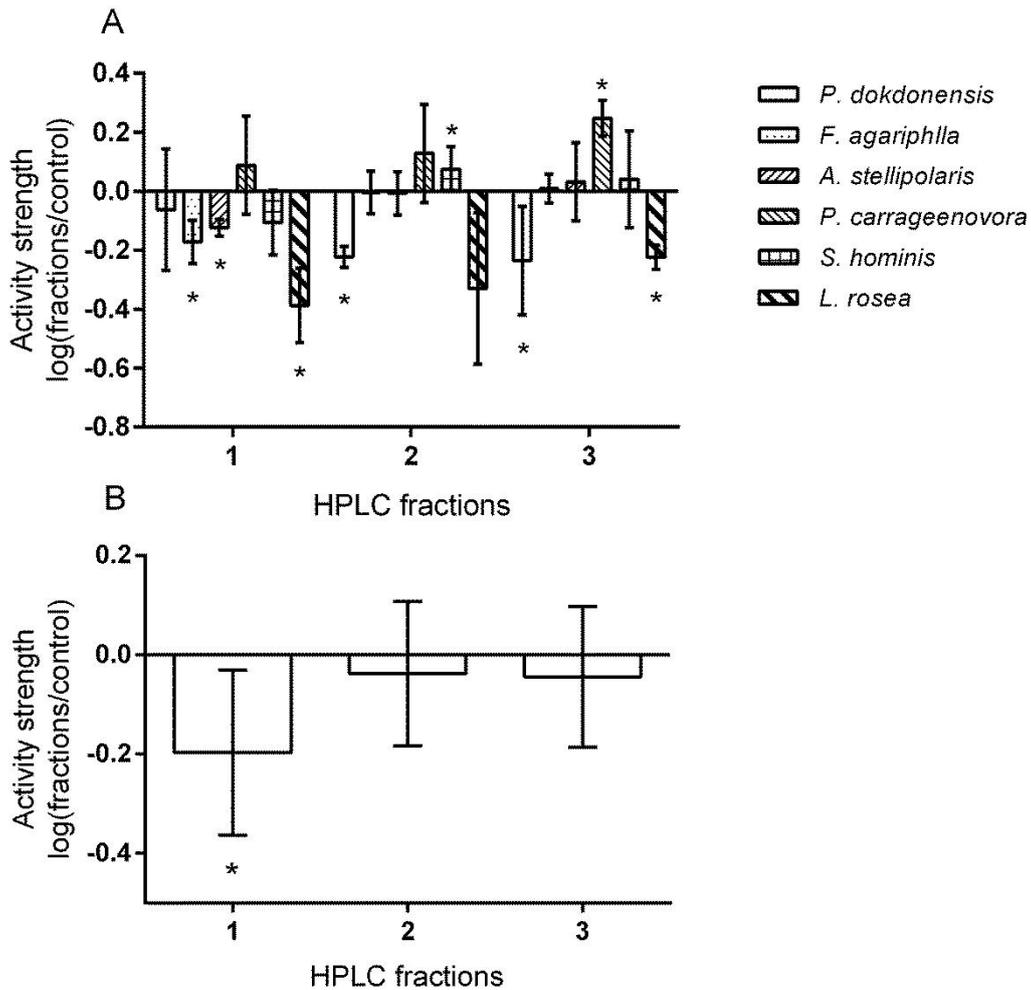


Figure 7. Structures of compounds identified in fraction 1 (Rosmarinic acid), 2 (luteolin-7-sulfate), and 3 (3.1 Diosmetin-7-sulfate; 3.2 Chrysoeriol-7-sulfate).

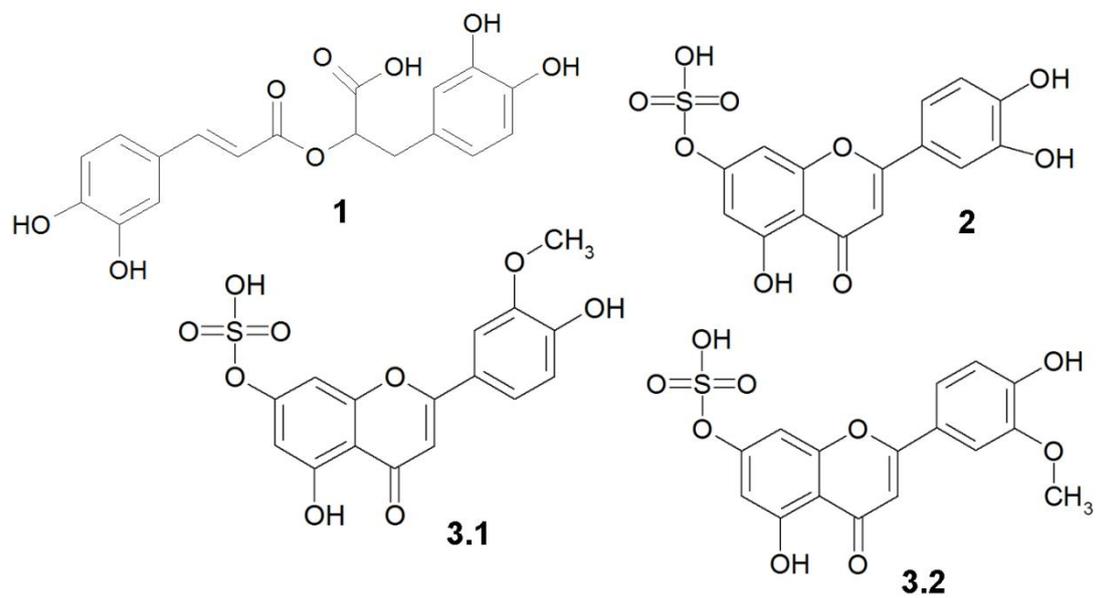
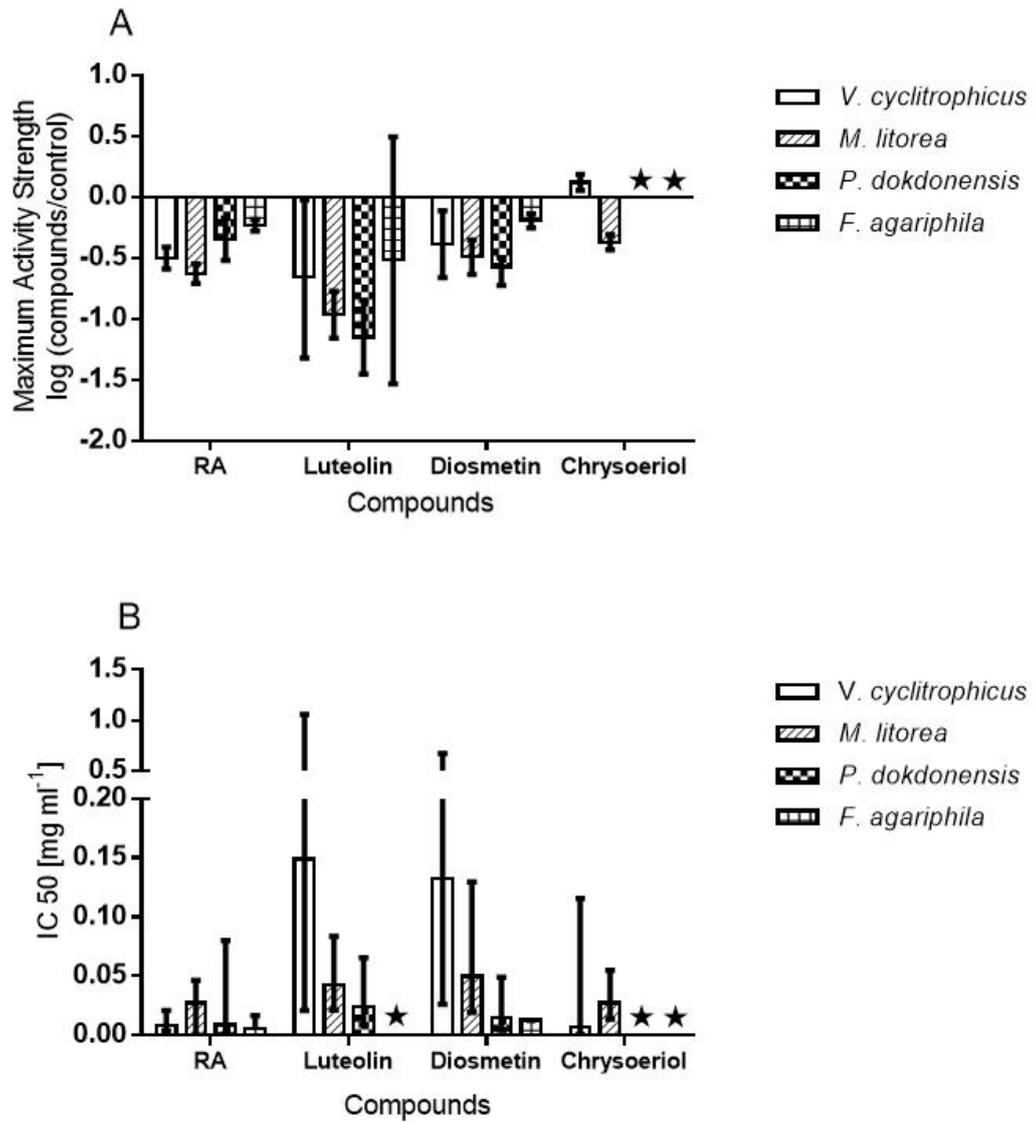


Figure 8. The maximal activity strength (A) and the necessary concentration to obtain 50 % of this strength ( $IC_{50}$ ) (B) as computed for four standard compounds (Mean $\pm$ 95% CI, n=4). RA = Rosmarinic acid. Stars indicate data that could not be computed in a meaningful way (see text for details).



**Heat waves accelerate the chemical defense of *Zostera marina* against microfoulers**

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## **Heat waves accelerate the chemical defense of *Zostera marina* against microfoulers**

### **Abstract:**

The ecology of marine habitat species *Zostera marina* L. is strongly influenced by the climate warming. In order to investigate the consequence of climate change on the chemical antifouling defense of *Z. Marina*, we incubated *Z. marina* plants in Kiel Outdoor Benthocosm system (KOB) under the stimulation of its natural living condition in Baltic Sea except for monthly heatwave treatments from May to August in 2015. As a result, all investigated variables except for antifouling defense capacity of *Z. marina* did not influenced by heatwave treatments, but had shown seasonal changing pattern which peak in July. The antifouling defense capacity was up-regulated during the incubation especially after the second heatwaves when defensive compounds Rosmarinic acid became the most dominant compounds on plants surface. Bacteria settled on *Z. marina* significantly correlated with defense activity and Rosmarinic acid surface contents, which hints the demand-driven antifouling defense. Meanwhile, we observed that repeated heatwave treatments result in weaker antifouling response, suggesting the possible adaptation of *Z. marina* to heat stimulation.

Key words: Chemical defense; *Zostera marina*; Rosmarinic acid; Heatwave treatments..

### **Introduction:**

Climate change may result in various consequences on marine species and communities. Global

and regional climate models predicted an increase of both mean temperatures and more frequency of heatwave events such as the 2003 heatwaves (Beniston et al. 2007). In Kiel Fjord, summer water temperature was predicted to increase of 3 to 5 °C and winter temperature could increase even 5 to 10 °C (BACC 2006). Communities and ecosystems in western Baltic Sea suffered from climate change, the vulnerability of key coastal species such as *Zostera Marina L.* in response to temperature change is important for the stability of marine environment in Kiel fjord (Ehlers et al. 2008).

Compared to terrestrial environments, coastal ecosystems are usually more vulnerable when they need to cope with abnormally high temperature. Individual marine species are often less adapted to fluctuating temperatures than terrestrial organisms, because the general buffering capacity of the ocean often prevents abrupt temperature change (Hoegh-Guldberg & Bruno 2010). At the same time, temperature stress effects on habitat forming species may result in changes of whole coastal environments (Holling 1973, Hughes et al, 2003).

In many parts of the world seagrasses are of great importance for the near shore marine ecosystems, and *Z. marina*, also known as eelgrass, is one of the most dominant seagrass species (den Hartog & Kuo 2007). The eelgrass ecosystem is very productive and diversified (den Hartog & Kuo 2007, Hemminga & Duarte 2000). Within this system, eelgrass provides a supportive nursery habitat for several fish and shellfish species that are of economic importance (Plummer et al. 2013, Orth et al. 2006) and the basis of a complex food web that supports all other organisms. Eelgrass meadows thus have numerous ecological functions that are important for the stability of the ecosystem and at the same time for human fisheries (Duarte 2002). Hence, loss of eelgrass

plants on a larger scale usually leads to serious consequences and devastation of the seagrass ecosystem (Hemminga & Duarte 2000, Williams & Heck 2001, Waycott et al. 2009). The currently observed decline of eelgrass in most coastal environments (Waycott et al. 2009) often appeared accelerated by warming. For example, a heat wave of 3 weeks during the summer of 2003 caused major losses and die-offs of eelgrass in Europe (Greve et al. 2003, Reusch et al 2005). Such heat waves are likely to happen more severely and frequently in the future (Beniston et al. 2007), which may cause further losses of European eelgrass. At the same time infectious diseases are often increased by high temperatures and also wasting disease of *Z. marina* has been suggested to be accelerated at high temperatures (Giesen et al. 1990, Vergeeret al. 1995), which might increase the sensitivity of eelgrass further. The optimum water temperature for eelgrass is between 10 and 20 °C, high temperature (25-30 °C) largely increased mortality and decrease photosynthesis rate of eelgrass plants (Nejrup & Pedersen 2008). Although the relatively high temperature in summer of current Baltic Sea (around 24 °C) tolerated eelgrass, but the possible temperature increase in following decades and the synergistic effects generated from low salinity in Baltic Sea would cause threats for the fitness of eelgrass plants (Salo & Pedersen 2014).

Marine macrophytes - including seagrass - are continuously threatened by epibiosis and fouling, due to their constant exposure to marine sessile live stages (Wahl 2009). Numerous marine bacteria, fungi, micro- and macroalgae, invertebrates etc. tend to colonize surfaces in the sea (Wahl 1989) and also seagrass leaves (Borowitzka et al. 2007, Marhaeni et al. 2011). Despite of a few beneficial effects on hosts in some cases (for example, through associational resistance, coating effects, uptake of nutrients from epibionts; Thornber 2007, Jormalainen et al. 2008,

Schumacher et al. 2007, Harder 2008), most of the consequences of fouling are detrimental for hosts, and seagrass is not an exception (Waycott and Duarte 2009, Hughes et al. 2009). For this reason seagrasses have developed strategies to defend themselves against epibionts and one of the strategies is the release of functional compounds that repel or kill bacteria (Marhaeni et al. 2011, Zidorn 2016), which usually mediate the early stages of macrofouling (Wahl 2009). Research concerning the chemical defense of seagrass has received much attention in the past years and various relevant compounds have been identified (Fusetani 2011, Zidorn 2016). Phenolic secondary metabolites from seagrasses have repeatedly been identified as bioactive compounds that may affect bacterial settlers (Iyapparaj et al. 2014) and macrofoulers (Davis et al. 1989) and in our previous study Rosmarinic acid was identified as the main defensive compound against bacterial settlers that is present at the surface of *Z. marina* (Chapter I).

The effect of abiotic environmental factors on the regulation of the biosynthesis of phenolic compounds is not very well understood. Temperature is an important factor that can influence the production of phenolic compounds in terrestrial plants (Løvdaal et al. 2010). Corresponding research with marine plants was rarely conducted, but Vergeer et al. (1995) suggested that a negative relationship between the content of phenolic defense compounds against the wasting disease pathogen *Labyrinthula zosterae* and temperature may exist in *Z. marina*. Obviously unfavourable environmental conditions could generally limit resources or the physiological capacity for chemical defenses in host macrophytes, as predicted by environmental stress models (Menge et al 2002, Weinberger et al. 2011). On the other hand, the chemical defense of marine plants could be also either activated or induced by biotic factors. For instance, tissue

consumption by grazers resulted in an increased defence of the seagrass *Cymodocea nodosa* against these grazers (Martinez-Crego et al. 2014). Such demand-driven defences cause lower energetic costs (Cronin 2001) and reduce the risk of enemy adaptation (Keen 1990). So far little evidence of demand-driven defenses in seagrasses or other marine macrophytes against epibionts exists (but see Rickert et al. 2016, Wang 2017 for seaweeds). However, this could be due to the circumstance that rigorous tests are often hampered because completely epibiont-free hosts are difficult or impossible to obtain (Weinberger 2007).

The goal of the present study was it to investigate heat wave effects on *Zostera marina*'s capacity for defense against microfoulers. Eelgrass was incubated in outdoor benthocosms under the same conditions as in natural assemblages, with the single exception that different heatwave treatments were applied. Control treatments without heat waves were compared to treatments with one simulated heatwave at the hottest time of the year. Additional treatments allowed it to investigate the possible weakening or adaptive effect of repeated heatwaves. Response variables studied were on one hand the densities of bacterial microsettlers on eelgrass and on non-living surfaces in the benthocosms and on the other hand the concentrations of compounds with antimicrofouling activity and in particular of Rosmarinic acid on the surface of eelgrass leaves.

## **Method and Material:**

### ***Material***

#### *Eelgrass and sediment*

*Z. marina* was collected by scuba diving at a water depth of 3-4 m from Falkenstein Beach at Kiel, Germany (54°38' N / 10°17' E) in May 2015. Sediment was collected at the same site and on the same day from the research vessel “Polarfuchs”, using a van Veen-grabber. The sampling site is located in the Kiel Fjord (southwest Baltic Sea), a brackish water environment, where the general salinity is around 16‰. *Z. marina* was transported in cooler boxes to the lab and maintained over night in 300 L tanks containing Baltic Sea water with salinity around 16 psu with waterflow at 1800 L/day in a climate room at 15 °C, under a light intensity of 20-30  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ .

### ***Experimental setup***

#### *Benthocosm system*

The experiment was conducted in the Kiel outdoor benthocosms facility (KOB). The KOB is a mesocosm system located on aluminum floats in the inner Kiel Fjord, in a distance of approximately 7.5 km from the collection site. The KOB allows for the control and manipulation of various environmental parameters in order to obtain target treatments (details about KOB see Wahl et al. 2015). Six benthocosm tanks with a volume of 3000 L (inner size: 2m  $\times$  2m  $\times$  0.9m) were applied in this experiment. Each tank was evenly divided into two subunits through a PVC wall. The separating wall only had a limited capacity for temperature isolation, therefore we always applied the same temperature treatment in both subunits within one tank. The water temperature in each tank was adjusted by a system of aquarium heaters and coolers. These were controlled by designed software that allows for the realization of complex temperature treatments. In total 12 controllers were applied to control and trace the temperature in each of the subunits and an additional sensor was used to measure the temperature in the Kiel Fjord (see figure S1).

Seawater was constantly pumped from the Fjord (water depth: 1 to 1.2 meter) into the tanks at an exchange rate of 1800 liter per day, while wave generators prevented stratification within the tanks. As a consequence, salinity in the benthocosms underwent the same fluctuations as salinity in Kiel Fjord surface water (Wahl et al. 2015). Also other environmental variables - such as light and pH – underwent the natural daily and seasonal fluctuation and were continuously measured by sensors.

#### *Experimental set-up*

Eelgrass was planted into plastic aquaria made of polystyrene (28cm×25cm×19cm; Savic, Heule Kortrijk, Belgium) that contained sediment at a height of approximately 10 cm. Each aquarium received six individual plants and eleven aquaria together containing 66 plants were placed at the bottom (70 cm water depth) of each benthocosm subunit. In addition various benthic invertebrates and algae that are common in the Kiel Fjord (e.g., *Idotea baltica*, *Littorina littorea*, *Gammarus* spp., *Mytilus edulis*, *Balanus improvisus*, *Fucus vesiculosus*, *Gracilaria vemiculophylla*) were inoculated into all benthocosm subunits at identical densities, in order to create a diverse benthic ecosystem. PVC panels each containing one glass slide were deployed as a proxy for bacterial settlement on non-living substrate.

#### ***Temperature fluctuation***

To set up the temperature regime in the tanks, a model of seawater temperature fluctuation from May to Sept in time intervals of 5 min was computed that represented the mean temperature fluctuation in the Kiel Fjord during the same time period in five preceding years (2010-2014). This temperature fluctuation model was then realized in two tanks (four subunits) as a control

treatment. The remaining four tanks received the same temperature treatment, but with temporary simulation of heatwaves. Three heatwaves (3HWs) at the end of May, July and August, each lasting 7 d with a maximum temperature increase of 5 °C relative to the base were applied in two tanks (four subunits), while only the last of these heatwaves (1HW) was applied in two other tanks (four subunits) at the end of August, when the seasonal temperature increase had reached its annual maximum (Fig. S1). Thus, with this experimental design the effect of one severe heatwave during the hottest time of the year could not only be compared to a regime without heatwaves, but also to the possible modulating effect of preceding (less severe) heatwaves.

### ***Samplings***

Samples were taken on the 6th of May (= start of the experiment), on the 7th of July (= after application of the 2nd HW) and on the 10th Aug (= end of the experiment). At these occasions two plants were sampled from each subunit and processed as described below.

### ***Surface extraction***

The ‘dipping method’ (de Nys et al. 1998) was applied to obtain compounds from the surface of sampled eelgrass leaves as described previously (Chapter I). Briefly, spin-dried leaves were dipped into 2-propanol for 5 s, which allowed to extract surface compound without disruption of epidermal cells. The concentration of compounds was related to the extracted surface volume, which was determined by multiplication of the leaf surface area with the mean thickness of 30 µm of the leaf surface boundary layer of marine plants (Wahl et al. 2010). The leaf surface was calculated from the leaf wet weight using the factor of 78.9903 cm<sup>2</sup> g<sup>-1</sup> that was described earlier

(Chapter I). The obtained surface extracts were filtered through glass fiber-filters with a mesh size of 0.2  $\mu\text{m}$  (Carl Roth, Karlsruhe, Germany), evaporated in vacuo and stored at  $-20\text{ }^{\circ}\text{C}$ . For all subsequent steps the extracts were redissolved in methanol at the same natural concentration. As described previously this change of solvent causes no loss of bioactivity and facilitates evaporation and HPLC analysis (Chapter I).

### ***Anti-settlement bioassay***

Bioassays were employed to quantify the antifouling activity of eelgrass surface extracts as described in Chapter I and in Saha et al. (2011). The bioassays were conducted with five isolates originating from stones on North Sea and Baltic Sea coasts (Saha et al. 2016) that had been conserved for two years at  $-80^{\circ}\text{C}$ . The isolates were *Alteromonas stellipolaris* (Strain AMAN1), *Pseudoalteromonas carrageenivora* (Strain AMAN4), *Marivita litorea* (Strain ANY8), *Polaribacter dokdonensis* (ANORD1) and *Vibrio cyclitrophicus* (ANORD3). All bacteria were incubated in liquid medium (5 g peptone (Carl Roth, Karlsruhe, Germany), 3 g yeast extract (Carl Roth, Karlsruhe, Germany), 16 g artificial sea salt and 1L tap water) at  $25\text{ }^{\circ}\text{C}$  for 1 or 2 days before they were applied in the assays.

To realize the assays 30  $\mu\text{l}$  of each extract were added into 8 wells on 96 well microtiter plates and diluted with 70 $\mu\text{l}$  of pure methanol. Control wells received 100  $\mu\text{l}$  of pure methanol instead. The solvent was then evaporated in a freeze-dryer and 100  $\mu\text{l}$  of bacterial suspension were added into 4 of each 8 wells while sterile seawater was added into the remaining four wells. Syto 9 (Invitrogen, Waltham, MA. USA) was applied to stain the DNA of attached bacterial cells (as described in Saha et al. (2011) and chapter 1) after 2 hours of incubation at 25 degree in darkness

and one rinsing with 100  $\mu$ l of sterile seawater. The fluorescence intensity was measured with a Hidex Chameleon IV plate reader (wave length of excitation: 477-491 nm, wave length of emission: 540 nm).

### ***Quantification of defense related compounds***

Rosmarinic acid was quantified by HPLC on a semi-preparative LichroCart 250/10 Purospher STAR-rp 18ec column (VWR, Radnor, USA), using a Varian 940-LC with diode array detection (Varian 940-LC, Agilent Technologies, Palo Alto, California, USA). The solvent system ranged from 0.1% formic acid (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in Acetonitrile (Sigma-Aldrich, St. Louis, Missouri, USA) to 0.1% formic acid dissolved in water, as described previously (Chapter I). The detection of rosmarinic acid was based on its light absorption at 350nm and concentrations were derived from peak areas after calibration of the system with purchased rosmarinic acid (Sigma-Aldrich, St. Louis, Missouri, USA) as described (Chapter I).

### ***Epibacterial abundance on eelgrass***

Biofilms from 1 cm<sup>2</sup> of *Zostera* surface (1 cm from the tips) were collected by swabbing with a sterile cotton tip, followed by vortexing the cotton tip for 30 secs in a sterile Eppendorf vial containing 1 ml of sterile-filtered seawater (SSW, 16psu). The relative abundance of diatoms (and any other possible photoautotrophs) in 100  $\mu$ L of subsample was determined by measuring the fluorescence of chlorophyll a at 477-491 nm (excitation) and 677 nm (emission), using a plate reader (Hidex Chameleon IV, Turku, Finland) and 96-well microtiter well plates (Greiner). Subsequently, the relative density of all microfoulers (including bacteria and diatoms) was determined by staining all the particles in the same 100  $\mu$ L subsample with the fluorescent

DNA-binding dye Syto 9, 0.005mM (Invitrogen GmbH). Following an incubation time of 10 min in darkness, fluorescence was subsequently measured (excitation 477-491 nm, emission 540nm), using the same plate reader. Replication was 4 fold per treatment. The first measurements provided data on treatment effects on the relative microalgal density at the algal surface, while the second measurement provided similar information on all epibiotic cells. Thereafter, the bacterial abundance was determined by subtracting the first measurement from the second one.

### ***Bacterial fouling pressure on non-living substrate***

To investigate bacterial fouling pressure, microscopic slides (Polysine slides) were exposed at a depth of about 50cm in each benthocosm unit containing the *Zostera* individuals. The slide were exposed before the experiment started (i.e. before beginning of first heatwave) and were sampled after each heatwave and before the beginning of 2<sup>nd</sup> and 3<sup>rd</sup> heatwave. At each sampling time point, the sampled slide was replaced by a new slide. Following sampling, 1cm<sup>2</sup> of the slides (1 cm from the top part) were collected by swabbing with a sterile cotton tip, followed by vortexing the cotton tip for 30 secs in a sterile Eppendorf vial containing 1 ml of sterile-filtered seawater (SSW, 16psu). Bacterial abundance was determined using the method described above.

### ***Statistical analysis***

A log effect ratio was used to calculate the defense activity strength of extracts from each plant. It is calculated as the logarithm of the ratio of target microbes settled in presence of extract and target microbes settled on solvent controls. Thus, negative values indicate an inhibitory effect of an extract against a target organism, while 0 would indicate absence of such an effect. The ratio of

density of bacteria settled on *Z. marina* plants to fouling pressure (measured as bacterial abundance on PVC panels) was calculated to represent the percentage of bacteria settled on *Z. marina* plants compare to potential bacterial fouling pressure. It represents the consequences of antifouling defense: higher values represent lower defense effects towards bacterial settlement.

Two-way-repeated-measures-ANOVA was used to investigate the effects of sampling time and heat wave treatments on the defensive activity against different bacteria, the production of rosmarinic acid in eelgrass, and the log-transformed densities of bacteria living on eelgrass plants and PVC plates. Shapiro-Wilk's test was used to test whether data within groups were normally distributed ( $p < 0.05$ ) and Levene's test was used to test whether homogeneity of variances was given ( $p < 0.05$ ). Tukey's HSD test was used to compare the means of single groups post hoc. The relationships between water temperature, fouling pressure and abundance of bacteria settled on eelgrass with general antifouling effects and concentrations of the defensive compound rosmarinic acid were analyzed with simple linear regression ( $p < 0.05$ ). All statistical analyses were conducted with the Statistica 8.0 software package (StatSoft, Tulsa, Ok., USA).

## **Results**

### ***Variation of fouling pressure and density of bacteria settled on eelgrass***

Bacteria settled on PVC panels were quantified as a measure of general fouling pressure. Their relative abundance varied significantly between single tanks (Repeated measures ANOVA,  $p < 0.05$ ), but not between the three treatments (Figure 1A, Table 1A). However, the general fouling pressure on eelgrass was altogether slightly higher in July than in May and August. Similar as with the fouling pressure, bacteria that settled on eelgrass peaked in July and had similar densities in May and August, but were unaffected by the heat treatments (Figure 3B, Table 3B). In May and August - but not in July - bacteria reached relatively lower densities on eelgrass surfaces than on PVC. This gets obvious when we regard the ratios of their abundances (Figure 1C, Table 1C). Also these settlement ratios were not significantly different between treatments. Correspondingly, a positive correlation was detected between fouling pressure and bacterial abundance on *Zostera* ( $r = 0.6493$ ,  $p < 0.0001$ , Fig. 1D).

### ***The Fluctuation of anti-bacterial defense activity***

The mean antibacterial activity of eelgrass against 5 bacterial isolates varied significantly with season (Table 2A). It increased significantly from the beginning of the incubation in May until the end of the second heatwave treatment in July, then it dropped back to an even lower level at the end of the third heatwave in August (Figure 2A). The heatwave treatments also had a significant influence on the defense activity (Table 2A). This difference was mostly generated by the last treatment, since a lower antibacterial activity was detected in the end of the experiment under ambient conditions than under the two conditions with heatwave application (Fig. 2A).

The 5 tested bacterial isolates can be divided into 3 groups based upon their different responses to heat treatments and sampling times (Figure 2B-F). The first response pattern was exhibited by two bacteria (*P. carrageenovora* (Man4) and *V. cyclitrophicus* (Nord3)) which were both deterred less in August than in the preceding months (Table 2B and 2C, Fig. 2B and 2C). Moreover, after the last sampling both isolates were less deterred by extracts from *Z. marina* raised under ambient conditions than by extracts originating from plants that had experienced one heat wave. Only in the case of *P. carrageenovora* the latter effect was significant over all three samplings. A second response pattern was detected in *M. litorea* (NY8) and *P. dokdonensis* (Nord 1). These two isolates were more deterred in July than in the preceding and the following months and exhibited no significant differences in their responses to extracts originating from plants that experienced different heat wave treatments (Table 2D and 2E, Fig. 2D and 2E). The last response pattern was shown by *A. stellipolaris* (Man1), which only exhibited a significantly weaker deterrence in May than in July in the ambient condition (but not in the 1 HW condition, which was not different from the ambient condition before August) (Figure 2F, Table 2F). Interestingly, samples collected in August from tanks that were subject to ambient temperature exhibited (not always significantly) less deterrence of all five bacterial isolates than samples that originated from tanks that were subject to heat waves. Moreover, four out of the five strains were (non-significantly) more deterred by extracts coming from *Z. marina* that had just experienced one HW than by extracts from plants that had experienced three repeated HWs.

### ***The variation of defense related compounds***

The concentration of RA on eelgrass surfaces from different heatwave treatments exhibited no significant differences (Figure 3, Table 3). However, the RA concentration differed among sampling times and decreased from May to August.

### ***Correlations among factors***

Although both antifouling activity and Rosmarinic acid concentration varied with season they were not significantly correlated when all three sampling time points were regarded (Fig. 4A), The same was true when only eelgrass samples collected in May or July were regarded (Figure 4B-C), but a negative relationship of the two variables existed in August ( $p < 0.0176$ , Figure 4D), suggesting that RA surface concentrations increased with increasing antifouling capacity of eelgrass in this month.

In contrast, however, the bacterial abundance on eelgrass strongly correlated with antifouling defense activity when all eelgrass samples were regarded ( $p < 0.0001$ , Figure 5A), but only weakly within the August samples ( $p < 0.0537$ , Figure 5D) and not at all within May and July (Figures 5B-C). No correlations were observed between the bacterial abundance on PVC panels and either antifouling defense activity or Rosmarinic acid concentration of eelgrass surface extracts – neither within single months, nor when all time points were regarded together (data not shown).

Similarly, the surface concentrations of RA also correlated with the number of settled bacteria on eelgrass in August ( $p < 0.014$ ), but not in May, July or when all three time points were

regarded together (Figure 6A-D).

## **Discussion**

The general design of our experiment allows for a comparison of heat wave effects and effects of seasonal warming. During incubation in control benthocosms without simulation of heat waves, the eelgrass plants experienced a relatively rapid temperature increase from 9.5°C in May to 18.3 °C in July, followed by a moderate increase to 19.4 °C in August. Heatwaves, in contrast, were characterized by a much more rapid increase by 5° C within few days and they were therefore generally expected to cause more pronounced effects than seasonal warming. However, most of the variables under investigation in our study exhibited no significantly different responses after different heatwave treatments, with the only exception of antifouling defense. In contrast, almost all of the variables clearly differed among sampling times and with few exceptions they exhibited a similar seasonal pattern, with an increase from May to July and a decrease thereafter until the end of the incubation.

For example, the numbers of potential bacterial foulers on eelgrass - measured as bacteria that settled on (undefended) PVC panels – were unaffected by heatwave treatments, but exhibited a seasonal pattern with higher densities in July compared to May and August. The fouling pressure in the Baltic Sea usually peaks in the middle of summer (Saha et al. 2011, Chapter III) and one possible explanation could be higher water temperatures in summer. However, a significant correlation between fouling pressure and water temperature (resulting from the combined effect of simulated seasonal warming and simulated heatwaves) was in our study also not detected (data not shown), which suggests that the seasonal pattern must be caused by other abiotic or biotic

variables.

Also the density of bacteria settled on eelgrass was not affected by HW treatments and exhibited a similar seasonal pattern as fouling pressure. In this case, however, the bacterial density correlated significantly with water temperature ( $r = 0.3434$ ,  $p < 0.041$ , data not shown), suggesting that the settlement of bacteria on eelgrass is to some degree promoted by high temperature. The chemical defense of plants is rarely comprehensive and continuous, in most cases it is dynamic and selective (Lankau 2007) and only microbes that are resistant to the chemical defense of eelgrass are capable to colonize the plant (Chapter I). In general bacteria tend to invade plants in higher numbers at high temperature, either because the bacteria are more active under this condition (Case et al. 2011) or because the hosts become more susceptible when they are weakened by heat stress (Vergeer et al. 1995). Similar densities of bacteria were observed on eelgrass and on PVC panels in July, while eelgrass was significantly less colonized than PVC panels in May and August and at first glance this could suggest that the defense of eelgrass collapsed in July. However, our bioassays detected no evidence of such breakdown (see below) and an increased susceptibility of eelgrass to bacterial settlement after heat stress also seems improbable because heat waves had no promoting effect on this settlement. Much rather, bacteria that are capable of settlement on eelgrass may have generally benefited from the specific combination of higher temperatures, and perhaps other conditions in the benthocosms in July.

However, the mean antifouling defense of eelgrass against bacteria was affected by heatwave treatments, and this was mainly due to differences among the treatments that were observed at the end of the experiment in August. Under conditions without HW application the

defensive capacity of eelgrass decreased from July to August, but this reduction was less pronounced when heat waves were applied. Interestingly, plants that experienced their first HW directly prior to the sampling in August exhibited the strongest defensive capacity, which suggests that HW treatment causes not a weakened, but an increased defense against microfoulers. Plants that experienced the third heatwave in August in most cases exhibited a mean defensive capacity that was between those of the ambient and the 1HW treatment. This difference was only in the case of one of the isolates that were tested, *A. stellipolaris*, statistically significant, but it nonetheless indicates that repeated heatwaves could not cause a stronger increase in defense than one single heatwave. Thus, preconditioning by repeated heatwaves did not result in a stronger, but in tendency in a weaker response. This could also explain the fact that no difference in the defensive capacities of differently treated eelgrass plants was observed in July, when plants from the 3HW treatment had just been exposed to the second (and thus to a repeated) HW, while the other two groups had received no HW yet.

Similar as the abrupt increase of water temperature during heatwaves, the long term temperature increase from May to July came along with an upregulated defense capacity against bacterial settlers. In contrast, a considerable reduction of this defense capacity was observed during the following period from July to August, when the water temperature increased only slightly, and simulation of a heatwave during this time made the reduction less severe. Thus, either the eelgrass plants required a steady temperature increase to be able to maintain the level of their defensive capacity or temperature was not the only factor that regulated seasonal changes in this capacity. Recent research already demonstrated that other factors, such as light intensity and

nutrient fluctuation may also influence the chemical defense of marine plants both individually and synergistically (Amsler 2008, Hay 2009, Rosenthal & Berenbaum 2012). Moreover, a demand-driven regulation of the chemical defense of eelgrass against microfoulers can also not be excluded and could explain these observations: Both antifouling defense activity and the density of bacteria settled on *Z. marina* peaked in July and reached their monthly maximum in August in 1HW treatments. As a consequence, both parameters correlated strongly when all sampling points were regarded and weakly when only August was regarded. Thus, a strong defense activity was generally observed when the density of microsettlers on *Z. marina* was high, which could hint at a stronger production of deterrent compounds in response to these microsettlers.

An induced or activated chemical defense of eelgrass or other aquatic macrophytes against bacterial settlers has to our knowledge not been described yet. However, vascular plants (Vera et al. 2011) and also algal macrophytes (Weinberger 2007) usually have innate immune receptors on the outer cell plasma membrane. These perceive highly conserved and not pathogen-specific, but microbe-specific defense elicitors (so-called microbe-associated molecular patterns), that often originate from the outer cell envelopes of microbes (Nürnberg et al. 2004). A relatively recent study demonstrated that such receptors apparently exist in the seagrass *Thalassia testudinum*, as it is capable of perceiving bacterial lipopolysaccharides (Loucks et al. 2013). Similar mechanisms could potentially be the basis of a recognition of microbial settlers in *Z. marina*.

Only three measured variables in our experiment did not reach a peak in July, but changed steadily with the seasonal temperature increase, and one of them was the concentration of the

identified deterrent compound rosmarinic acid. Promoting effects of temperature increase on the antifouling defenses of marine macrophytes have been described before. For instance, surface concentrations of the deterrents Fucoxanthin and DMSP on the brown seaweed *Fucus vesiculosus* were higher at increased temperature (Saha et al. 2012). However, rosmarinic acid concentrations decreased with the seasonal increase in water temperature from May through July to August, and this could explain the corresponding seasonal decrease in the deterrent activity against *A. stellipolaris* and *P. dokdonensis*. In contrast, the deterrent activities against all other bacteria showed no seasonal patterns that corresponded with the seasonality of rosmarinic acid. This suggests that additional deterrent compounds that underwent other seasonal concentration dynamics must have been active and it confirms our previous conclusion that rosmarinic acid cannot be the only compound that deters microsettlers from the surface of *Z. marina* (Chapter I).

Likewise, the differences in deterrence observed after different heat wave treatments cannot be fully explained with changes in Rosmarinic acid concentrations: In August antifouling defense was the strongest after application of one heatwave and the weakest under simulated ambient conditions, and mean Rosmarinic acid concentrations also decreased in this order, which was reflected in a significant correlation of both variables. However, heat wave treatments in August had no significant effect on Rosmarinic acid concentrations and correlations between Rosmarinic acid concentration and antifouling activity were not detected in May and July or when the full experimental period was regarded. Obviously Rosmarinic acid primarily affected the deterrent activity of eelgrass toward the end of the experiment. A positive correlation was also detected in August between the abundance of bacteria settled on eelgrass plants and the RA

surface concentration, suggesting that the above discussed increase in antifouling capacity in the presence of high numbers of settlers in August was largely due to an increase in Rosmarinic acid.

In conclusion, compared to seasonal environmental change heat wave treatments only had a moderate effect on the chemical antifouling defense of eelgrass in this experiment. Application of a single HW in August during the warmest period caused an increase of antifouling activity, while this increase was less pronounced in plants that had experienced preceding heat waves, which suggests that an adaptation to heat stimulation took place. Earlier heatwaves in July had no detectable effect on antifouling and it remains unclear whether this was due to the generally increased defense capacity of eelgrass in July (that perhaps could not be increased much further), to the fact that the heatwave applied in July was a second heatwave (that perhaps resulted in a less pronounced effect) or to the fact that other compounds than Rosmarinic acid (that were perhaps simply not affected by the heatwave treatment) apparently dominated the deterrent surface chemistry of eelgrass. In addition, bacterial abundance on eelgrass to some degree correlated with Rosmarinic acid content and defense activity, which could hint at a demand-driven defense against microfoulers by Rosmarinic acid. Thus, even after transient exposure to water temperatures of 25°C *Zostera marina* from the Baltic Sea seems capable of a dynamic management of its surface defense chemistry, despite the fact that increased mortality and reduced growth were previously observed with plants from the same region that were maintained for six weeks at this temperature (Nejrup & Pedersen 2008). In this light a collapse of eelgrass resistance to epibionts will probably not be the most prominent effect of future global warming.

## Acknowledgements

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## Figure legends:

Figure 1: Fluctuation of A: bacterial fouling pressure (measured as bacterial density non-living PVC substrate); B: bacterial density on *Z. marina*; C: bacterial density on *Z. marina* (shown in (B)) relative to bacterial fouling pressure (shown in (A)) and (D) correlation between fouling pressure and bacterial abundance (mean  $\pm$  95% CI). Different Greek letters indicate sampling times that differed significantly in a Tukey post hoc-test ( $p < 0.05$ ).

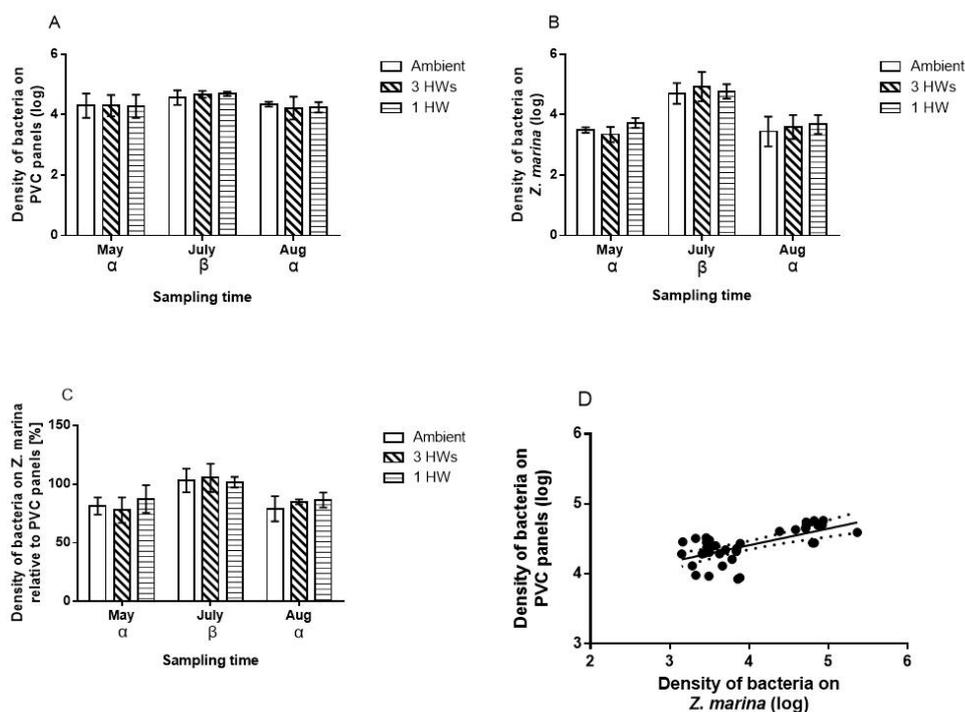


Figure 2: Antisettlement activity of propan-2-ol surface extract from *Z. marina* against A: five different bacterial isolates (mean of B to F); B: *A. stellipolaris*; C: *P. carrageenovora*; D: *M. litorea*; E: *P. dokdonensis*; F: *V. cyclitrophicus* tested at 1-fold of natural concentration in response to three different heatwave treatments at three sampling points (mean  $\pm$  95% CI; in (A):  $n = 5$ , in B-F:  $n = 12$ ). Increasingly negative values indicate increasing activity strength. Sampling times,

treatments and treatments at individual sampling times that are significantly different (Tukey post hoc test,  $p < 0.05$ ) are marked by caps, Greek letters and small Latin letters, respectively.

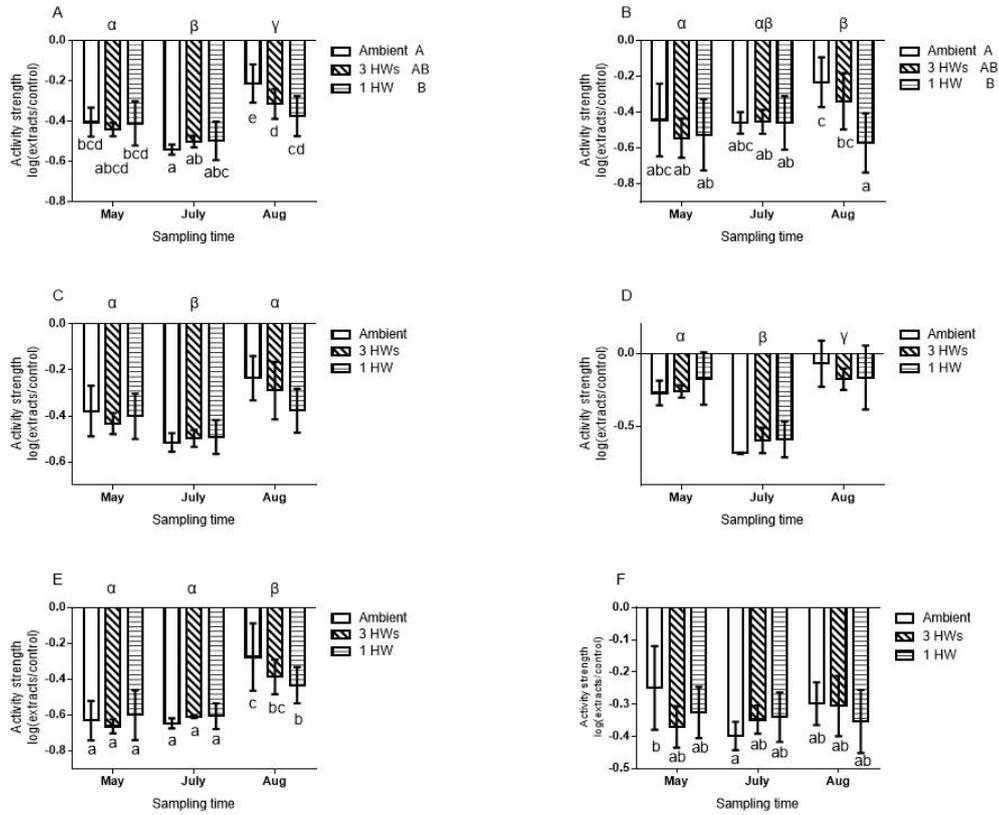


Figure 3: Concentrations of Rosmarinic acid on the surface of eelgrass plants (mean±95% CI). Greek letters indicate months with significantly different concentrations.

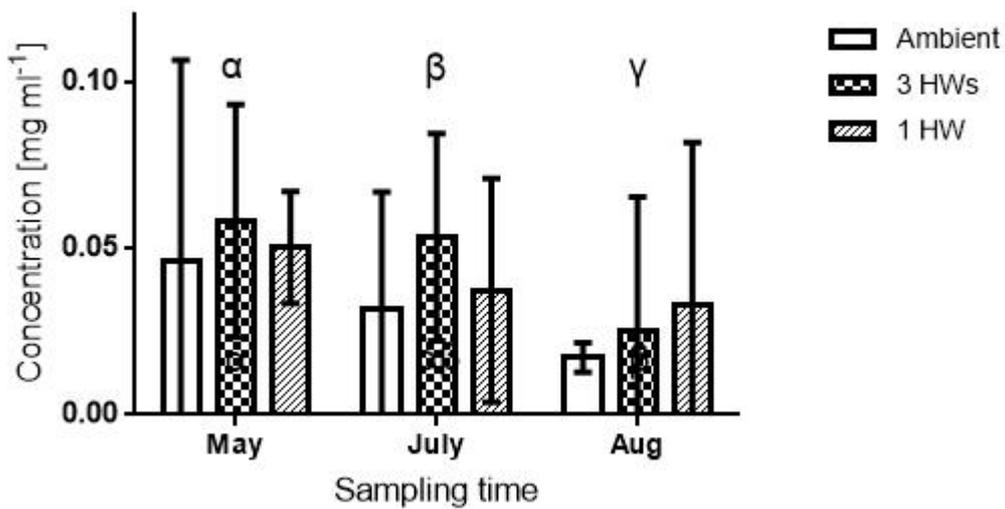


Figure 4: Pearson correlations of defense capacity of eelgrass and surface concentration of rosmarinic acid on eelgrass in (A) all samples ( $r=-0.2851$ ,  $p<0.0919$ ); (B) samples collected in May ( $r=0.3287$ ,  $p<0.2968$ ) (C) samples collected in July ( $r=0.4253$ ,  $p<0.168$ ) and (D) samples collected in August ( $r = -0.6678$ ,  $p < 0.0176$ ).

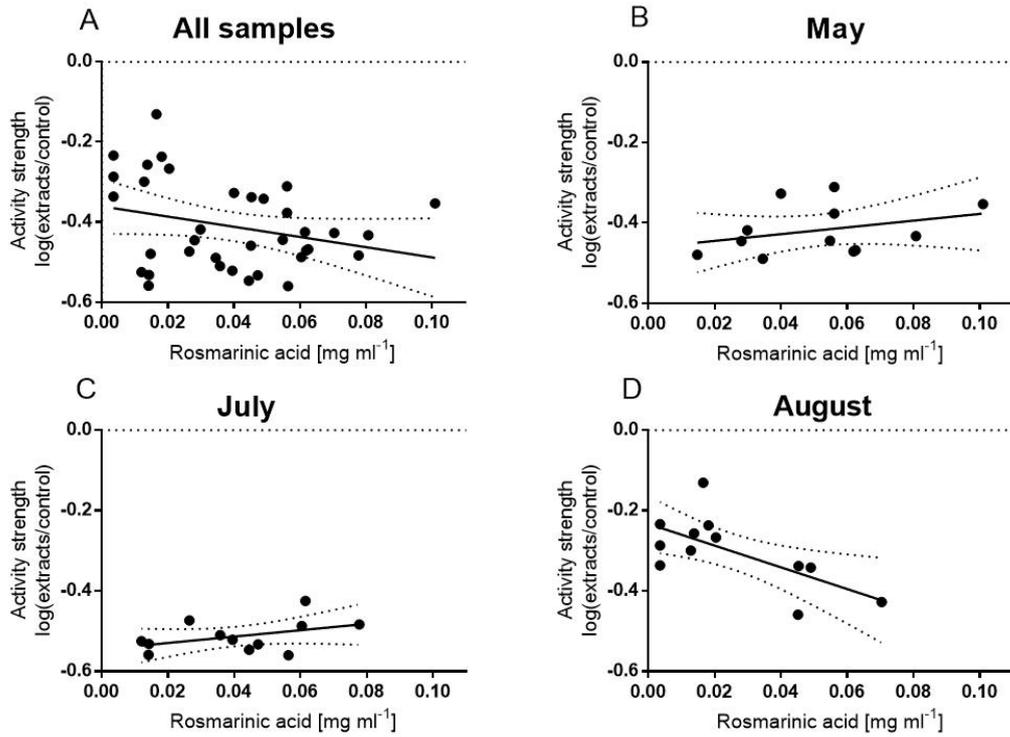


Figure 5: Pearson correlations of defense capacity of eelgrass and density of bacteria settled on eelgrass in (A) all samples ( $r=-0.645$ ,  $p<0.0001$ ); (B) samples collected in May ( $r=0.416$ ,  $p<0.179$ ) (C) samples collected in July ( $r=0.3014$ ,  $p<0.341$ ) and (D) samples collected in August ( $r = -0.5686$ ,  $p < 0.0537$ ).

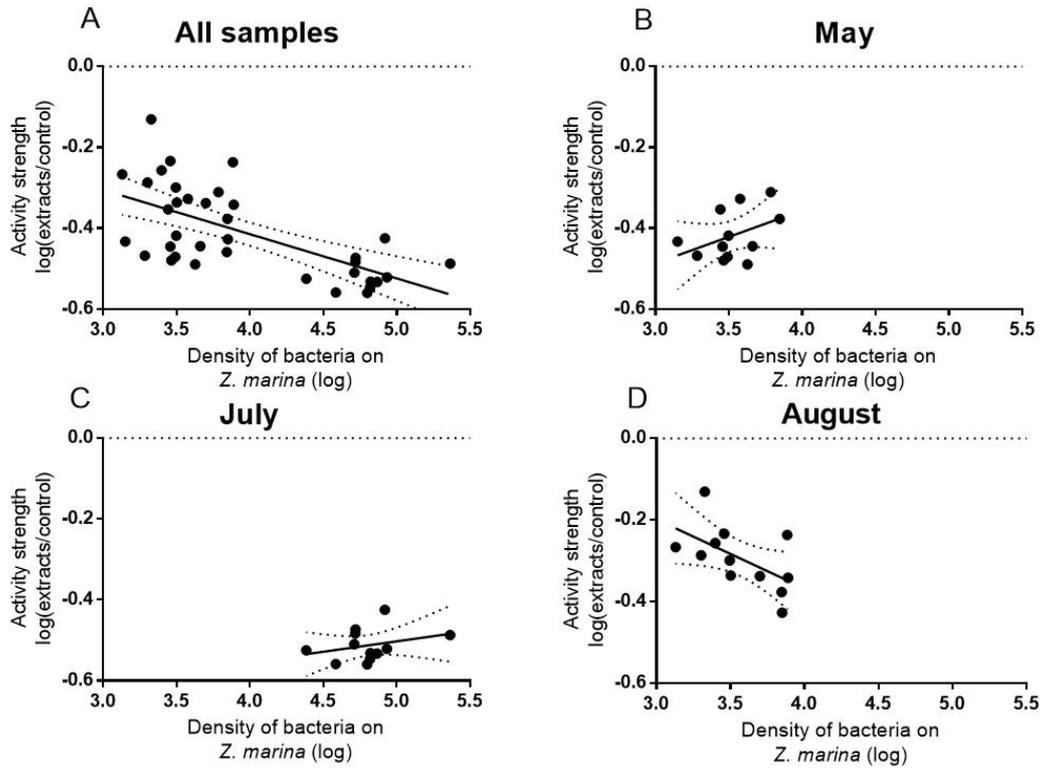
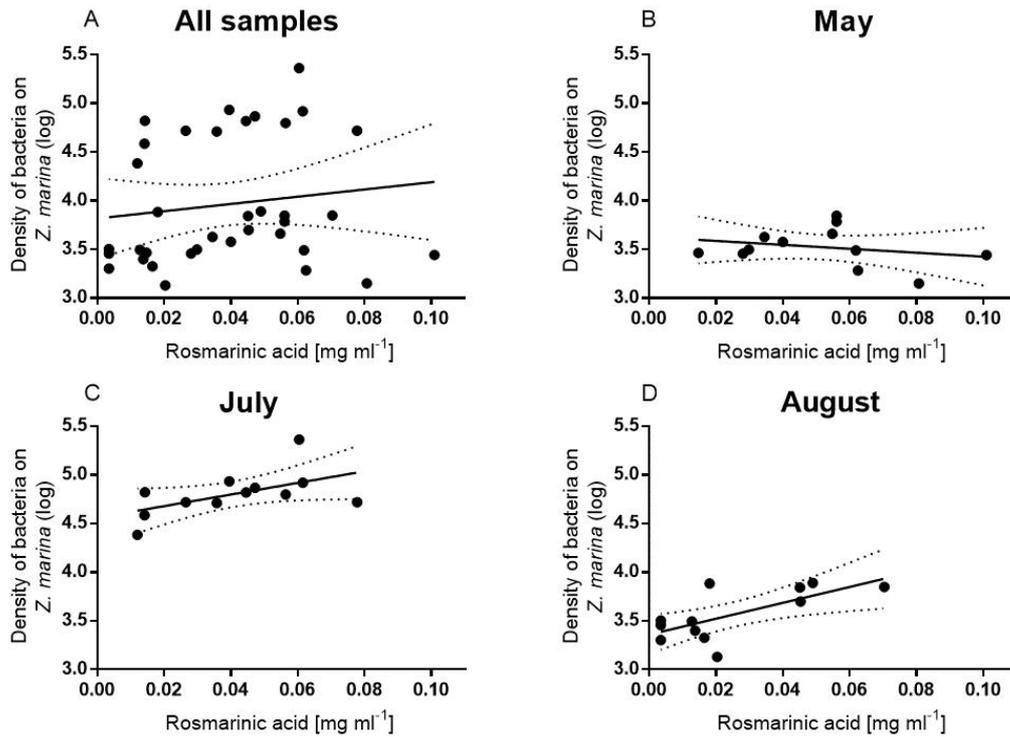


Figure 6: Pearson correlations of density of bacteria settled on eelgrass and surface concentration of RA on eelgrass in (A) all samples ( $r=0.1414$ ,  $p<0.4109$ ); (B) samples collected in May ( $r=-0.2492$ ,  $p<0.4346$ ) (C) samples collected in July ( $r=0.5447$ ,  $p<0.0671$ ) and (D) samples collected in August ( $r = 0.4693$ ,  $p < 0.014$ ).



**Table legends:**

Table 1: Two-way-repeated measures ANOVA of A: bacterial fouling pressure (measured as bacterial density on non-living PVC substrate; see Fig. 1A); B: bacterial density on *Z. marina* (see Fig. 1B); C: bacterial density on *Z. marina* relative to bacterial fouling pressure (see Fig. 1C).

Treatment was used as between-measures factor and Month was used as within-measures-factor.

	Effect	SS	Degr. Of Freedom	MS	F	P
A	Intercept	697.8254	1	697.8254	20514.19	<0.0001
	Treatment	0.0002	2	0.0001	0.00	0.9974
	Error	0.3062	9	0.0340		
	Month	1.0482	2	0.5241	18.55	<0.0001
	Month*Treatment	0.0756	4	0.0189	0.67	0.6217
	Error	0.5085	18	0.0283		
B	Intercept	566.4868	1	566.4868	8985.591	<0.0001
	Treatment	0.1907	2	0.0954	1.513	0.2714
	Error	0.5674	9	0.0630		
	Month	12.6256	2	6.3128	180.978	<0.0001
	Month*Treatment	0.3274	4	0.0819	2.347	0.0935
	Error	0.6279	18	0.0349		

C	Intercept	290778.4	1	290778.4	5910.895	<0.0001
	Treatment	92.8	2	46.4	0.943	0.4248
	Error	442.7	9	49.2		
	Month	3348.5	2	1674.2	39.004	<0.0001
	Month*Treatment	172.5	4	43.1	1.005	0.4308
	Error	772.7	18	42.9		

Table 2: Statistical results of repeat measured ANOVA of antifouling defense against A: overall antibacterial activity; B: *A. stellipolaris*; C: *P. carrageenovora*; D: *M. litorea*; E: *P. dokdonensis*; F: *V. cyclitrophicus* tested at 1-fold of natural concentration on bacteria in response of heatwaves treatments at three sampling points.

	Effect	SS	Degr. Of		F	P
			Freedom	MS		
A	Intercept	6.0698	1	6.0698	4644.334	<0.0001
	Treatment	0.0114	2	0.0057	4.377	0.0470
	Error	0.0118	9	0.0013		
	Month	0.2725	2	0.1363	47.371	<0.0001
	Month*Treatment	0.0497	4	0.0124	4.318	0.0127
	Error	0.0518	18	0.0029		
	B	Intercept	7.2116	1	7.2116	951.3581
Treatment		0.1192	2	0.0596	7.8684	0.0106
Error		0.0682	9	0.0076		
Month		0.0938	2	0.0469	5.1909	0.0166
Month*Treatment		0.1460	4	0.0365	4.0388	0.0165
Error		0.1626	18	0.0090		
C		Intercept	6.4628	1	6.4628	2197.928
	Treatment	0.0208	2	0.0104	3.544	0.0733
	Error	0.0265	9	0.0029		
	Month	0.1179	2	0.0590	13.675	0.0002
	Month*Treatment	0.0389	4	0.0097	2.257	0.1033
	Error	0.0776	18	0.0043		
	D	Intercept	3.9669	1	3.9670	2030.453
Treatment		0.0094	2	0.0047	2.401	0.1460
Error		0.0176	9	0.0020		
Month		1.5745	2	0.7872	94.383	<0.0001
Month*Treatment		0.0634	4	0.0158	1.899	0.1544
Error		0.1501	18	0.0083		

E	Intercept	9.6542	1	9.6542	4989.740	<0.0001
	Treatment	0.0034	2	0.0017	0.891	0.4436
	Error	0.0174	9	0.0019		
	Month	0.8463	2	0.4231	109.764	<0.0001
	Month*Treatment	0.0491	4	0.0123	3.182	0.0384
	Error	0.0694	18	0.0039		
F	Intercept	3.9681	1	3.9681	2011.360	<0.0001
	Treatment	0.0051	2	0.0026	1.296	0.3202
	Error	0.0178	9	0.0020		
	Month	0.0163	2	0.0082	2.755	0.0904
	Month*Treatment	0.0402	4	0.01	3.390	0.0311
	Error	0.0533	18	0.003		

Table 3: Statistical results of repeat measured ANOVA analysis of Rosmarinic acid concentration on the surface of eelgrass during the incubation.

Effect	SS	Degr. Of Freedom	MS	F	P
Intercept	46651.13	1	46651.13	90.2616	<0.0001
Treatment	1193.07	2	596.53	1.1542	0.358
Error	4651.60	9	516.84		
Month	4335.65	2	2167.83	3.7621	0.0431
Month*Treatment	633.45	4	158.36	0.2748	0.8904
Error	10372.06	18	576.23		

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## Supplements:

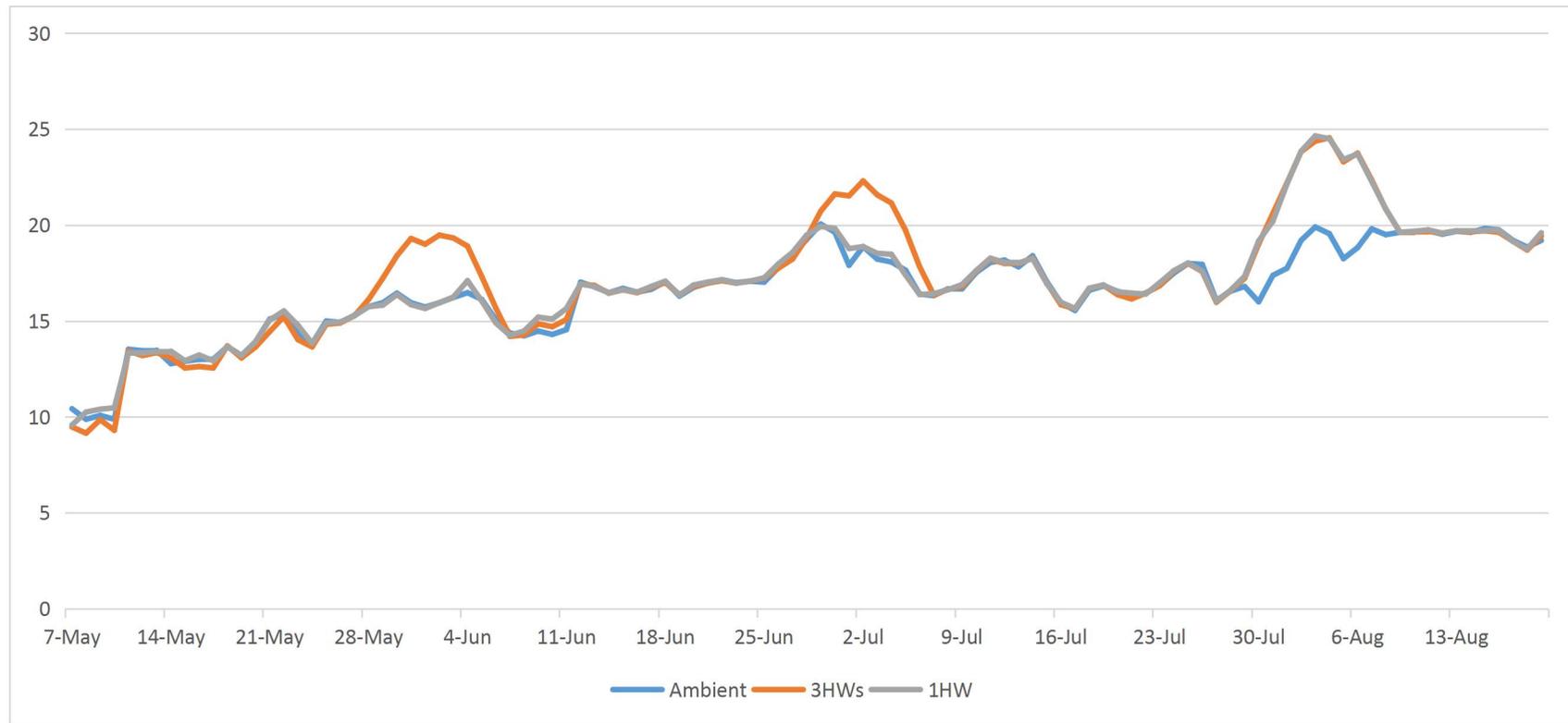


Figure S1. Change of Temperature in Kiel Outdoor Benthocosm (KOB) system.

**Seasonal variations in the anti-bacterial defense activity of *Zostera marina* L. correlate with seasonal variations in fouling pressure and density of microepibionts**

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# **Seasonal variations in the anti-bacterial defense activity of *Zostera marina* L. correlate with seasonal variations in fouling pressure and density of microepibionts**

## **Abstract**

In the marine environment microfouling is usually subject to seasonal fluctuation. We therefore assessed the seasonality of chemical antifouling defense and surface concentrations of a previously identified defense compound, Rosmarinic acid, on *Z. marina*. All examined variables exhibited a similar fluctuating pattern and peaked in summer, while they tended to be lower in spring and autumn. The seasonality of both defense activity and Rosmarinic acid surface concentration was significantly correlated with the seasonal fluctuation of fouling pressure, which suggests that *Z. marina* plants in the Baltic Sea can acclimatize their defensive level to the relatively high bacterial fouling pressure in summer. Besides of biotic factors the seasonal change of environmental factors, such as nitrogen supply and in particular temperature also affected the defensive level, either directly or through indirect effects on the microbial settlers.

Key words: *Zostera marina*; seasonal fluctuation; antifouling defense; Rosmarinic acid.

## Introduction

Seagrasses, marine flowering plants, are widely distributed across the globe (Orth et al. 2006).

*Zostera marina*. L is one of the most dominant species in northern European and American coastal area and also in the Baltic Sea, where eelgrass beds extend from 2 to 14 m below the mean sea surface level (Wium-Andersen & Borum 1984). Eelgrass and other seagrasses provide various ecological services to the environment of coastal waters (Wright & Jones 2007). The roles of seagrass in the ecosystem are vital due to its fundamental functions in food web formation, its altering effect on nutrients cycling, and its importance as nursery habitat for many other organisms (Hemminga & Duarte 2000).

In addition, seagrasses provide a substratum for epiphytic organisms and may suffer from negative effects of epibiosis. The development of epibiosis in the marine environment generally passes four stages (Wahl 1989): macromolecules randomly stick to the surface, which leads to an accumulation of energy resources and nutrients that are attractive to microbes. As a consequence the formation of biofilms – composed of bacteria, fungi, protists and other microscopic organisms - is facilitated. These provide the interface for further settlement of micro- and macrocolonizers, such as animals and algae (Wahl 2009, Harder 2008). Epibionts (fouling organisms) and basibionts (substrate organisms) may interact directly, both in beneficial and detrimental ways (Wahl 2009). Examples of advantageous effects of fouling on the host exist. For instance, the chemicals generated by epibionts may help hosts to escape from predation, since prey is typically identified through chemical cues (Stoecker 1978, Feifarek 1987). In addition, some well defended epibionts can protect basibionts from pathogen (Saro et al. 2012, Barbosa et al. 2009). Despite of

that, in most cases the negative impact of epibiosis outweighs the positive impact and basibionts suffer from competition for nutrients (Bryars et al. 2011, Romero et al. 2006), reduced photosynthesis due to epiphytic shading (Muñoz & Fotedar 2010), increased susceptibility to infection (Goecke et al. 2010) or predation (Da Gama et al. 2008). Epibionts on plants could also be feeding cues for grazers (Karez et al. 2000); for example the attachment of bryozoans altered the susceptibility of the red seaweed *Cryptonemia seminervis* to grazers (da Gama et al. 2008). The epibionts on marine macrophytes may also cause direct tissue damage by colonization of the surface and subsequent penetration into thallus tissues (Gonzalez & Goff 1989).

To cope with epibionts, marine macrophytes have evolved diversified and specific antifouling defenses that are often adapted to specific foulers (Wahl 2009, Qian et al. 2013, Scardino & de Nys 2011). The chemical defense against fouling is long known and believed to be an evolutionary response of hosts that was necessary for their survival (Sammarco & Coll 1992). Various secondary metabolites with a potential for chemical antifouling defense have been discovered in marine macrophytes. They can be non-toxic deterrents or compounds that inhibit settlement, growth, survival or reproduction of foulers (Dobretsov et al. 2006, Wahl et al. 1998). For instance Saha et al. (2011) demonstrated the inhibitory function of fucoxanthin from the brown seaweed *Fucus vesiculosus* on bacterial microsettlers; In seagrass, biologically active phenolic metabolites were repeatedly discovered, and proposed to be functional in the chemical defense against fouling (Prabhakaran et al. 2012), herbivory (Vergés et al. 2007), microbes (Harrison & Durance 1995, Jensen et al. 1998 etc. In a previous study we isolated rosmarinic acid and other phenolic compounds that are responsible for the defense against bacterial settlers from

the surface of the seagrass *Zostera marina*, which proved the ecological relevance of phenolics with antifouling activity on eelgrass surfaces (see chapter I).

Phenolic compounds in plants are considered as carbon storage compounds that may accumulate when plants are photosynthetically active, but nutrient depleted (Bryant et al. 1987). This corresponds with observations of negative correlations between the concentration of phenolic compounds and the nitrogen content in eelgrass tissues (Vergeer et al 1995). Other environmental factors that could alter photosynthesis rates - such as light intensity, temperature, salinity etc. – may also affect the concentrations of phenolics in seagrass (Arnold et al. 2012). As a consequence, the concentration of phenolics in seagrasses often varies with season (Harrison & Durance 1989, Ravn et al. 1994).

Similarly, an impact of abiotic factors (Amade & Lemee 1998; Helio et al. 2004; Marechal et al. 2004) and a seasonality of the antifouling defense of marine macrophytes has also been observed in many studies (Helio et al. 2004, Rickert et al, 2016). For instance, the antifouling defense of *Fucus vesiculosus* exhibited a clear seasonal fluctuating pattern and a higher defense activity was observed in summer than in other seasons (Saha et al. 2011). The mechanism behind this pattern was not clearly revealed, but the authors interpreted it as an adaptation to seasonal changes in abiotic factors and related changes in fouling pressure. Similar work on *Z. marina* is still missing, but the above mentioned evidence of seasonal variation in tissue concentrations of rosmarinic acid and other phenolic compounds (Harrison & Durance 1989, Ravn et al. 1994) – combined with the knowledge that rosmarinic acid plays a role as deterrent against microsettlers (Chapter I) – suggest that a seasonality of antifouling defense in eelgrass may also exist.

In addition to abiotic factors, biological factors may theoretically also regulate the antifouling defense of marine plants. There is a growing body of evidence indicating that defense induction against biological enemies such as herbivores and pathogens exists in marine macrophytes (Weinberger 2007, Steele and Valentine 2012, Arnold et al. 2008). Such induced defense could be beneficial for the host in several respects. For example, it would reduce the metabolic costs of defenses (Cronin 2001), and the risk of adaptation of enemies to these defenses (Keen 1990). Unambiguous direct evidence of an induced defense of marine macrophytes against epibionts has not yet been reported, but this does not mean that it is inexistent.

In the present study we monitored seasonal fluctuations in the chemical antifouling defense of *Zostera marina* L., using an antibacterial settlement bioassay as indication system. The pre-identified phenolic defense compound rosmarinic acid was also quantified, in order to verify that previously observed seasonal dynamics of tissue concentrations (Ravn et al. 1994) can also be observed in surface concentrations and to test the hypothesis that the relative contribution of rosmarinic acid to the overall antifouling defense of *Z. marina* changes with season. Fluctuations in the abundance of bacteria living on *Z. marina* and on adhesive glass slides - representing settled bacteria and the bacterial fouling pressure, respectively - were measured to test the hypotheses that antifouling defense and/or rosmarinic acid excretion of eelgrass correlate with fouling pressure.

## **Method and Material**

### ***Collection and preparation of samples***

*Zostera marina* L. plants were collected monthly from four locations in Falckenstein beach at Kiel,

Germany (54°38'N/10°17'E) from May to November in 2015 that were in 50 m distance from each other. The sampling site is located in the southwest Baltic Sea, where the general salinity is around 16‰. *Z. marina* were collected in Ziploc bags, transported to the lab in a cooler box and extracted immediately afterwards.

### ***Surface extraction of samples:***

The surface compounds of *Z. marina* were obtained by the 'dipping method' (de Nys et al. 1998). Fresh *Z. marina* leaves were dipped into propan-2-ol for 5 s, which allows the solvent to extract surface compounds without breaking the epidermal cells (Chapter I). The concentration of surface compounds was related to the surface volume, which was calculated by multiplication of surface area with the mean thickness of the leaf surface boundary layer of marine plants (30µm) (Wahl et al. 2010). As described in Chapter I, the leaf surface was calculated from the wet weight, using the factor 78.9903 cm<sup>2</sup> g<sup>-1</sup>. Crude extracts were filtered through 0.2µm glass fibre-filter (Carl Roth, Karlsruhe, Germany) and then evaporated in vacuo at 30°C before they were stored in a freezer (-20 °C). Prior to all following steps the surface extracts were redissolved in methanol (Carl Roth, Karlsruhe, Germany) as described in chapter I.

### ***Abiotic factors:***

Data for temperature in 1 m water depth, and salinity in 4 m water depth were constantly measured from May to November 2015 at Kiel light house (distance to the experimental site: 1.5 km) in time intervals of 1 hour and kindly provided by BSH. Radiation between 300 and 3000 nm was constantly logged above the sea surface at Kiel-Düsternbrook (54°19.8' N, 10°9.0' E; distance

to the experimental site: 7.5 km) in time intervals of 5 min and kindly provided by the meteorology department of GEOMAR. Those data were measured as  $W\ m^{-2}$  and transformed to photosynthetically active radiation measured as  $\mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ , following Weinberger et al. (2008). Of all these logged data only the mean of the week before eelgrass sampling was used in statistical analyses. Weekly means of temperature peaked in August and those of light intensity in June, while those of salinity oscillated between 15 and 19 psu (Supplementary Information Figure S1A-C). Water samples were collected monthly from each sampling point, filtered through  $0.2\ \mu\text{m}$  glass fibre-filters and analyzed for their content of nitrate plus nitrite, nitrite only, ammonium and phosphate by Dr. Rheena Schumann (Biologische Station Zingst, University of Rostock). The monthly mean of ammonium peaked in July, that of nitrate decreased from May to November, that of nitrite showed an opposite trend with a minor peak in July and that of phosphate was considerably higher in November than in all other months (Supplementary information figure S1D-G).

### ***Anti-bacterial-settlement bioassays***

In order to quantify the antifouling activity of eelgrass surface extracts, we used the anti-bacterial-settlement bioassays described in Chapter I and in Saha et al. (2011). Briefly, four bacterial isolates applied in these bioassays were isolated from stones of the North Sea and Baltic Sea upper subtidal and lower intertidal zones (Saha et al. 2016) and had been conserved in  $-80^{\circ}\text{C}$  for two years. They were *Alteromonas stellipolaris* (strain AMAN1), *Loktanella rosea* (strain AFALK1), *Polaribacter dokdonensis* (strain ANORD1) and *Bacillus aquamaris* (strain AMAN10). All bacteria were cultivated in liquid medium containing 25 g sea salt, 5 g peptone (Carl Roth,

Karlstuhe, Germany), and 3 g yeast extract(Carl Roth, Karlstuhe, Germany) in 1 L deionized water at 25 °C in darkness for 1-2 days before bioassays were conducted.

For these assays 30 µl of each extract and 70 µl of pure methanol – for solvent controls 100 µl of methanol - were added into multi-well plates and the solvent was evaporated in a freeze-dryer. Then 100 µl of bacterial suspension in seawater were added into 4 of the 8 wells and 100 µl of seawater were added into the remaining 4 wells as a control of extract fluorescence. The wells were incubated for 2 hours at 25 °C at 100 rpm. Then unattached cells were rinsed by seawater, the remaining bacterial cells were stained with Syto 9 and their fluorescence was measured in the Hidex Chameleon plate reader. All extracts were tested at 1-fold natural concentration, which was calculated as described in chapter 1.

### ***Quantification of defense related compounds:***

The quantification method for the defense related compound Rosmarinic acid by HPLC was also described in Chapter I. A semi-preparative LichroCart 250/10 Purospher STAR-rp 18ec column (VWR, Radnor, USA) together with a Varian 940-LC with diode array detection (Varian 940-LC, Agilent Technologies, Palo Alto, California, USA) was used as the HPLC system. The solvents ranged from 0.1% formic acid (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in Acetonitrile (Sigma-Aldrich, St. Louis, Missouri, USA) to 0.1% formic acid dissolved in water. Rosmarinic acid was detected as light absorption at 350nm, and its concentration was calculated on the base of peak area and calibration of the system with defined quantities of purchased rosmarinic acid (Sigma-Aldrich, St. Louis, Missouri, USA) as described in Chapter I.

### ***Estimation of bacterial density on *Z. marina*:***

The relative density of bacteria on the surface of eelgrass was estimated in each sampling. To do this four individual eelgrass leaves were randomly collected during each sampling, and transported to the lab in seawater in individual sterile tubes in a cooling container. In a sterile hood a disk with a diameter of 8 mm were excised from each leaf with a sterilized cork borer, to obtain leaf area of 2 cm<sup>2</sup> from each leaf. Attached bacteria were then swabbed from the disk surface with sterile cotton tips, which were soaked in 1ml of sterilized seawater in 2ml Eppendorf vials (Eppendorf-Elkay, Shrewsbury, MA, USA). The bacteria were suspended by vortexing for 30 s and 100 µl of the resulting suspension were transferred into 96 well plates (flat bottom, Greiner®). The bacteria suspended in each well were stained with Syto 9 (Invitrogen, Waltham, MA, USA) as described in Saha et al. (2011). The relative bacterial density was then determined with a plate reader as fluorescence intensity (Excitation wavelength: 477-491 nm, emission wavelength: 540 nm).

### ***Fouling pressure:***

To estimate the seasonal macrofouling pressure, HistoBond® adhesive glass slides (Marienfeld Lauda-Königshofen, Germany) which were fixed on plastic panels were deployed vertically and horizontally at 1m depth in the sampling points on Falckenstein beach monthly from May to Nov in 2015, collected after one month and transported to the lab in a cooler box in Falcon tubes containing seawater. Relative densities of bacteria on these slides were measured using the same methodology as described before, except that bacteria were swabbed from an area of 2 cm<sup>2</sup> on glass slides, which was marked with a sterile cork borer.

### ***Statistical analysis:***

The anti-bacterial-settlement activity of eelgrass surface extracts was calculated as a log effect ratio of bacterial strains settled in the presence of extracts and in presence of the solvent control (Saha et al. 2011). Therefore a value of 0 indicates no defense activity, while positive and negative values indicate attracting and repelling effects of surface extracts on bacterial settlers, respectively. The “general effect” of extracts was calculated as the mean of antisetlement activity against all four bacteria within each plot. Percentages of bacterial density on eelgrass relative to the bacterial density on PVC panels (fouling pressure) were calculated in order to evaluate the overall resistance of eelgrass to bacterial foulers.

Repeated measures ANOVA was used to analyze the monthly change of defense activities against bacteria, of Rosmarinic acid surface concentration and of densities of bacteria settled on *Z. marina* and on PVC panels. Shapiro-Wilk’s test and Levene’s test were used to test for normal distribution and homogeneity of variances ( $p < 0.05$ ), Tukey’s HSD test was used to compare the means of single groups *post hoc*. Simple linear regression analysis was used to analyze the relationships between defensive compounds, bacterial densities and the general antifouling defense activity ( $p < 0.05$ ). All before mentioned statistical analyses were conducted using the software Statistica 8.0 (StatSoft, Tulsa, OK, U.S.A.) Multiple regression models were developed with R studio (R studio. Inc, Boston, MA , U.S.A), using the STEP procedure implemented in R to analyze the synergistic effects of abiotic factors (including salinity, temperature, four different nutrients and light intensity) on the production of rosmarinic acid in *Z. marina* and the general defense activity ( $p < 0.05$ ).

## **Results**

### ***Fouling pressure, settled bacteria on eelgrass and the ratio***

The fouling pressure by potential bacterial settlers was in this experiment indicated by the abundance of bacteria on PVC panels that were exposed in the environment two weeks before each sampling. This fouling pressure exhibited a clear seasonal pattern (Figure 1A), with a significant peak in the mid of summer (July and August; Repeated measures ANOVA, Table 1A). A similar seasonal pattern was also detected in the abundance of bacteria that settled on eelgrass leaves; although this abundance was generally lower (Figure 1B). It increased from early spring to a peak in July, decreased steadily until October and increased non-significantly to November (Repeated measures ANOVA, Table 1B). Corresponding with the overall similarity among the seasonal patterns, fouling pressure and numbers of bacteria settled on eelgrass correlated significantly (Figure 1D) and the density of bacteria settled on eelgrass relative to the density on PVC panels varied relatively little, but tended to be lower when fouling pressure was relatively high in August (Fig. 1C, Table 1 C).

### ***Chemical defense:***

Four bacterial isolates were applied in anti-bacterial settlement inhibition bioassays, in order to quantify antifouling activity on eelgrass. The defense strength against all four bacteria was rather low in May and June (Figure 2). It increased – in three out of four cases significantly - from June to July and this was followed by non-significant decreases until November (Repeated measures ANOVA, Table 2A-D). The mean defense activity against all four isolates basically exhibited the

same pattern (Repeated measures ANOVA, Table 2E) and it was significantly lower in May than in July and August and significantly lower in June than in any other month (Figure 2E).

### ***Variation of compounds***

The mean surface concentrations of rosmarinic acid followed a similar seasonal changing pattern as fouling pressure, but differences among months were not significant (Figure 3, Repeated measures ANOVA, Table 3).

### ***Correlations***

#### *Correlations between bacterial fouling pressure and antifouling defense:*

Bacterial fouling pressure exhibited a significant correlation with the mean antifouling defense, which tended to be higher when the fouling pressure was also high (Linear regression,  $p < 0.05$ , Figure 4A, Table 4). In addition, bacterial fouling pressure was even stronger correlated with the defense activity against one of the four tested bacterial strains, *L. rosea*; (Figure 4C, Table 4). Bacterial fouling pressure was also correlated with the lagged mean antifouling defense that was observed 1 month after the fouling pressure (Figure 4B, Table 4), but with lower significance ( $p = 0.0376$  compared to  $p = 0.0256$ ). The antifouling activity against one of the four isolates, *P. dokdonensis* (Figure 4D, Table 4) was also significantly correlated with the fouling pressure in the previous month. As was to be expected, no significant correlations were detected between the defense activity strength and the density of bacteria on PVC panels one month later (data not shown).

### *Correlations between the bacterial density on eelgrass and antifouling defense*

Also the density of bacteria settled on eelgrass surfaces was significantly correlated with the mean defense activity against four bacterial isolates (Linear regression,  $p < 0.05$ , Figure 5A, Table 4) and with the defense activity against two individual bacterial isolates (*B. aquamaris* and *L. rosea*) (Figure 5C,E, Table 4). As with fouling pressure, a significant correlation between the density of bacteria settled on eelgrass and the mean defense activity one month later was also observed, but this lagged correlation was weaker ( $p = 0.0237$  compare to  $p = 0.0116$ , Figure 5A-B). The opposite phenomenon was observed with defense against the isolate *B. aquamaris* only, which exhibited a stronger correlation with the bacterial abundance in the preceding month ( $p = 0.01$ ) than in the current month ( $p = 0.047$ ), Figure 5C-D). In addition, the defense against *A. stellipolaris* was also significantly correlated with the density of bacteria that had settled in the preceding month (Figure 5F, Table 4). Similar as with the density of bacteria on PVC panels, the density of bacteria on eelgrass leaves was not significantly correlated with the antifouling defense activity in the preceding month (data not shown).

### *Correlations between fouling pressure and rosmarinic acid surface contents:*

The content of defensive compounds on the surface of eelgrass was correlated with fouling pressure in the same month and even stronger with fouling pressure in the preceding month (Figure 6, Table 4,  $p = 0.012$  compared to 0.016).

### *Correlations between abiotic factors and antifouling defense related variables*

Based on the Akaike Information Criterion (AIC) a multiple regression model including the four

factors nitrite concentration, nitrate concentration, temperature and ammonium concentration but not light, salinity and phosphate concentration was the least complex model that could predict the monthly variation of mean antissettlement defenses of *Z. marina*, although ammonium had no significant impact on the response ( $p < 0.05$ ; Table 5A). In contrast, temperature was identified as the only of the same seven factors that was needed to predict the monthly variation of rosmarinic acid in these extracts (Table 5B) and the surface concentration increased with increasing temperature (Figure 7).

Two different regression models were developed to predict the bacterial density on *Z. marina*. One of them included all abiotic environmental factors except temperature, but within this model only light, nitrate concentration and salinity had a significant impact on the density ( $p < 0.05$ , Table 5C). The second model included fouling pressure (determined as density of bacteria on PVC panels) and the mean antiouling activity of *Z. marina* surface extracts, but only the first of the two factors had a significant impact ( $p < 0.05$ ; Table 5D). Altogether, the model based on six abiotic factors was statistically more significant than the model based on two fouling-related factors ( $p = 0.0018$  vs  $0.0047$ ).

## **Discussion:**

A clear seasonal pattern was observed in almost all the variables that were measured in this study and indicators of microfouling as well as indicators of antimicrofouling defense were overall higher in summer than in winter and spring. For instance, the bacterial fouling pressure on *Z. marina* increased from May to a peak in August and dropped again afterwards. The reason could

be increased bacterial growth in summer, since higher temperatures usually accelerate metabolic activities (Rao 2010, Zaccone et al. 2014) and water temperature also reached its maximum in August. In addition, dissolved organic carbon resources released from macrophytes and feasible nitrogen resources are also usually higher in summer (Barron et al. 2003, Tyler et al. 2001, Tyler & McGlathery 2006), which typically leads to high cell numbers of bacteria in the marine environment and as a consequence in increased fouling pressure on eelgrass. This is not the first time that an increased fouling pressure in summer was observed in the Kiel Fjord, the same seasonal pattern was also described in Saha et al. (2011) and Rickert et al. (2016). The seasonal pattern of bacterial densities on the eelgrass surface was largely similar to that of the fouling pressure, but less variable and both patterns correlated significantly. However, in most months the mean number of settled bacteria was lower on eelgrass plants than on PVC panels, which confirms the existence of an antimicrofouling defense in *Z. marina* plants. Interestingly, the ratio of settled bacteria on eelgrass to settled bacteria on PVC panels varied (although non-significantly) with season and lower percentages of the available bacterial settlers settled on eelgrass in summer - when the potential bacterial foulers were most abundant - compared to other seasons. Similarly, the general defense activity of eelgrass surface extracts against four bacterial isolates indicated a stronger deterrent activity in summer.

However, the general defense activity exhibited significant correlations both with the bacterial fouling pressure and the number of bacteria that settled on eelgrass. A high defense activity strength was observed when bacterial settlement was high, suggesting that eelgrass adjusts its defense activity to the demand. In addition, high densities of bacteria on eelgrass and high

fouling pressure were also correlated with a relatively higher antisetlement activity in the following month, while the density of epiphytic bacteria was not correlated with the antisetlement activity in the preceding month. Thus, the intensity of bacterial settlement possibly influenced the defense activity in the following month, but not vice versa. Both algal macrophytes (Weinberger 2007) and terrestrial plants (Vera et al. 2011) are able to recognize bacterial pathogens through relatively unspecific defense elicitors that are typically constituents of the outer cell envelope of pathogenic and non-pathogenic microbes and that activate cellular immune responses when they are recognized. It is not known yet whether such an innate immune system also exists in *Z. marina*, but recent research demonstrated that the seagrass *Thalassia testudinum* is capable to recognize bacterial lipopolysaccharides (Loucks et al. 2013). Thus, a similar mechanism could also exist in *Z. marina* and then possibly explain the relation between fouling pressure and defense activity in this experiment. As indicated by the multiple regression shown in Tab. 5D the bacterial fouling pressure nonetheless has a stronger impact on the number of bacteria that settle on eelgrass than the defense activity, which probably explains the fact that we found no reduced bacterial density when the defense activity was relatively high.

Although rosmarinic acid was identified as the ecologically most relevant defense compound of eelgrass against microfoulers in our previous study (Chapter I) its surface concentration only correlated significantly with the defense activity against one out of four tested bacterial isolates (*Loktanella rosea*) in this experiment. Thus, the seasonal variation in the general deterrence of bacteria can obviously not be fully explained by seasonal changes of rosmarinic acid concentrations alone and other compounds in addition to rosmarinic acid were also active, which

confirms the findings described in chapters I and II. For instance, in Chapter II rosmarinic acid could also not explain the full variation of antifouling defense in a benthocosm experiment and in Chapter I we have isolated in addition to rosmarinic acid, flavonoids with deterrent activity from the surface of eelgrass, although their repellent effects were minor due to the low surface concentration.

In contrast, the surface concentration of rosmarinic acid was correlated with the fouling pressure and eelgrass tended to produce/excrete more rosmarinic acid to the surface when the fouling pressure increased. Such a relationship was also detected when the rosmarinic acid concentration was correlated with the fouling pressure in the preceding month, suggesting that eelgrass possibly adapted the surface concentration to the fouling pressure and this adaptation lasted for four weeks, perhaps in a similar manner as described above for the adaptation of the antimicrofouling defense. Our findings about the seasonality of rosmarinic acid on the surface of *Z. marina* seem not in agreement with a previous study by Ravn et al. (1994) that described the seasonality of rosmarinic acid in eelgrass tissue. In the mentioned earlier research, rosmarinic acid tissue concentrations were high in spring and low during summer and fall, while we found an opposite pattern of surface concentrations with (non-significantly) more rosmarinic acid in summer and autumn than in spring and late autumn. If we ignore the circumstance that Ravn et al. (1994) analyzed tissue samples that were dried at temperatures as high as 80°C - which is not appropriate for phenolic compounds because they become unstable at high temperature and can degrade (Palma et al. 2001) – the discrepancy could result from the fact that tissue and surface concentrations need not to follow the same dynamics. In fact, the tissue content of phenolic

compounds (including rosmarinic acid) in eelgrass is normally much higher than the surface concentration (Chapter I) and high surface concentrations could possibly come along with a relative reduction of tissue concentrations, given that all rosmarinic acid on the surface must be excreted from the tissue. However, simultaneous measurements of surface and tissue rosmarinic acid concentrations would be necessary to test this hypothesis.

Both the deterrent activity and the rosmarinic acid content of eelgrass surface extracts correlated not only with densities of bacterial settlers and/or the fouling pressure, but also with the water temperature (Table 5A and 5B) and both measures in tendency increased when the temperature increased (Fig. 7, not shown for the deterrent activity). Only the deterrent activity was in addition also affected by the availability of different forms of inorganic nitrogen (Table 5B) and it increased in tendency when  $\text{NO}_2$  concentrations were low,  $\text{NO}_3$  concentrations were intermediate or (non-significantly)  $\text{NH}_4$  concentrations were high (data not shown). Given that the different specimens of inorganic nitrogen exhibited very different trends their possible impact on the defensive activity was probably not related with the general supply of inorganic nitrogen and is for this reason difficult to explain. However, the fact that inorganic nitrogen supply only affected the general defense capacity and not the production of Rosmarinic acid suggests that it may have mainly influenced the defensive effect contributed by other compounds than rosmarinic acid.

Several ecological theories, such as the carbon-nitrogen balance hypothesis (Bryant et al. 1987) and the resource availability hypothesis (Coley 1985, Bazzaz 1987)) suggest that the production of carbon-rich secondary metabolites like rosmarinic acid is limited by the availability

of resources. According to these theories growth should dominate the carbon allocation in plants as long as the conditions are favorable for it. When growth is limited by certain factors, carbon and energy will increasingly be allocated to other biological processes, such as defense. Therefore, one might hypothesize that high temperatures in summer accelerated the production of phenolic compounds in summer because eelgrass growth was to some degree inhibited. Eelgrass in temperate areas can be reduced in summer (Lee et al. 2005) and negative effects of high temperature on the physiology of eelgrass have been demonstrated (Moore & Jarvis et al. 2008, Moore et al. 2012, Nejrup & Pedersen 2008). However, the weekly mean water temperatures in our study did not exceed 18 °C (Figure 7), while growth inhibition of Baltic Sea eelgrass was only observed when weekly mean temperatures exceeded 25 °C (Nejrup & Pedersen 2008). In this light, the observed accelerating effects of increased temperature in our study can probably not be regarded as a result of resource limitation due to temperature stress. Much rather, they were probably a consequence of the fact that fouling pressure increased during the warm season.

In conclusion, in 2015 the chemical antifouling defense of eelgrass underwent seasonal variation and peaked in summer, when the abundance of both real and potential bacterial foulers was the highest. Possibly *Z. marina* perceived the increased abundance of bacteria and reacted with an up-regulation of defense-related compounds. Other defensive compounds than rosmarinic acid may have been involved in the antifouling defense either individually or synergistically, because the seasonality of rosmarinic acid was not significantly correlated with the defense activity except for the activity against *L. rosea*. Besides of the biotic factors environmental factors such as nitrogen supply and in particular temperature also affected the chemical defense of

eelgrass plants and we can not decide with certainty whether this was a direct effect on eelgrass or an indirect effect through acceleration of the fouling pressure, but the fact that stressful temperature conditions were not observed during the experiment seems to exclude the possibility of stress-induced effects..

### **Acknowledgements**

This work was supported by the Chinese Scholarship Council (CSC) under grant number 201206330077 to Chi Guan. M. Saha acknowledges funding from the DFG excellence cluster “Future Ocean” under grant number CP1215.

### **Figure legends:**

Figure 1: Seasonal variations of A: bacterial fouling pressure (measured as bacterial density on PVC panels); B: bacterial density on *Z. marina*; C: bacterial density on *Z. marina* (shown in B) relative to bacterial fouling pressure (shown in A) and D: correlation between bacterial fouling pressure and bacterial density on *Z. marina* ( $r=0.8278$ ,  $p<0.0001$ ). In A-C mean $\pm$ 95% CI,  $n=4$ , different letters indicate months that are significantly different in a Tukey post hoc-test ( $p<0.05$ ).

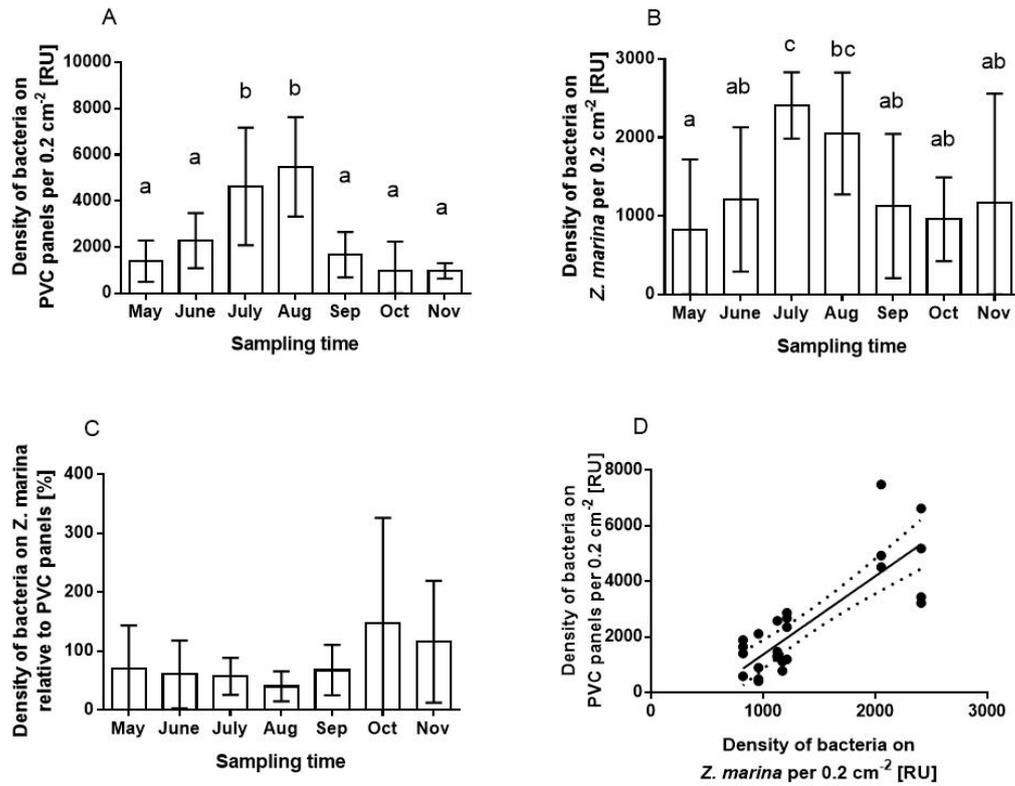


Figure 2: Seasonal variation of the antisettlement activity of propan-2-ol surface extract from *Z. marina* against A: *B. aquimaris*; B: *A. stellipolaris*; C: *L. rosea*; D: *P. dokdonensis* and E: all four bacteria (mean of A to D) (Mean±95%CI, n=4). Increasingly negative values indicate increasing activity strength. Months that are significantly different (Tukey *post hoc* test,  $p < 0.05$ ) are marked by different letters.

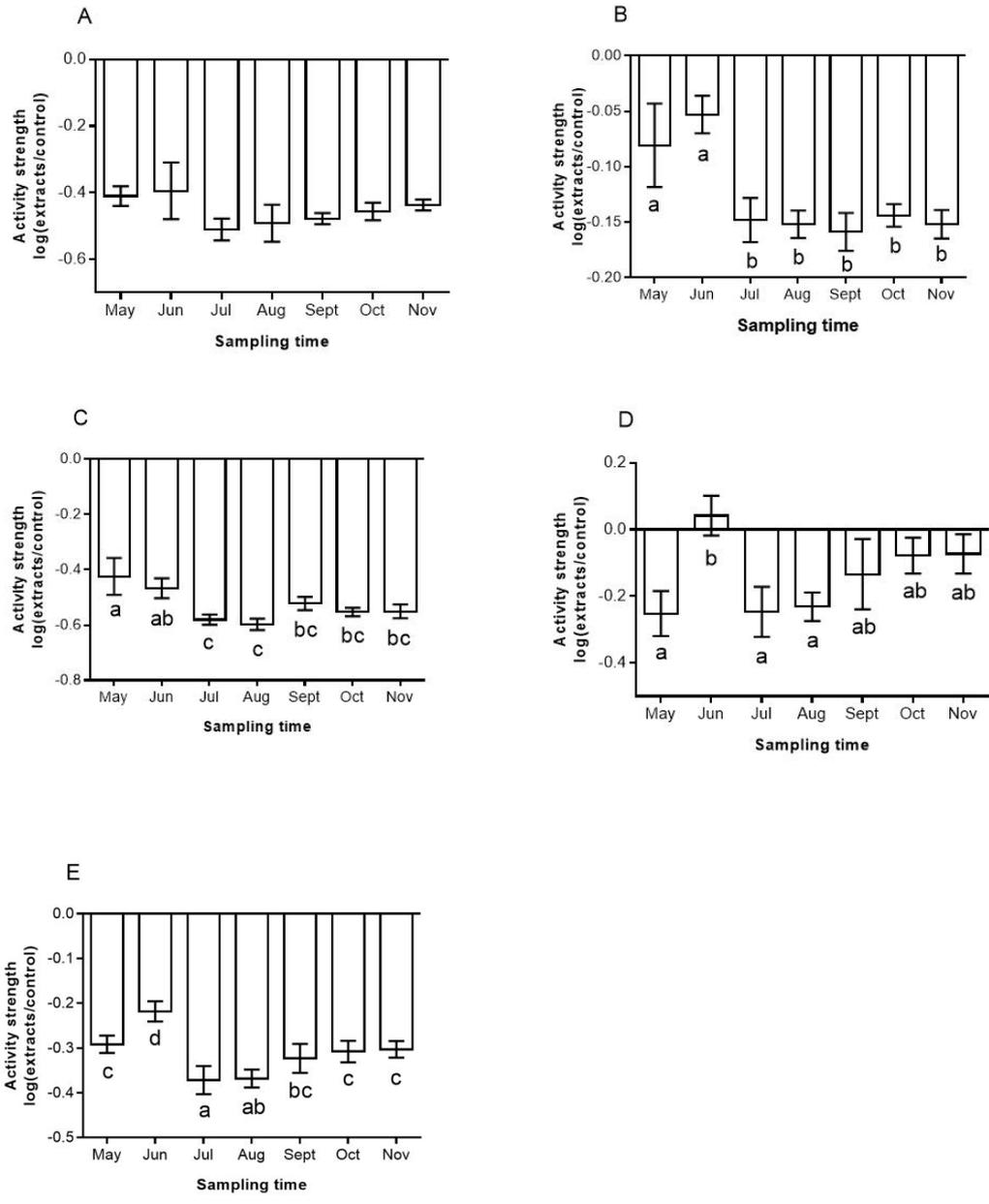


Figure 3: Seasonal variations of rosmarinic acid concentration on the surface of *Z. marina* (Mean±95%CI, n=4).

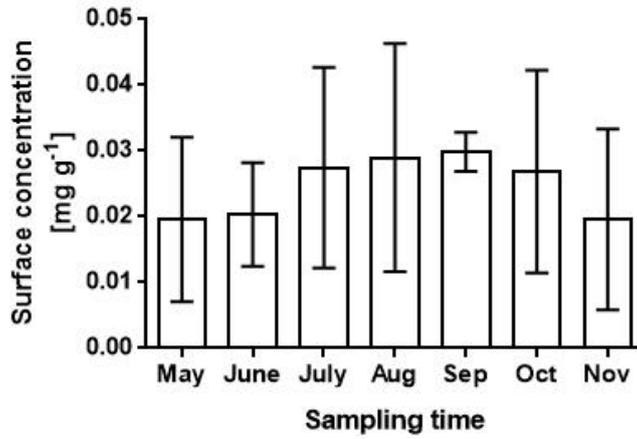


Figure 4: Pearson correlations of bacterial fouling pressure on *Z. marina* and (A) mean defense activity against 4 bacterial isolates; (B) lagged mean defense activity against 4 bacterial isolates; (C) defense activity against *L. rosea* and (D) lagged defense activity against *P. dokdonensis* of *Z. marina* surface extracts (Mean $\pm$ 95%CI, n=4).

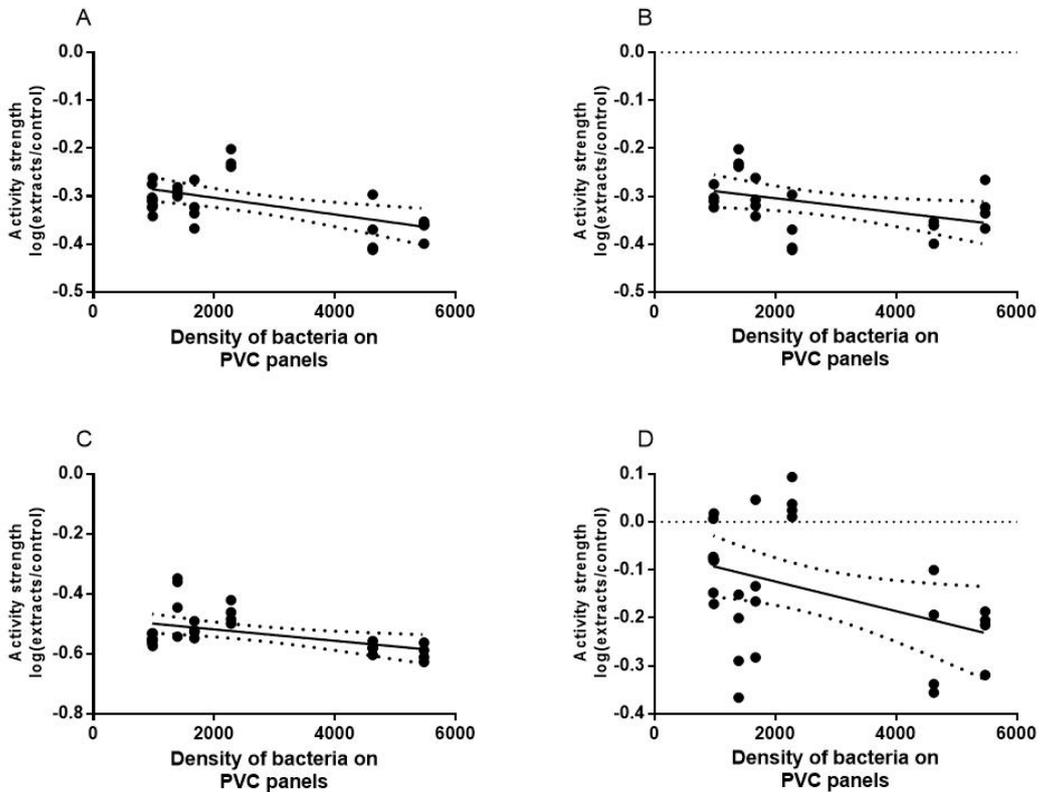


Figure 5: Pearson correlations of the abundance of bacteria settled on *Z. marina* and (A) general

defense activity; (B) general defense activity one month later; (C) defense activity against *B. aquamaris*; (D) defense activity against *B. aquamaris* one month later; (E) defense activity against *L. rosea* and (F) defense activity against *A. stellipolaris* one month later of *Z. marina* surface extracts (Mean $\pm$ 95% CI, n=4).

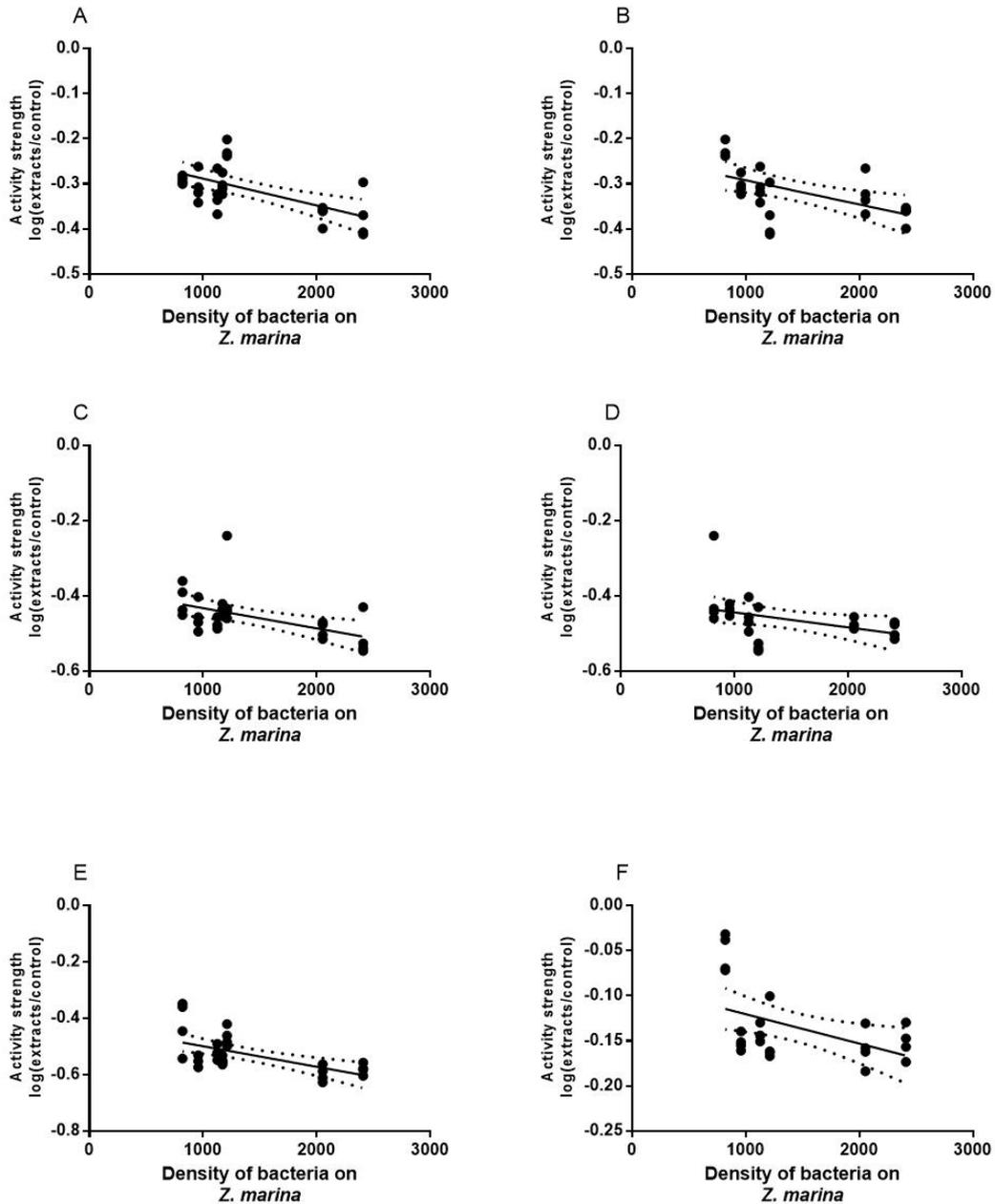


Figure 6: Pearson correlations of bacterial fouling pressure and (A) Rosmarinic acid surface concentration; (B) Rosmarinic acid surface concentration one month after quantification of the

fouling pressure (Mean $\pm$ 95%CI, n=4).

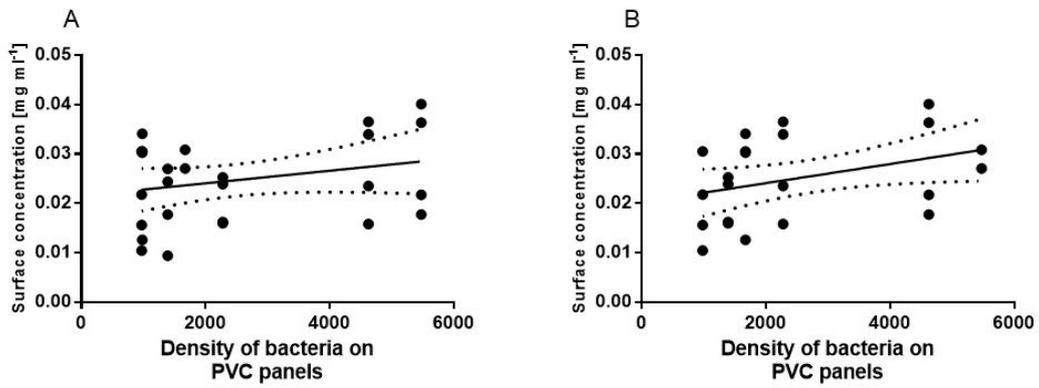
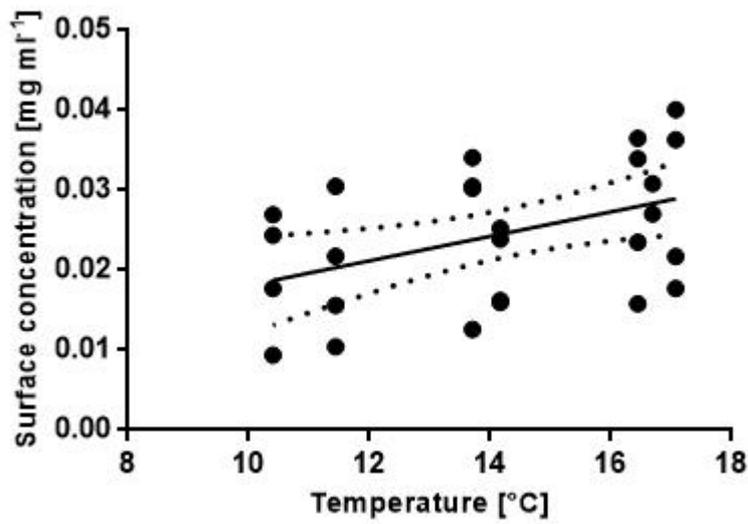


Figure 7: Pearson correlation of rosmarinic acid surface concentration and temperature (p=0.0159;

Dotted lines represent the 95%CI).



### Table legends:

Table 1: Repeated measures ANOVA of A: bacterial fouling pressure (measured as bacterial

density on PVC substrate; see Fig. 1A); B: bacterial density on *Z. marina* (see Fig. 1B); C: bacterial density on *Z. marina* relative to bacterial fouling pressure (see Fig. 1C) in different months.

		SS	DF	MS	F	P
A	Intercept	172931151	1	172931151	81.2247	0.0029
	Error	6387136	3	2129045		
	Month	79883772	6	13313962	19.0246	<0.0001
	Error	12596887	18	699827		
B	Intercept	54269905	1	54269905	79.1181	0.0030
	Error	2057806	3	685935		
	Month	8559980	6	1426663	5.7564	0.0017
	Error	4461122	18	247840		
C	Intercept	177998.3	1	177998.3	46.36561	0.0065
	Error	11517.0	3	3839.0		
	Month	34337.6	6	5722.9	1.9249	0.1316
	Error	53516.4	18	2973.1		

Table 2: Repeated measures ANOVA of chemical defense against A: *B. aquamaris*; B: *A. stellipolaris*; C: *L. rosea*; D: *P. dokdonensis*. E: the mean of four different bacteria (mean of A to D) of *Z. marina*. Within-measures factor was the month of sampling.

		SS	DF	MS	F	P
A	Intercept	5.7717	1	5.7717	4604.625	<0.0001
	Error	0.0038	3	0.0013		
	Month	0.0441	6	0.0073	2.674	0.0492
	Error	0.0494	18	0.0027		
B	Intercept	0.4503	1	0.4503	2517.795	<0.0001
	Error	0.0005	3	0.0002		
	Month	0.0425	6	0.0071	13.065	<0.0001
	Error	0.0098	18	0.0005		
C	Intercept	7.8046	1	7.8046	7108.191	<0.0001
	Error	0.0033	3	0.0011		
	Month	0.0933	6	0.0155	8.928	0.0001
	Error	0.0313	18	0.0017		
D	Intercept	0.5419	1	0.5419	68.5033	0.0037
	Error	0.0237	3	0.0079		
	Month	0.2952	6	0.0492	5.9727	0.0014
	Error	0.1483	18	0.0082		
E	Intercept	8.1757	1	8.1757	5435.142	<0.0001
	Error	0.9169	3	0.3056		

Month	0.1941	6	0.0323	21.507	<0.0001
Error	0.1158	77	0.0015		

Table 3: Repeated measures ANOVA of rosmarinic acid concentrations on *Z. marina* surfaces.

Within-measures factor was the month of sampling.

	SS	DF	MS	F	P
Intercept	0.0171	1	0.0171	118.5662	0.0017
Error	0.0004	3	0.0001		
Month	0.0005	6	<0.0001	1.5810	0.2097
Error	0.0010	18	<0.0001		

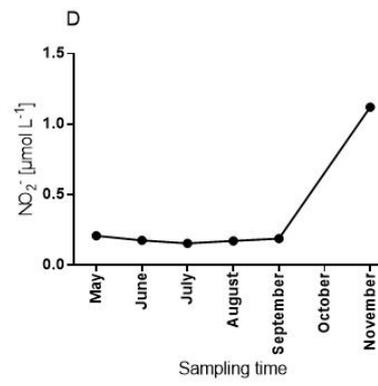
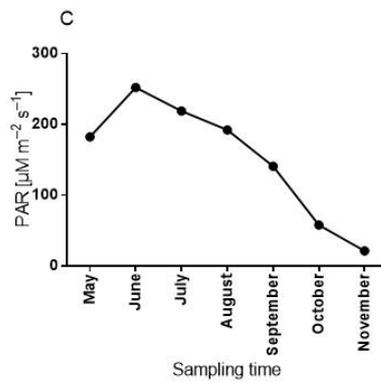
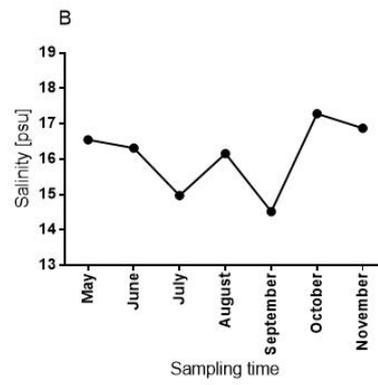
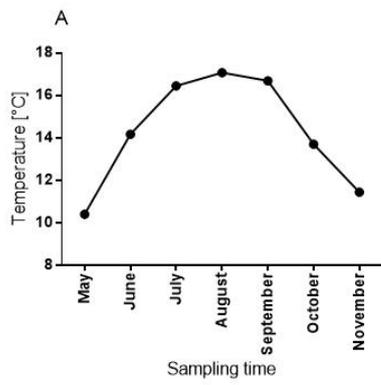
Table 4: Statistical results of Pearson correlations of Bacterial densities on *Z. marina* (BD) and on PVC plates (FP) with the defense activities of *Z. marina* surface extracts against four different bacterial isolates, the mean of these activities and the Romarinic acid concentration (RA) in these extracts. In (A), bacterial densities were correlated with activities of extracts sampled in the same month. In (B), bacterial densities were correlated with activities in extracts sampled one month later (= lagged measures).

A.	Bacteria Density (BD)		Fouling Pressure (FP)		RA contents (RA)	
	R	p-value	R	p-value	R	p-value
<i>B. aquamaris</i>	-0.3784	<b>0.0471</b>	-0.3179	0.0992	-0.2565	0.1877
<i>A. stellipolaris</i>	-0.3209	0.0959	-0.1413	0.4731	-0.328	0.0879
<i>L. rosea</i>	-0.4174	<b>0.0271</b>	-0.4407	<b>0.0189</b>	-0.43	<b>0.0224</b>
<i>P. dokdonensis</i>	-0.308	0.1108	-0.2995	0.1215	-0.0568	0.7742
General effects	-0.4702	<b>0.0116</b>	-0.4211	<b>0.0256</b>	-0.2967	0.1252
	BD to RA		FP to RA			
	0.1758	0.3709	0.4509	<b>0.016</b>		
B.	Bacteria Density (BD)		Fouling Pressure (FP)			
	R	p-value	R	p-value		
<i>B. aquamaris</i>	-0.5153	<b>0.01</b>	-0.3664	0.0782		
<i>A. stellipolaris</i>	-0.448	<b>0.0281</b>	-0.3869	0.0618		
<i>L. rosea</i>	-0.2703	0.2014	-0.2284	0.283		
<i>P. dokdonensis</i>	-0.3771	0.0693	-0.4194	<b>0.0413</b>		
General effects	-0.46	<b>0.0237</b>	-0.426614	<b>0.0376</b>		
	FP to lagged BD		BD to lagged RA			
	0.8596	<b>0.0381</b>	0.1514	0.3021		
	RA to lagged BD		FP to lagged RA			
	-0.3974	0.0545	0.5	<b>0.013</b>		

Table 5: Multiple linear regression models to explain (A) the general antisetlement activity of *Z. marina* surface extracts in different months, (B) the rosmarinic acid content in these extracts and (C, D) the bacterial density on *Z. marina* leafs in different months. The models were selected with the STEP procedure implemented in R. They are the least complex models without loss of information (based on the Akaike Information Criterion (AIC)) that could be derived from initial models containing seven different abiotic factors in (A) to (C) and the two factors fouling pressure and mean antisetlement activity strength in (D).

A		Df	Sum Sq	Mean Sq	F value	P
	NH4	1	0.003564	0.003564	3.2467	0.0875
	NO3	1	0.036928	0.036928	33.6427	<b>0.0001</b>
	Temp	1	0.014374	0.014374	13.0951	<b>0.0018</b>
	NO2	1	0.006761	0.006761	6.1591	<b>0.0226</b>
	Residuals	19	0.020856	0.001098		
B		Df	Sum Sq	Mean Sq	F value	P
	Temperature	1	0.0004	0.0004	7.5427	<b>0.0118</b>
	Residuals	22	0.0012	0.0001		
C		Df	Sum Sq	Mean Sq	F value	P
	PO4	1	340743	340743	1.2886	0.2721
	Salinity	1	1344377	1344377	5.0841	<b>0.0376</b>
	Light	1	4942563	4942563	18.6916	<b>0.0005</b>
	NO2	1	828775	828775	3.1342	0.0946
	NH4	1	223201	223201	0.8441	0.3711
	NO3	1	1686026	1686026	6.3762	<b>0.0218</b>
	Residuals	17	4495253			
D		Df	Sum Sq	Mean Sq	F value	P
	FP	1	4800251	4800251	12.1066	<b>0.0022</b>
	GE	1	734242	734242	1.8518	0.188
	Residuals	21	8326443	396497		

## Supplements:



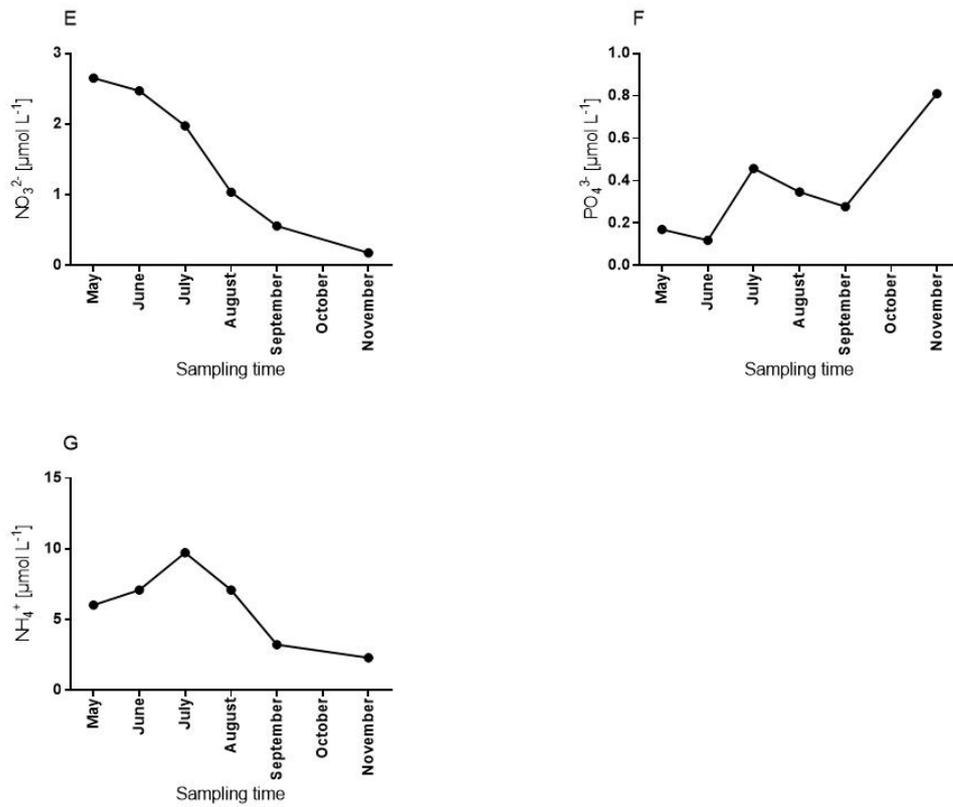


Figure S1. Seasonal change of A. Temperature; B. Salinity; C. Photosynthetically active radiation (PAR); D.  $\text{NO}_2^-$ , E.  $\text{NO}_3^{2-}$ , F.  $\text{PO}_4^{3-}$ ; G.  $\text{NH}_4^+$  in Falckenstein Kiel. In A. to C. the presented data are means of the week before eelgrass sampling, in D. to G. the data are for samples that were collected on the sampling day.

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## **General Discussion:**

### ***Chemical antifouling defense of eelgrass***

In Chapter I, I demonstrated the chemical defense of *Z. marina* against bacterial foulers: Surface extracts of eelgrass repelled bacteria from the settlement on their surface. The bacterial settlement on living substratum is the first stage of the formation of a biofilm and the capacity to inhibit bacterial settlement to some degree represents the antifouling defense capacity of eelgrass. In this chapter I proved that eelgrass surface compounds rather repel than kill bacterial foulers, since bacterial growth was unaffected by these compounds at different concentrations. Rosmarinic acid and two sulphated flavonoids were subsequently separated from the extracts and their antifouling activity was proven (Chapter I). The non-toxic antifouling properties of these compounds could be interesting for practical applications, including antifouling strategies in the shipping industry.

In order to cope with heavy fouling, marine plants have evolved various defense systems, in which highly diverse secondary metabolites are produced and excreted to the surface to reduce the attachment of bacteria (Treutter 2006, Lattanzio et al. 2006). Several theories tried to explain the non-toxic antifouling defense of marine plants, and some of them are related to bacterial quorum sensing (Dobretsov et al. 2009). Bacterial quorum sensing (QS) is a mechanism of cell-cell communication and gene regulation through chemicals (autoinducers), which allows bacteria to coordinate their behavior as a system (Melissa et al. 2001). The phenomenon was first described in two marine luminous bacterial species *Vibrio fischeri* and *Vibrio harveyi* (Eberhard et al. 1981, Neilson & Hastings 1979) and later widely discovered in terrestrial and marine bacterial

species (Bauer et al. 2005, Waters & Bassler 2005). Autoinducers mediate the communication among bacterial cells and regulation their gene expression, which is essential for the formation and maturation of biofilms. For instance, the regulators enable pathogenic bacteria to maintain a low level of virulence gene expression during the first stage of biofilm development and therefore to avoid to trigger a host defense (Rice et al. 2005). The bacterium *P. aeruginosa* terminates biofilm formation at the micro-colony stage if the LasI-dependent HSL autoinducer is inhibited (Davies et al. 1998). Marine organisms - including marine plants - have developed approaches to interfere with and block bacterial quorum sensing signals, in order to control the growth and development of bacterial communities (Zhang & Dong 2004, Skindersoe et al. 2008). Halogenated furanones isolated from the red alga *Delisea pulchra* are the potential inhibitors of quorum sensing-regulated behaviors (including virulence gene expression, bacterial motility, biofilm formation etc.) in Gram-negative bacteria (Steinberg & De Nys 2002). Those furonones bind to the autoinducer receptors of bacterial envelopes to prevent the correct folding of a protein, which disrupts the signal mediated regulation (Koch et al. 2005). In several bacteria phenolic compounds cause structural changes of receptor kinase and they thereby inhibit quorum sensing regulation (Stephenson et al. 2000). In addition to their relation with QS-regulation, phenolic compounds were also demonstrated to be functional in other ways. For instance, a flavanone isolated from the plant *Sophora exigua* was believed to reduce the fluidity of the bacterial membrane (Tsuchiya & Inuma 2000) and the antimicrobial activity of three phenolic compounds separated from the roots of *Clycyrrhiza inflata* was due to an inhibitory effect on the oxidation of NADH in bacterial membrane preparations and to further inhibition of the respiratory electron transport chain

(Haraguchi et al. 1998). However, the exact mechanisms behind the antibacterial effects observed in the present study could only be revealed by further comprehensive research.

### ***Phenolic compounds on eelgrass:***

Not all bioactive compounds that are present in eelgrass crude extracts were necessarily detected in the present study, since combined metabolites could have synergistic effects on the plant defense capacity that may not be observed when separated compounds are tested at natural concentration. Other compounds that were previously discovered in eelgrass - including zosteric acid, caffeic acid and other non-phenolic compounds - could also be relevant for the antifouling defense (Enerstvedt et al. 2016). For instance, Zosteric acid was repeatedly proven to fend off fouling organisms in a non-toxic way, but not detected in the present work (Hany et al. 2004, Haslbeck et al 1996, Newby et al. 2006). However, possibly Zosteric acid was present at other seasons of the year, when the material for chapter I was not collected. This could be an explanation why the seasonality of rosmarinic acid surface concentrations on eelgrass was not correlated with antifouling defense activities in chapters II and III.

Although there are possibilities that non-phenolic compounds exist and participated in the antifouling defense of eelgrass, phenolic compounds and especially rosmarinic acid (RA) were the most dominant defense compounds on eelgrass in our research. Phenolic compounds exist widely distributed both in the land and marine plant kingdoms (Balasundram et al. 2006) and their antiviral (Hu et al. 2013), antibacterial (Rauha et al. 2000, Pereira et al. 2007), antigrazing (Boeckler et al. 2011, Cipollini et al. 2008) and antioxidant (Rice-Evans et al. 1997) effects have

often been demonstrated. The biosynthesis of phenolic compounds in seagrass was not studied in detail yet, but we can infer it from its terrestrial counterparts. For instance, the synthesis of RA in terrestrial plants originates from the two amino acids precursors (L-Phenylalanine and L-Tyrosine). The two aromatic amino acids are transferred into two intermediate precursors (t-coumaroyl-CoA and 4-Hydroxyphenyllactic acid, respectively) after several enzymatic steps and then coupled by ester formation to construct the main skeleton of rosmarinic acid (Figure 2). The ester-forming process is specifically catalyzed by rosmarinic acid synthase (RAS) (Petersen & Simmonds 2003). The biosynthesis of RA is a branch of the general phenylpropanoid pathway and at most four enzymes are specific among all 8 enzymes that are involved (Petersen et al. 2009). Intermediary precursors that are also derived from L-phenylalanine, such as 4-Coumaric acid and 4-Coumaroyl-CoA, participate in the biosynthesis of other phenolic compounds, including flavonoids, coumarins etc. (Petersen & Simmonds 2003) (Figure 2). Thus, the production of those phenolic compounds overlaps with the basic metabolism of plants and there are studies that hypothesize that one of the basic functions of phenolic compounds within plants is carbon and energy storage if nutrients - especially nitrogen – are in shortage (Buchsbaum et al. 1990, Mattson et al. 2005, Goecker et al. 2005).

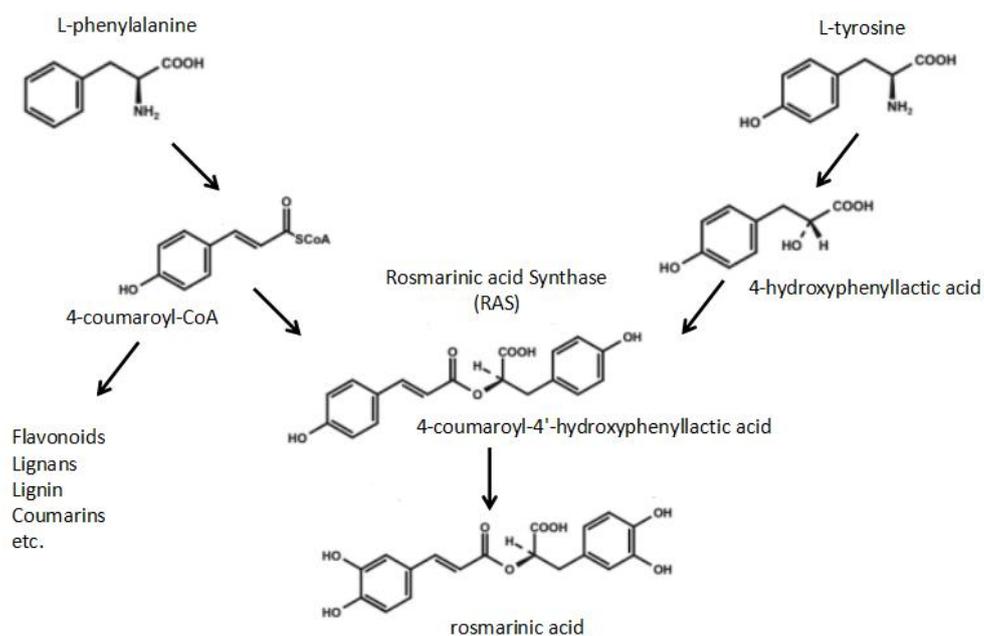


Figure2. Brief biosynthetic pathways of Rosmarinic acid in plants. Detailed pathway see the description of Petersen et al. in 2009.

A few studies directly describe mechanisms that explain certain biological activities of RA and of some other phenolic compounds that have a similar structure, such as quercetin and apigenin. For instance, RA was proven to have antioxidant activity and to scavenge Reactive Oxygen Species (ROS) from cells (Osakabe et al. 2004, Huang & Zheng 2005) and quercetin reduces the energy flow on bacterial membranes through inhibition of ATPase activity and it hence reduces the bacterial mobility (Wu et al. 2008, Mirzoeva et al. 1997). Such properties were suggested by the authors to enable RA to repel bacterial adhesion on surfaces and to prevent the formation of biofilms on terrestrial plants, and they could also be the mechanisms behind antifouling defense effects in my research.

For the defense against bacterial settlers the content of defense compounds on surfaces is ecologically more relevant than their content in tissues. In Chapter I, I compared their content in

eelgrass tissue and in eelgrass surface extracts for the first time and the results suggest that tissue concentrations are on average ten times higher than in surface extracts. Therefore, compounds such as sulfated flavonoids would be functional at the tissue concentration, but they would perform not or less at the surface concentration. Therefore we can conclude that surface extracts are more suitable than tissue extracts for the study of antifouling defenses. The biosynthesis of phenolic compounds occurs in tissue cell, while these chemicals must be excreted onto the tissue surface to perform their defense activity (Pohnert 2004). The cellular mechanisms behind the excretion process are not clear yet.

### ***Regulation of antifouling defense:***

Secondary metabolites are the basis of a chemical defense in various marine plants (Rosenthal & Berenbaum 2012, Schmitt et al. 1995) and they are normally by-products of primary biological processes, such as photosynthesis, respiration and amino acid metabolism. Therefore, abiotic and biotic environmental factors often influence their production (Akula et al. 2011, Sudatti et al. 2011, Løvdal et al. 2010) and correspondingly regulate chemical defense (Sudatti et al. 2011, DeLucia et al. 2012). Marine plants have often developed complex and sophisticated chemical defense systems, in which various defense compounds work either individually or synergistically to cope with highly-diverse epiphytic communities (Goecke et al. 2010, Jormalainen & Honkanen 2008). Various chemicals - including terpenoids and steroids (White et al. 2008, Tsoukatou et al. 2007), fatty acids (Baze et al. 2009, Bhattarai et al. 2007), amino acids (Diers et al. 2004, Ortlepp et al. 2007) and alkaloids (Diers et al. 2006, Nakamura et al. 1982, Dahlström & Elwing 2006) -

have been identified as compounds that are bioactive against marine fouling organisms. The biosynthesis of such defensive metabolites is usually costly in terms of metabolic resources and energy and plants have to allocate the necessary resources to the defense system at the cost of other processes, such as growth (Huot et al. 2014). Therefore it is often beneficial if the defensive level does not constantly remain high and if the defense strength is adjusted when it is necessary. For instance, the content of three defense related compounds of the brown seaweed *Fucus vesiculosus* was up or down-regulated when the alga was exposed to different levels of temperature and light conditions (Saha et al. 2014) and also the antigrazing chemical defense of the brown seaweed *Lessonia nigrescens* was demonstrated to decrease under severe light limitation (Pansch et al. 2009).

The regulatory mechanisms of chemical defenses are much better known in terrestrial plants than in their aquatic counterparts. Chemical energy saved in the form of carbohydrates must be fixed by photosynthesis before it can be applied in either growth or defense and the choice of plants is largely influenced by environmental stresses (Huot et al. 2014). The concentration of some small plant molecules that act as defense hormones - such as salicylic acid and jasmonic acid - typically changes when plants sense environmental changes. Accumulation of those molecules transmits the signal to downstream levels, which in the end results in an up-regulation of the defense activity (Erb et al. 2012, Wasternack 2014). However, the mechanisms of defense (and especially antifouling) regulation in seagrasses still remains unexplored.

The work presented in this dissertation provides evidence that antifouling defense of *Z.*

*marina* is subject to regulation, both under artificial and natural living conditions. In chapter II, I incubated *Z. marina* in the Kiel Outdoor Benthocosm system with and without heat wave treatments that were characterized by temperature increases of up to 5 °C for 1 week during incubation. A significant difference of the chemical defense activity was observed between eelgrass plants that were and were not exposed to heat waves, which indicated that the plants tends to develop a stronger defense capacity on their surface when they encounter heat stress. However, the increase after heat stress is relatively moderate compared to the response to seasonal change. Most of the variables that were traced both in cultivated (Chapter II) and natural living conditions (Chapter III) exhibited clear seasonal changing patterns. For example, the bacterial fouling pressure both in the Kiel Outdoor Benthocosms and in natural stands in the Baltic Sea remained relatively low in spring and fall, but peaked in summer. The significant correlation between fouling pressure and bacterial abundance on eelgrass suggests that the seasonality of bacteria that settle on eelgrass surfaces may be driven by the variation of bacterial fouling pressures in the environment. The seasonal change of fouling pressure is related to temperature and to bacterial traits that are relevant for their settlement (Zaccone et al. 2014). The growth and development of bacterial foulers increased, because of the synergistic effects of environmental parameters in summer (Rao et al. 2010, Zaccone et al. 2014). High temperature probably accelerated metabolic activities and the high content of dissolved carbon and nitrogen resources that was released from macrophytes in summer probably also allowed the bacterial density to increase (Barron et al. 2003, Tyler & McGlathery 2006), which in sum lead to a relatively high fouling pressure on eelgrass.

At the same time, a similar seasonal change in the defense activity was also observed in

the two experiments. This suggests that eelgrass has a capacity to adapt its defense behavior to the seasonal variation of fouling pressures. Apparently their defense capacity is regulated based on the demand in a specific situation. For instance, the defense activity of eelgrass incubated in the Kiel Outdoor Benthocosms was the highest in July, when fouling pressure reached a peak (Chapter II) and potential bacterial foulers in the Kiel Fjord were most abundant in July and August (2015), when at the same time antifouling defense activity also reached its maximum during these two months (Chapter III). Plants are usually able to perceive the existence of pathogens and to adapt their defense accordingly (Huot et al. 2014). The innate immune system, which is the basis of this perception, was first explored in terrestrial plants and recognizes certain highly conserved molecular patterns that indicate the presence of pathogens (Boller & Felix 2009, Farnell et al. 2003, Remer et al. 2003). These patterns - that are not only present on pathogens but also on the surfaces of other microbes – are perceived by membrane associated pattern recognition receptors of plants (PRPs) (Remers et al. 2003, Monaghan & Zipfel 2012) that trigger cellular adjustments - such as an up-regulation of reactive oxygen species, calcium influx, activation of protein kinases etc. – that result in an elimination of microbes from the plant surface (Boller & Felix 2009, Dodds & Rathjen 2010). Similar mechanisms involving recognition by receptors and subsequent induced defense responses occur also in some brown and red macroalgal species (Cosse et al. 2009). So far it is not clear whether such mechanisms also exist in *Z. marina*, but the seagrass *Thalassia testudinum* was proven to be capable to recognize bacterial lipopolysaccharides (Loucks et al. 2013). In this light it is not impossible that eelgrass has also developed receptors that mediate innate immunity and can activate defensive mechanisms against microbial fouling, which would

explain the observed correlations of antifouling defense activity with both fouling pressure and eelgrass-associated bacteria (Chapter III).

Rosmarinic acid, the most abundant and ecologically relevant compound on the eelgrass surface, was used to monitor the fluctuation of defenses under different environmental conditions (Chapter II, III). The surface concentration of RA was not altered by heat treatments, and it was not particularly well correlated with the general defense activity during three months of incubation (Chapter II). Moreover, the seasonality of RA surface concentrations was not corresponding to the seasonal variation of defense activities of eelgrass in the Kiel Fjord (Chapter III). This supports our previous conclusion that RA can not be the only compound that deters microsettlers from the surface of *Z. marina* (Chapter I). Individual or combined effects of other compounds - such as sulphated flavonoids or zosteric acid - in surface extracts could also play a role in the seasonal adaptation of eelgrass defenses to biotic and abiotic factors. Unlike the physical defense, chemical antifouling defense only selectively repels certain species or groups of bacteria. This is indicated by findings from Chapter I, where I demonstrated that surface extracts of eelgrass were not able to indiscriminately deter all tested bacterial species from settlement. Instead, bacteria isolated directly from eelgrass leaves were not or less repelled by eelgrass surface extracts than bacteria originating from stones.

Another interesting phenomenon is the circumstance that temperature-defense regulation in eelgrass plants seems to be accelerated by environmental stress. The up-regulation of the chemical defense after heatwaves was less pronounced when the heat treatments were repeated (Chapter II). The reason could be that the accumulation of defensive compounds after the first

stimulation slowed down when more stimulations occurred. Such observations have been made in land plants (Jump & Penuelas 2005, Davis & Shaw 2001). For instance, the content of defense regulation signals - such as Jasmonic acid (JA) and Salicylic acid (SA) - was enhanced when plants were exposed to high temperature (DeLucia et al. 2012) and antioxidant flavonoids and pathogen-related defense compounds that are under regulation of either SA or JA were induced subsequently (Djanaguiraman et al. 2011). The increase of defense activity in response to both herbivores and pathogens is usually less rapid when the plant hormones have reached certain levels (Maimbo et al. 2007). Although SA and JA were demonstrated to be elicitors of chemical defenses in many terrestrial plants (Arimura et al. 2009, Thaler et al. 1999), they are less likely to participate in the same way in defense regulation in seagrass. None of the two compounds was so far discovered in seagrass and application of the two chemicals in a natural environment of seagrass failed to trigger an up-regulation of defense related compounds (Steele et al. 2005). However, the observations made in my experiments suggest that induction and regulation systems exist in eelgrass, although more work at the molecular level will be necessary to make identify them.

In conclusion, the chemical antifouling defense of *Z. marina* was proven in this project, Rosmarinic acid is the main compound, but not the unique compound that is related in the defense. The compounds that are produced in the tissue of eelgrass and excreted onto the surface to selectively repel bacteria from settlement. Two flavonoids and zosteric acid were also identified in eelgrass surface extracts during the fractionation process in this project and the existence . However the defense activity of combined fractions was not stronger, but reduced compared to the

defense activity of crude extracts when both were tested at the same concentration. This suggests that other important defense related compounds were not present in the fractions. Given that propan-2-ol was used to extract compounds from the eelgrass surface in this project, I could not collect compounds with high polarity. However, a possible function of such highly polar compounds in antifouling of eelgrass is not impossible. Therefore an extraction method should be developed in the future that allows it to obtain the full spectrum of compounds on the eelgrass surface. This could allow for the identification of the full spectrum of all relevant compounds, which would be essential for the subsequent analysis of the interplay of different compounds during defense induction and regulation. The circumstance that high temperature stimulates antifouling defense demonstrates that other abiotic factors influence the defense activity of eelgrass. Both the defense activity and RA surface concentrations exhibited a seasonal changing pattern under natural living conditions, which was also affected by temperature. Besides of abiotic factors, the change of antifouling defense in eelgrass were also correlated with bacterial fouling pressure in the living environment, which suggests that an induced defense toward bacterial foulers may exist.

Given that this project started from the idea that ecologically relevant compounds should be investigated I nearly only monitored the content of defensive chemicals on the eelgrass surface. A comparative study of surface and tissue concentrations in response to environmental triggers could be more informative in the future. Such an approach could help to understand not only the ecological effects and cause, but also the regulation and excretion processes. Last, but not least, my work provides first evidence of an inducible antifouling defense in eelgrass. However, more

work on the molecular and metabolic level would be needed in the future to really demonstrate this, for example through the identification of relevant receptor systems in eelgrass epidermal cells that can regulate an upregulation of the defense metabolites..

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## **Erklärung**

Hiermit erkläre ich, dass ich die vorgelegte Dissertation mit dem Titel '*Zostera marina* and epibionts: the dynamics of chemical defenses.' selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt und habe die Dissertation nach den Regeln guter wissenschaftlicher Praxis verfasst.

Kiel, den \_\_\_\_\_