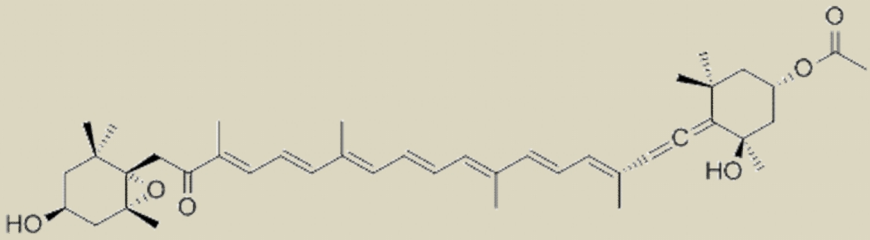
ANTI-SETTLEMENT DEFENCE OF *FUCUS VESICULOSUS*
Chemistry and Ecology



Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftelichen Fakultät
der Christian-Albrechts-Universität zu Kiel

Vorgelegt von
Mahasweta Saha
Kiel 2011

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Der Dekan

Dedicated to my fiancé Arijit

Contents

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Summary

Bacteria are omnipresent in the marine environment. For example, in the Kiel Fjord (Baltic Sea) 0.7 to 2.24 x 10^6 bacterial cells are present in one ml of seawater. Marine organisms are thus continuously exposed to high densities of bacteria, some of which tend to settle and colonise living surfaces. Some associations between host and bacteria could be neutral or mutualistic, for example algae associated bacteria may protect their host from fouling. But mostly, bacterial epibionts have a number of negative impacts on their hosts, such as providing positive settlement cues to macrofoulers. Although macroalgae constitute a potential substratum for surface colonisation, many of them remain largely free from heavy fouling, presumably due to surface properties or release of antifouling chemicals. In the Baltic Sea this has also been observed in the ecologically important alga *Fucus vesiculosus* (Phaeophyceae), which remains free from heavy fouling during most of the year. So far there was no indication of a mechanical or structural defence against epibionts in this alga, which suggested that one or several chemical defence mechanisms may exist.

The chemical defence of macroalgae has been a theme of literature over two decades. But so far, most of these effects have been demonstrated in studies investigating total tissue extracts instead of testing ecologically relevant surface extracts or surface metabolites at natural concentrations, which actually affect the fouling organisms in nature. Further, only very few among the studies which so far tested surface based compounds at ecologically relevant concentrations targeted the first phase of fouling, i.e. bacterial settlement. It was a main aim of my work to study the effect of surface-associated metabolites on microbial settlement, using the rockweed *Fucus vesiculosus* as a model organism.

Since the control of bacterial fouling on macroalgae is of substantial ecological importance, it is highly relevant to characterize the nature and dynamics of potential bacterial settlement inhibitors - the first line of defensive compounds against microfoulers. In *F. vesiculosus*, polyphenols have been suggested as fouling deterrents. However, a bioassay-guided structure elucidation of the alga's metabolites deployed in defence against bacterial settlement has been realized for the first time in my study, and a role of polyphenols in antimicrobial defence of *F. vesiculosus* was not detected. Using different chromatographic techniques followed by bioassays, I was instead able to isolate and identify an active lipophilic metabolite: fucoxanthin (Chapter I). Subsequently, I proved its surface presence. This was followed by an investigation of the metabolic provenance through segregation of fucoxanthin originating from *Fucus* and fucoxanthin originating from surface associated diatoms. Further

Summary

I quantified the compound on algal tips and on whole individuals, in order to know whether it is sufficiently concentrated on the algal surface to warrant an inhibitory effect on bacterial settlers. With the help of this comprehensive study, I was able to report a novel defence strategy of *Fucus*. In chapter II, I proceeded to investigate the active polar metabolites on *Fucus* surfaces and reported DMSP and proline to be the hydrophilic metabolites contributing to an inhibition of bacterial settlement. This was the first study reporting the role of DMSP and of the amino acid proline as algal antifoulants. The combined results of chapter I and II provides a picture of multiple chemical defence strategies of *Fucus vesiculosus* in an ecological context. In chapter III, I show how the algal defence based on these inhibitors may be affected by environmental factors/ shifts such as high temperature and low light. Based on the surface concentrations of the three settlement inhibitors the defence capacity of *F. vesiculosus* appears to be only moderately affected by potential stressors. Finally, I report on the seasonal and geographical variation of antibacterial defence of the alga (Chapter IV). The anti-settlement defence showed a temporal variation with a peak activity in late summer/ autumn and also showed a strong and consistent difference between sites throughout the year.

In summary this thesis highlights the capability of the Baltic foundation species *Fucus vesiculosus* for chemical defence against microfoulers, as well as the natural defence variability with site and season and the alga's responses toward simulated environmental conditions. The study thus provides numerous new insights into algae-bacteria interactions and their dynamics and it helps in better understanding of the alga's reaction towards bacterial epibiosis in response to potential abiotic stressors.

Zusammenfassung

Bakterien sind im marinen Lebensraum weit verbreitet. In der Kieler Förde finden sich beispielsweise 0.7 bis 2.24 x 10^6 Bakterienzellen in einem ml Meerwasser. Meeresorganismen sind also ständig einem relativ hohen Besiedelungsdruck durch solche Bakterien ausgesetzt, die lebende Oberflächen als Substrat nutzen können. Die Beziehungen zwischen Wirt und Bakterium sind manchmal neutral oder sogar mutualistisch, beispielsweise können manche algenassoziierte Bakterien ihren Wirt vor der Besiedlung durch andere Aufwuchsorganismen schützen. Öfter haben bakterielle Besiedler aber negative Effekte, beispielsweise indem sie durch die Freisetzung von Signalstoffen weitere Aufwuchsorganismen anlocken. Obwohl die Oberflächen von Makroalgen potentiell ein Substrat für Aufwuchsorganismen darstellen, sind viele Algen weitgehend frei von Aufwuchs, möglicherweise aufgrund ihrer Oberflächeneigenschaften oder weil sie aufwuchshemmende Metabolite abgeben. In der Ostsee wurde dies an der ökologisch wichtigen Algenart *Fucus vesiculosus* (Phaeophyceae) beobachtet, die während des größten Teil des Jahres fast frei von Aufwuchs ist. Bisher gibt es keine Anzeichen für eine mechanische oder strukturell bedingte Abwehr dieser Alge gegen Epibionten, so daß die Existenz chemischer Verteidigung - möglicherweise auch multipler chemischer Verteidigungsstrategien – nahe lag.

Die chemische Verteidigung von Makroalgen gegen Aufwuchsorganismen wird seit zwei Jahrzehnten in der wissenschaftlichen Literatur behandelt. Die meisten der vorliegenden Arbeiten beschreiben aber Effekte von Gewebeextrakten, die sich in ihrer Zusammensetzung und Konzentration meistens deutlich von Oberflächenextrakten unterscheiden und Aufwuchsorganismen unter natürlichen Bedingungen daher nur selten beeinflussen. Die wenigen mit natürlich konzentrierten Oberflächenextrakten durchgeführten Arbeiten berücksichtigten darüber hinaus meist nicht die Verteidigung gegen bakterielle Besiedlung, obwohl diese den notwendigen ersten Schritt bei der Entwicklung von Aufwuchsgemeinschaften darstellt. Ein Hauptziel der vorliegenden Arbeit bestand deshalb darin den Effekt von an der Oberfläche vorhandenen Abwehrmetaboliten zu untersuchen, wobei *Fucus vesiculosus* als Modellorganismus verwendet wurde.

Die Notwendigkeit zur Beschreibung des Charakters und der Dynamik von Metaboliten, die die bakterielle Besiedelung von *Fucus vesiculosus* hemmen, ergibt sich aus der Tatsache dass diese Abwehrstoffe die erste Verteidigungslinie gegen Aufwuchs in einem Organismus von

erheblicher ökologischer Bedeutung darstellen. In vorhergehenden Untersuchungen an *F. vesiculosus* wurde eine Rolle von Polyphenolen als Aufwuchs hemmende Abwehrstoffe vorgeschlagen. Eine Bioassay-geleitete Strukturaufklärung der relevanten Algenmetaboliten in der Verteidigung gegen bakteriellen Aufwuchs wurde jedoch in der vorliegenden Arbeit erstmalig durchgeführt und ergab keinen Hinweis auf eine Rolle von Polyphenolen. Durch Einsatz verschiedener chromatographischer Techniken in Kombination mit Bioassays konnte vielmehr der lipophile Metabolit Fucoxanthin isoliert und als Abwehrstoff identifiziert werden (Kapitel I). Anschliessend wurde seine Anwesenheit auf der Algenoberfläche überprüft und bestätigt. Es folgte eine getrennte Quantifizierung von direkt aus *F. vesiculosus* und von aus epiphytischen Diatomeen stammendem Fucoxanthin. Ausserdem wurden Fucoxanthin-Konzentrationen auf Thallusspitzen und auf ganzen Thalli bestimmt und es wurde getestet ob derartige Konzentrationen ausreichen um verschiedene potentiell auf *Fucus* siedelnde Bakterien zu hemmen. Durch diese umfassenden Arbeiten konnte eine neue Verteidigungsstrategie in *F. vesiculosus* identifiziert werden.

Kapitel II beschreibt die Identikation von DMSP und Prolin als hydrophilere Metaboliten, die ebenfalls zur Hemmung bakterieller Besiedler auf *Fucus*-Oberflächen beitragen. Beide Metaboliten werden hier erstmalig als Algen-Abwehrstoffe gegen Aufwuchs vorgeschlagen. Die Ergebnisse der Kapitel I und II zusammen machen deutlich dass *Fucus vesiculosus* multiple chemische Verteidigungsstrategien gegen Aufwuchs besitzt. In Kapitel III wird gezeigt wie die Verteidigung der Alge mittels der verschiedenen Inhibitoren durch Umweltveränderungen wie Temperaturerhöhung oder Lichtverminderung beeinflusst wird. Insgesamt scheinen die Oberflächenkonzentrationen der drei Inhibitoren und damit die Verteidigungsfähigkeit von *F. vesiculosus* nur geringfügig durch Faktoren wie Temperatur oder Licht beeinflusst zu sein.

Dennoch existiert eine jahreszeitliche und geographische Variabilität in der antibakteriellen Verteidigung der Alge (Kapitel IV). Die Abwehr gegen bakterielle Besiedelung erreichte ihren Höhepunkt im Spätsommer und Herbst und war ganzjährig deutlich unterschiedlich wenn Algen aus verschiedenen Freilandpopulationen verglichen wurden.

Die vorliegende Arbeit bestätigt und erklärt also die Fähigkeit der habitatbildenden Ostseealge *Fucus vesiculosus* zur chemischen Verteidigung gegen bakterielle Aufwuchsorganismen. Die Arbeit beschreibt darüber hinaus die Variabilität dieser Verteidigung zwischen Populationen, Jahreszeiten und verschiedenen experimentellen Bedingungen. Die Dynamik von Algen-Bakterien-Wechselwirkungen wird von einer neuen

Warte aus dargestellt, die es insbesondere erlaubt die Abwehr gegen Aufwuchs unter variierenden nichtbiotischen Stressbedingungen besser einzuschätzen.

General Introduction

Marine organisms, especially those living attached and in shallow depths, often have to cope with drastic change in environmental conditions/ multiple stresses – a situation enhanced by ongoing global change (e.g. Chapin et al. 2000). This change may consist of strong deviation of physico-chemical variables from the physiological optimum, or pressure exerted by competitors, consumers, parasites, pathogens or epibionts. These abiotic and biotic factors do not interact in isolation (e.g. Wahl et al. 2011). There are interactions that cause non-additive effects (Breitburg et al. 1999; Folt et al. 1999; Vinebrooke et al. 2004). Influence of these factors are synergistic when their combined effect is larger than predicted from the sizes of the responses to each factor alone, and antagonistic when the cumulative impact is smaller than expected (Folt et al. 1999). Thus, the fitness and survival of marine organisms may be impacted by the interactive effects of – 1) abiotic factors (e.g. light reduction, heat stress), 2) biotic factors (e.g. epibiosis and herbivory) (Rohde et al. 2008).

Epibiosis is one of the most important 'surface' interactions of the host that may act as an important ecological lever by modifying interactions and by amplifying or buffering environmental stress (Wahl 2008). Unlike its terrestrial counterpart, aquatic organisms are involved in a number of processes through their body surface- gases, micro or macro nutrients or ions are absorbed or excreted; chemical, mechanical and visual cues are received or transmitted; radiation is absorbed; defensive metabolites are transmitted (Wahl 2009). Spores, gametes, zygotes or larvae often get released from their parents' body thorough localised rupture of the body surface (Wahl 2008). Additionally different types of biotic and abiotic stressors that an organism may experience, i.e. salinity and temperature fluctuations, UV radiation, desiccation, pollutants, are first experienced and transferred through the body surface (Wahl 2009). Thus, getting densely colonized at the functional interface will clearly modulate most interactions of the host and can have a community wide impact (Wahl 2008).

Sessile life-style: the key to epibiosis

The unique characteristics of water (e.g. high density and viscosity) have lead to the evolution of a sessile mode of life among marine organisms which – for dispersal – often depend on a pelagic larval stage (Wahl 2009). 'Space', i.e. settlement substratum, often is an important limiting factor in such environments (Dworjanyn et al. 2006a). Such environments are also characterised by competition for light, nutrients and other resources (Wahl 2009). The demand: supply ratio being high especially for space, any freely available animate or inanimate surface in natural seawater is rapidly colonized by sessile organisms. This colonisation process of a solid substrate (living or dead) is called 'fouling' (Wahl 1989). Wahl (1989) defined epibioses as a non-symbiotic, facultative association between a substrate organism (basibiont) and a non-motile organism (epibiont) attached to the outer surface of the basibiont. The process of fouling has been described by classical (Davis et al. 1989) and dynamic models (Clare et al. 1992; Maki and Mitchell 2002). Basically, fouling occurs in four principal stages (Wahl 1997; Railkin 2004) (Figure 1). An initial adsorption of organic molecules (e.g. polysaccharides and peptides, 'conditioning' or 'chemical fouling') is followed by the formation of a primary biofilm consisting mainly of pioneer bacteria, which provide further cues to other bacteria, diatoms, protozoans and fungi. They condition the surface and begin settling on it, thus composing a secondary biofilm thereafter followed by the settlement of macrofoulers (Wieczorek and Todd 1998). Macrofoulers may also produce chemicals that may influence further settlement of conspecifics or other species (e.g. Railkin 2004), ultimately leading to the formation of a complex, dynamic and diversified epibiotic community. These steps generally occur sequentially but there is no such hardbound rule (Wahl 1989). However, the development of a macromolecular conditioning film always takes place which in turn alters the properties of the substratum leading to energy accumulation at

the interface in addition to being a food source of carbon and other compounds, attracting pioneer bacteria (Characklis and Cooksey 1983).

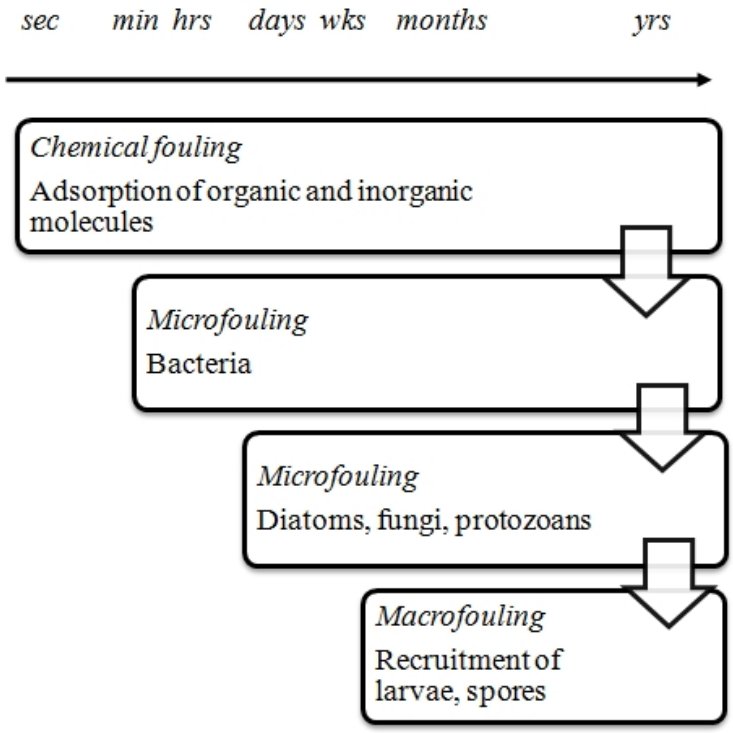

Figure1. The classical model depicting four stages of successional fouling. Adapted from Wahl 2009.

Epibiosis: the consequences

The microfoulers

Some kind of host-microbe associations could be mutualistic (Lemos 1985; Boyd et al. 1999; Armstrong et al. 2001). For example, bacteria isolated from the surface of a tunicate inhibited the settlement of tunicate and barnacle larvae exposed to the bacteria as biofilms in petridishes (Holmstrom et al. 1992; Armstrong et al. 2001). Additionally, some seaweed requires surface bacteria to develop properly, a typical morphology being exhibited when grown in axenic culture (Tatewaki et al. 1983; Armstrong et al. 2001). Other kinds of associates are clearly detrimental to hosts (Lane and Kubaneck 2008). Epibiotic microbial films may act as a filter controlling the molecules liberated by the basibiont or the

compounds reaching the basibiont from surrounding water (e.g. Saroyan 1968). Thus, the nearby surface concentrations of O_2, CO_2, H+ and nutrients may be regulated by the presence of biofilms (Araujo-Jorge et al. 1992; Thevanathan et al. 2000; Railkin 2004) which may benefit or damage the host. Biofilms of a certain thickness may insulate the basibiont from essential resources (e.g. Costerton et al. 1987) or prevent the detection by symbionts or mates (Wahl 2009). Such insulation may help the basibiont from being detected and consumed by predators or being located by detrimental epibionts or pathogens (Gilturnes and Fenical 1992). There could be some associational resistance but there is also the risk of 'shared doom', i.e. the chance that predation gets aggravated by macro epibionts (Manning and Lindquist 2003; Wahl and Hay 1995).

Bacteria: the first colonisers

Bacterial attachment represents the very first phase of microbial colonisation. The ability to colonize surfaces provides bacteria with important advantages, including (i) increased access to nutrients, (ii) protection against toxins (e.g., biocides, heavy metals, or UV radiation), (iii) maintenance of extracellular enzyme activities, and (iv) shelter from predation (Dang and Lovell 2000). While some bacteria are beneficial (see above for details) (Boyd et al. 1999; Armstrong et al. 2001) others are mainly detrimental, causing diseases and involving fouling associated fitness costs (e.g. D'Antonio 1985; Correa 1997; Ruesink 1998). Primary bacterial colonisers could be deleterious to the host by providing inductive cues to the larvae and spores of macrofoulers (Unabia and Hadfield 1999). These can cause extensive tissue damage and in their turn pave the way for pathogenic bacteria (Sawabe et al. 1998). By altering the physical and chemical properties of the basibiont, the biofilm in turn alters the interactions that are regulated through the basibiont's surface properties (Wahl et al. 2010). Bacterial epibionts along with macroscopic foulers may also affect the host's fouling sensitivity

(Holmstrom & Kjelleberg 1999; Dobretsov 2008) or its susceptibility to grazers, which may increase pathogenic infection through grazing wounds (Wahl 1997). As indicated by the number of published results- compared to the beneficial effects most consequences of bacterial colonisation have been found to be negative.

Algae: a potential host and its defence strategy

In the marine environment, one important source of substrata for colonisation are macroalgae. Being restricted to the photic zone, they provide optimal growth conditions to many epiphytic organisms (Harder 2008). Their three-dimensional structure offers a significant amount of surface in benthic marine habitats (Seed 1985). Macroalgae provide permanent or temporary shelter and sediment traps (Williams and Seed 1992). They release large amounts of organic carbon into the surrounding environment providing nutrients to micro-organisms (Armstong et al. 2001; Lane and Kubanek 2008) trigerring chemotactic behaviour of bacteria (Paul and Puglisi 2004; Goecke at al. 2010). Most of the primary metabolites such as proteins, peptides, carbohydrates and amino acids are known as inducers of microbial colonisation (Steinberg et al. 2002). Thus, their surface provides a protected microniche suitable for bacterial colonisation and reproduction (see Goecke et al. 2010 and references therein). Therefore, macroalgae are continuosly challenged by micro-organisms (Weinberger et al. 1997) and are apparently very much susceptible to epibiosis (e.g. Harder et al. 2004).

Although macroalgae provide seemingly favourable conditions to many kinds of biofoulers, most of them often remain free from heavy fouling (Potin et al. 2002). This suggests that seaweeds have developed mechanisms to keep deleterious foulers at bay (Lane and Kubanek 2008). One mode of preventing colonisation is through physical defence, e.g., through production of a mucilaginous covering or outer cell layer shedding (Nylund and Pavia 2005). Algae may also prevent colonisation through the oxidative burst, i.e. the production of

reactive oxygen species (Weinberger and Friedlander 2000). Often they strongly depend on their chemical repertoire, i.e. chemical defence, to ward off foulers (Potin 2008). A wide variety of anti-bacterial compounds or fractions have been reported from macroalgae. Halogenated furanones from the red alga *Delisea pulchra* are known to inhibit the quorum sensing process, thereby restricting bacterial colonisation (de Nys and Steinberg 2002; Mannfield et al. 2002). The genus *Caulerpa* is known to produce acetylene sesquiterpenoid esters and terpenoinds with anti-algal and anti-bacterial properties. Methanolic extract and a sulphono-glycolipid (genus *Enteromorpha*), lipid extract and steroidal glycosides (genus *Codium*); chloroform extract and phlorotannins (genus *Sargassum*), methanolic and choloroform extracts and unsaturated fatty acids (genus *Lobophora*); elatol, deschloroelatollaurencienyne, brominated diterpene, iso-obtusol and halogenated metabolites (genus *Laurencia*) have been reported to be anti-bacterial (reviewed in Bhadury and Wright 2004). Most of these studies have been conducted with total algal extracts, thus not demonstrating the ecological relevance of these compounds. However, few studies have reported some ecologically relevant surface based anti-bacterial compounds (de Nys et al. 1998; Paul et al. 2006; Nylund et al. 2008; Saha et al. 2011).

Variability of defence

Quantitative variation of secondary metabolites is common (Van Alstyne et al. 2001). Secondary metabolite levels in seaweeds have been correlated with or found to be influenced by abiotic factors like light intensity (Cronin & Hay 1996; Pavia et al. 1997; Sudatti et al. 2011), nutrient levels (Yates & Peckol 1993; Cronin & Hay 1996), desiccation (Renaud et al. 1990), and salinity (Pedersen 1984). Biotic factors such as grazing have also been found to affect nature and level of defences (Van Alstyne 1988; Yates & Peckol 1993; Cronin & Hay 1996).

Fouling pressure (together with other biotic factors) and abiotic factors vary with season, location and habitat and so does the defence strength of the host vary (Hellio et al. 2004). Macroalgae are known to modify their morphology or chemistry and thereby respond plastically to environmental variations (Hemmi and Jormalainen 2004). For example, phlorotannin, known for its putative ecological functions, shows variation in response to abiotic factors such as salinity, nutrient and light, UV and herbivore pressure (reviews by Targett and Arnold 1998; Amsler and Fairhead 2006; Jormalainen and Honkanen 2008). In addition phlorotannin content and composition may be dependent on the genetic equipment of an organism (Jormalainen and Honkanen 2004). Similarly, the concentration of dimethlysulphopropionate (DMSP), which is known to have a number of ecological and physiological functions, varies geographically and taxonomically (Alstyne & Puglisi 2007). In macroalgae the DMSP concentration significantly increases from the tropics to the poles in the northern hemisphere but not in the southern hemisphere and low DMSP concentrations have been reported from red and brown algae when compared to green algae (Alstyne & Puglisi 2007). Field and laboratory based experiments reported plasticity in DMSP production of *Codium fragile,* with an increase in tissue DMSP content with increasing light intensity and sea urchin grazing and decrease in temperature (Lyons et al. 2010).

Although chemical defences offer a number of advantages, their generation might be related to various costs. For example, the production of furanone in the red alga *Delisea pulchra* has been found to be negatively correlated with its fecundity (Dworjanyn et al. 2006b). Similarly, phlorotannin production has been found to be negatively correlated with growth of *Fucus vesiculosus* (Jormalainen & Ramsay 2009). However, there have been reports which found no evidence of defence costs in seaweeds (e.g. Pansch et al. 2009). Targett and Arnold (2003) argued that the production of phlorotannin may be driven by other primary functions than

defence and in such cases, where secondary metabolites play multiple roles, the hypothesis of growth/defence tradeoff would probably overestimate the cost of defence (Targett and Arnold 2003). Even if costs incur they may not matter when resources are not limiting (Cronin 2001). If there are costs of defence and when resources are limited, then the expected increase of stresses/ shifting of environmental factors (e.g. light reduction, high temperature) is likely to jeopardize defences with a severe feedback regarding the condition of a habitat-forming alga such as *Fucus* (see review by Wahl et al. 2011 for details) . If *Fucus* disappears this may lead to a massive re-structuring of communities with subsequent shifts in ecosystem services.

Study organism

The rockweed *Fucus vesiculosus* Linnaeus (Phaeophyceae) [Figure 2] has a monophasic diploid life history. Haploid sperm and eggs are produced in numerous conceptacles within each of several large receptacles on apical tips of thallus branches. Reproduction is iteroparous, with dioecious species. Eggs are fertilized close to (or on) the female and the diploid zygotes typically settle and mature within meters of the female parent (for details see Wahl et al. 2011 and references therein).

F. vesiculosus occurs in the cold and temperate zones of the North Atlantic. It is the dominant habitat forming perennial species and is vertically distributed from 0- 3m in the subtidal zone of the western Baltic Sea.

Figure 2. *Fucus vesiculosus* (photo courtesy of Uli Kunz)

F. vesiculosus provides food for numerous herbivores, a substratum for epibionts, shelter for many associated species, plays an important role in biogeochemical cycles (e.g. Rangeley & Kramer 1995) and provides other valuable ecosystem services (Rönnbäck et al. 2007). During the past decades, *F. vesiculosus* has retreated from the deeper parts of its former distribution range in the Baltic Sea (Vogt and Schramm 1991), which is presumably due to the combined action of eutrophication and epibiosis (Rohde et al. 2008). High bacterial density in the Kiel fjord (0.7 to 2.24 x 10^6 ml^{-1} seawater, mean of monthly samplings between 2005 and 2008, H.J. Hoppe and R. Koppe, pers. comm.) makes the alga prone to the bacterial coloniser pool, settlement of which may in turn promote further settlement of fungi, algal spores and larvae of macrofoulers. Given the disadvantages of being fouled, *F. vesiculosus* is found to modulate this process and remain only covered with a thin film of micro epibionts through most of the times of a year, which hints at the likely presence of a defence capacity in the

alga (Lachnit et al. 2010; Wahl et al. 2010; Saha et al. 2011). However, in the recent years the alga is not faring well and fouling tends to be more, especially in summer (M. Wahl pers. comm.). The threatened existence of this habitat forming alga makes it essential to study its fouling control mechanisms, in order to understand the interactions of *F. vesiculosus* with micro and macrofoulers in the face of environmental change (Saha et al. 2011).

Thesis outline

By the beginning of this research in 2008, surface exuded metabolites containing phlorotannins have been identified in *F. vesiculosus* to be inhibitors of barnacle settlement (Brock et al. 2007). However, the role of phlorotannins have been highly controversial and also until then, no such specific surface based metabolites representing the first line of defence against fouling i.e. bacterial settlement have been identified from this alga in an ecological perspective.

Identification of surface-bound antibacterial compounds

Since bacterial fouling is the initial step of colonisation it was of utmost importance to **identify active metabolite(s) against bacterial settlement- the first milestone of this research.**

Following an initial fractionation and thereafter bioassay of very polar (water soluble), polar (MeOH soluble) and non-polar (Hexane soluble) extract, in chapter I the active non-polar surface extract of the alga has been investigated. A strong activity of the non-polar extract raised the 1st question: **What are the chemical fraction(s) and finally active metabolite(s) responsible for this activity?**

Identification of the active non-polar metabolite was not enough to prove its ecological relevance and thus lead to the immediate challenge- my next important question: **What is the origin of this compound- *Fucus* itself or surface associated diatoms?**

The sources being identified, it was very important to know: **What are the ecologically relevant concentrations of this compound and whether it is sufficiently concentrated on the algal surface to exhibit an activity?**

After having identified, segregating the origin, quantifying and testing the active non-polar compound, the next target was to investigate the similarly active polar extract (Chapter II). Thus, the 2nd relevant question of my thesis was: **What are the surface associated anti-bacterial polar metabolite(s) of *F. vesiculosus*?**

The metabolites being identified it was essential to know at what concentrations does the alga deploy them on its surface i.e. the natural concentrations with which the bacteria actually interact. So the question was: **What are the surface concentrations of these active polar metabolites?**

As two active amino acids (along with another active metabolite) were spotted in the active fraction, another question was: **Is this anti-settlement activity of amino acids structure specific to certain amino acids or a generalised one for all amino acids?**

Defences and abiotic stress/ factors (temperature, light)

The information on the identity, source, concentration of these active metabolites and the sensitivity of certain bacterial strains towards these inhibitors was available from chapter I and II. As a second part of my thesis, I particularly asked how the anti-settlement defence potential of this alga against bacteria varies with environmental shifts like disruptive temperature stress and light limitation. Thus my next goal (Chapter III) was: **How do these active metabolites vary quantitatively with regard to temperature and light?**

Natural variability of defences in season and site

As fouling pressure is known to vary with season, region and habitat, I raised the last question of my thesis- whether the alga's **anti-settlement defence against bacteria exhibits variability** with season and site and possibly mirrors the variability of threat.

The identification of ecologically relevant active metabolites along with their source, natural surface concentration etc. was a major breakthrough of this research, which further allowed studying the effect of abiotic stress factors on these active algal metabolites. And a further parallel study on the seasonal and geographical variation of antibacterial defence, proved the existence of defence variability of this alga in an ecological context.

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Surface-associated fucoxanthin mediates settlement of bacterial epiphytes on the rockweed *Fucus vesiculosus*

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Abstract

The chemical defence against microfouling in the brown seaweed *Fucus vesiculosus* was investigated and an inhibitor of bacterial settlement was isolated by bioassay-guided fractionation of non-polar surface extracts. UV-vis and mass spectrometry were used to identify the compound as the carotenoid fucoxanthin. The metabolite was tested at the natural concentration (in a surface volume based assay) against the settlement of four bacterial strains isolated from *F. vesiculosus* and 11 strains isolated from co-occurring algae and marine sediment. Surface concentrations between 1.4 and 6 µg cm^{-2} resulted in 50% inhibition of four of these isolates, which were studied in more detail using a surface area-based assay, while a fifth isolate proved to be less sensitive. The presence of fucoxanthin on the surface of *F. vesiculosus* was demonstrated with two different surface extraction methods. Fucoxanthin was detected at concentrations between 0.7 and 9 µg cm^{-2} on the algal surface. Fucoxanthin was still present at the algal surface after removal of associated diatoms through mechanical cleaning and germanium dioxide treatment and was thus mainly produced by *F. vesiculosus* rather than by diatoms. Thus, the photosynthetic pigment fucoxanthin appears to be ecologically relevant as a surface-associated antimicrobial agent, acting against the settlement of bacteria on the surface of the macroalga *F. vesiculosus*.

Keywords: chemical defence; *Fucus*; fucoxanthin; epibiotic bacteria; anti-bacterial activity; fouling modulation.

Introduction

Benthic marine environments are diverse and characterised by immense competition for light, space, nutrients and other resources (Wahl 2009). In such habitats, many macroalgae provide a substratum that is rich in organic material and a safe micro-niche for microbial colonization and reproduction as well as the settlement of other organisms. Associations between microbes and macroalgae can be mutualistic, providing benefit to both the basibiont and the epibiont. For example, some epiphytes are known to produce allelochemicals which in turn can inhibit further settlement, growth or germination of potential fouling organisms (Egan et al. 2001; Dobretsov et al. 2006). The algae might also benefit from micronutrients that are provided by the biofilm components (Ericcson and Lewis 1953). Other kinds of associations are disadvantageous to the host, involving disease and fouling associated fitness costs (da Gama et al. 2008; Wahl 2008). Bacterial colonisers could provide inductive cues to the larvae and spores of macrofoulers (Holmstrom and Kjelleberg 1999; Unabia and Hadfield 1999; Dobretsov et al. 2009). As the larvae and spores develop, they can cause extensive tissue damage, hinder gaseous exchange and photosynthesis (Wahl 1989, 2008) and pave the way for pathogenic bacteria (Sawabe et al. 1998). Algae must either tolerate fouling organisms or develop barriers such as sloughing of the outermost layers (Nylund et al. 2005) or production of chemical defences (Wahl 1997; Weinberger and Friedlander 2000; Steinberg et al. 2001). Defensive metabolites can be produced either by the seaweed itself (Kubanek et al. 2003; Nylund et al. 2008) or by host-symbiotic microorganisms (Egan et al. 2001).

The ability of benthic marine algae to inhibit fouling by the release of chemicals from their surface at the micro- and macro-scale has been investigated over many years (eg de Nys et al. 1998; Steinberg and de Nys 2002; Nylund et al. 2008). A wide range of biologically active compounds are produced by marine macrophytes (reviewed in Qian and Fusetani 2010), which potentially hamper the attachment, settlement, survival, growth and reproduction of microorganisms, larvae or spores. So far, these effects have been mainly demonstrated in studies that investigated the effects of total tissue extracts (Hellio et al. 2000; Viano et al. 2009). In these investigations, the ecological relevance of natural antifouling agents is often unknown since whole cell metabolites might not be present on the surface of the macroalgae and therefore are not available to the fouling organisms. However, some studies have selectively tested surface-associated meta- bolites at ecologically relevant concentrations (eg Dworjanyn et al. 2006; Sudatti et al. 2008).

The bladder wrack, *Fucus vesiculosus*, inhabits cold and temperate zones on both sides of the North Atlantic. As a perennial species, *F. vesiculosus* often forms dense belts and typically grows in the upper intertidal, while in large parts of the tidal Baltic Sea it inhabits the first meters of the subtidal zone. *F. vesiculosus* provides food for numerous herbivores, a substratum for epibionts, shelter for many associated species and other valuable ecosystem services (Rönnbäck et al. 2007). During the past decades, *F. vesiculosus* has retreated from the deeper parts of its former distribution range in the Baltic Sea (Vogt and Schramm 1991), which is probably due to the combined action of eutrophication and epibiosis (Rohde et al. 2008). The threatened existence of this habitat-forming alga in the face of environmental change makes it essential to understand its interactions with micro- and macrofoulers.

Bacterial densities in the Kiel fjord vary from 0.7 to 2.24 x 10^6 ml^{-1} seawater (mean of monthly samplings between 2005 and 2008, H.J. Hoppe and R. Koppe, personal communication), thus imparting a strong colonisation pressure on *F. vesiculosus*. But,

strikingly, the alga remains free from heavy scale fouling throughout most of the year (Lachnit et al. 2010; Wahl et al. 2010). Therefore, it is likely that the alga modulates its fouling community structure probably either by producing allelochemicals (Brock et al. 2007) or through components of the microbial community present on its surface.

The chemical defence of *F. vesiculosus* against epibionts has been studied by Brock et al. (2007), Lachnit et al. (2010) and Wahl et al. (2010), and polyphenolic metabolites have been proposed as fouling deterrents (McLachlan and Craigie 1964). The study presented here aimed to detect and identify metabolite(s) that inhibit bacterial settlement on *F. vesiculosus* surfaces. Bacterial strains isolated from *F. vesiculosus* and co-occurring algae as well as from marine sediment were used as test organisms in order to find metabolite(s) active against ecologically relevant bacteria. Surface-associated antimicrobial metabolites that target bacterial settlement were specifically investigated. Bioassay-guided fractionation was used to isolate surface-associated fucoxanthin. The study suggests a dual role of this metabolite as a pigment and as a secreted defence compound.

Materials and methods

Organisms

Algal material

For fractionation 7.7 kg (spin dried, wet weight) of the brown alga *F. vesiculosus* were freshly harvested from the subtidal zone of Kiel Fjord, Germany (54˚26’ N/ 10˚11’ E) in December 2009. Further samples were collected for other experiments from Gelting, Germany (54˚48’ N/9˚44’ E) in July 2010 and in Poel, Germany (54˚01’ N/11˚28’ E) in September 2010. The plants were individually sealed in zip-lock bags and transported to the laboratory in a cool box. To avoid desiccation and damage, the plants were held in a saturated seawater atmosphere prior to extraction.

Bacteria

Fifteen different bacterial isolates were used as test organisms in settlement assays. They were *Cytophaga* KT0804 (isolated from *Halidrys siliquosa* and also detected on *Saccharina latissima*); *Bacillus aquimaris* (isolated from *Halidrys siliquosa* and also detected on *Desmarestia aculeata* and *Ahnfeltia plicata*); *Cobetia marina* (isolated from seawater), *Flavobacterium* sp., *Rheinheimera baltica*, *Koprimonas byunsamensis*, *Shewanella baltica* (all four isolated from *F. vesiculosus*); *Ulvibacter littoralis*, *Alphaproteobacterium* DG1293, *Vibrio* sp. siga 198 (all three isolated from Fucus serratus); *Pseudoalteromonas* BSw200058, *Pseudoalteromonas* sp. 135Xa1, Alteromonadaceae E1 (all three isolated from the red alga *Polysiphonia stricta*); marine sediment bacterium ISA 7311 and *Marinobacterium rhizophila* (isolated from marine sediment) (strains were isolated and identified by F. Symanowski, unpublished data). The strains were maintained as cryostocks at -80ºC.

Isolation and characterization of fucoxanthin

Surface specific extraction of the algal material

The surface associated metabolites of *F. vesiculosus* were extracted using the 'dipping technique' of de Nys et al. (1998). Fronds of *F.vesiculosus* were collected and surface extracted. For this purpose the algal thalli were dipped for 10s in a stirred mixture of methanol and hexane 1:1 (v/v) (see Lachnit et al. 2010). Larger thalli had to be cut prior to dipping. Care was taken that the cut end had no contact with the solvent mixture, in order to avoid extraction of intracellular compounds. The prepared extract was immediately filtered through a GF/A filter (Whatman, Ø = 15mm) to remove particles and the solvent was evaporated under vacuum at 30 ºC. The resulting residue was re-dissolved in hexane to yield the non-polar metabolites. This step was repeated until the hexane appeared colorless. In a similar manner, the residue remaining after treatment with hexane was then taken up in methanol to extract the rest of the metabolites. The final residue remaining after methanol

treatment was dissolved in water. Solvent controls were prepared by evaporating equivalent amounts of the solvents required for surface extraction and subsequent chromatographic steps. For the bioassays, all extracts and solvent controls were taken up in dimethylsulphoxide (DMSO) and tested at natural concentrations, expressed as amount [of metabolites] per ml [*F. vesiculosus* surface volume extracted].

Extracted surface volume

The extracted surface volume was calculated as the algal surface area x 30 mm (estimated thickness of the surface boundary layer, in which the translocation of molecules is affected by ionic interactions or hydrogen bonds (Wahl et al. 2010)). With the exception of the material used for fractionation, the surface areas of the plants were determined by imaging. For this purpose, the individuals of *Fucus* were spread under a plexiglass panel and photographed beside a benchmark area. Pixels representing the benchmark area and *Fucus* were counted with the imaging software Image J (National Institute of Health, Bethesda, Maryland, USA). Pilot studies indicated that 1 g of algal wet weight corresponded to *ca* 25.57 cm^2 (SD ± 1.88) of algal surface area (T. Lachnit, personal communication). Since the surface area determination of 7.7 kg of seaweed material was impractical, the total surface area of the *F. vesiculosus* biomass extracted for fractionation was determined by multiplying the wet weight of 7.7 kg by 25.57 $cm^2\ g^{-1.}$

Anti-settlement assays

Cytophaga KT0804 and *B. aquimaris* were used in all tests, while all other isolates were only tested against the pure compound. The bacterial strains were grown in nutrient media (5 g peptone + 3g yeast in 1l of filtered seawater) for 20–22 h and when necessary (to achieve optimal cell density) for 48–72 h at 20˚C. Prior to the assays, the optical density (OD) of the bacterial cultures was determined with a Beckman Du® 650 spectrophotometer at a wavelength of 600 nm, using pure medium as blank. All the working cultures had an OD in

the range of 0.5–0.8. Cultures were diluted to the mentioned OD range with sterile seawater (SSW) when necessary. All settlement assays were conducted in multi-well plates (96 wells, flat bottom, Greiner®). In each well, bacterial cells in suspension were mixed with algal extract, fucoxanthin, DMSO or solvent control. The multi-well plates were placed on a shaking table (100 rpm) at 20˚C and bacteria were allowed to attach for 1 h. The bacterial suspension was then removed from the wells and non-attached cells were eliminated by gently rinsing twice with sterile filtered seawater. The attached cells were quantified by staining (10 min) with the fluorescent DNA-binding dye, Syto 9, Invitrogen, GmbH. The fluorescence was subsequently measured (excitation 477–491 nm, emission 540 nm) in a plate reader (Hidex Chamaeleon, Turku, Fi) as a proxy for bacterial settlement in terms of cell density.

Surface volume-related assay

All crude and fractionated extracts, as well as fucoxanthin, were tested at natural concentrations, identical to the concentrations present in the algal surface volume. In these assays 97 µl of bacterial suspension were transferred to a well. Three µl of DMSO containing an extract or fucoxanthin at 33.3 fold natural concentration were added so that the tested compounds were present at natural concentration in the final mixture. The bacteria were never exposed to DMSO at concentrations > 3%, as DMSO at 5% or more in some cases proved to be toxic. Bacterial suspensions in DMSO only served as controls when crude extracts were tested. In order to compensate for possible solvent effects, DMSO containing fractionated solvent residue for each solvent used were controls when fractionated extracts were tested. For the preparation of solvent residue fractions, solvent containing no extract was fractionated in an identical manner as extract.

Bioassay guided fractionation by gravity column chromatography (GCC)

Bacterial settlement inhibition assays were used to guide chromatographic fractionation and purification of the non-polar extract of *F. vesiculosus*. In the first fractionation step GCC on Silica gel 60 (Merck, 2 x 30 cm) was used. The fractions were eluted with n-hexane, chloroform ($CHCl_3$) and ethyl acetate (EtOAc) and collected on the basis of eluent color. The fractions were analysed by thin layer chromatography (TLC) (Alugram Sil G, 0.20 mm, Macherey Nagel; mobile phase 1:1 hexane: $CHCl_3$ (v/v)). The TLC plates were observed under UV light (254 nm) and pure fractions were maintained separately. Impure fractions and fractions of similar chemical profile were combined and re-chromatographed. This yielded seven fractions, including the pure fractions (fraction GCC-A to GCC-G), which were tested against solvent residue controls.

Purification by high performance liquid chromatography (HPLC)

The inhibitory GCC fraction was further fractionated by HPLC, using a Macherey Nagel Nucleodor Si column (10 mm x 25 cm) (gradient: 100% n-heptane, 10 min; linear gradient to 100% EtOAc for a further 20 min, flow rate of 4 ml min^{-1}) on a Varian 940-LC with integrated photodiode array detector (PDA) and a connected Varian evaporative light scattering detector (ELSD). The injection volume was 100 µl and the fraction collection interval was 1 min. Fractions showing peaks in PDA (254 nm and 280 nm) or ELSD chromatograms were tested in the anti-settlement assay. One of the inhibitory fractions was further purified using the same HPLC system, preparative column, flow rate and mobile phase as above (gradient: start at 70% n-heptane/30% EtOAc, then a linear gradient to 100% EtOAc for a total of 10 min). The fraction collection interval was 0.5 min. The fractions were again tested for effects on bacterial settlement, using all 15 bacterial isolates mentioned above.

Characterisation of the anti-bacterial compound

Analytical LC-MS measurements were carried out on an UPLC-MS system equipped with a 2996 PDA detector and a qTOF micro ESI-TOF (Waters) with a gradient of solvent A (water acidified with 0.1% formic acid (v/v) and 1% acetonitrile (v/v)) and solvent B (acetonitrile acidified with 0.1% formic acid (v/v)) 0 min at 0% B; 0.5 min to 50% B; 5 min to 100% B; 5.5 min 100% B; 6 min 0% B. Column parameters were: BEH C18 (2.1650 mm), particle size 1.7 mm. ESI-parameters were: capillary voltage of 3000 V (ESI- positive), collision energy 5 V. UV-VIS-PAD-parameters were: recording speed 20-spectra s^{-1} and an acquisition range from 190–800 nm. The structure of the active compound was elucidated by evaluation of the ESI-pos MS andMS/MS spectra and analysis of the descriptive UV-Vis spectrum. Final comparison with a commercially available standard fucoxanthin (Cayman chemicals, Hamburg, Germany) proved the structure of the natural product, which eluted with identical retention time and exhibited identical UV and MS properties.

Presence of fucoxanthin on* F. vesiculosus *surfaces

Comparison of surface extraction techniques

In order to verify the results obtained with the 'dipping' extraction technique, surface specific extrac- tion was also conducted using a 'swabbing technique' (Schmitt et al. 1995). After collection, the plants were spin dried in a salad spinner for 30 s and swabbed with cotton tips. These were extracted in a 1:1 (v/v) mixture of hexane: methanol for several hours until they appeared white. The resulting extract was filtered through GF/A filters and rotary evaporated to dryness and then re-dissolved in hexane. Extracts prepared by swabbing and dipping technique were analysed by NP-HPLC for fucoxanthin (Macherey Nagel Nucleodor Si column 10 mm x 25 cm) on a Varian 940-LC with PDA detector (450 nm), using commercial fucoxanthin as a standard; n-heptane/EtOAc was used as a mobile phase with a flowrate of 4

ml min^{-1} (gradient: 100% n-heptane, 10 min; linear gradient to 100% EtOAc for a further 20 min).

Microscopic monitoring of cells after surface extraction

Algal tips of ca 2–4 cm^2 were either swabbed with cotton tips or dipped for 10 s into solvent as described above, rinsed with sterile filtered seawater and stained with 0.05% Evans blue in seawater for 30 min. The tips were monitored under the microscope for the presence of lysed cells (Weinberger et al. 2005). Tips of untreated individuals served as a negative control, while algal tips dipped in hexane/methanol (1:1) for 60 s served as a positive control.

Natural surface concentration of fucoxanthin

F. vesiculosus individuals were collected from Poel in September 2010 and individual surface extracts of apical tips were made using the 'swabbing technique'. The fucoxanthin content was quantified using preparative NP-HPLC. The surface areas of the plants were determined by ImageJ.

Origin of fucoxanthin

Since diatoms present in *F. vesiculosus* biofilm may contribute to surface extracted fucoxanthin, the concentrations of this compound on the surface of *Fucus* were compared for individuals with a natural and reduced density of associated diatoms. In order to reduce the diatom density, *F. vesiculosus* individuals (n=3) were maintained in 3 l conical flasks containing 2.5 l sterilised filtered seawater supplemented with 0.179 mg l^{-1} GeO_2 (Markham and Hagmeier 1982). Control individuals were maintained in the same way, but without GeO_2. All individuals were maintained for 10 days under continuous aeration at 16˚C, with a 16:8 h (light/dark) regime at 20 µmol m^{-2} s^{-1}. *Fucus* individuals under GeO_2 treatment were also swabbed with a paper towel prior to the incubation, in order to mechanically remove diatoms. The biofilm on the control plants was kept intact. The health status of the treated thalli was monitored daily. At the end of the experiment, the algae were photographed for

image analysis and surface extracted by the 'dipping technique' as described before. Prior to extraction, ca 0.5 cm^2 were cut from an old part of both the treated and control plants and observed under the microscope for the presence of diatoms. After extraction (all individuals were pooled), fucoxanthin obtained from GeO_2 treated and untreated plants was compared to the commercial standard fucoxanthin by analytical NP-HPLC as described above. For quantification, fucoxanthin obtained from treated and untreated algae was purified by preparative NP-HPLC, weighed and tested at its natural concentration.

Surface area-related assay

Commercial fucoxanthin was also tested as a su rface coating for its anti-settlement activity. Fucoxanthin (in acetonitrile) was pipetted into microtiter plate wells and evaporated so that the bottom and side walls of the wells were coated with surface concentrations of fucoxanthin in the range of natural concentrations, and 100 µl of bacterial suspension were added. As a control, the wells were impregnated with solvent residue.

Statistical analyses

The strength of extract, fraction or compound effects on bacterial settlement were expressed as the log effect ratio (ie decimal logarithm of the ratio of target strain settlement in the presence vs absence of extract; Wahl et al. 2004). A log effect ratio value of 0 indicates that the tested extract had no effect on settlement, whereas a negative log effect ratio value indicates an inhibitory and a positive log effect ratio value a promoting effect, respectively. One-way ANOVA was used to analyse the effect of crude extracts, GCC and HPLC fractions on settlement of individual bacterial strains. Shapiro– Wilk's test was used to test for normal distribution ($p < 0.05$), while Levene's test was used to test for homogeneity of variance ($p < 0.05$). Datasets not fulfilling the criteria of homoscedasticity were Box- Cox transformed using the software Minitab 12.2 (Minitab Inc., State College, PA, USA). Post hoc comparisons were made using Tukey's honest significant difference test (HSD) when

required ($p < 0.05$). The t-test was used to compare the effect of fractions G27a and G27b, and to compare effects of fucoxanthin prepared from GeO_2 treated and untreated plants on bacterial settlement. All statistical tests were conducted using the computer program Statistica, (StatSoft, Tulsa, OK, USA). For the test of surface-coated fucoxanthin, functions that described best fits of the data were computed by iterative adaptation, using the software package Prism 4 (GraphPad, La Jolla, CA) and the logistic function Y=Min+(Max-Min)/(1+10^(log(EC_{50})-X)). In this function X and Y represent fucoxanthin concentration and settlement response, respectively. Min, Max and EC_{50} are constants describing minimal and maximal responses and the necessary concentration for half-maximal inhibition of settlement, respectively.

Results

***Bioassay guided fractionation of apolar surface extract of* F. vesiculosus**

Crude hexane and methanol extracts inhibited the settlement of *Cytophaga KT0804* and *B. aquimaris*, with mean log effect ratio values of –1.17 and -1.01 (Figure 1). A lower inhibitory effect (mean log effect ratio value of -0.17) was observed with the total water extract. There was no statistically significant difference between bacterial settlement inhibition among the methanol and hexane extracts, while both differed significantly from the total water extract (One-way ANOVA (*Cytophaga KT0804*, n = 3, F = 18, p = 0.0028; *B. aquimaris,* n = 3, F = 90, $p < 0.001$) and Tukey's HSD).

The hexane extract was chosen for further fractionation. GCC of this extract yielded seven fractions GCC-A to GCC-G, out of which fraction GCC-G showed an inhibitory effect. The effect of fraction GCC-G was dose-dependent and increased when tested at twice the natural concentration (Figure 2i).

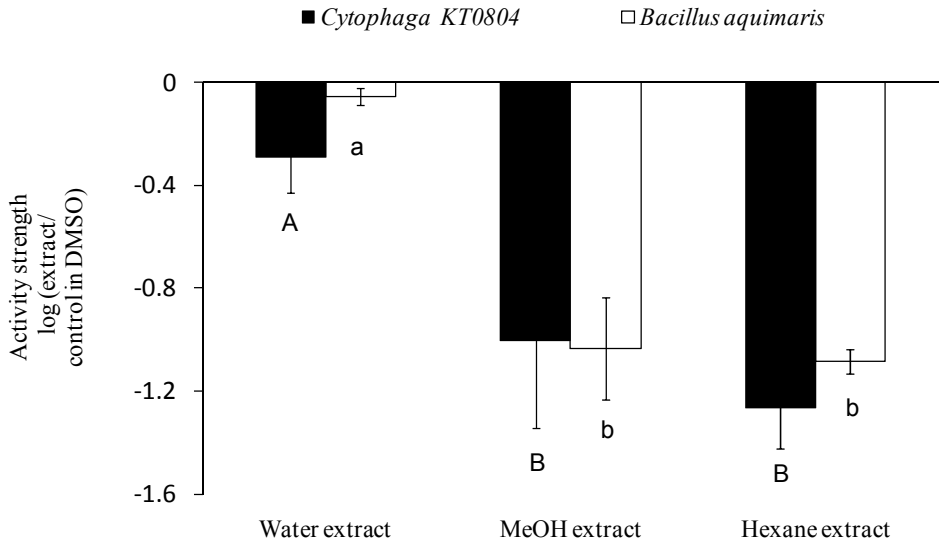

Figure 1. Anti-settlement activity of total water, methanol and hexane surface extract of *Fucus vesiculosus* (mean ±SE, n = 3). Different capital and small letters indicate significantly different treatment responses of *Cytophaga KT0804* and *Bacillus aquimaris* (Tukey's test, $p < 0.05$).

The other fractions GCC-A to GCC-F had a weak effect, no effect at all or even a positive effect on the settlement of two bacterial strains (Figure 2ii). Fraction GCC-G was statistically significantly different from all the other fractions when tested at natural (*Cytophaga KT0804,* $n = 4$, $F = 59$, $p < 0.001$; *B. aquimaris,* $n = 4$, $F = 43$, $p = 0.002$, one-way ANOVA, Tukey's HSD, Figure 2i) and twice the natural concentration against both strains (*Cytophaga KT0804,* $n = 3$, $F = 42$, $p < 0.001$; *B aquimaris*, $n = 3$, $F = 32$, $p < 0.001$, one-way ANOVA, Tukey's HSD, Figure 2ii) .

An analytical NP-HPLC profile with PDA and ELSD detectors of fraction GCC-G revealed the presence of two major and one minor compound peaks. They were collected as different preparative NP-HPLC fractions (fraction G23, G24 and G27). Fraction G27 produced a

significant inhibitory effect (*Cytophaga KT0804,* n = 3, F = 122, p < 0.001; *B. aquimaris, n = 3,* F = 14, p = 0.002, one-way ANOVA, Tukey's HSD, Figure 3).

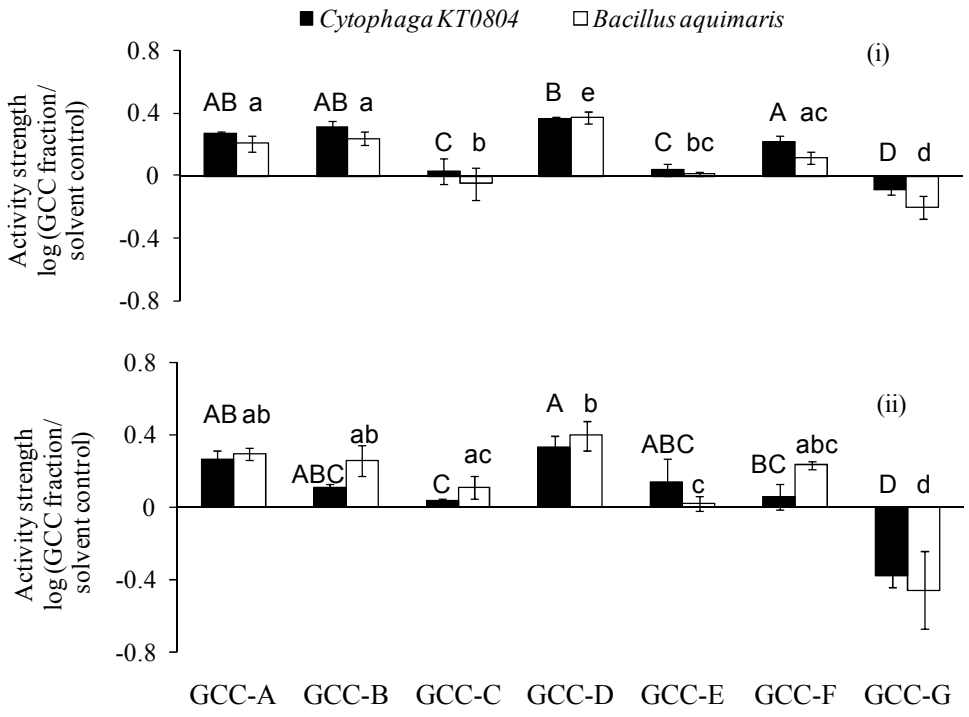

Figure 2. Anti-settlement activity of NP-GCC hexane fractions (A-G) of hexane surface extract of *Fucus vesiculosus* (mean ±SE, n = 3 for (i) and 4 for (ii)).The fractions were tested at natural concentration (i) and twice the natural concentration (ii). Different capitals and small letters indicate significantly different treatment responses of *Cytophaga KT0804* and *Bacillus aquimaris* (Data in (i) and (ii) were tested independently, Tukey's test, p<0.05 in (i) and p < 0.01 in (ii))

Analytical NP- HPLC of fraction G27 showed the presence of two minor peaks, which were fractionated. Fraction G27a showed a more pronounced anti-settlement effect (log effect ratio value ranging from – 0.45 to – 0.01) while G27b had a minor effect (log effect ratio value ranging from – 0.29 to + 0.05) (t-test, p < 0.05, Figure 4i). However, the two sediment strains were inhibited more by fraction G27b when compared to G27a. Additionally, fraction G27a had less inhibitory effect (log effect ratio ranging from – 0.02 to - 0.12 on the bacterial strains isolated from the surface of the alga itself (Figure 4ii, t-test, p > 0.05).

Characterisation of the isolated compound

UPLC-MS analyses of fraction G27a showed the presence of the tetraterpenoid fucoxanthin. After gradient optimization for the UPLC-PAD-MS, chromatograms of fraction G27a shown were obtained (Supplementary information Figure S1). The resulting UV-Vis spectra revealed the typical broad absorption band at 450 nm, which as attributed to a polyunsaturated system. The ESI-pos mass spectra showed clear evidence from the pseudo-molecular ions $[M+H^+]^+$ and $[M+Na^+]^+$ that the active molecule has a molecular monoisotopic mass of 658.4 Da. These data suggested fucoxanthin, which was found previously in *F. vesiculosus* (Hertzberg et al. 1977). To test this hypothesis, a comparison of retention time and spectral properties of the fraction G27a with a commercial standard was performed. The comparison of all the analytical parameters (Mass-, UV-Vis spectra and retention time) was in perfect accordance with the standard (data not shown).

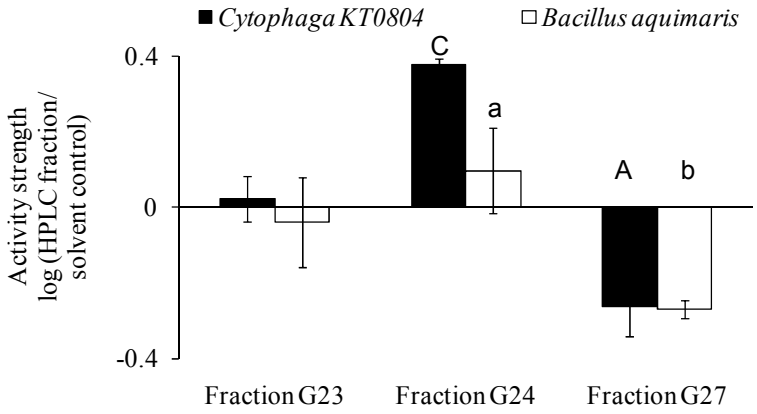

Figure 3. Anti-settlement activity of NP-HPLC purified fractions of NP -GCC active fraction G (mean ±SE, n= 3). Different capitals and small letters indicate significantly different treatment responses of *Cytophaga KT0804* and *Bacillus aquimaris* (Tukey's test, $p < 0.05$)

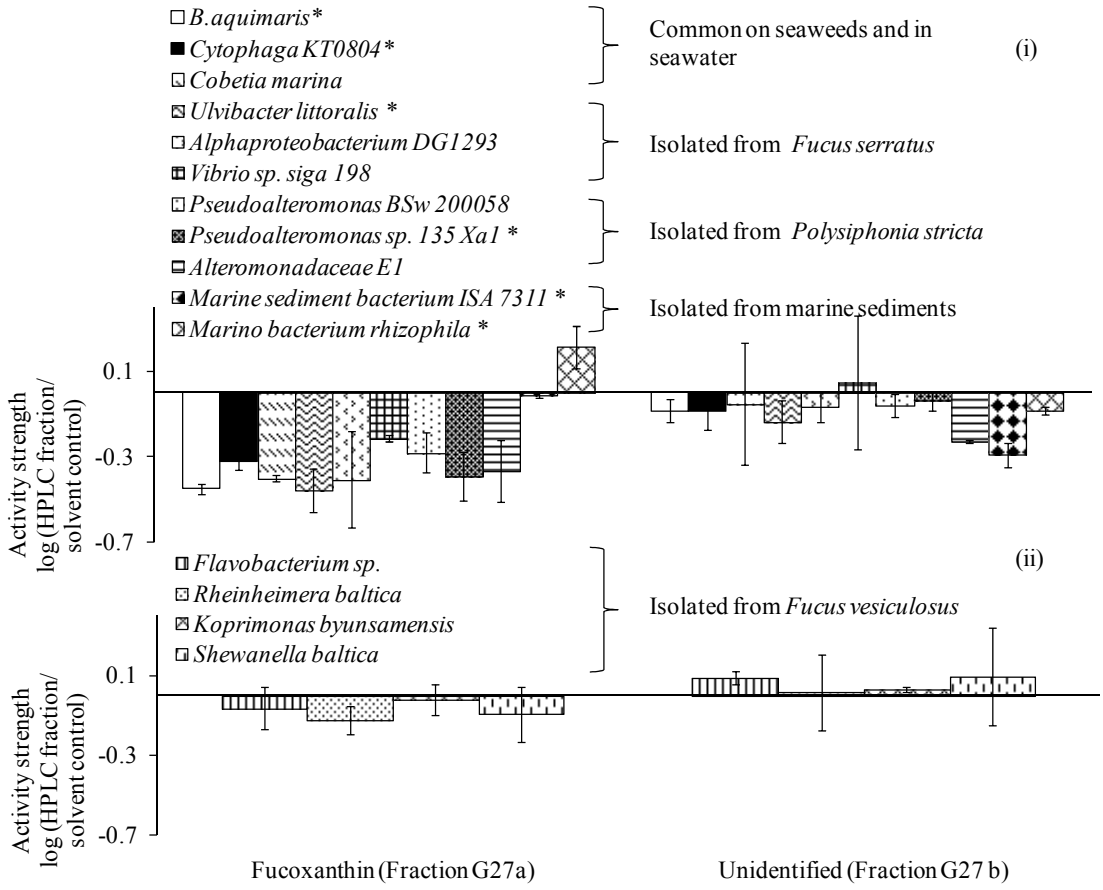

Figure 4. Anti-settlement activity of a fraction containing fucoxanthin and an unidentified fraction against 11 bacterial strains isolated from different marine environments (i) (mean ±SE, n = 2 or 3, t-test, $p < 0.05$) and 4 bacterial strains isolated from *Fucus vesiculosus* (ii) (mean ± SE, n = 2, t-test, $p > 0.05$). Significantly different effects of the two fractions on each bacteria are indicated by asterisks.

Most of the NP-HPLC profiles showed the presence of one, two or sometimes three additional isomers along with the major contribution of the trans isomer (Supplementary Information Figure S2). The 'cis isomers' were formed as isolation artefacts, resulting from light or heat exposure (Haugan and Jennings 1992).

Surface presence of fucoxanthin

A comparison of the NP-HPLC profiles of fucoxanthin extracted with 'dipping technique' and 'swabbing technique' and standard fucoxanthin resulted in matching chromatograms (Supplementary Information Figure S3). Microscopic investigation after treatment with Evans blue did not reveal the presence of any lysed cells after the application of both

extraction techniques when compared to severely damaged and undamaged controls (Supplementary Information Figure S4). The average natural surface concentration of fucoxanthin on the algal tip surfaces extracted by the 'swabbing technique' was found to be 693 ng cm^{-2} (S.D = ±182).

GeO_2 treated algae did not look unhealthy or morphologically different when compared to control algae. Microscopic investigation of the treated algae revealed no contaminating diatoms, whereas they were present on the control algae. NP-HPLC profiles of extracts obtained by dipping confirmed the presence of fucoxanthin on the surface of *F. vesiculosus*, even after elimination of the diatoms (data not shown). Control (untreated) algae yielded 9.07 µg cm^{-2} of fucoxanthin whereas the GeO_2 treated algae yielded 5.68 µg cm^{-2}, indicating the presence of approximately 1.6 times more fucoxanthin on *F. vesiculosus* with its intact biofilm components, including diatoms. There was no statistically significant difference in the inhibitory effects of fucoxanthin extracted from the GeO_2-treated and untreated algae when tested at natural concentration (t-test, $p > 0.05$).

Anti-settlement activity of surface coated fucoxanthin

In the concentration range between 0.3 and 10 µg cm^{-2} surface-impregnated fucoxanthin inhibited the settlement of *Cytophaga KT0804*, *Bacillus aquimaris*, *Alteromonadaceae A1* and *Ulvibacter littoralis* in a dose dependent manner (Figure 5). Based on best-fitting logistic functions the maximal settlement inhibition of all four isolates was in the range between -0.51 and -0.69, while the necessary dose of fucoxanthin for 50 % settlement inhibition was computed to be between 1.4 and 6.0 µg cm^{-2} (Table 1). The marine sediment bacterium ISA 7311 was less sensitive towards fucoxanthin. Only at the highest tested concentration settlement of this isolate was significantly reduced (One-way-ANOVA and Tukey-test, $p < 0.05$; Figure 5). As a consequence, the maximal settlement inhibition and EC_{50} could not be computed for this strain.

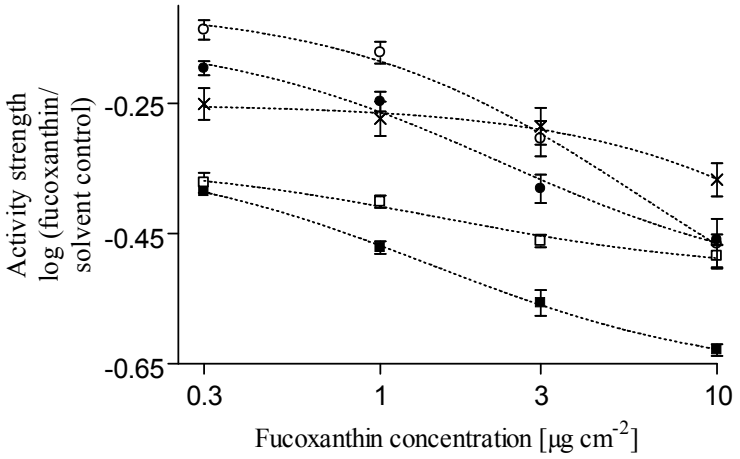

Figure 5. Anti-settlement activity of surface coated Fucoxanthin against *Cytophaga KT0804* (●), *Bacillus aquimaris*(○), *Ulvibacter littoralis* (■), Alteromonadaceae E1 (□), and Marine sediment bacterium ISA 7311 (X). Mean ± SE, n = 4, lines represent best fitting logistic functions. See table 1 for details of best fitting functions.

Table1. Maximal settlement inhibition (+95% CI) by and EC_{50} (95% CI in brackets) of fucoxanthin as computed from the best fitting logistic functions for four different bacterial isolates. See figure 5 for further details.

Strain tested	Maximal settlement inhibition [log (fucoxanthin/ solvent control)]	EC_{50} [µg cm^{-2}]	r^2 of fit
Cytophaga KT0804	-0.54±0.08	2.3 (1.0-5.2)	0.9314
Bacillus aquimaris	-0.69±0.31	6.0 (1.5-23.0)	0.8877
Ulvibacter littoralis	-0.67±0.05	1.4 (0.7-1.9)	0.9475
Alteromonadaceae E1	-0.51±0.05	1.5 (0.3-7.6)	0.7759

Discussion

An initial investigation of the total non-polar (hexane), polar (methanol) and very polar (water) surface extracts hinted at the presence of both active non- polar and polar inhibitors of bacterial settlement at the surface of *F. vesiculosus*. Chemical analysis of the polar fraction provided evidence for one and/or two anti-bacterial active polar metabolite(s), suggesting a multiple chemical defence strategy of the alga. The bioassay-guided fractionation of the total non-polar surface extract resulted in isolation of the tetraterpenoid fucoxanthin, which was the most inhibitory compound among the lipophilic metabolites. At the natural concentration, the purified compound proved to have a significant inhibitory effect on 9 of the 11 bacterial strains tested, indicating its wide range antibacterial activity. The compound was active when tested at natural per volume concentration and also when tested at natural per area concentrations against five of the strains tested. The results regarding theactivity strength of fucoxanthin are generally in accordance with the study of Viano et al. (2009) which was conducted with total tissue extracts from the Mediterranean brown alga *Dictyota* sp. However, the anti-settlement activity strength is species specific. Fucoxanthin had a significant effect on almost all the bacterial isolates tested, with the exception of the strains isolated from *F. vesiculosus* itself. This possibly explains why these strains are present on the surface of *F. vesiculosus*.

The structure of fucoxanthin, which is the major xanthophyll of brown algae and diatoms, was first elucidated by Bonnet et al. (1969). Fucoxanthin is an accessory allenic carotenoid pigment that is found in the chloroplasts of brown algae and other heterokonts. All-E (3S,5R,6S,3’S,5’R,6’R)-fucoxanthin is the only naturally occurring isomer in brown algae; the mono-Z isomers (13-Z,9’-Z and 13’-Z) were shown to be isolation artefacts formed in solution (Andrie´ Haugan and Liaaen-Jennings 1992). NP-HPLC suggested that the cis isomers of fucoxanthin were also detected in the present study, but their position was not

determined. Recently the anti-plasmodial, anti-inflammatory (Heo et al. 2010), anti-pigmentary (Shimoda et al. 2010) and UV-protective effects of this metabolite have been demonstrated. Fucoxanthin has also been reported to be one of the natural inducers of coral larval metamorphosis in a study conducted by Kitamura et al. (2007). The role of fucoxanthin as an anti-microbial metabolite has been described in Viano et al. (2009) against *Pseudoalteromonas* sp D41 and by Gerasimenko et al. (2010). The possible ecological role of this metabolite was not investigated, as all the studies referred to above reported the effect of total algal tissue extracts. Also, the latter pharmacological study only tested against ecologically irrelevant non-marine bacteria (but see Lachnit 2010 for test against marine bacteria). An interesting aspect of the present study is the finding of fucoxanthin on an algal surface. Secretion of this metabolite by *Fucus* or other brown macroalgae was not reported previously and it has been considered that fucoxanthin solely functions as a pigment. This is reminiscent of the unconventional anti-bacterial (Nakamura et al. 2002) and anti-protozoan (Matz et al. 2008) role of the pigment violacein, as well as the dual role of polyphenolics as feeding deterrents (Hay 1996) and as UV shading components in the cell walls of brown algae (Schoenwaelder and Clayton 1999). Two different surface extraction methods confirmed the presence of fucoxanthin at the algal surface. The 'dipping technique' is based upon direct contact of alga (and its surface microlayer) with solvent. It therefore allows for a relatively complete extraction of surface-bound compounds (de Nys et al. 1998), but it possibly poses an elevated risk of membrane disruption. Therefore the 'swabbing' extraction technique was also applied. This method is only based upon mechanical removal of surface-bound metabolites with cotton swabs, and is possibly more gentle, but less complete than the 'dipping' method. Both methods resulted in the extraction of fucoxanthin, while microscopic observation showed the algal epidermis was intact and therefore fucoxanthin was not contaminated by extraction from chloroplasts. Fucoxanthin was also extracted from algal

surfaces with and without the presence of associated diatoms. However, a 1.6-fold higher concentration was detected when diatoms were present, suggesting that the host alga contributes fucoxanthin to the surface, but the extraction also picks up cellular or surface fucoxanthin from diatoms. It has been shown that microalgae present on macroalgal surfaces may lose their structural integrity through mechanical damage resulting from grazing, surf, osmotic shock or bacterially-induced lysis. Such impacts are known to lead to the release of free fatty acids from the phospholipids of cell membranes and galactolipids of chloroplasts in damaged diatoms (Pohnert 2002; d'Ippolito et al. 2004; Wichard et al. 2007) and could possibly also cause release of fucoxanthin. Thus, harbouring a thin film of epiphytic diatoms might support the chemical defence of macroalgae against bacterial settlement. This kind of defence could be considered to be metabolically inexpensive for the host. However, similar inhibitory effects were detected after application of fucoxanthin extracted from F. vesiculosus that had been treated with GeO_2, which suggests that even in the absence or a reduced presence of diatoms, the metabolite is present at a sufficient concentration to hinder bacterial settlement.

Approximately 13 times more fucoxanthin was detected on the surface of whole plants (extracted by the 'dipping technique') as compared to tips (extracted by 'swabbing technique'). This discrepancy may result either from different extraction efficiencies of both methods or from the fact that a part of the fucoxanthin bound to the algal surface is contributed by associated diatoms, which are usually less abundant on apical parts compared to more mature parts of rockweeds (Cundell et al. 1977). The settlement of four out of five bacterial isolates from the coastal marine realm (none of them originating from *Fucus*) proved to be 50% inhibited when fucoxanthin was present at surface concentrations between 1.4 and 6.0 $\mu g cm^{-2}$. A fifth isolate proved to be less sensitive and was only affected at fucoxanthin concentrations of 10 $\mu g cm^{-2}$ or more, while it was almost unaffected by *Fucus* extract

at the natural concentration. Given that concentrations of up to 9.01 μgcm^{-2} were detected on *Fucus* surfaces, fucoxanthin appears to be an ecologically relevant defence compound in *F. vesiculosus.*

Until now, only a few studies have measured the surface concentrations of algal antifouling metabolites. Palisadin A and aplysistatin have been reported to be present on the surface of *Laurencia obtusa* at a concentration of < 1 ng cm^{-2} (de Nys et al. 1998). Concentrations ranging from 100–500 ng cm^{-2} of halofuranones were detected on the surface of the red alga Delisea pulchra and have been found to be sufficiently high to deter ecologically relevant bacteria (Dworjanyn et al. 1999). Compared to these compounds fucoxanthin appears to be less efficient, as higher concentrations were necessary for settlement inhibition. The reduced efficiency, however, is apparently compensated by an increased availability of the photosynthetic pigment fucoxanthin. Studies on the red alga *Asparagopsis armata* have shown that halocarbons like bromoform (0.58% to 4.3% of dry weight) and dibromoacetic acid (0.02% to 2.6% of dry weight) are present at high concentrations on the algal surface (Paul et al. 2006). To the authors' knowledge, this study represents the first surface quantification of an anti-bacterial compound from a brown alga (but see Brock et al. 2007 for the quantification of phlorotan nin, which has been speculated to be an inhibitor of barnacle settlement). In contrast to some of the above mentioned studies, it has not yet been possible to demonstrate the mode of action of fucoxanthin on bacterial settlement (as, for example, by the haloge- nated furanones from *D. pulchra*). In conclusion, it is demonstrated here that a metabolite that was hitherto considered to have a primary function as a photosynthetic pigment can also play a major role in the surface defence chemistry of brown algae. This is due to apparent releasemechanisms that lead to enhanced surface concentrations of the metabolite. This unprecedented role of pigments represents a novel defence strategy in marine algae. The finding of fucoxanthin as the only ecologically relevant lipophilic

metabolite on the surface of *F. vesiculosus* implies that it is the most important nonpolar com- pound active against bacterial surface colonisation. The work conducted so far on *F. vesiculosus* has provided an insight into an algal–bacterial interaction through chemical modulation of bacterial fouling, and thus requires more detailed future investigation.

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Supplementary Information

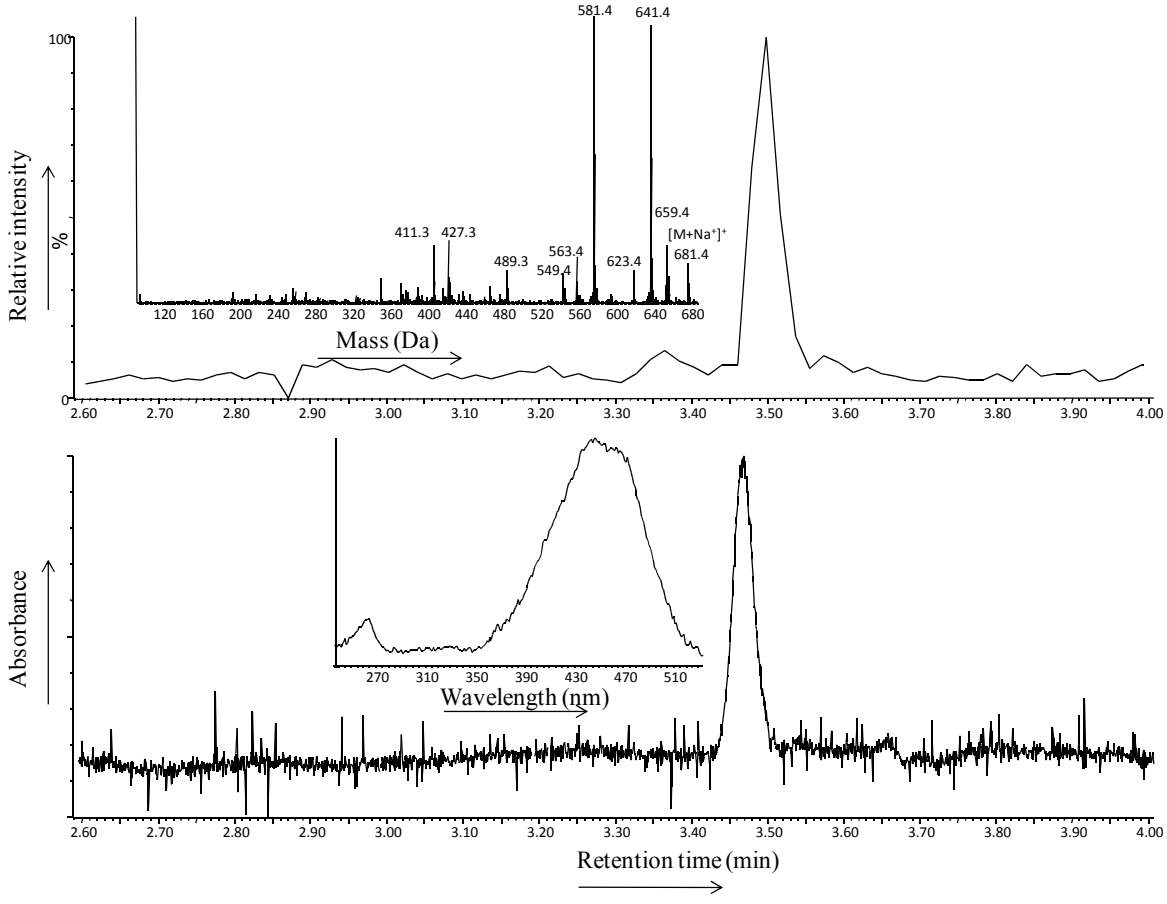

Figure S1. UPLC-MS analyses of the pure fraction G27a. UV-VIS-photodiodearray detection (lower chromatogram, detection 190-500 nm with insert of UV spectrum at 3.48 min). Upper chromatogram ESI-pos-MS (m/z = 659.4) with insert of the corresponding MS.

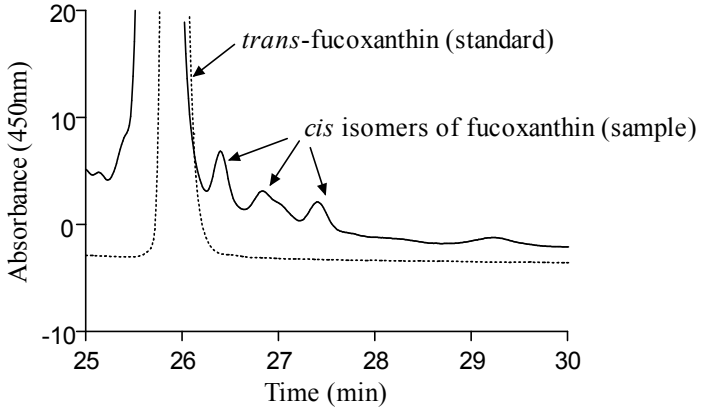

Figure S2. NP-HPLC profile comparison for isomers among standard fucoxanthin (dotted line) and fucoxanthin obtained from non-polar surface extract (sample) prepared by 'dipping technique'(solid line). Gradient: 10mins of 100% *n*-Heptane; linear gradient to 100% EtOAc until 30mins.

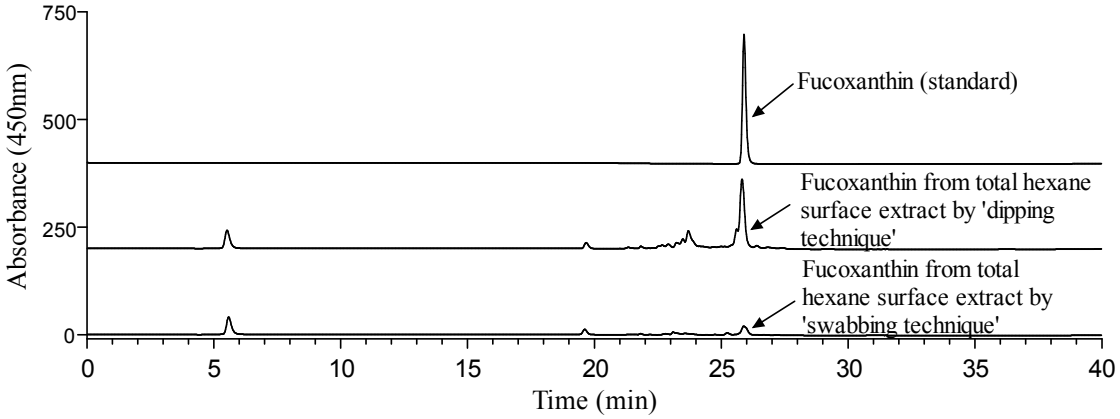

Figure S3. NP-HPLC profile comparison among standard fucoxanthin and fucoxanthin obtained from *Fucusvesiculosus*with two different extraction methods. Gradient: 10mins of 100% *n*-Heptane; linear gradient to 100% EtOAc until 30mins.

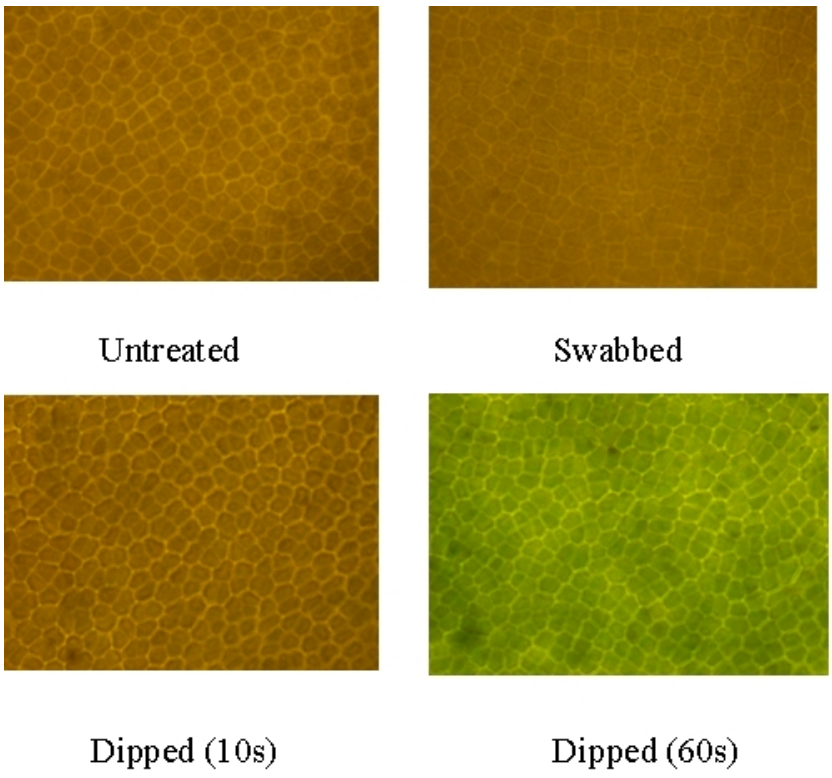

Figure S4.Thallus surface of un-treated and treated (swabbing and dipping technique (10secs and 60secs)) *Fucus vesiculosus*. Incorporation of Evans blue at 60secs indicated cell lysis.

Polar metabolites from the surface of the brown alga *Fucus vesiculosus* inhibit bacterial settlement

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Abstract

It has been previously demonstrated that polar and non-polar surface extracts of the brown alga *Fucus vesiculosus* inhibit bacterial settlement at natural concentrations. Fucoxanthin has been identified as the active metabolite present in the non-polar extract. In the present study we describe the bioassay guided identification of the active metabolites from the polar fraction. Chromatographic separation on a size-exclusion liquid chromatography column (SEC) and bioassays identified an active fraction that was further investigated using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). This fraction contained the metabolites dimethylsulphopropionate (DMSP), proline and alanine. The antibacterial activity was caused by DMSP and proline. The metabolites were further quantified on the algal surface. DMSP and proline were detected in the range of 0.12 to 1.08 ng cm^{-2} and 0.09 to 0.59 ng cm^{-2}, respectively. These metabolites were tested in a concentration range of 0.1 to 1000 ng cm^{-2} against the settlement of five bacterial strains isolated from co-occuring algae and sediment. DMSP and proline surface concentrations between 0.005 to 0.38 and 0.01 to 0.13 ng cm^{-2}, respectively resulted in 50% inhibition of five and four of these strains, respectively. Thus, this study shows that DMSP and proline

have an ecologically relevant role as surface inhibitors against bacterial settlement on *F. vesiculosus*.

Keywords: Chemical defence, *Fucus*, DMSP, proline, anti-bacterial, anti-fouling.

Introduction

Marine macroalgae produce a diverse range of chemical compounds that play a significant ecological role in the marine environment including protection against natural enemies (for example, microbes and herbivores), settlement cues and competitive interactions (reviewed in Paul et al. 2006). Chemical compounds from seaweeds are also known to inhibit foulers which include bacteria as primary colonisers (e.g. Sieburth and Conover 1965).

Seaweeds provide a microniche rich in nutrients that promotes bacterial colonisation, which in turn can have detrimental effects on the host (Littler & Littler 1995; Sunairi et al. 1995; Sawabe et al. 1998; Vairappan et al. 2001). Thus, there should be a demand for defence against bacteria in seaweeds and chemical antifouling defence has been reported in a number of studies (e.g. de Nys et al. 1991; Schmitt et al. 1995). However, ecological roles of specific algal inhibitors against microbes have been demonstrated for few species (Kubanek et al. 2003; Paul et al. 2006; Nylund et al. 2008; Saha et al. 2011). Furanones from *Delisea pulchra* have been quantified and studied as antifoulants (de Nys et al. 1998; Dworjanyn et al. 1999). However, less is known about the role of algal metabolites as settlement inhibitors of microbes (but see Paul et al. 2006; Nylund et al. 2010; Saha et al. 2011 for reports on microbial inhibitors).

In the Western Baltic, the perennial rockweed *F. vesiculosus* occurs mainly between mean sea surface level and 3m depth. A variety of abiotic stress factors are common in this habitat and some of them have been predicted to increase in the course of ongoing climate change (Wahl et al. 2010). Presumably because of eutrophication and epibiosis, the alga has evinced

a dramatic decline in spatial range and biomass and has already retreated from its earlier distribution range in the Western Baltic (Vogt & Schramm 1991; Wahl et al. 2010). The jeopardised existence of this alga in the course of climate change makes it important to understand the detailed interaction of this alga with its potential foulers.

In spite of high bacterial densities in the Kiel fjord [0.7 to 2.24 x 10^6per ml seawater (mean of monthly samplings between 2005 and 2008), H.J. Hoppe & R. Koppe, pers. comm.], this alga usually manages to remain largely free from fouling throughout most of the year, hinting at the presence of chemical defence mechanisms (Brock et al. 2007; Saha et al. 2011).

A previous study on the chemical anti-bacterial defence of *F. vesiculosus* revealed the surface presence of non-polar and polar active metabolites against bacterial settlement. Both hexane (non-polar extract) and methanol (polar extract) surface extracts of *F. vesiculosus* inhibited bacterial settlement strongly and to a similar extent, while the water (very polar extract) surface extract was less efficient (Saha et al. 2011). Investigation of the non-polar extract has led to the identification of the compound fucoxanthin as surface inhibitor in *F. vesiculosus* (Saha et al. 2011). Until now mainly non-polar compounds have been reported as algal anti-foulants (de Nys 1998; Schmitt et al. 1995; Nylund et al. 2008; but see Harder et al. 2004 for report on polar compounds). This may be due to the fact that non-polar compounds have a better chance of accumulation at algal surfaces than polar compounds (Jennings and Steinberg 1997), which in turn may have a higher probability of diffusion into the surrounding water column.

The present study is focussed on the identication of the metabolites responsible for the anti-settlement activity in the MeOH surface extract of *F. vesiculosus* and in assessing their possible ecological role as natural antifoulants against bacterial settlement. Bioassay-guided fractionation of MeOH extracts was used, in order to test extractable polar metabolites for

their antibacterial activity. It was also investigated whether, and at what concentrations, the deterrent metabolites are presented in nature to colonising bacteria by determining surface concentrations of the metabolites on algae collected in the field. Isolated bacteria from seaweeds co-occurring with *F. vesiculosus* were used as test organisms, in order to test the antimicrobial effects of metabolites on ecologically relevant bacteria. Bacterial strains present on the surface of *F. vesiculosus* were not investigated because they were expected to be relatively insensitive (see Saha et al. 2011 for details).

Materials and methods

Algal material and extraction of metabolites

Specimens of *F. vesiculosus* were collected from the littoral zone of Kiel Fjord, Germany (54°26'N/10°11' E) in December 2009 and transported to the laboratory in a cool box. For surface quantification, the algal individuals (n=4) were collected from Laboe, Germany (54°40'N/10°21'E) in March 2011. For the quantification of active metabolites, young algal fronds up to a maximum length of 10 cm were extracted. Prior to extraction, the algae were held in saturated seawater atmosphere to avoid dessication and damage. For surface extraction algal fronds were dipped for 10 secs into a mixture of MeOH (methanol): hexane (1:1 v/v) as described in Saha et al. (2011) and the resulting extract was concentrated to dryness under reduced pressure. The residue was extracted in hexane to remove non-polar metabolites that inhibit bacterial settlement, such as fucoxanthin. The step was repeated until the solvent appeared colorless. The remainder after this hexane treatment was taken up in MeOH to yield the polar metabolites. The final residue after this MeOH treatment contained highly polar compounds and was dissolved in water.

Bacteria and anti-settlement assay

Cytophaga KT0804 (isolated from the brown alga *Halidrys siliquosa* and also detected on the brown alga *Saccharina latissima*) and *Bacillus aquimaris* (isolated from *Halidrys siliquosa* and also detected on the brown alga *Desmarestia aculeate* and the red alga *Ahnfeltia plicata*) were used in all the bioassays, in order to screen the activity of the extracts/compounds against gram-negative and gram-positive microorganisms, respectively. Three additional strains isolated from different algal species and marine sediment were used to test pure dimethylsulphopropionate (DMSP) and proline: *Ulvibacter littoralis* (isolated from the brown alga *Fucus serratus*), *Alteromonadaceae E1* (isolated from the red alga *Polysiphonia stricta*) and bacterium ISA 7311 (isolated from marine sediment) (Saha et al. 2011).

The bacterial strains were grown in nutrient media (5 g peptone + 3 g yeast in 1 L filtered seawater) for 18 to 20 h. Prior to the assays, the optical density (OD) of the bacterial cultures was determined with a Beckman Du® 650 spectrophotometer (λ 600 nm). The working cultures had an OD in the range of 0.5-0.8. The bacteria in suspension were confronted in multi well plates (96 wells, flat bottom, Greiner®) with algal extract, size-exclusion chromatography fractions (SEC), or solvent controls. The 96 well plates were incubated for 1 h on a shaking table (100 rpm) at 20ºC. After that, the bacterial suspension was removed from the wells and unattached cells were eliminated by gently rinsing twice with 100 µL of sterile filtered seawater (SSW). The attached cells were quantified by staining (10 min) with the fluorescent DNA–binding dye Syto 9 (0.005mM) (Invitrogen, GmbH). The fluorescence was subsequently measured (excitation 477-491 nm, emission 540nm) in a plate reader (Hidex Chamaeleon, Turku, Fi) as a proxy for bacterial settlement in terms of cell density.

Bioassay guided fractionation

Polar extract was fractionated using SEC on Sephadex LH20 (65 x 2 cm, Sigma-Aldrich, Germany) using isocratic elution with MeOH (UV detector 254nm, Biorad biologic

chromatography system). The fractions were collected at a time interval of 5 min (flow rate 2.5mL min^{-1}), pooled on the basis of observed UV peaks (Figure 1) and tested for settlement inhibiting activity.

Surface volume based assay with SEC fractions

The SEC fractions were tested at their natural concentration identical to the concentration present in the algal surface volume, which was calculated as described in Saha et al. 2011. Ninety-seven µL of bacterial suspension were added to the wells. Three µL of extract /SEC fractions (dissolved in DMSO) present at 33.3 fold of natural concentration were added to the suspension so that the tested fractions in the final mixture were diluted to their natural concentration. Bacteria were never exposed to DMSO concentrations over 5%, in order to prevent toxic effects (Wahl et al. 2010). DMSO containing fractionated solvent residue was taken as a control when SEC fractions were tested. This was done to assure that solvent presence was not an artefact confounding the results. To prepare SEC solvent residue fractions, solvent containing no extract was fractionated in a similar manner as the MeOH extract.

Analysis of the bioactive SEC fraction and quantification of settlement inhibiting compounds

The active LH20 fraction was evaporated until dryness and dissolved in CD_3OD. Structure elucidation was based on 1D- and 2D- NMR spectroscopy and comparison with synthetic DMSP hydrochloride. DMSP hydrochloride was synthesised by applying general procedures (see Chambers 1987). This metabolite could, however not explain all observed signals in the NMR (see Supplementary Information Figure S1). The active LH20 fraction was further analyzed by GC-MS after silylation with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) in pyridine.

A GCT-Premier (Waters Micromass, UK) time-of-flight mass selective detector coupled to an Agilent 6890N gas chromatograph was used for analyzing the derivatised samples.

Injection to the GC was done in split mode (split ratio 1). A DB5-MS column from Agilent (29.5 m x 0.25 mm and 0.25 µm film thickness) was used for separation. The carrier gas was helium 5.0. Constant flow mode was chosen with a flow of 1 mL He/min and 1 µL of the sample was injected into the system. The injector and the transfer line were kept at 280°C. The oven temperature was initially held at 40°C for 5 min and then increased by 30°C/min to 305°C, this temperature was held for 5 min. Metabolites were identified using the NIST library and the identity of the amino acids proline and alanine was verified by co-injection with derivatised commercially available amino acids.

An Aquity UPLC^{TM} BEH HILIC column(1.7 m, 2.1 mm×50 mm) was used for LC separation and a Q-ToF Micro mass spectrometer (Waters Micromass, UK) was used for detection and quantificationof the amino acids and DMSP according to Spielmeyer and Pohnert 2010.

Surface quantification of DMSP, proline and alanine

Quantification of DMSP in the surface extracts was done according to Spielmeyer and Pohnert (2010). Quantification of proline and alanine was done using the same LC-MS method after external calibration with three concentrations of the commercially available amino acids.

Surface area based test of settlement inhibiting components

After the identification of settlement inhibiting components these were further tested as a surface coating for their anti-settlement activity. Amino acids were bought from Sigma Aldrich, Germany. DMSP was prepared as per Chambers et al. (1987). DMSP or proline (dissolved in MeOH), as well as alanine, valine or isoleucine (all dissolved in water) were pipetted in 96 well plates and the solvent was evaporated in order to coat the bottom and side walls of the wells with different concentrations in the range of natural concentrations. 108µL (to the wells coated with DMSP and proline) and 100µL (to the wells coated with alanine,

valine and isoleucine) of bacteria suspension was added. MeOH and SSW were used as a solvent controls, respectively.

Statistics

The activity strength of extract, SEC fractions and the individual compounds was expressed as log effect ratio (Wahl et al. 2004). A log effect ratio of 0 indicates no effect on bacterial settlement, whereas a negative value indicates settlement inhibition and a positive value indicates a pro-settlement effect. 1 way ANOVA was conducted to compare log effect ratio distributions. Homogeneity of variances was tested using Levene's test ($p < 0.05$). Shapiro-Wilk's test was used to test for normal distribution ($p < 0.05$). Post-hoc comparisons were made using Tukey's honest significant difference test (HSD) ($p < 0.05$). The computer program Statistica, (StatSoft, Tulsa, OK, U.S.A.) was used to conduct all statistical tests. For the analysis of surface coated compound effects, functions that described best fits of the data were computed by iterative adaptation, using the software package Prism 4 (GraphPad, La Jolla, CA) and the logistic function Y=Min+(Max-Min)/(1+10^(log(EC_{50})-X)). In this function X and Y represent compound concentration and settlement response, respectively. Min, Max and EC_{50} are constants describing minimal and maximal responses and the necessary concentration required for 50% inhibition of settlement, respectively.

Results

Bioassay guided fractionation of the polar extract

SEC of the *Fucus* MeOH extract produced from hexane extraction residue yielded five fractions SEC1 to SEC5 (Figure 1), out of which SEC3 produced the maximum inhibitory effect (*Cytophaga KT0804,* $n = 3$, $F = 12$, $p < 0.001$; *B. aquimaris,* $n = 3$, $F = 5$, $p < 0.02$, one-way ANOVA, Tukey's HSD, Figure 2).

Chemical analysis of SEC fraction 3

The ^{1}H-NMR spectrum of fraction SEC3 (Supplementary Information Figure S1) exhibited signals characteristic for DMSP. HSQC, COSY and HMBC measurements confirmed this structure. The structure was verified by comparison with an authentic standard.

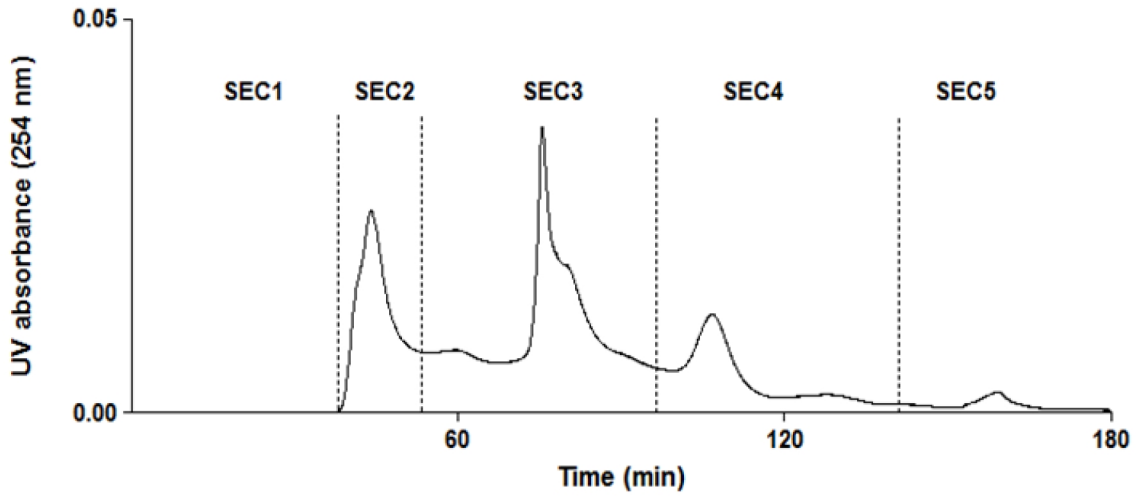

Figure 1. Bioassay-guided size-exclusion fractionation of MeOH surface extract of *F. vesiculosus*. Chromatographic parameters: Sephadex LH20 (2 × 65 cm); 1mL injection; isocratic elution with MeOH at 2.5 mL min^{-1}. Vertical dotted lines indicate the fraction combination.

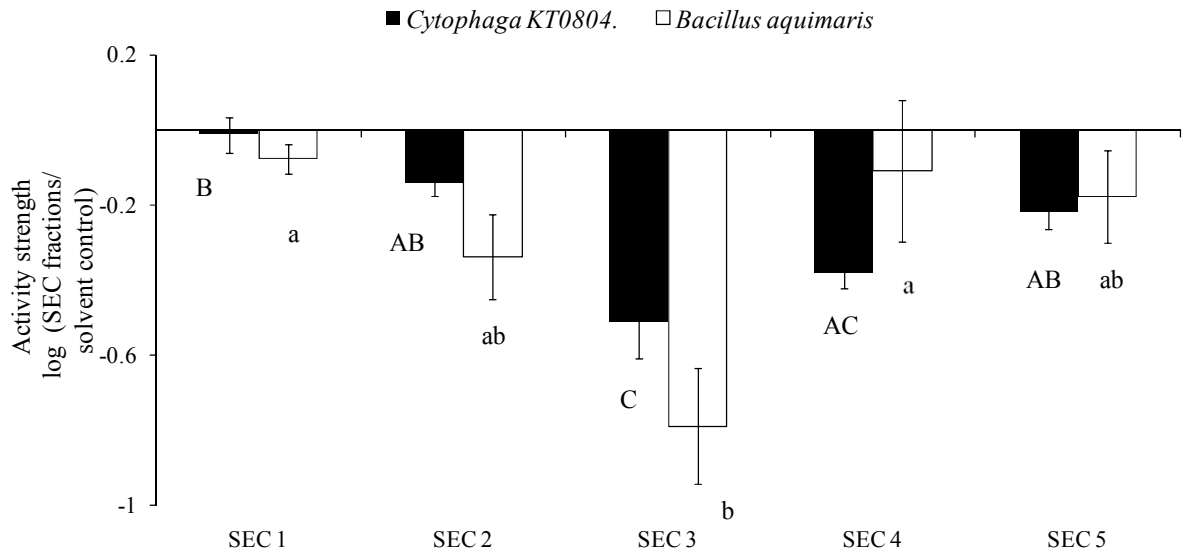

Figure 2. Anti-settlement activity of SEC fractions of MeOH surface extract (mean ±SE, n = 3). Different capitals and small letters indicate significantly different treatment responses of *Cytophaga KT0804* (black bars) and *Bacillus aquimaris* (white bars) (Tukey's test, $p < 0.05$).

Structure elements of two amino acids were also detectable in the ^{1}H-NMR-spectra of the active fraction. By comparison of the chemical shifts from these two spin systems suggested the presence of alanine and proline. The comparison of the proton and carbon shifts showed good accordance with literature values. No indication for a peptide bond of DMSP with an aminoacid could be detected in the HMBC data suggesting a mixture of these three dominant metabolites.

To verify the presence of free alanine and proline the sample was evaporated to dryness and redissolved in 1 mL pyridine. 10 µL MSTFA was added and the sample was heated for 1h to 40 °C and injected directly to the GC-MS system. The mass spectra and retention times were in perfect consonance with amino acid standards derivatised according to the same protocol (Supplementary Information Figure S2 and S3).

Surface quantification of DMSP, proline and alanine

The natural concentrations of DMSP, proline and alanine were found to be in the range of 0.12- 1.08 ng cm^{-2} (mean 0.40 ng +/- S.D. 0.45), 0.09- 0.59 ng cm^{-2} (mean 0.45 ng +/- S.D. 0.43) and 0.09- 1.25 ng cm^{-2} (mean 0.47 ng +/- S.D. 0.54) respectively.

Anti-settlement activity of surface coated DMSP, proline, alanine, valine and isoleucine

Surface coated DMSP inhibited the settlement of all tested strains in the concentration range between 0.1 and 1000 ng cm^{-2} (Figure 3). Based on best-fitting logistic functions the maximal settlement inhibition of all five isolates was in the range between -0.08 and -0.59, while the necessary dose of DMSP for 50 % settlement inhibition was computed to be between 0.005 and 0.38 ng cm^{-2} (Table 1). The two most sensitive strains were *U. littoralis* and Alteromonadaceae E1, which were maximally inhibited by all DMSP concentrations that were tested. The two least sensitive strains were *Cytophaga KT0804*, which was 50% inhibited at a concentration of 0.38 ng cm^{-2}, and Marine sediment bacterium ISA 7311,

which was 50% inhibited at a 5 times lower DMSP concentration, but which was generally relatively little affected.

Also surface coated proline inhibited the settlement of all tested strains in the concentration range between 0.1 and 1000 ng cm^{-2} (Figure 4). The maximal settlement inhibition of all five isolates was in the range between -0.12 and -0.55 and the required dose of proline for 50% settlement inhibition was computed to be between 0.01 and 1.66 ng cm^{-2} depending on the bacterial isolate (Table 2). As with DMSP, *Cytophaga KT0804* required the highest dose of proline for 50% inhibition and ISA 7311 was generally relatively little affected.

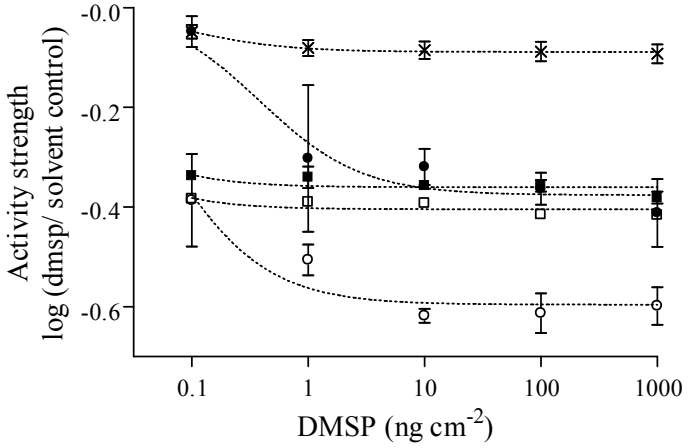

Figure 3: Anti-settlement activity of surface coated DMSP against *Cytophaga KT0804* (●) and *B. aquimaris* (○), *U. littoralis* (□), Alteromonadaceae E1 (■) and Marine sediment bacterium ISA 7311 (X). Mean ± SE, n = 4, lines represent best fitting logistic functions. See table 1 for details of best fitting functions.

Alanine inhibited the settlement of *Cytophaga KT0804* and *Bacillus aquimaris* when tested in the concentration range between 0.984 and 984 ng cm^{-2} (Figure 5). Based on best-fitting logistic functions the maximal settlement inhibition effect of alanine for *Cytophaga KT0804* and *Bacillus aquimaris* were -0.28 and -0.25 respectively and the required dose for 50% settlement inhibition was 18.11 and 9.72 ng cm^{-2} (Table 3).

Table 1: Maximal settlement inhibition (±95%CI) by and EC_{50} (95%CI in brackets) of DMSP as computed from best fitting logistic functions for five different bacterial isolates. See Figure 3 for further details.

Strain tested	***Bacillus aquimaris***	***Cytophaga KT0804***	***U. littoralis***	**Marine sediment bacterium ISA7311**	***Alteromonadaceae E1***
Maximal settlement inhibition [log (DMSP/ solvent control)]	-0.59 (-0.64 to -0.54)	-0.37 (-0.46 to -0.28)	-0.40 (-0.41 to -0.39)	-0.08 (-0.10 to -0.07)	-0.36 (-0.37 to -0.34)
EC_{50} [ng cm^{-2}]	0.05 (0.02 to 0.12)	0.38 (0.06 to 2.15)	0.005 (0.002 to 0.012)	0.08 (0.01 to 0.41)	0.007 (0.001 to 0.02)
r^2 of fit	0.45	0.44	0.32	0.21	0.12

Table 2: Maximal settlement inhibition (±95%CI) by and EC_{50} (95%CI in brackets) of proline as computed from best fitting logistic functions for five different bacterial isolates. See Figure 4 for further details.

Strain tested	***Bacillus aquimaris***	***Cytophaga KT0804***	***U. littoralis***	**Marine sediment bacterium ISA7311**	***Alteromonadaceae E1***
Maximal settlement inhibition [log (proline/ solvent control)]	-0.55 (-0.59 to -0.50)	-0.44 (-0.48 to -0.40)	-0.44 (-0.46 to -0.43)	-0.12 (-0.14 to -0.10)	-0.26 (-0.29 to -0.23)
EC_{50} [ng cm^{-2}]	0.01 (0.001 to 0.07)	1.661 (0.94 to 2.9)	0.01 (0.005 to 0.025)	0.13 (0.04 to 0.40)	0.09 (0.04 to 0.22)
r^2 of fit	0.08	0.87	0.31	0.38	0.47

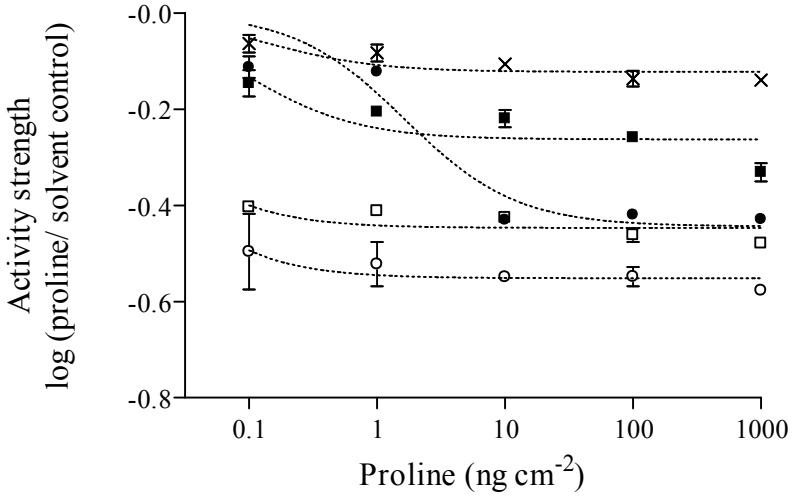

Figure 3: Anti-settlement activity of surface coated proline against *Cytophaga KT0804* (●) and *B. aquimaris* (○), *U. littoralis* (□), Alteromonadaceae E1 (■) and Marine sediment bacterium ISA 7311 (X). Mean ± SE, n = 4, lines represent best fitting logistic functions. See table 2 for details of best fitting functions.

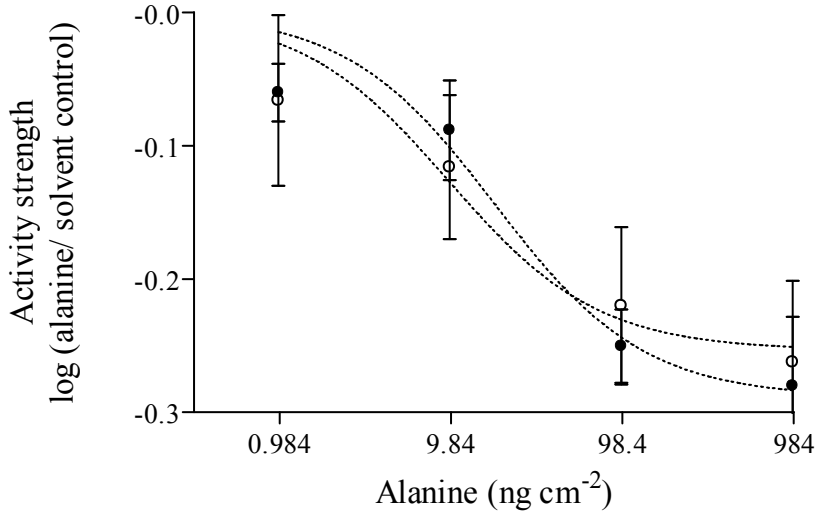

Figure 5: Anti-settlement activity of surface coated alanine against *Cytophaga KT0804* (●) and *B. aquimaris* (○). Mean ± SE, n = 3, lines represent best fitting logistic functions. See table 3 for details of best fitting functions.

Table 3: Maximal settlement inhibition (±95%CI) by and EC_{50} (95%CI in brackets) of alanine as computed from best fitting logistic functions for two different bacterial isolates. See Figure 5 for further details.

Strain tested	***Bacillus aquimaris***	***Cytophaga KT0804***
Maximal settlement inhibition [log (alanine/ solvent control)]	-0.25 (-0.35 to -0.15)	-0.28 (-0.36 to -0.21)
EC_{50} [ng cm^{-2}]	9.72 (1.18 to 79.93)	18.11 (5.06 to 64.8)
r^2 of fit	0.42	0.73

To verify that the effects of alanine and proline were structure specific and not general to amino acids we also tested valine and isoleucine, which did not show any significant anti-settlement activity. Instead, valine had a tendency of probiotic effect at lower concentrations, particularly for the strain *Cytophaga KT0804*. Isoleucine showed a strong probiotic effect on *Cytophaga* whereas it was probiotic for *Bacillus aquimaris* at lower concentrations (Supplementary Information Figures S4 and S5).

Discussion

Bioassay guided fractionation of the methanolic extract of *F. vesiculosus* produced three hydrophilic/ polar metabolites, dimethylsulphopropionate (DMSP), proline and alanine, which all inhibited bacterial settlement. Despite the fact that the NMR indicated that the compounds were present in the extract with nearly identical concentration, no indication for a peptide linkage between the metabolites was detected in NMR and MS data. It cannot be excluded that the three components results from the cleavage of a peptide initially produced by the alga. This would however not be due to the sample handling since NMR analysis of the reference peptide captopril that underwent similar treatment as the extract, showed no hydrolysis during the procedure (data not shown). The compounds in the mixture were not only active when they were tested together (in terms of surface volume), but they also

showed activity when tested individually as a surface coating. Proline and several other amino acids have previously been detected at surfaces of *F. vesiculosus* (Lachnit et al. 2010) and the presence of DMSP in tissue of *F. vesiculosus* has also been reported (Howard & Russell 1995; Lyons et al. 2007).

The methionine derived secondary metabolite dimethylsulphopropionate has several different physiological and ecological functions in marine algae. DMSP along with its associated compounds has so far been shown to function as a cryo-protectant and as an anti-oxidant (Karsten et al. 1996; Sunda et al. 2002; Van Alstyne 1988). Enzymatic cleavage of DMSP leads to the production of dimethlysulphide (DMS) and acrylic acid (Cantoni and Anderson 1956) and may be catalysed by numerous marine bacterial taxa (Howard et al. 2008). DMS plays a role in contributing biogenic sulphur into the atmosphere annually (Andreae 1986). Further, DMS and acrylic acid play a role in the anti-grazing defence of marine algae (Van Alstyne et al. 2001b; Lyons et al. 2007). Our study reports for the first time the surface based role of DMSP as an algal antifoulant. An earlier study of Jackson and Stukey (2007) of the cord grass *Spartina alterniflora* ruled out an effect of DMSP as anti-foulant on ephiphytic algae. A spatial, temporal and taxonomic variation in the presence of this sulphonium compound among macroalgae has been reported (Blunden et al. 1992; Van Alstyne and Puglisi 2007; Lyons et al. 2010). DMSP has been quantified in studies of macroalgae and coral reef invertebrates (Howard & Russell 1995; Alstyne and Slattery 2006; Lyons et al. 2010) and has repeatedly been reported to be particularly present in green algae, which often contain more than ten times as much DMSP as *F. vesiculosus*. The quantification studies with macroalgae were based on the total dry weight and to our knowledge no surface based quantification has so far been realised. A DMSP surface concentration ranging from 0.12 to 1.08 ng cm^{-2} was found for *F. vesiculosus* in the present study. The five tested bacterial strains were inhibited by 50 % when DMSP was present at surface concentrations between

0.005 and 0.38 ng cm^{-2}. Certain heterotrophic bacteria have been found to be attracted to microscale pulses of DMSP (Seymour et al. 2010). Certain bacteria are known to use DMSP as dominant nutrient and metabolize it very quickly (Dickschat et al. 2010). Thus, the alga might be expected to use DMSP in maintaining a specific bacterial community only, similar as observed for the strain specific activity strength of fucoxanthin against bacterial settlement (Saha et al. 2011). DMSP thus seems to be an ecologically relevant anti-bacterial compound on *F. vesiculosus.*

Quite a few studies have demonstrated the anti-microbial activity of proline rich peptides (Yang et al. 2009). In our study an anti-bacterial role of proline, surface associated in *F. vesiculosus* at concentrations of up to 0.59 ng cm^{-2}, has been found. Four out of the five tested bacterial strains (not *Cytophaga KT0804*) were inhibited by 50 % when proline was present at surface concentrations between 0.01 to 0.13 ng cm^{-2}, which shows that this amino acid is clearly present at a sufficient concentration to deter bacterial settlement on the algal surface.

Alanine inhibited settlement of *Cytophaga KT0804* and *B. aquimaris* by 50% at concentrations of 18.11 and 9.72 ng cm^{-2} respectively. Given that a concentration up to 1.25 ng cm^{-2} has been detected on *F. vesiculosus*, it seems that this amino acid only has a feeble anti-bacterial effect when compared to DMSP and proline. The low natural surface concentration of alanine may probably not be absolute and may vary with different environmental factors. To the author's knowledge an anti-bacterial effect of alanine from an algal surface has not been reported yet. In contrast to proline and alanine, valine and isoleucine had a probiotic effect which indicates that the anti-bacterial activity is specific and is not generalised for all amino acids. Interestingly, those probiotic effects were detected at lower rather than higher concentrations, which seem to hint at interacting antibiotic effects at higher concentrations.

Table 4. Phyologenetic specificity and concentrations of surface associated anti-bacterial compounds (reported until now).

Compound	**Compound concentration**	**Phyologenetic specificity**	**Literature**
Halogenated furanones	100 to 500 ng cm^{-2}	Gram negative	de Nys et al. 1998, Maximilien et al. 1998, Dworjanyn et al. 1999
1,1,3,3-tetrabromo-2-heptanone	3.6 µg cm^{-2}	Gram positive	Nylund et al. 2008
Fucoxanthin	0.7 to 9 µg cm^{-2}	Gram positive and negative	Saha et al. 2011 (chapter I)
DMSP	0.12 to 1.08 ng cm^{-2}	Gram positive and negative	Saha et al. (chapter II)
Proline	0.09 to 0.59 ng cm^{-2}	Gram positive and negative	Saha et al. (chapter II)

The deterring activity of DMSP, proline and specifically alanine is concentration dependent and their concentration may vary, probably regulated by genetic, temporal and/or spatial conditions, which might then regulate whether an individual would be well defended or not. Further, the effects of DMSP, proline and alanine apparently vary when different bacterial species are tested. Compared to less polar surface associated settlement inhibitors in algae DMSP, proline and alanine all reach relatively low concentrations in the range between 0.09 and 1.25 ng cm^{-2}. Fucoxanthin was detected at concentrations between 0.7 and 9 µg cm^{-2}, furanones from *Delisea pulchra* at 100 to 500 ng cm^{-2} (de Nys et al 1998; Dworjanyn et al. 1999) and 1,1,3,3-tetrabromo-2-heptanone in *B. hamifera* at 3.6 µg cm^{-2} (Table 4). This difference between polar and nonpolar compounds may possibly be due to differential solubilities in the surrounding water. Nonetheless, the polar compounds identified here are obviously sufficiently concentrated to affect associated microorganisms.

In conclusion, this study shows that DMSP and proline along with the non-polar metabolite fucoxanthin (Saha et al. 2011) have an ecologically relevant role as natural anti-foulants in *F.vesiculosus*, whereas the third metabolite alanine was not found to be sufficiently concentrated on the surface of *Fucus* to produce an ecologically relevant inhibitory effect.

F.vesiculosus thus uses a multiple defence strategy against microfoulers. Interestingly, all the deterrents detected so far in this algae are relatively widely spread or even universally present among macroalgae and it may therefore be expected that they could be relevant for the antimicrobial defence of other species than *F. vesiculosus* alone. This is particularly the case for DMSP, which reaches more than ten times as high tissue concentrations in green seaweeds as in *F. vesiculosus* (Lyons et al. 2010) and could thus potentially be more concentrated on green algal surfaces, as well.

Acknowledgements

This study was supported by a PhD scholarship (A077 1469) to M. Saha from the German academic exchange service (DAAD).

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Supplementary Information

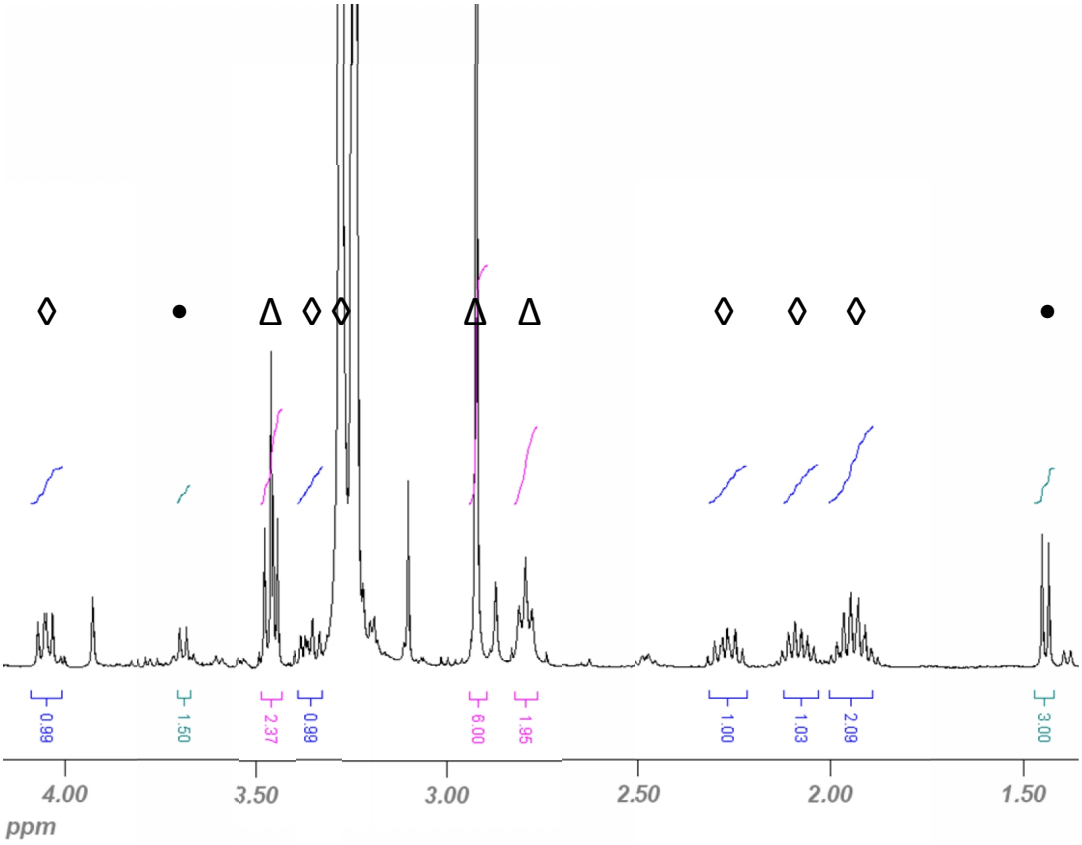

Figure S1. ^{1}H-NMR-spectrum (600MHz, CD_3OD) of the active LH20 fraction: alanine (•), proline (◊), DMSP (Δ). One proton of proline falls together with the solvent signal. Given values of integrals represent intensities within the compounds, not between different compounds.

DMSP

^{1}H-NMR (600 MHz, CD_3OD) δ ppm 3.49 (t, J = 6.77 Hz [2H]), 2.93 (s, [6H]), 2.74 (t, J = 6.79 Hz [2H])

^{13}C-NMR (150 MHz, CD_3OD) δ ppm 175.08, 41.88, 30.21, 26.33

Proline

^{1}H-NMR (600 MHz, CD_3OD) δ ppm 4.05 (dd, J = 8.48, 6.61 Hz [1H]), 3.42-3.34 (m [1H]), 3.22-3.17 (based on COSY and HSQC), 2.28 (m [1H]), 2.09 (m [1H]), 2.01-1.88 (m [2H]).

^{13}C-NMR (150 MHz, CD_3OD) δ ppm 176.75, 62.46, 47.38, 30.24, 24.95

Alanine

^{1}H-NMR (600 MHz, CD_3OD) δ ppm 3.67 (m [1H]), 1.45 (d, J = 7.20 Hz [3H]).

^{13}C-NMR (150 MHz, CD_3OD) δ ppm 176.28, 51.59, 17.00

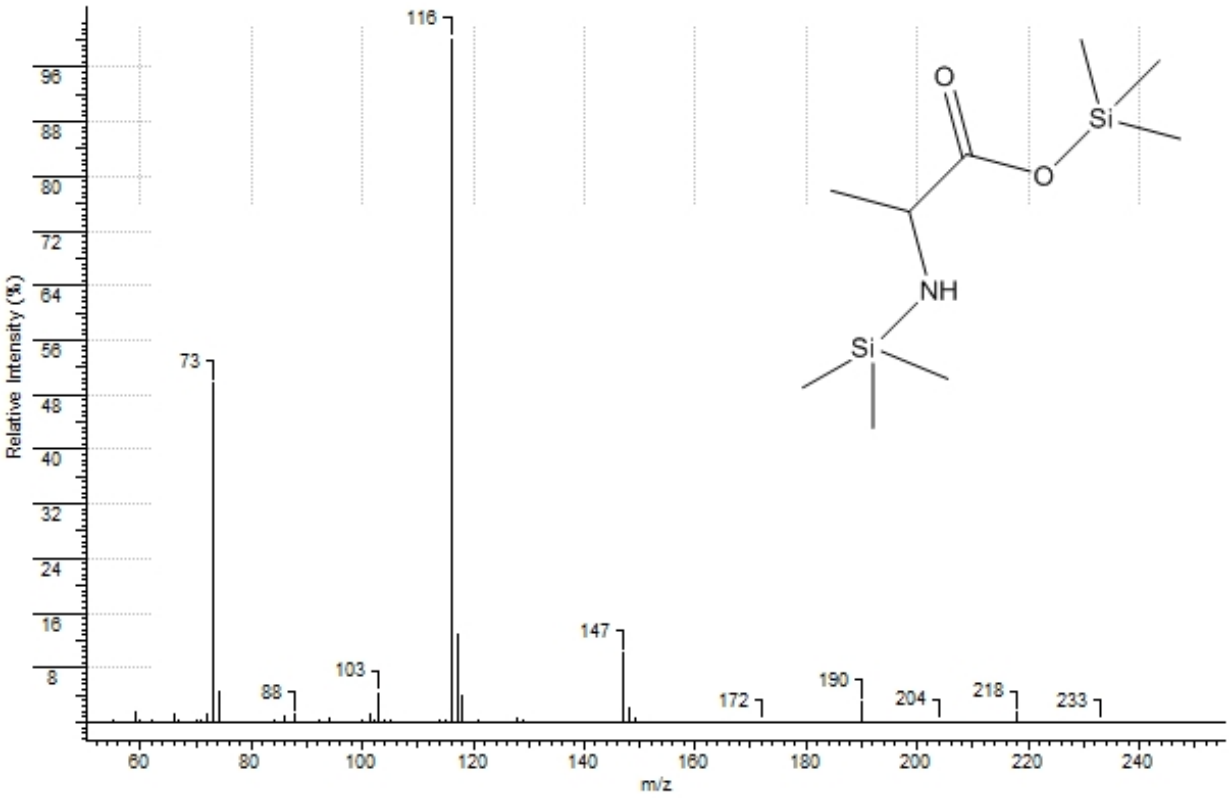

Figure S2. Mass spectrum of alanine derivatised to (*S*)-trimethylsilyl 2-(trimethylsilylamino) propanoate.

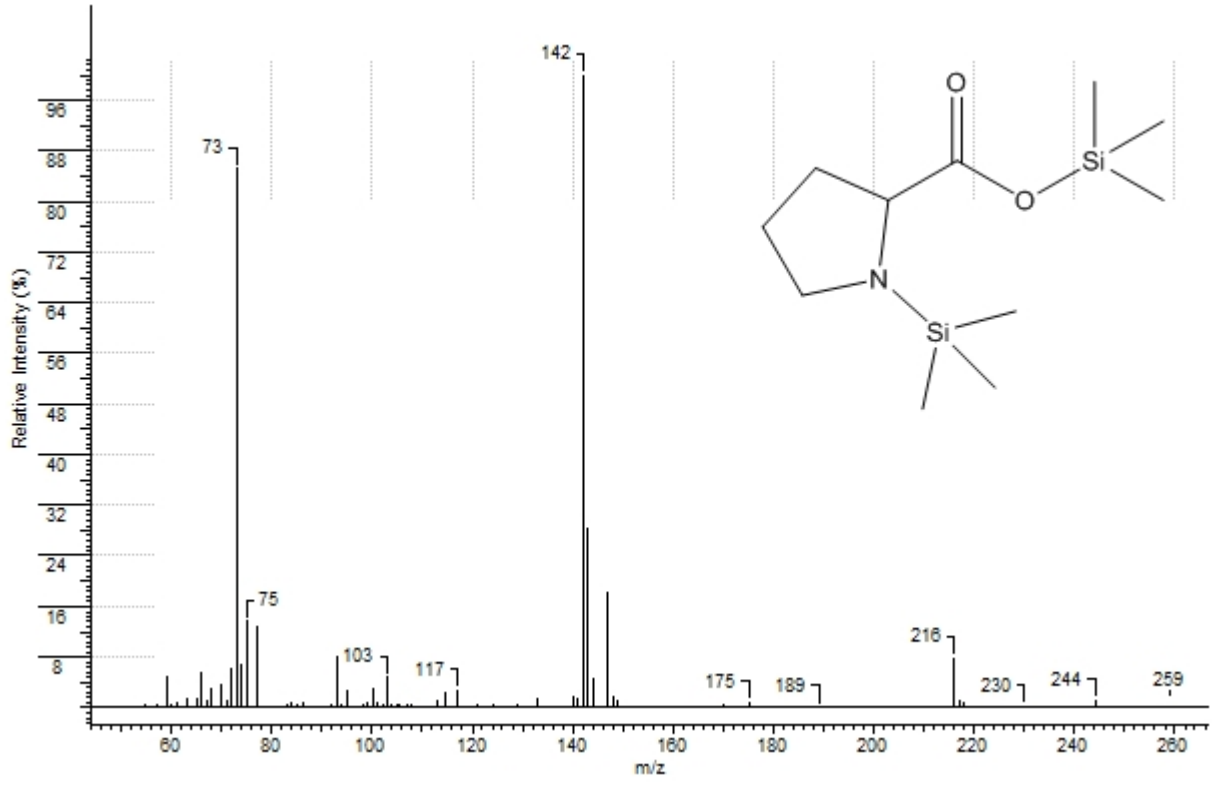

Figure S3. Mass spectrum of proline derivatised to (*S*)-trimethylsilyl 1-(trimethylsilyl) pyrrolidine-2-carboxylate.

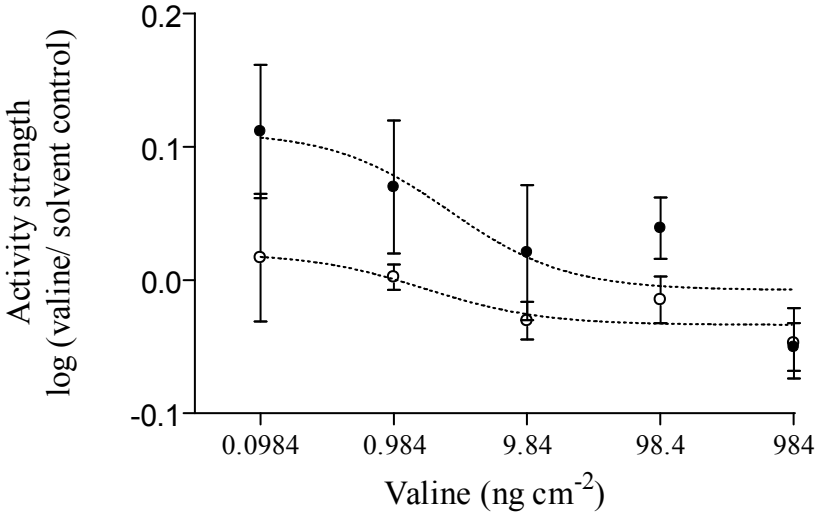

Figure S4. Pro-settlement activity of surface coated valine against *Cytophaga KT0804* (●) and *B. aquimaris* (○). Mean ± SE, n = 4, lines represent best fitting logistic functions.

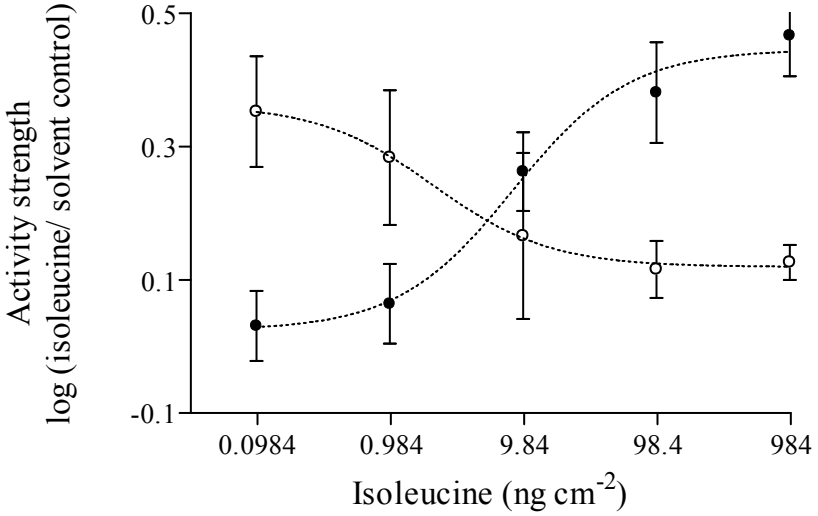

Figure S5. Pro-settlement activity of surface coated isoleucine against *Cytophaga KT0804* (●) and *B. aquimaris* (○). Mean ± SE, n = 4, lines represent best fitting logistic functions.

Effects of light and temperature stress on the anti-bacterial defence chemistry of the brown alga *Fucus vesiculosus*

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Abstract

Previous reports have demonstrated the activity of fucoxanthin, DMSP and proline as surface inhibitors against bacterial settlement in the brown alga *Fucus vesiculosus*. This alga also was shown to vary in its defence strength with season and site. We here investigated how the alga modifies the surface concentration of the mentioned bioactive metabolites and thereby its antibacterial defence properties under the influence of different levels of temperature and light. To this aim we incubated *F. vesiculosus* in indoor mesocosms at different temperature conditions (5 to 25°C) and in outdoor mesocosms under differently reduced sunlight, respectively. Both experiments showed that extrinsic factors may provoke a variation in the concentration of the three metabolites. At 20˚C, as well as under full sun light and in complete darkness, the DMSP concentration alone was high enough to warrant for a sufficient anti-settlement activity against bacteria. At the other tested temperature and light conditions DMSP was less concentrated, but still sufficient for a partial inhibition of bacterial settlement. In contrast, proline was in most treatments not sufficiently concentrated for such inhibition and the same was generally observed with fucoxanthin. However, the combined activity of the 3 defence lines was sufficient enough to substantially reduce bacterial settlement since under all tested conditions, atleast one compound- in most cases DMSP- was present at inhibitory concentration. Based on the surface concentrations of the three compounds the defence

capacity of *F. vesiculosus* against bacterial settlement appears as only moderately impacted by the potential environmental stresses of shading and warming.

Keywords: *Fucus*, DMSP, proline, fucoxanthin, anti-settlement, light, temperature.

Introduction

Fouling is ubiquitous in the marine environment and varies temporally and spatially in terms of quality (i.e. species composition) and quantity (Wahl et al. 2010). Biological surfaces, especially seaweeds, provide a microniche rich in nutrients and offer a favourable substratum to micro and macro colonisers (Lane and Kubaneck 2008). Fouling of a living surface may incur a number of beneficial or – more frequently - detrimental effects on an algal host since it alters the mechanical, chemical and physical properties of the alga and may affect the exchange processes through the surface (Wahl 2008). Fouling may also have some more indirect effects, as it often makes the basibiont more susceptible to drag or shading (Rohde et al. 2008), increases its susceptibility to further fouling (Dobretsov 2008) and grazing (Wahl & Hay 1995; Korpinen et al. 2007) or affects the host's access to resources (Wahl 2008).When negative fouling effects prevail the algal hosts should develop defence strategies to control the colonisation of foulers. Such defences may be mechanical, or based on the production of chemical compounds (Wahl 1989; de Nys& Steinberg 1999; Saha et al. 2011). Variation in the production of these chemicals is common and shows geographical or seasonal patterns (Van Alstyne et al. 2001). Sources of variation could be genotypic (Haavisto et al. 2010) or variable environmental factors like light, temperature or presence or absence of grazers (Karsten et al. 1992; Yates & Peckol 1993).

The brown alga *Fucus vesiculosus* is known to be chemically defended against microfoulers (Wahl et al. 2010). The alga harbours fucoxanthin, dimethylsulfopropionate (DMSP) and proline on its surface which may inhibit bacterial settlement (Saha et al. 2011 and Saha et al., submitted/ Chapter II). A recent study also demonstrated a spatial and temporal variation in the anti- settlement defence of this alga against bacteria (Saha et al., submitted/ Chapter IV).

The production of defence metabolites is usually expected to cause energetic costs. For example, the production of settlement inhibiting halofuranones in the red alga *Delisea pulchra* is negatively correlated with its fecundity (Dworjanyn et al. 2006). Similarly, the production of phlorotannins - confirmed deterrents of certain herbivores (Toth and Pavia 2000) and suspected deterrents of fouling organisms (Jennings and Steinberg 1997; Brock et al. 2007) – has been reported to be negatively correlated with growth of the rockweed *Fucus vesiculosus* (Jormalainen & Ramsay 2009). However, there have been reports which found no evidence of defence costs in seaweeds (e.g. Pansch et al. 2009) and a meta-analysis by Strauss et al. (2002) concluded that evidence of defence costs was only detected in approximately 50-60% of all studies conducted on terrestrial plants. Compounds such as phlorotannins have multiple roles in marine plants, including UV protection, and their production may therefore be driven by other needs than defence (Targett and Arnold 2003). In such cases, where defence compounds have additional functions, their tissue or surface concentration may appear unaffected by resource limitation (e.g. Pohnert et al. 2007). This could be the case with all three anti-settlement compounds from *Fucus*, as fucoxanthin is also the main accessory pigment of photosynthesis, while proline and DMSP have osmoregulatory functions (Bjørnland and Liaaen-Jensen 1989; Edwards et al. 1987; Kirst1996). Both light limitation stress and disruptive temperature shift stress have been shown to limit the capacity of *F. vesiculosus* for induced antiherbivore defence (Weinberger et al. 2011). The purpose of the present study was to investigate whether there is a similar effect of potential stressors on the presence of bacterial settlement inhibitors on the algal surface. To this aim surface concentration of fucoxanthin, DMSP and proline were quantified after different temperature and light treatments and compared to the necessary EC_{50} doses for settlement inhibition of potential microfoulers of *Fucus* (Saha et al. 2011; Saha et al., submitted/ Chapter II). In this way it could be deduced how the anti-settlement defence capacity of *F. vesiculosus* is impacted by different abiotic conditions. In *F. vesiculosus* light limitation and the resulting

reduction of CO_2 fixation is usually reflected in a reduced concentration of the primary carbon storage compound mannitol (Weinberger et al. 2011). The mannitol content and the photosynthetic quantum yield was used as proxies of light limitation stress, in order to estimate the stress level of different test conditions. To determine the relative effects of the light treatments on the microfouling status at the algal surface, the epibiotic cell abundance was investigated. The two experiments differed in several aspects and hence the surface concentration results are meant to be interpreted within the experiments and not to be compared between the experiments.

Materials and methods

General experimental setup

Fucus vesiculosus (Linnaeus) was cultivated in two successive monofactorial experiments at independently controlled temperature and light conditions. The effect of different water temperatures at identical light conditions was compared in a "temperature experiment", while the effect of different light intensities at identical water temperatures was investigated in a "light experiment".

Algal material

Samples of *Fucus vesiculosus* used in the temperature experiment were collected from the littoral zone of Kiel Fjord, Germany (54°26'N/10°11' E) in October 2009. Samples were collected from Laboe, Germany (54°23'N/10°12' E) in March 2011 for the light experiment. The algae were individually sealed in zip-lock bags and transported to the laboratory in a cooler box.

Temperature experiment

Twenty-five *Fucus vesiculosus* individuals were maintained separately for 28d in 25 indoor aquaria (25 L) in a temperature constant room. Water was replaced once a week by seawater (16 psu) composed of one third filtered fjord water mixed with two-third of artificial seawater. This was done in order to limit diatom growth by nutrient dilution. Five different water

temperature levels were tested: 5, 10, 15, 20 and 25˚C, and five replicate aquaria were maintained at each of these temperatures. Room temperature was 15°C, and higher temperatures were obtained through aquaria heaters (Schego GmbH), while lower temperatures were obtained through Aqua Medic (Aqua Medic GmbH, Bissendorf) coolers. All aquaria were maintained at a light intensity of 110 $\mu molm^{-2}s^{-1}$ (SD ± 5), with 8:16 light: dark cycle. This daily dose is well above low-irradiation and below high-irradiation stress levels (e.g. Weinberger et al. 2011).

Light experiment

The setup consisted of an outdoor seawater system of 30 aquaria (20 L each). Seawater from Kiel fjord constantly circulated through these aquaria (40 L h^{-1}). Side and bottom walls of the aquaria were covered with black plastic bags, in order to exclude diffused light. Six different treatment levels were tested: full sunlight (mean 247 $\mu molm^{-2}s^{-1}$; SD ± 4.24), 44% (achieved through shading with 1 layer of mosquito gauze (Max Bahr GmbH, Kiel)), 23% (2 layers), 13% (3 layers), 5% (4 layers) of natural sunlight and complete darkness. Five replicate aquaria were maintained at each of these light conditions with random distribution. The water temperature and light intensity was measured at 30 min intervals with loggers (HOBO®, Onset Computer Corporation, USA) in two out of five replicates. The mean water temperature under all treatments was 5˚C (SD ±0.09˚C).The algae were placed individually into these aquaria and acclimated to the new environmental conditions for 4 days before the start of the experiment. The duration of the treatment was 18d.

Quantification of fucoxanthin, DMSP and proline

After the temperature or light treatment, young algal tips of approximately 4-5 cm length were surface extracted (10 secs dipping in a stirred mixture of 1:1 MeOH: hexane). The extracts were vaccum dried in a rota-evaporator and fractionated into non-polar (hexane) and polar (MeOH) extracts as described in Saha et al. (2011) and stored at -20 ˚C. Fucoxanthin was quantified using a Macherey Nagel Nucleodur analytical normal phase Si column (4.6 mm x

25 cm) (gradient: 100% n-heptane, 10 min; linear gradient to 100% EtOAc for a further 20 min; flow rate 1 ml min^{-1}) on a Varian 940-LC with integrated photodiode array detector (PDA) at 450 nm. A calibration curve of peak areas with eight concentrations of standard fucoxanthin (Cayman chemicals, Hamburg, Germany) was used. Quantification of DMSP was done by LC-MS according to Spielmeyer and Pohnert (2010). Proline quantification was done using the same LC-MS parameters and external calibration with three concentrations.

Quantitative analysis of biofilm

To quantify the microfouling status (diatoms and bacteria) in different light levels on the 10^{th} and 18^{th} day of the light experiment, biofilms from 1 cm^2 of algal surface (tips) were harvested by swabbing with a sterile cotton tip, followed by vortexing the tip for 30 sec in an eppendorf vial containing 1 ml of sterile seawater (SSW). The relative abundance of diatoms (and any other possible photoautotrophs) in 100 µL 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) 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 µL subsample with fluorescent DNA–binding dye Syto 9, 0.005mM (Invitrogen GmbH). Following an incubation time of 10 mins (dark), the fluorescence was subsequently measured (excitation 477-491 nm, emission 540nm), using the same platereader. Replication was 10 fold per treatment (n=9 for bacterial abundance quantification). The first measurements provided data on the relative effects of the treatments on microalgal density at the algal surface, while the second measurement provided similar information on all epibiotic cells.

PAM (pulse amplitude modulation) measurement

In order to monitor the overall physiological state of *F. vesiculosus* on the 18^{th} day of the experiment PAM fluorometry was used. Three thallus tips per individual were incubated in

darkness for 5 min and photosynthetic yield values were recorded, using a Diving-PAM (Waltz GmbH) (light intensity 11, gain 8).

Analysis of mannitol

The mannitol content was quantified in order to determine whether light reduction resulted in carbon limitation (e.g. Weinberger et al. 2011). After the light experiment, six individuals, each representing a different light treatment level, were freeze-dried, ground and stored at -20 ˚C. Mannitol was extracted and analyzed as described in Vas'kovskii&Isai (1972), but periodate oxidation was stopped after 10 s.

Statistical analysis

One-way ANOVA was used to analyse the effects of temperature and light on the DMSP, proline and fucoxanthin content. One-way ANOVA was further used to analyse quantitative differences in microfouling pressure during the light treatment. Shapiro– Wilk's test was purposed to test for normal distribution ($p< 0.05$), while Levene's test was used to test for homogeneity of variance ($p<0.05$). Datasets not fulfilling the criterium of homoscedasticity were Box- Cox transformed using the software Minitab 12.2 (Minitab Inc., State College, PA, USA). Post hoc comparisons of DMSP, proline and fucoxanthin content variation among different temperature or light treatments were made using Tukey's honest significant difference test (HSD) ($p<0.05$). Regression analysis was used to analyse the relationship between tissue mannitol content and different light conditions. Co-relation analysis was used to analyse the relationship between diatom abundance and fucoxanthin concentration.The computer program Statistica (StatSoft, Tulsa, OK, U.S.A.) was used to conduct all statistical tests.

Results

Temperature experiment

After two weeks of incubation the highest tested water temperature of 25°C visibly exerted stress upon *F. vesiculosus*, as the apical tips in one out of five individuals started to decay.

Such decay was detected in four out of five individuals after three weeks and the symptom progressed further, so that only old parts were alive in all five individuals after four weeks of incubation (data not shown). No decay was observed at 5, 10 and 20°C, while one individual at 15°C also showed the symptom from the 2nd week on, although to a lesser degree. Epiphytic algae – mainly diatoms of the genus *Melosira*- were observed in all treatments, and their density generally increased during the first three weeks of the experiment. Epiphytism increased with temperature and after three and four weeks the algae incubated at 5°C still appeared relatively clean (bearing only few macroscopically visible algal filaments), while those incubated at 25°C were massively overgrown.

Quantification of DMSP, proline and fucoxanthin

Natural concentrations of DMSP and proline quantified from apical tips of the algae exposed to different temperatures ranged between 0.15 and 0.96 ng cm^{-2} and between 0.004 and 0.01ng cm^{-2}, respectively. Fucoxanthin concentrations in the apical tips ranged from 18 to 400 ng cm^{-2}. The surface DMSP concentration varied significantly among temperature levels (1- way ANOVA, F= 3, p=0.03). While there was no significant correlation of DMSP content with temperature, it tended to be highest at intermediate temperatures. DMSP was significantly more concentrated at 20 ˚C than at 25˚C (1- way ANOVA, Tukey's HSD, p< 0.05, Figure 1). The surface proline content did not vary significantly with temperature (1- way ANOVA, p= 0.05). As with DMSP, no significant correlation of surface proline content and temperature was observed, however it tended to decrease with warming. Fucoxanthin surface concentration varied significantly with temperature (1- way ANOVA, F=42, p<0.001, Figure 3), and increased with warming (Tukey's HSD, p< 0.05). Detailed results of compound concentration at the different treatment levels are given in Table 1.

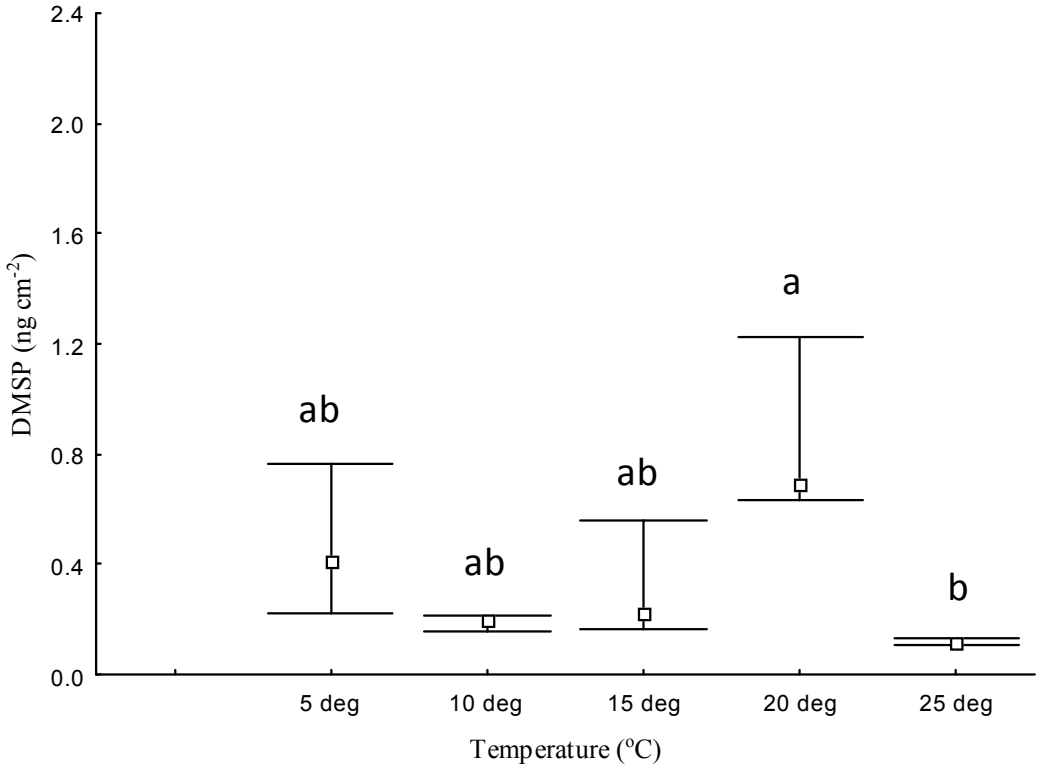

Figure 1. Variation of surface DMSP concentration in *F. vesiculosus* treated under different temperature conditions for 28d. Different letters indicate different temperature treatment responses in DMSP concentration (Tukey's test $p<0.05$). Median (central symbol), n=5, interquartile range.

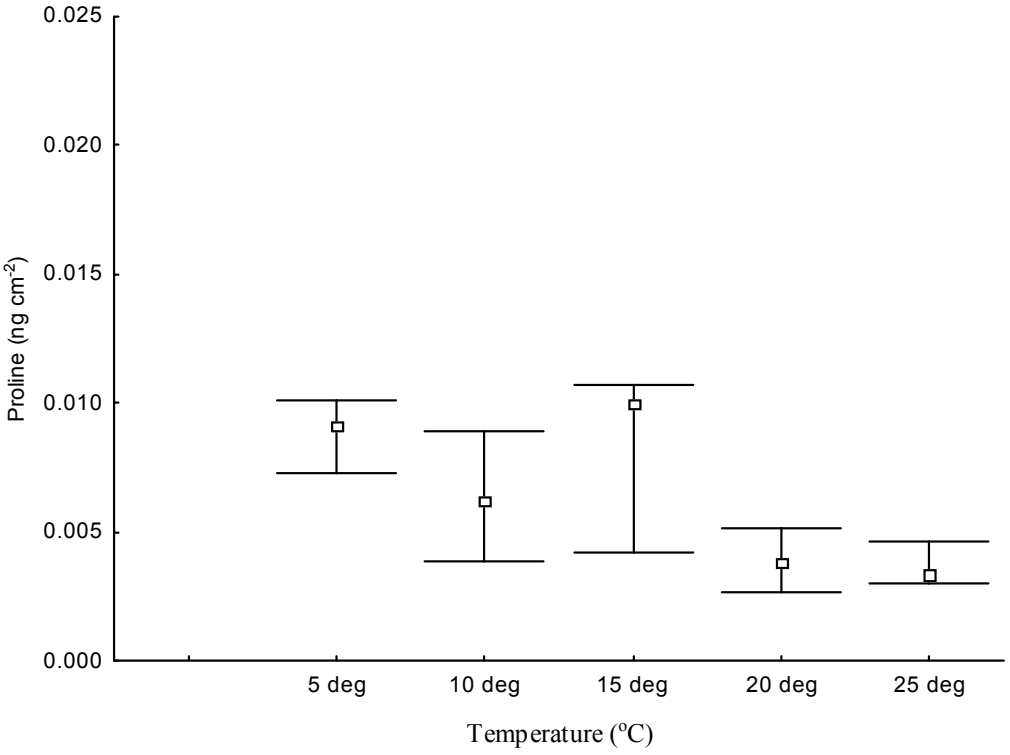

Figure 2. Variation of surface proline concentration in *F. vesiculosus* treated under different temperature conditions for 28d. Median (central symbol), n=5, interquartile range.

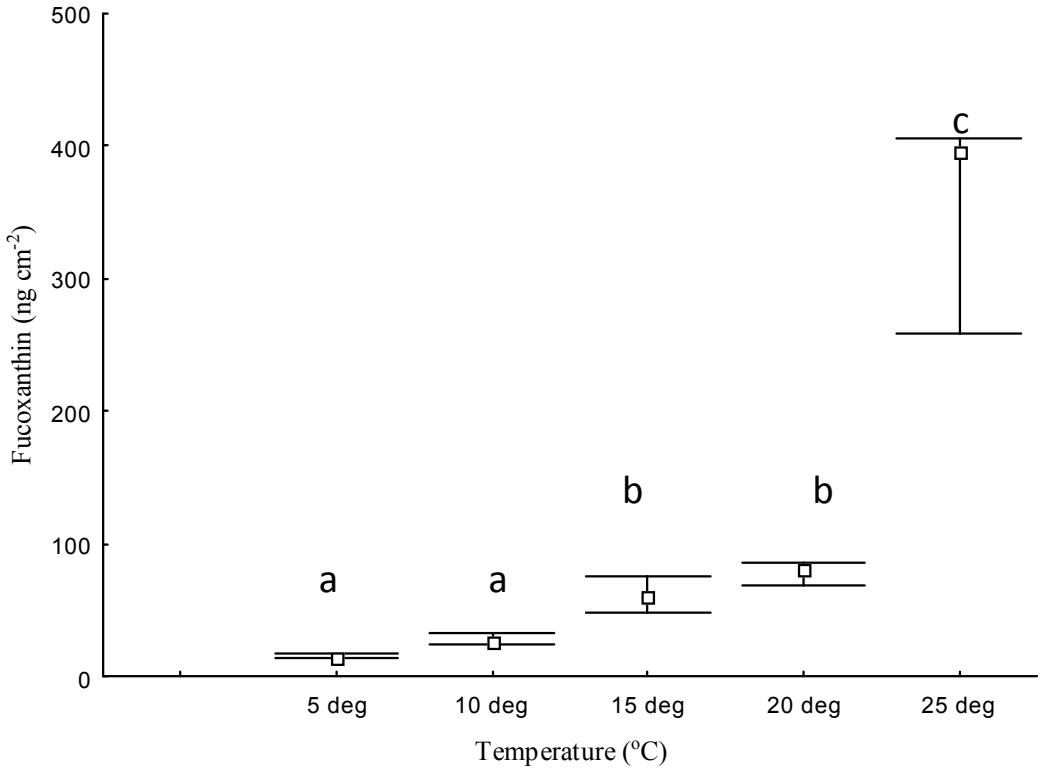

Figure 3. Variation of surface fucoxanthin concentration in *F. vesiculosus* treated under different temperature conditions for 28d. Different letters indicate different temperature treatment responses in fucoxanthin concentration (Tukey's test $p<0.05$). Median (central symbol), n=5 (n=4 for 10 ˚C), interquartile range.

Light experiment

Regression analysis showed that there was a significant positive relationship between the available light energy and the mannitol content in differently light treated individuals of *F. vesiculosus* ($y = -0.025x + 0.765$, $R^2= 0.88$, $p<0.05$, Supplementary Information Figure S1). In contrast, there was no statistically significant difference in the photosynthetic yield of differently treated algae (data not shown).

The diatom density didnot differ significantly among the light levels (1- way ANOVA, F=1.6, p=0.17, Supplementary Information Figure S2). However, a co-relation analysis showed a significant positive corelation between diatom density and fucoxanthin content among the different treatments ($y = 22.279 + 0.10376x$, $R^2= 0.92$, $p<0.05$, Supplementary Information Figure S3). The total density of bacteria did not differ significantly among the light levels (1-way ANOVA, F= 2.3, p= 0.06, Supplementary Information Figure S4).

Quantification of DMSP, proline and fucoxanthin

The natural concentrations of DMSP and proline on surfaces of the apical tips of light treated algae were found to be in the range of 0.03- 0.45 ng cm^{-2} and 0.01-0.03ng cm^{-2}, respectively. Fucoxanthin concentration in the apical tips ranged from 146 to 353 ng cm^{-2}.

The surface DMSP content varied significantly with different light intensities (1- way ANOVA, F= 4, p=0.009, Tukey's HSD, Figure 4) with highest and lowest concentrations under dark and 44% light conditions respectively (Table 1). In contrast, there was no significant variation of proline surface concentrations among differently light treated individuals (1- way ANOVA, F= 0.75, p=0.59, Figure 5). Fucoxanthin varied significantly among different light intensities with highest concentration at 23% light regime (1- way ANOVA, F= 4, p=0.01, Tukey's HSD, Figure 6). See table 1 for details.

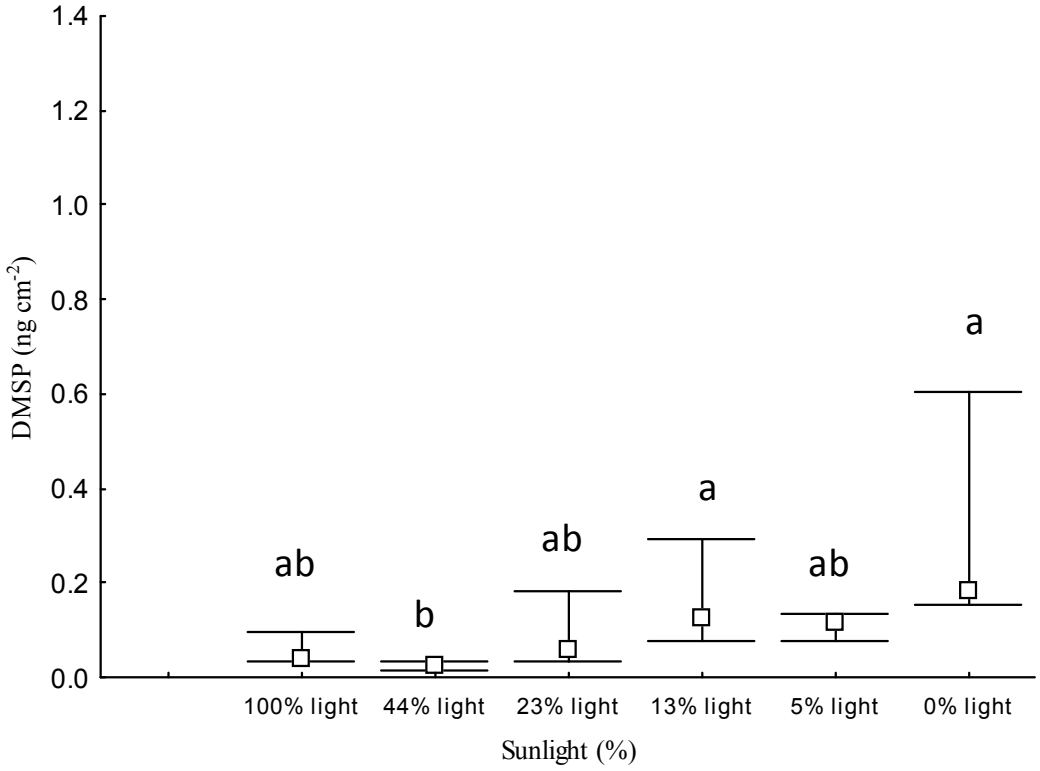

Figure 4. Variation of surface DMSP concentration in *F. vesiculosus* treated under different light conditions for 18d. Different letters indicate different light treatment responses in DMSP concentration (Tukey's test $p<0.05$). Median (central symbol), n=5, interquartile range.

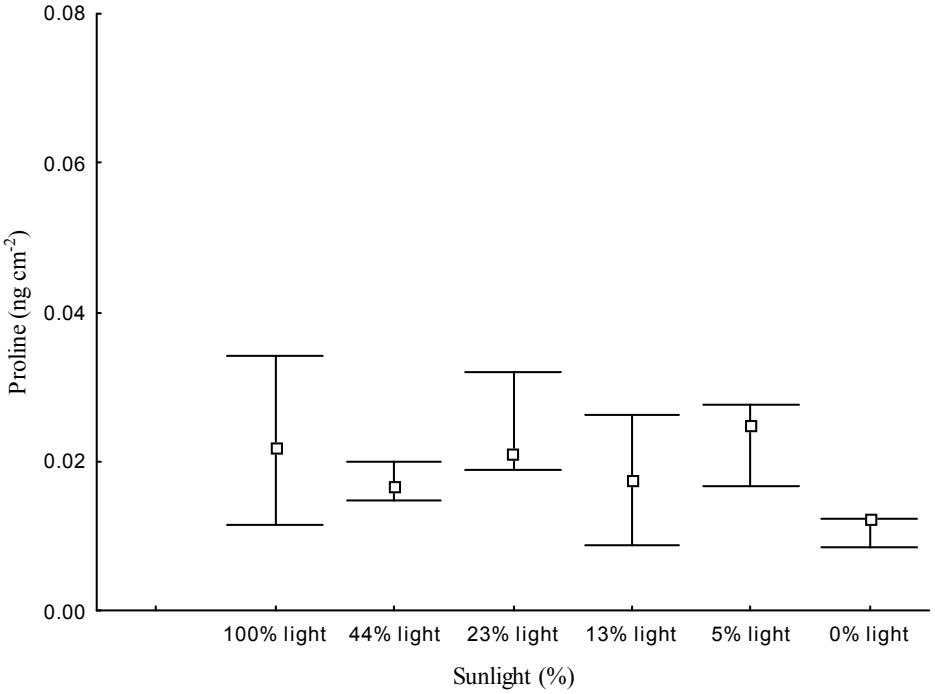

Figure 5. Variation of surface proline concentration in *F. vesiculosus* treated under different light conditions for 18d. Median (central symbol), n=5, interquartile range.

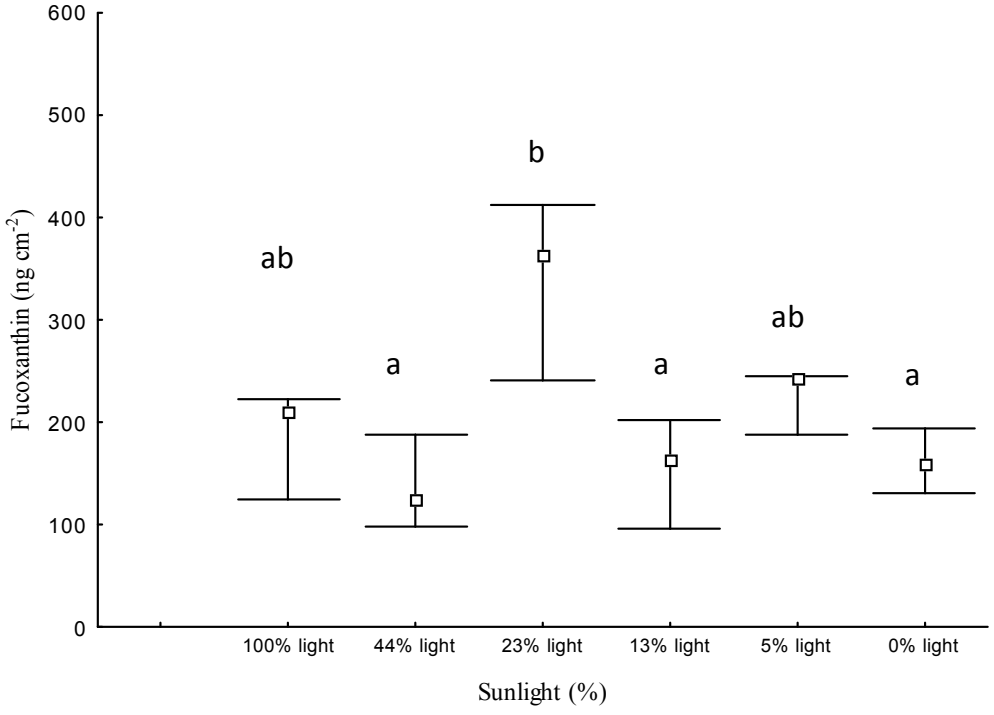

Figure 6. Variation of surface fucoxanthin concentration in *F. vesiculosus* treated under different light conditions for 18d. Different letters indicate different light treatment responses in fucoxanthin concentration. Median (central symbol), n=5, interquartile range.

Table1. Variation in mean surface concentrations of DMSP, proline and fucoxanthin in *F. vesiculosus* at different temperature and light treatments. SD is given in brakets.

	Detected concentrations (Chapter I & II)	*EC50*	*Mean concentrations detected (ng cm^{-2} of apical tips)[temp. treatment]*					*Mean concentrations detected (ng cm^{-2} of apical tips) [light treatment]*					
			5˚C	***10˚C***	***15˚C***	***20˚C***	***25˚C***	***0%***	***5%***	***13%***	***23%***	***44%***	***100%***
DMSP	0.12 to 1.08 ng cm^{-2} (apical tips)	0.005 to 0.38 ng cm^{-2}	0.46 (0.30)	0.17 (0.07)	0.59 (0.75)	0.96 (0.76)	0.16 (0.13)	0.45 (0.43)	0.1 (0.04)	0.17 (0.10)	0.1 (0.09)	0.03 (0.16)	0.3 (0.57)
Proline	0.09 to 0.59 ng cm^{-2} (apical tips)	0.01 to 0.13 ng cm^{-2}	0.009 (0.002)	0.006 (0.003)	0.01 (0.006)	0.004 (0.001)	0.004 (0.001)	0.01 (0.006)	0.025 (0.01)	0.02 (0.04)	0.03 (0.12)	0.02 (0.17)	0.03 (0.39)
Fucoxanthin	0.7 to 9 µgcm^{-2} (apical tips to whole alga)	1.4 to 6 µgcm^{-2}	18 (11.08)	29 (6.81)	74 (37.84)	84 (22.53)	400 (187.04)	159 (84.36)	206 (63)	150 (187)	353 (94)	146 (101)	192 (85.21)

Discussion

The surface concentrations of two out of three anti-settlement metabolites of *F. vesiculosus* varied both with temperatures and with irradiation. Thus, in indoor and outdoor experiments DMSP and fucoxanthin concentrations at the thallus surface of *F. vesiculosus* varied among different temperature and light conditions, while the concentration of proline remained constant. Different metabolite concentrations were found under comparable factor settings in the two experiments, possibly resulting from the differences in experimental set-up and/or in the seasons. Overall, DMSP and fucoxanthin were less concentrated in the light experiment, while the inverse was true for proline.

In the temperature experiment severe stress resulting in the decay of apical thallus parts was apparently exerted when *F. vesiculosus* was incubated for more than one week at 25°C. In the habitat the alga were collected from, 20°C is considered the highest surface water temperature that may pertain for several weeks (Weinberger et al. 2011). No such morphological symptoms of stress were observed in the light experiment, and also PAM fluorometry indicated no physiological stress. However, the mannitol content of *F. vesiculosus* decreased in a linear manner with decreasing light, indicating a significant limitation of photosynthetic CO_2 fixation under low light conditions (Weinberger et al. 2011). It should be noted, however, that even after 18d of severe light limitation, mannitol content had only decreased by less than 25%, possibly illustrating the alga's capacity to survive dark northern winters.

In previous studies with field collected material, DMSP has been detected on *Fucus* surfaces at concentrations in the range of 0.12 to 1.08 ng cm^{-2} (Saha et al., submitted/ Chapter II). In the present study, differently temperature treated samples of *Fucus* contained DMSP at similar concentrations, with the highest concentrations at 20˚C (Figure 1, Table 1). As a trend, DMSP surface concentrations in *F. vesiculosus* increased with temperature, unless

stressfully high temperatures (e.g. 25°C) are reached. The particularly low surface concentration of DMSP at 25°C despite particularly strong fouling by epiphytic diatoms indicates that DMSP was not produced by these epiphytes. This low concentration under temperature stress further corresponds with the environmental stress hypothesis (EST) and with similar reports for some other defence compounds (e.g. elatol in *Laurencia dendroidea*, Sudatti et al. 2011). The EST predicts that the concentration of defensive compounds can decrease in stressed plants (e.g. Cronin 2001). The increase of *Fucus* DMSP with temperature (up to a stressful threshold) contrasts to *Codium fragile* DMSP (Lyons et al. 2010). However, unlike the studies on green algae we worked with surface concentrations (relevant for antifouling) and not with tissue concentrations of DMSP (relevant for cryoprotection). In *C. fragile* as in other green algae the tissue DMSP content increases with decreasing temperature, since DMSP in these organisms plays a role as cryoprotectant (Karsten et al. 1996). However, the tissue concentration of DMSP in *Fucus* is considerably lower than in green algae (Blunden et al. 1992), making a role as cryoprotectant less probable. In *F. vesiculosus* low temperatures could also result in high tissue content, but low surface concentration of DMSP since in the cold season the need for cryoprotection increases while the need for antifouling protection decreases. Such a hypothetical shift of function of DMSP between seasons has not been studied yet but might be worth investigating.

DMSP has previously been shown to inhibit settlement of three out of five potential bacterial microfouler strains (*Ulvibacter littoralis,* Alteromonadaceae E1 and marine sediment bacterium ISA 7311; all isolated from rockweed dominated habitats in the Baltic Sea) at a surface concentration of 0.005 ng cm^{-2} or more, while the strains (*Cytophaga KT0804*, *Bacillus aquimaris*; also isolated from rockweed dominated habitats) required higher DMSP concentrations in the range between 0.005 and 0.38 ng cm^{-2} for 50% inhibition (Saha et al., submitted/ Chapter II). Based upon the mean concentrations that were detected in our study

the former three strains would be largely inhibited by DMSP at all tested temperature conditions, including 25°C (Table 1). In contrast, the latter two strains would be largely repelled by DMSP during the summer only. This corresponds with the results of a recent study which reported a particularly strong anti-settlement activity of *F. vesiculosus* surface extracts against *Cytophaga KT0804* and *B. aquimaris* in summer, irrespective of the geographic location (Saha et al., submitted/ Chapter IV).

Overall, the surface DMSP content of *F. vesiculosus* was lower in the light experiment than in the temperature experiment (Table 1), with relatively high mean concentrations at100% and 0 % light. The variability at 100% was particularly high, while significantly less DMSP was detected on *F. vesiculosus* that was maintained at 44% of sun light, as compared to 13 % and 0 % (Figure 4), which suggests that a certain tendency towards increased presence of DMSP at low light may exist. In *Codium fragile* (Lyons et al. 2010) and many other algae (e.g. Karsten et al. 1992), tissue DMSP usually increases with light intensity, often due to its ecophysiological role as an antioxidant. We do not know, however, whether this is also reflected in higher surface concentrations of DMSP. A multitude of marine organisms including certain heterotrophic bacteria are known to be attracted to DMSP (Seymour et al. 2010). Certain bacteria are known to metabolize DMSP quickly (Dickschat et al. 2010). Differences in the bacterial community composition under differing light treatments might account for, or at least contribute to, the detected concentration differences among different light conditions, as well as for the considerable variability within light treatments (Figure 4).

At the DMSP concentrations found, the above mentioned three more sensitive strains of microfoulers would be repelled under all tested light regimes, while the two less sensitive strains, *Cytophaga KT0804* and *B. aquimaris* would be partly inhibited and largely inhibited, respectively by DMSP under full sunlight and largely inhibited in complete absence of light.

A light limitation of DMSP-based defence in *F. vesiculosus* was thus not detected, although light reduction resulted in significantly reduced CO_2 fixation.

In a former study proline was detected on *Fucus* apical tips at concentrations in the range between 0.09 and 0.59 ngcm^{-2} (Saha et al., submitted/ Chapter II). In the present temperature treatment study proline was detected at much lower concentrations (Table 1) with no significant differences among the treatments (see Figure 2, Table 1). Similar proline concentrations were detected in the light treatment study (Table 1), again without significant differences among the treatments (Figure 5). Proline concentrations of 0.01 ng cm^{-2} or more, sufficient for a partial inhibition of all but the most resistant strains (Saha et al., submitted/ Chapter II), were present on the surface of *F. vesiculosus* under all conditions in the light experiment, but only at 15 ˚C in the temperature experiment.

In a previous study (Saha et al. 2011) fucoxanthin has been detected at a concentration of 0.7 µg cm^{-2} on *Fucus* apical tips. Considerably lower surface concentrations were detected in the temperature experiment described here. They increased with temperature from 5°C to 25°C, with a five-fold increase between 20°C and 25°C (Table 1, figure 3). Epiphytic diatoms which contribute to the fucoxanthin accumulation at the surface of *F. vesiculosus* (Saha et al. 2011) were particularly abundant at 25°C and relatively rare at 5°C. Fucoxanthin was present at surface concentrations between 159 and 353 ng cm^{-2} on the apical tips of light treated algae and showed no steady change in either direction with decreasing light intensities (see Figure 6). A significantly higher concentration of fucoxanthin under 23% of sunlight might have been caused by a particularly high abundance of diatoms found on *F. vesiculosus* maintained at this irradiation level. Indeed, fucoxanthin concentration related significantly and positively to diatom abundance. Diatoms apparently contributed an important amount of surface fucoxanthin in both experiments.

Fucoxanthin surface concentrations of 1.4 to 6 µg cm^{-2}caused a 50% settlement inhibitionin four out of five bacterial test strains (*Ulvibacter littoralis,* AlteromonadaceaeE1, *Cytophaga* KT0804 and *B. aquimaris*), while the fifth isolate (marine sediment bacterium ISA 7311) showed a similar effect only above 6 µg cm^{-2} (Saha et al. 2011). Both in the light and the temperature experiment fucoxanthin was always detected at concentrations below 6 µg cm^{-2}. Only a feeble contribution of fucoxanthin to antisettlement defence can, thus, be assumed in both experiments.

Overall, DMSP was the most relevant defence metabolite in both the temperature and light treatment, as it was present at sufficient concentrations for moderate to strong inhibition of relevant bacterial microfoulers at all tested temperature and light. Proline contributed to this defence in the light experiment only, fucoxanthin under none of the test conditions. The polar compounds proline and DMSP can be expected to possibly act additively or synergistically. When total polar surface extract of *F. vesiculosus* was tested without prior fractionation and purification it had a stronger inhibitory effect on bacterial settlement than DMSP and proline alone, which could be explained through synergisms among the identified or even among unidentified components (see Saha et al. 2011 for details). Fucoxanthin concentrations on whole algal surfaces were found to be 9 µg cm^{-2} on (Saha et al. 2011). The compound appears thus to be more concentrated and thus relevant for anti-settlement defence on older thallus parts than on tips. Thus, there is a potential for differential contribution to antifouling defence of polar and non-polar inhibitors on different thallus parts. However, this hypothesis also needs to be tested in detail.

The defence system of *F. vesiculosus* against microfoulers is of multiple nature and composed of at least three metabolites which differ regarding their concentration of different thallus parts, their sensitivity to environmental conditions and possibly other factors. Under all test conditions the natural surface concentration of at least one of the defensive

metabolites was high enough to substantially reduce settlement of all bacterial strains tested. Thus, while the concentration of single compounds may be reduced under certain adverse conditions, the compound antifouling defence of *F. vesiculosus* appears not to be significantly affected by limitation stress or disruptive stress, including complete absence of light for 18 d and 25°C for 4 weeks. Complex defence systems composed of several (independent) mechanisms may not only make the antifouling defence more efficient and less vulnerable to co-adaptation as suggested earlier (e.g. Wahl 2008) but also less sensitive to environmental stress.

Acknowledgements

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Supplementary Information

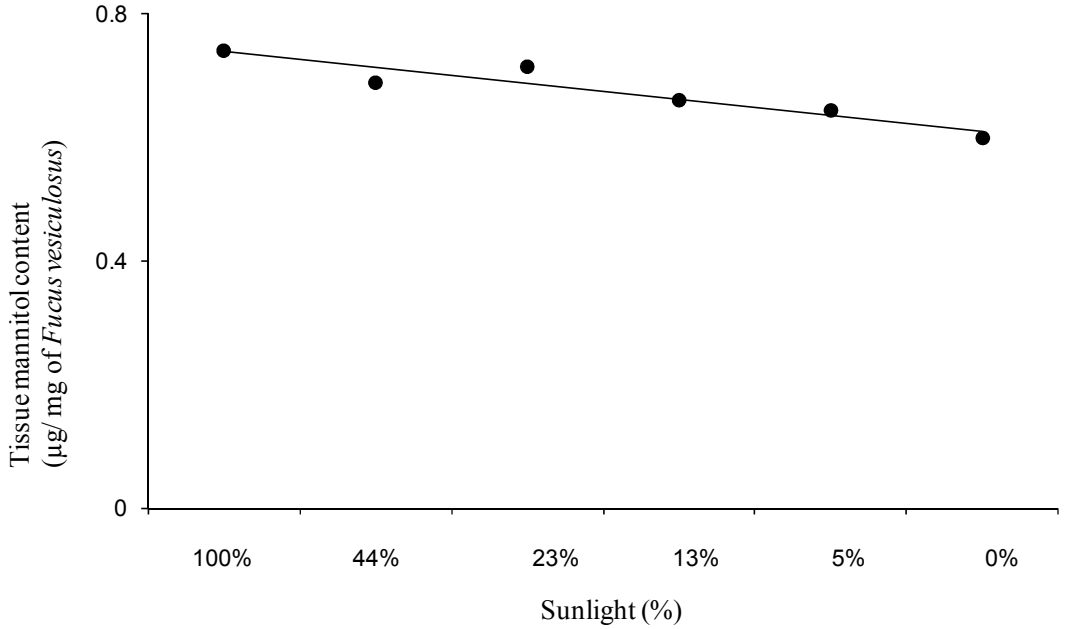

Figure S1. Relationship between tissue mannitol content and light among differently light treated individuals of *F. vesiculosus* (R^2 = 0.880, $p<0.05$). Straight line: best fitting linear function (y = -0.025x + 0.765).

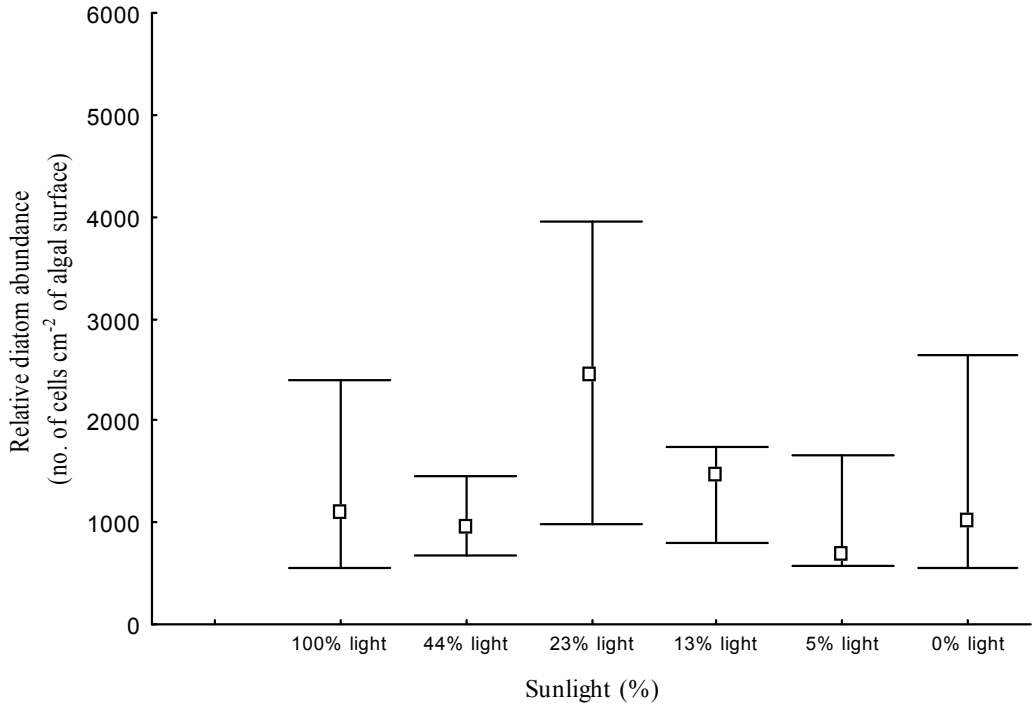

Figure S2. Variation of diatom abundance among differently light treated *F. vesiculosus* individuals. Median (central symbol), n=10, interquartile range.

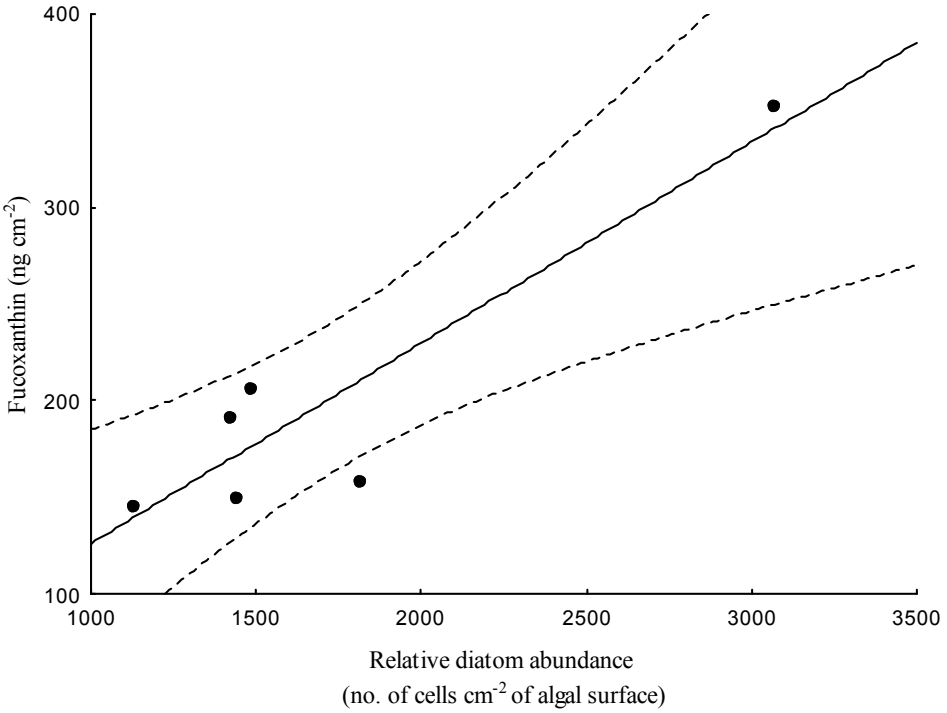

Figure S3. Relationship between surface fucoxanthin concentration and relative diatom abundance cm^{-2} of algal surface among differently light treated individuals of *F. vesiculosus* (R^2= 0.92, $p<0.05$). Straight line: best fitting linear function (y=22.279 + 0.10376*x). Dotted lines: 95% CI.

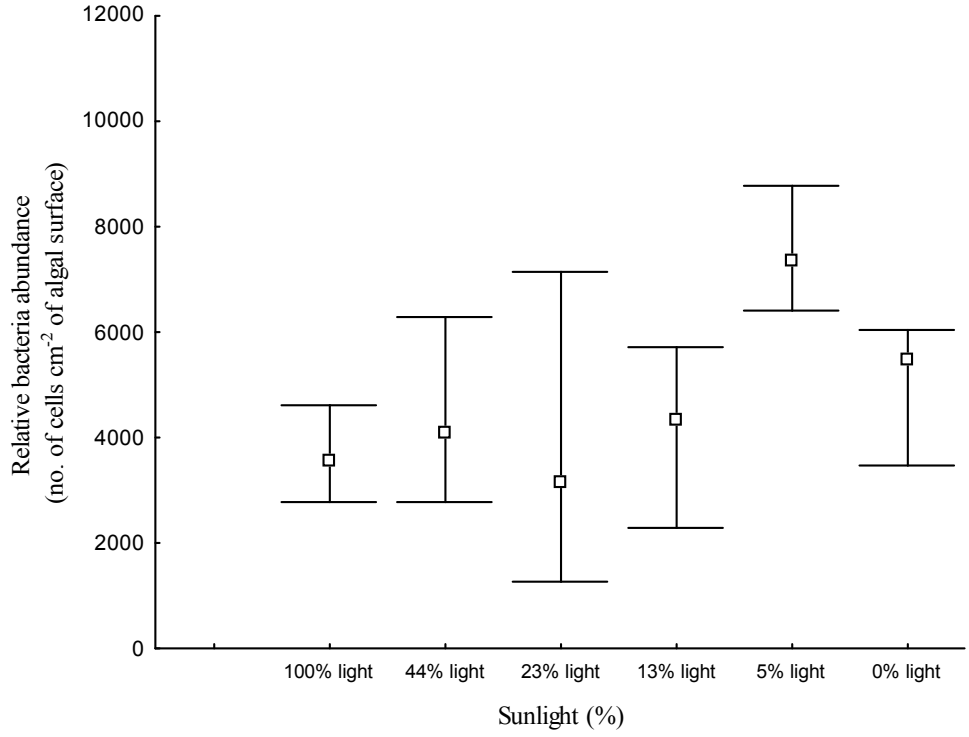

Figure S4. Variation of bacteria abundance among differently light treated *F. vesiculosus* individuals. Median (central symbol), n=9, interquartile range.

Spatial and temporal variation in anti-bacterial activity of the brown alga *Fucus vesiculosus*

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Abstract

Previous studies have demonstrated that the brown macroalga *Fucus vesiculosus* contain polar and non-polar compounds with antifouling activity against marine bacteria. Studies related to chemical defence mechanism of both terrestrial and marine organisms suggest that they vary widely in the production of chemical defences in relation to biotic and abiotic factors, season and location. Until now, insufficient attention has been paid to evaluate the possible spatial and temporal variation of anti-fouling activity in an ecological context. The present study aimed to investigate the seasonal and geographical variation in the anti-settlement activity of surface extracts of *F. vesiculosus* against bacteria. The antibacterial activity was found to be temporally variable and reached a peak in late summer/ autumn corresponding to maximal values for water temperature, light and fouling pressure. Antisettlement activity also showed a consistent and strong difference between sites throughout the year. This study is the first to report spatial and temporal variation of antifouling defence originating from ecologically relevant surface associated compounds.

Keywords: *Fucus*, anti-bacterial, chemical defence, seasonal variation, geographical variation, antifouling.

Introduction

Microbes constitute an important component of the earth's terrestrial and aquatic environment (Whitman et al. 1998). Their number in the marine and freshwater environment could be enormous. An average ml of seawater contains up to 10^7 viruses, 10^6 bacteria, 10^3 fungi, 10^3 microalgae, and 10 to 100 microscopic larvae and spores (Cole 1982; Jennings & Fenical 1994). The aquatic environment facilitates the development of microbes and the formation of biofilms on surfaces (e.g. Weinberger 2007). All submerged marine surfaces are continuously challenged by colonizing micro-organisms (Zobell and Allen 1935). This microbial film (Costerton et al. 1995) formed, for instance, on a macroalga's thallus replaces the original interface between the host organism and its environment by a new epibiotic interface with often dramatically different physical, chemical, mechanical, topographical and biological properties (e.g. Wahl 1997). The presence of an epibiotic biofilm can be expected to affect many or most biotic interactions of the host (e.g. Wahl et al. 2010). The primary bacterial colonisers could, for instance, be deleterious to the algae by providing inductive cues to the larvae and spores of macrofoulers leading to macrofouling (Unabia & Hadfield 1999) with all associated consequences for the host (e.g. Wahl 2008). Microbial pathogens may cause extensive tissue damage (e.g. Sawabe et al. 1998). Also, the epibiotic biofilm may hinder gaseous exchange, reduce the intensity of incoming radiation and thereby reduce photosynthetic activity of the algae (Wahl 2008). Bacterial epibionts along with the macroscopic foulers may affect the host's access to resources, its fouling sensitivity (Holmström & Kjelleberg 1999; Dobretsov 2008) or its susceptibility to grazers with grazing wounds in turn increasing the probability of pathogenic infection (Wahl 1997). Most consequences of micro and macro foulers are disadvantageous for marine algae. In such a scenario a control over the fouling process is generally expected and should be of selective advantage (Dworjanyn et al. 2006a).

It has often been hypothesised that macroalgae posses chemical defence in order to combat foulers (de Nys et al. 1998; Nylund et al. 2008; Saha et al. 2011). The production of these defence chemicals might involve energy costs which may compete with other metabolic budgets (e.g. Harvell 1998) although the existence of defence-associated costs has rarely been

demonstrated for marine algae (e.g. Pansch et al. 2009 but see Dworjanyn et al. 2006b; Jormalainen & Ramsay 2009 for costs of chemical defence). If costs exist and when energy is a limiting factor defence production "on demand" (i.e. tuned to fouling pressure) should be selectively favoured (Tollrian & Harvell 1999).

Some authors have studied the variation in chemical (Steinberg and Van Altena 1992; Culioli et al. 2002) or biological activity (Hellio et al. 2004) in benthic organisms at different seasons. While the rockweed *Fucus vesiculosus* is known to upregulate or downregulate its anti-herbivory defence as a function of grazing pressure (e.g. Rohde & Wahl 2008) not much information is available for the anti-fouling defence variation of the alga (but see Wahl et al. 2010). Since the biotic and abiotic factors, such as energy available to the alga and fouling pressure, vary among seasons and sites, an ability to regulate its antifouling defences might be expected to have evolved. The up-regulation of (costly) defence may affect the nutritional quality of the host, the level of other energy consuming processes such as growth or reproduction, and via exuded infochemicals affect nearby community dynamics (e.g. Hellio et al. 2004; Wahl 2008). Despite this ecological potential of regulated antifouling defences, until now no such efforts have been made to study spatial and temporal variation in surface based anti-bacterial activity of marine macroalga. In previous studies (Saha et al. 2011 and chapter II) it was demonstrated that *F. vesiculosus* possesses surface associated non-polar (fucoxanthin) and polar metabolites (DMSP and proline) with anti-bacterial activity. In this present study, the seasonal and geographical variation in the anti-bacterial activity of *F. vesiculosus* has been evaluated in samples collected monthly from two physically distinct geographical locations. As fouling is a surface based process, chemical activity originating from the algal surface and not the whole cell extract has been investigated. Antifouling assays were conducted using organisms known to colonize surfaces immersed in these marine habitats.

Materials and methods

Abiotic underwater regime

The temperature and salinity regime between 0.5-1.5 m depth (Poel) and 1-2.5 m depth (Gelting) was monitored at 1 hr intervals with loggers (HOBO®, Onset Computer Corporation) from March 2009 to Feb 2010 at Gelting and March 2010 to Feb 2011 at Poel (except April 2010). Gelting features a higher salinity and a lower temperature (yearly mean 15 psu, yearly mean 8ºC, March 2009-Feb 2010; Supplementary Information Figure S1a) when compared to Poel (yearly mean 9 psu, mean 9ºC, March 2010-Feb 2011; Supplementary Information Figure S1b).

Fouling regime

To estimate the seasonal macrofouling pressure, settlement panels were deployed vertically and horizontally at 1m depth in the Kiel fjord (54°26'N/10°11'E) monthly from 2005-2008 (data courtesy of R. Schütt, IFM-Geomar). Monthly bacterial density at Boknis Eck [(54°45'N/9°83'E), 1m depth] were assessed as a coarse proxy for microbial fouling pressure in a separate project from 2005-2008 (data courtesy of H.G. Hoppe & R. Koppe, IFM-Geomar).

Algal material and surface specific extraction

In order to study potential temporal and spatial variation in chemical defence production, *Fucus vesiculosus* was collected monthly from two locations: Gelting, Germany (54°48' N/9°44' E) and Poel, Germany (54°01' N/11°28' E). The algae were individually sealed in zip-lock bags and transported back to the laboratory in a cooler box. The algae were held in a saturated seawater atmosphere (for a maximum of 12 hours) prior to extraction in order to avoid desiccation and damage. The algal branches were dipped for 10 secs into a mixture of MeOH (methanol): Hexane (1:1 v/v) as described in Saha et al. (2011) and the resulting extract was reduced under vaccum at 30 ˚C.

Five alga individuals were collected and extracted for each month and from each site (except n=4 for samples collected from Poel in March and April 2010 and from Gelting in April and November 2010).

Calculating the volume of the extracted surface-associated boundary layer

The extracted surface volume was calculated as the algal surface area (in cm^2) x 0.003 cm [estimated thickness of the surface boundary layer, (see chapter I for details)].

Bacteria

Fucus vesiculosus extracts were tested for inhibitory effect against the settlement of two strains of marine bacteria typical for biofilm on macroalgae: the gram negative bacterium *Cytophaga KT0804* (isolated from the brown macroalga *Halidrys siliquosa* and also detected on the brown macroalga *Saccharina latissima*) and the gram positive bacterium *Bacillus aquimaris* (isolated from the brown macroalga *Halidrys siliquosa* and also detected on the brown macroalgae *Desmarestia aculeate* and the red alga *Ahnfeltia plicata*).

Anti-settlement assay

Bacterial strains were grown in nutrient media (5 g peptone + 3 g yeast in 1 L filtered seawater) for 18 to 20 h. Prior to the assays, the optical density (OD) of the bacterial cultures was determined with a Beckman Du® 650 spectrophotometer (λ 600 nm). Bacterial cultures with an OD range of 0.5-0.8. were used. Settlement assays were conducted in multi-well plates (96 wells, flat bottom, Greiner®). Ninety-seven µL of bacterial suspension were added to the wells. Four µL of extract dissolved in DMSO (dimethylsulphoxide) at the 25 fold natural concentration was added to the suspension so that the tested extract in the final mixture were present at their natural concentration. Bacteria were never exposed to DMSO concentrations higher than 5%, in order to prevent toxic effects (pilot study, Wahl et al. 2010). The bacteria in suspension were confronted in multi well plates with algal extract. Wells with DMSO and bacterial suspension served as controls (n=8). The well plates were incubated for 1 h on a shaking table (100 rpm) at 20ºC. After that, the bacterial suspension

was removed from the wells by overturning the plates and unattached cells were eliminated by gently rinsing twice with 100µL of sterilised filtered seawater. The attached cells were quantified by staining (10 min in the dark) with the fluorescent DNA–binding dye Syto 9 (0.005mM) (Invitrogen, GmbH). The fluorescence as a proxy for the abundance of settled bacteria was subsequently measured (excitation 477-491 nm, emission 540 nm) using a plate reader (Hidex Chamaeleon, Turku, Fi). Each extract (n=5) was sub-replicated three fold for the purpose of settlement assay. To determine the auto-fluorescence values of the extracts itself, each extract was replicated once.

Statistical analysis

The anti-settlement activity strength of extract on bacterial settlement was expressed as the log effect ratio (i.e. decimal logarithm of the ratio of target strain settlement in the presence vs. absence of extract; Wahl et al. 2004). A log effect ratio value of 0 (i.e. equal number of bacteria in wells with extract and in wells without extract) indicates that the tested extract had no effect on settlement, whereas a negative log effect ratio value indicates an inhibitory and a positive log effect ratio value indicates an attractive effect, respectively. Thus, a log effect ratio of -1 represents a 10 fold reduction whereas a value of +1 represents a 10 fold enhancement of bacterial settlement due to the extract. Two-way ANOVA was used to analyse the effect of seasonality and site on the antifouling activity of the macroalgae. Shapiro– Wilk's test was used to test for normal distribution ($p< 0.05$), while Levene's test was used to test for homogeneity of variance ($p< 0.05$). A paired t-test (grouped by months) was used to compare the activity strength variation among sites independently of season. The computer program Statistica (StatSoft, Tulsa, OK, U.S.A.) was used to conduct all statistical tests.

Results

Fouling regime

Macrofouling pressure had a distinct seasonal pattern describing a polynomial curve ($y = -1.908\ x^2 + 24.35x - 20.73$, $R^2=0.86$, Figure 1). Bacterial density also showed a clear seasonal pattern describing a polynomial curve ($y = -0.045x^2 + 0.589x + 0.145$, $R^2=0.86$, Figure 2).

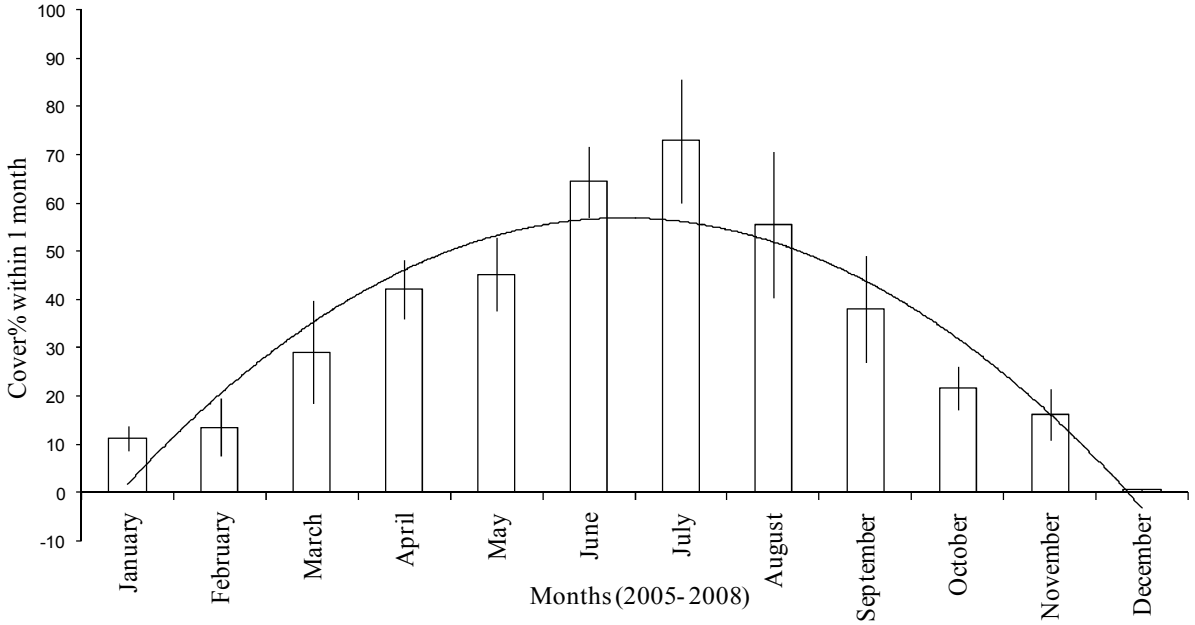

Figure 1. Mean seasonal macrofouling pressure (% cover) from 2005-2008 at Kiel fjord (1m depth). Data courtesy of R. Schütt, IFM-Geomar. Error bars are ±SE.

Temporal variation in anti-bacterial defence

The change of activity during the course of a year described a sinus curve for both the locations [$y = -1.34 + 0.2275*\cos(0.512*x-2.47)$, $R^2= 0.64$, Poel and $y = -1.03+0.13*\cos(0.467*x-1.64)$, $R^2= 0.44$, Gelting]. The anti-settlement activity of *Fucus vesiculosus* for the two bacterial strains showed a clear seasonal pattern with strongest repellence in late summer and autumn, and weaker repellence in winter and spring. This pattern was consistent among the two locations (Figure 3, Table 1). At both sites, antibacterial activity in late summer/autumn was about 2.5 times stronger than in winter/spring (Figure 3).

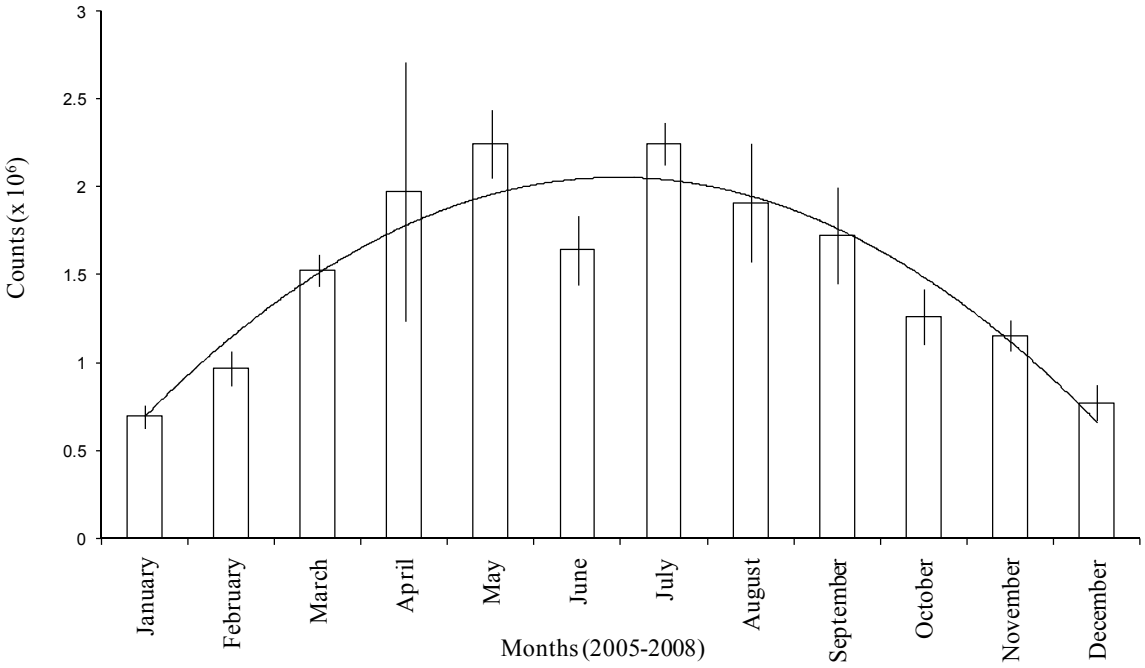

Figure 2. Mean seasonal bacterial density at Boknis Eck (1m depth). Error bars are ±SE. Data courtesy of H.G. Hoppe & R. Koppe, IFM-Geomar.

Table 1. Effect of season and site on anti-settlement defence of *Fucus vesiculosus* against bacteria (2-way ANOVA)

ANOVA	SS	df	MS	F	p
Site	3.5653	1	3.5653	75.407	0.000
Month	3.0752	11	0.2796	5.913	0.000
Site*Month	0.7906	11	0.0719	1.52	0.136
Error	4.539	96	0.0473		

Spatial variation in anti-bacterial defence

The anti-settlement activity was site-dependent with Poel extracts being more active than Gelting extracts at all seasons (Figure 3, Table 1). On average, algae from Poel were about twice as repellent as algae from Gelting (paired t-test, $p<0.001$, Table2).

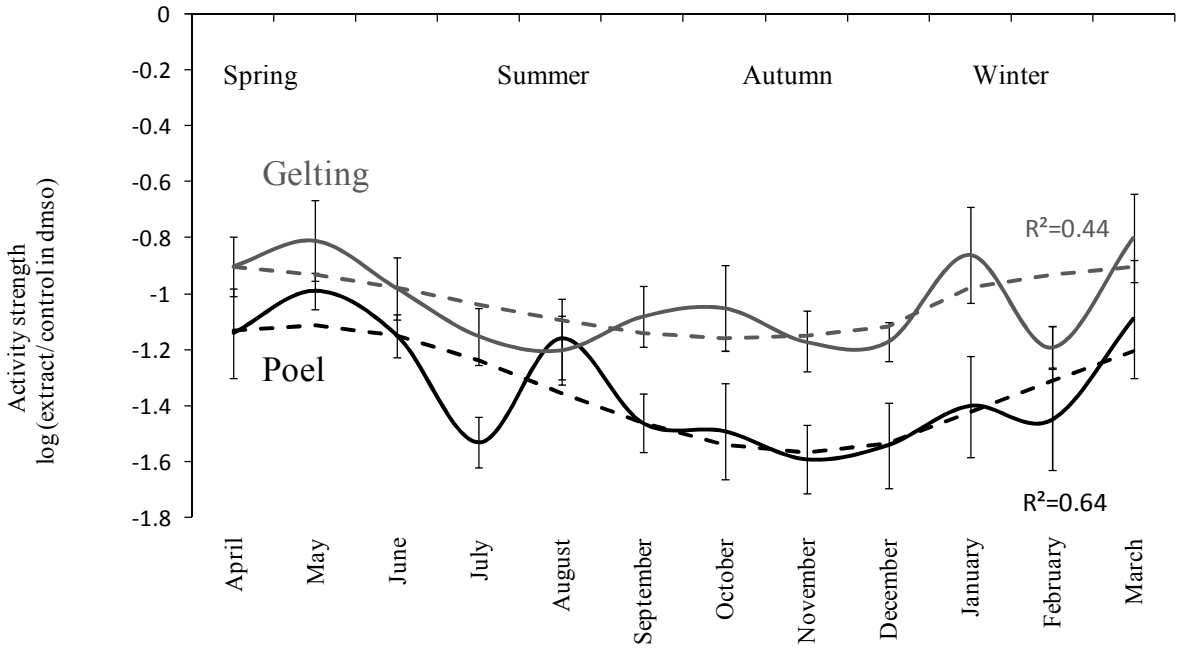

Figure 3. Seasonal and geographical variability (Gelting: grey; Poel: black) in mean antibacterial defence of *F. vesiculosus* against *Cytophaga KT0804* and *B. aquimaris* [n=10; n=8 for April, Nov (Gelting) and March, April (Poel)]. Error bars are ± 95%CI.

Table 2. Mean activity strength of extracts against *Cytophaga KT0804* and *B. aquimaris* (expressed as log effect ratio) collected monthly from March'10 to Feb'11. 95% CI in brakets.

Month		**Poel**	**Gelting**
April	Spring	-1.14 (0.16)	-0.90 (0.10)
May		-0.99 (0.06)	-0.81 (0.14)
June		-1.15 (0.07)	-0.98 (0.11)
July	Summer	-1.53 (0.09)	-1.15 (0.10)
August		-1.16 (0.14)	-1.20 (0.12)
September		-1.46 (0.10)	-1.08 (0.11)
October	Autumn	-1.49 (0.17)	-1.05 (0.15)
November		-1.59 (0.12)	-1.17 (0.10)
December		-1.54 (0.15)	-1.17 (0.06)
January	Winter	-1.40 (0.18)	-0.86 (0.17)
February		-1.45 (0.18)	-1.19 (0.07)
March		-1.09 (0.21)	-0.80 (0.15)

Discussion

In this paper we have shown seasonal and geographical variation in surface extract (non-polar, polar and very polar metabolites combined) of *Fucus vesiculosus* with respect to the anti-bacterial activity against two marine biofilm-forming bacteria (*Cytophaga KT0804* and *Bacillus aquimaris*). A clear trend in seasonal variation of anti-bacterial activity was observed with extracts of algae collected from July to December being the most active. A previous study has identified the presence of active non-polar (hexane soluble) and polar metabolites (MeOH soluble) on the surface of *F. vesiculosus* (Saha et al. 2011). Fucoxanthin has been found to be present in the range of 0.7- 9 μg cm^{-2} of algal surface in algae collected from Gelting (in July 2010) and Poel (in September 2010). This article also reports on the differences in fucoxanthin concentrations between thallus regions (apical tip versus whole alga) and between *F. vesiculosus* thallus with versus without epibiotic diatoms. Approximated 13 times more fucoxanthin was detected on the whole alga when compared to the apical tips alone. Additionally, there is evidence that algae experimentally exposed to higher temperatures featured elevated levels (by a factor of 22) of fucoxanthin on their thallus (Chapter III). The polar metabolites with antimicrofouling activity in *F. vesiculosus*, DMSP and proline also vary quantitatively with light and temperature (Chapter III). A recent study by Lyons et al. 2010 has demonstrated the spatial and temporal variation of tissue DMSP content in *Codium fragile* where the alga undergoes a seasonal cycle with DMSP content peaking in late winter or early spring.

A study on the anti-bacterial activity of the total cell extract of *F. vesiculosus* (i.e. not exclusively the compounds exposed at the thallus surface as in the present study), in contrast to this study has provided highest antimicrofouling defences in late winter and spring (Wahl et al. 2010). The findings of the two studies are not necessarily in conflict, since the activities in the whole-alga-extracts may be due to defence compounds stored within cells, while the activities in the surface extracts are factually deployed and "working". Possibly, the high spring concentration of stored defence of the whole alga extracts of the previous study is partially due to the low rate of surface deployment and, consequently, usage of these defences

in spring as found in the present study. These apparently, but not really, contrasting results underscore the importance to distinguish between antifouling activities found in surface extracts (active defence) and those found in whole-alga extracts (potential defence).

The observed seasonal variability of antifouling activity may be driven by a whole consortium of factors which in their turn feature seasonal variability. If the production of the antifouling defences incurs metabolic costs, it might be jeopardized when energy is low (e.g. winter, Lehvo 2001) and/ or when other essential processes such as growth (mainly early summer) and reproduction (mainly spring) drain on stored or newly produced energy (Wahl et al. 2010). If the deployment of defences at the thallus surface is triggered by microfouling pressure (e.g. numbers of bacteria settling per unit area and unit time), then this trigger should be strongest when bacteria are most numerous and most active in the water column, i.e. between April and September in Kiel Bight (H.G. Hoppe & R. Koppe, pers. comm, IFM-Geomar). The contribution of diatoms to the surface concentration of fucoxanthin should, in turn, correlate to the number and activity of diatoms on the thallus surface which might be highest in early summer when shading by plankton and ephemeral algae is still relatively low. The combined action of these factors may well produce the seasonal pattern of surface associated antimicrofouling defences observed.

Antimicrobial activity of *F. vesiculosus* also showed a geographical variation. Algae collected from Poel were always more active than those from Gelting. Such spatial variation in antifouling activity may be explained by different local environmental conditions. Factors like salinity, light, temperature, UV, grazing, nutrients and hydrodynamic conditions are known to affect chemical defence production in brown seaweeds (Hemmi et al. 2004; Macaya et al. 2005; Yun et al. 2007). Gelting and Poel differ in several environmental factors. Most conspicuously, Gelting features a higher salinity when compared to Poel. Tissue concentration of proline (one of the polar antimicrofouling compounds of *F. vesiculosus*) increases dramatically in *Enteromorpha intestinalis* during both short term and long term salinity acclimation (Edwards et al. 1987). DMSP also is used to adapt to long term salinity changes in *Enteromorpha intestinalis* (Edwards et al. 1987). It is unknown whether

similar processes enhance DMSP and proline concentration in the Gelting relative to the Poel population. But possibly due to a better solubility in sea water of polar metabolites their half-life at the thallus surface and thus their defence efficacy might be reduced as compared to the non-polar fucoxanthin. The Poel algae often are temporarily exposed to air or dwell just a few cms below the water surface (K. Maczassek and S. Stratil pers. comm, IFM-Geomar, Kiel) making them vulnerable to UV and temperature stress at certain times of the day. A previous study has demonstrated the UV-protective role of fucoxanthin (Bianchi et al. 1997). Additionally, transient exposure to air permits a conspicuous accumulation of antifouling products at the thallus surface (Brock et al. 2007). An enhanced concentration of fucoxanthin at the thallus surface resulting from temporary emergence might explain the higher activity of Poel extracts when compared to Gelting. Additionally, algae collected from Poel were more fouled by diatoms and green filamentous algae during certain months (particularly Feb, June and Sept) in comparison to Gelting (pers. obs.). A higher abundance of epibiotic diatoms might have contributed to a concentration of fucoxanthin at the thallus surface of the host.

To conclude, while the nature of abiotic and biotic mechanisms remain speculative at the moment, we have identified a clear and consistent seasonal pattern of antimicrofouling defences in the brown macroalga *Fucus vesiculosus* in addition to the geographical variation of defence in an ecological context. This pattern is consistent with seasonal fluctuations of resources and demands for this kind of defence. It illustrates that the dynamic nature of chemical defences found in the lab (antifouling: Wahl et al. 2010, antifeeding: Weinberger et al. 2011) can also be recognized in the field. The underlying mechanisms (e.g. the putative regulatory influence of fouling pressure), however, remain to be elucidated.

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Supplementary Information

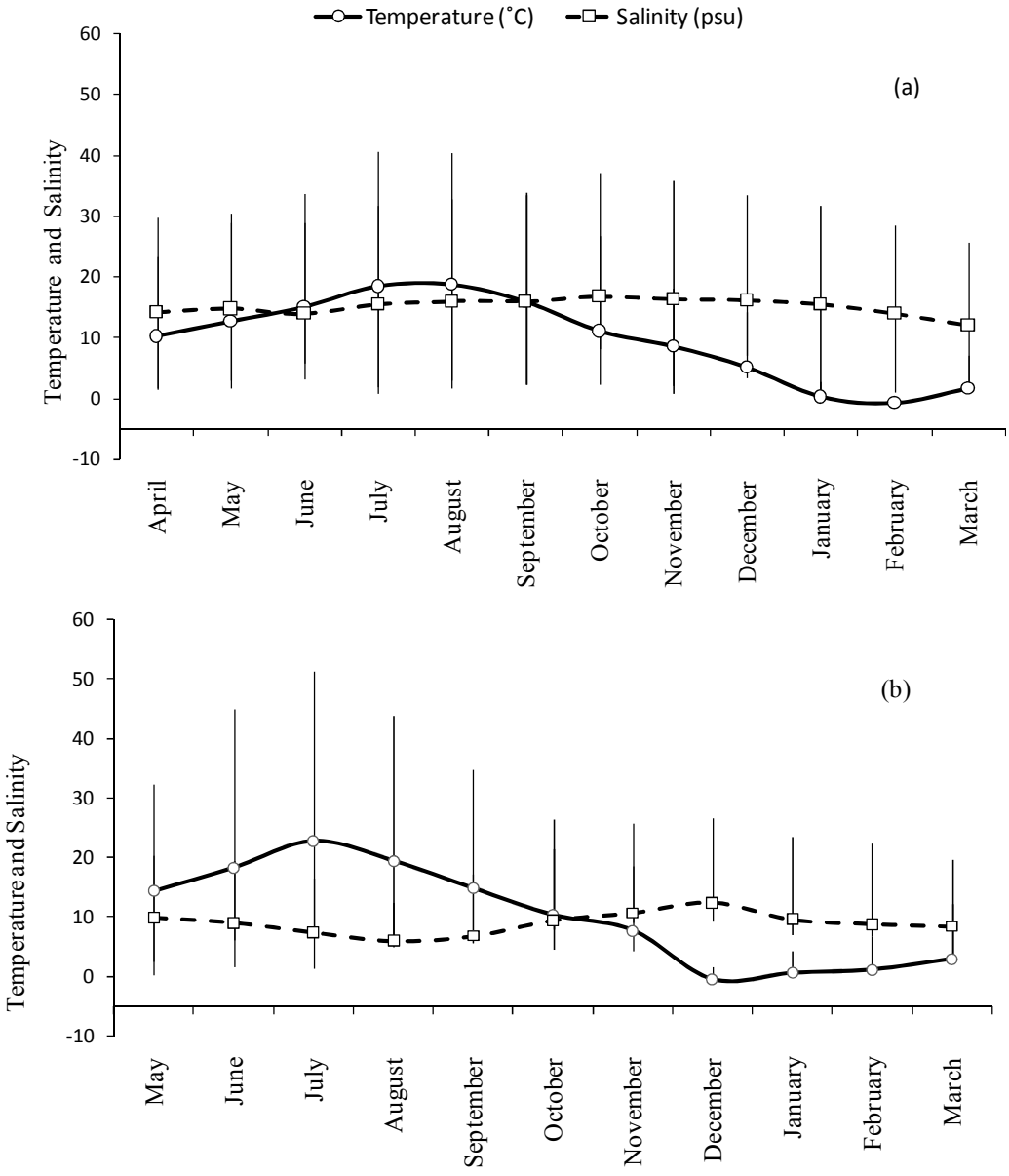

Figure S1(a) and (b). Abiotic underwater regime for temperature and salinity at Gelting (a) and Poel (b) between March 2009 to Feb 2010 at Gelting and March 2010 to Feb 2011 at Poel. Mean (central symbol), and extremes (min.–max.: thin lines) of temperature and salinity.

General Discussion

Surface associated deterrents of Fucus vesiculosus

Existence of chemical antifouling defence of the brown alga *Fucus vesiculosus* has been reported previously by Lachnit et al. 2010 (using surface extracts) and Wahl et al. 2010 (using surface extracts mostly). Until now, only phlorotannins have been suggested as anti-fouling compounds from this alga, having anti-algal and anti-barnacle larval activity (McLachlan and Craigie 1964; Brock et al. 2007). Among these two studies, only Brock et al. (2007) worked with surface associated and exuded metabolites from *Fucus vesiculosus* and reported that surface extracts had no significant effect on cyprid settlement and exuded metabolites containing phlorotannins consistently inhibited settlement. However, the role of phlorotannins in brown alga is highly controversial and phlorotannins have been suggested to have both primary and secondary roles as herbivore deterrents, digestion inhibitors, antibacterial agents and UV-screens (for details see Targett and Arnold 2003 and references therein). The role of phlorotannins being controversial it was important to identify ecologically relevant 'surface associated' compounds from the alga and to identify settlement inhibitors that target the very crucial and initial stage of fouling, i.e. bacterial colonisation. Fouling being a surface based process, only surface bound metabolites were extracted and tested against bacterial settlement (Chapter I and II). The approach of surface extraction was chosen in order to (a) avoid confounding activities from intracellular metabolites that might be produced for other roles (for e.g. anti-feeding defence) and (b) allow for an ecological assessment of metabolites deployed at the surface which would be in reach of the potential colonizers. An initial fractionation and bioassay experiment allowed for a preview of the nature of these bioactives. The very polar extract (water soluble) did not have a significant anti-settlement effect against bacteria. Sephadex G-10 chromatography of the water soluble

extract has led to the identification of a pro-settlement fraction and thus the fraction was not investigated further (data not shown). Using different chromatographic techniques followed by bioassays, I was able to isolate a pure active non-polar metabolite which was further identified to be fucoxanthin (Chapter I). The anti-microbial effect of fucoxanthin, isolated from the total cell extract of different brown algae (*Dictyota* sp. and *Laminaria* sp. respectively) against the marine biofilm forming strain *Pseudoalteromonas* sp D41 and against non-marine bacteria has been reported earlier by Viano et al. (2009) and Gerasimenko et al. (2010), respectively. Thus, the possible role in surface mediated antifouling was not investigated. This thesis has been the first to report the ecological function of fucoxanthin. In contrast to Lachnit (2010) (where surface extract was tested), the surface presence of fucoxanthin has been proven here and also a detailed investigation on the origin of this primary metabolite has been realized. Using the 'swabbing' technique (Schmitt et al. 1995) the existence of fucoxanthin on the surface of *F. vesiculosus* was demonstrated. With a GeO_2 inhibition experiment (to reduce diatom density) I was able to segregate the origin of fucoxanthin between the alga and its surface associated epibiotic diatoms and to prove an apparent surface deployment of this compound by *F. vesiculosus* itself. It was essential to demonstrate the metabolic provenance of this compound because (a) fucoxanthin has a 'primary' photosynthetic role and is mainly stored in the chloroplasts - when present at the outer algal surface, it probably might have some 'secondary' purpose - and (b) surface fucoxanthin could stem from the host alga or from epibiotic diatoms. Release of fucoxanthin by *Fucus* or any other brown algae has not been demonstrated before. Fucoxanthin had a siginificant inhibitory effect when tested at its natural concentration on almost all the bacterial isolates isolated from co-occuring algae and marine sediment. Thus it was possible to demonstrate its wide range anti-settlement activity against bacteria. Additionally, the

presence of certain strains on the algal surface could be explained by their reduced sensitivity towards fucoxanthin.

Until now only Nakamura et al. (2002) and Matz et al. (2008) have reported an anti-bacterial and anti-protozoan role of a pigment, violacein from a psychrotrophic bacterium (very closely related to *Janthinobacterium lividum*) and from biofilm bacteria, respectively. This thesis reports for the first time the same novel defence strategy, i.e. use of a pigment in a secondary function, but for a marine alga.

A basic question with respect to antifoulants is whether these metabolites are present at or near the surface of the organism at ecologically effective concentrations. Examples of inhibitors that have been localized and quantified at or near the surface are relatively few. Vesicles from the gland cells present on the surface of *Delisea pulchra* (red alga) contain furanones and release the compound at sufficient concentrations to deter common epibionts (de Nys et al. 1995, 1998; Maximilien et al. 1998). Terpenoids from the surface extracts of the brown seaweed *Dictyota menstrualis* deter settlement of the bryozoan *Bugula neretina* at natural concentrations (Schmitt et al. 1995). 1,1,3,3-tetrabromo-2-heptanone has been found to inhibit bacterial colonisation on the surface of the red alga *Bonnemaisonia hamifera* (Nylund et al. 2008). Some algal metabolites, in contrast, either do not appear to be present on the surface (de Nys et al. 1998) or are not present at sufficiently high concentrations (Jennings and Steinberg 1997; Steinberg and de Nys 2002) to produce an effect. The Brazilian red seaweed *Laurencia obtusa,* which produces the sesquiterpenoid eltaol has been found to inhibit foulers (da Gama et al. 2002) and consumers (Pereira et al. 2003) when natural tissue (not surface) concentrations of the compound were tested. But when Sudatti et al. (2008) investigated and tested the surface concentration of elatol, the compound was not

active against barnacle and mussel settlement. Thus, surface fucoxanthin quantification on the algal tips and whole thalli (including the tips) was realized in chapter I.

Most of the surface deterrents identified so far have been mainly non-polar compounds like terpenoids from *Dictyota menstrualis*, furanones from *Delisea pulchra,* elatol from *Laurencia obtusa* (Schmitt et al. 1995; de Nys et al. 1995, 1998; Maximilien et al. 1998; but see Harder et al. 2004 for a report of polar compounds). These compounds have a small molecular size (Steinberg et al. 2002) and - being non-polar – they can adhere to the surface of the producing organism (Jennings and Steinberg 1997). In contrast, polar compounds may have a higher rate of diffusion into the surrounding seawater in absence of strong inter ionic interactions or hydrogen bonds. I have therefore investigated the active polar inhibitors of bacterial settlement from *Fucus vesiculosus* in chapter II of my thesis, in addition to the well known non-polar modes of allelochemical defence against epibiosis. Sephadex LH20 chromatography of the active polar extract (MeOH soluble) allowed me to isolate an inhibitory fraction which when further analysed revealed the presence of three dominant metabolites: DMSP (dimethyl sulphopropionate) and the amino acids proline and alanine. These metabolites were not only active when tested singly in a surface area based assay but also when tested together in a surface volume based assay. The methionine derived sulphonium compound DMSP has a number of putative ecological and physiological roles in marine micro and macroalgae and invertebrates, e.g. as cryoprotectant, osmo-regulator or anti-grazing compound (Van Alstyne 1988; Karsten et al. 1996; Sunda et al. 2002). In contrast to the study of Jackson and Stukey (2007), who found no effect of DMSP from the cord grass *Spartina alterniflora* on the productivity of epiphytic algae, an anti-fouling activity of DMSP at natural concentration is reported here for the first time. Certain heterotrophic bacteria have been found to be attracted to microscale pulses of DMSP (Seymour et al. 2010).

DMSP is used as a dominant nutrient and metabolized very quickly by certain bacteria (Dickschat et al. 2010). Thus, the alga seems to use DMSP in maintaining a specific bacterial community only, similar as observed for the strain specific anti-bacterial activity range of fucoxanthin (Chapter I). A further novel aspect was the detection of the amino acid proline as an anti-settlement compound. Proline rich peptides have been reported to posses anti-microbial and antibiofilm activity in non-marine studies (Dean et al. 2011; Scocchi et al. 2011). Yang et al. (2010) reported the presence of a proline rich dipeptide having cytotoxic properties from the marine sponge *Callyspongia* sp. To my knowledge, no such anti-settlement role of proline has been reported until now.

Surface quantification of these active metabolites (DMSP, proline and alanine) was of crucial importance to validate the activity of these metabolites at natural concentrations on the algal surface. DMSP and proline were found to be sufficiently concentrated but alanine, although having an anti-settlement activity, was not concentrated enough to have an ecological effect. The low natural surface concentration of alanine may probably not be absolute and may vary with different environmental factors. It was also essential to know whether the activities of proline and alanine are specific or whether simply all amino acids interfere with bacterial settlement. A general effect of potentially essential nutrients, such as amino acids, on bacterial settlement could not per se be considered as impossible. However, valine and iso-leucine exhibited a pro-settlement effect, which proved the anti-settlement specificity of proline and alanine.

As with fucoxanthin, the activity and activity strength of DMSP and proline was variable among different strains. Out of the five tested strains, three and four strains were more sensitive to DMSP and proline, respectively. In this study, I didnot find any evidence of phlorotannins as bacterial settlement inhibitors which might be confirming the results of Jennings and Steinberg (1997) where phlorotannins had negligible impact on patterns of

epiphytism on the kelp *Ecklonia radiata.* However, in this thesis the surface extracts of *F. vesiculosus* were not tested against cyprid larvae or other macrofoulers, which were found to be sensitive toward phlorotannin (Brock et al. 2007; see discussion further up).

Interestingly, all the bacterial settlement inhibiots detected from this alga are relatively widely spread or might be even universally present among macroalgae. It may therefore be expected that they could be relevant for the antimicrobial defence of other species than *F. vesiculosus* alone. This is particularly the case for DMSP, which reaches more than ten times as high tissue concentrations in green seaweeds as in *F. vesiculosus* (Lyons et al. 2010) and could thus potentially be more concentrated on green algal surfaces, as well.

Defence and abiotic factors/ stressors

Variations in plant secondary metabolites are frequent and are influenced by a number of environmental factors like nutrients, salinity, light and temperature (Karsten et al. 1992; Yates and Peckol 1993; Kamiya et al. 2010; Pavia and Toth 2000). Although defensive compounds can enhance performance, reduce mortality and limit the competition an alga experiences, their production is usually expected to be associated with a number of costs (Lyons et al. 2010). For example, growth of *F. vesiculosus* has been negatively correlated with phlorotannin production (Jormalainen & Ramsay 2009). However, no evidence of defence costs has also been found in certain seaweeds (e.g. Pansch et al. 2009). As discussed earlier, phlorotannins have multiple functions in marine plants and their production might be regulated by their primary roles (Targett and Arnold 2003). In cases where defensive compounds have additional functions, resource limitation may not jeopardize their tissue or surface concentration (e.g. Pohnert et al. 2007). This could be the case for the three anti-settlement compounds from *Fucus*, i.e., fucoxanthin, DMSP and proline, which are known to

have primary roles as photosynthetic pigment and osmoregulators, respectively (Bjørnland and Liaaen-Jensen 1989; Edwards et al. 1987; Kirst 1996).

In this study (Chapter III) I have assessed the influence of disruptive temperature shifts (in indoor mesocosms) and of natural light limitation (in outdoor mesocosms) on fucoxanthin, DMSP and proline concentrations. DMSP was found to be significantly elevated at 20˚C in comparison to 5, 10, 15 and 25˚C. DMSP was down-regulated at the highest temperature of 25˚C, which corresponds to the environmental stress hypothesis (e.g. Cronin 2001) and was similar to the results of Sudatti et al. 2011, who reported that concentrations of the secondary metabolite elatol decreased at a relatively higher temperature of 35˚C. The increase of DMSP with temperature was in contrast to the results of Lyons et al. (2010), who found increasing tissue DMSP with decreasing temperatures in the green algae *Codium fragile* and suggested that this was due to the role of DMSP as cryo-protectant in green algae (Karsten et al. 1996). A role of DMSP as cryoprotectant may be less probable as it is considerably lower in *Fucus* than in green algae (Blunden et al. 1992). Also, the surface and tissue concentrations of DMSP in *F. vesiculosus* have not been correlated yet, and low temperatures may therefore in theory result in a relatively high tissue content, but in a low surface concentration of DMSP. Many microalgae and green algae are known to be DMSP (e.g. Kasamatsu et al. 2004) producers. Since *Fucus* bears epiphytic diatoms and at times associated filamentous green algae on its surface, a part of the compound may have been contributed by these epiphytes. But a particularly low DMSP concentration was detected at a higher temperature of 25°C despite stronger fouling pressure by epiphytic diatoms. In the light experiment too, although surface fucoxanthin concentration was positively co-related to the diatom abundance no such co-relation was observed among DMSP and diatom abundance. This might indicate that DMSP was not contributed by the host's epiphytes. In the temperature experiment both proline and fucoxanthin were detected below the necessary concentration for halfmaximal

settlement inhibition (EC_{50}), which had been determined in the previous study (Chapter II). A tendency towards elevated DMSP surface contents was observed under low light conditions, except at 100% sun light, where the variability was particularly high. These results are in contrast to earlier studies, in which tissue DMSP increased with increasing light intensities in *Codium fragile* (Lyons et al. 2010) and other algae (e.g. Karsten et al. 1992), probably due to its role as an anti-oxidant. Although a not much higher proline concentration was detected when compared to the previous results (Chapter II), proline was present above the EC_{50} dose under all light conditions. Fucoxanthin was in all treatments present below the EC_{50} concentration.

It might be possible that DMSP and proline act in synergy. In chapter I when the total polar extract was tested without prior fractionation and purification had a higher activity strength against bacteria settlement when compared to DMSP and proline tested singly. This could be explained through synergisms among the identified or even among unidentified components.

In chapter I fucoxanthin was detected at a higher surface concentration on whole algae than in chapter III on apical tips. Thus there could be a possibility that fucoxanthin is more present and active in the older parts of the thallus compared to the tips. There could thus be a possible interchange of roles among the polar and non-polar deterrents and different thallus parts and the hypothesis need to be tested for.

Overall, under all tested temperature and light conditions at least one deterrent, DMSP, was present above the threshold concentration to warrant a strong inhibition or at least partial inhibition of the settlement of relevant bacterial microfoulers (see Chapter III for details). Taken all together, the anti-settlement defence of *F. vesiculosus* against bacteria appears not as significantly affected by limitation stress or disruptive stress.

Such kind of defences could be non-costly (e.g. Kearsley & Whitham 1992) and may come cost free with regard to anti-epibiosis (Wahl et al. 1998) because in addition to serve as secondary defensive substances, primary metabolites such as fucoxanthin, DMSP, proline have evolved for other important physiological and ecological roles, similar to the phlorotannins of brown algae (Targett and Arnold 2003). Since the production of these chemicals may be driven by their other primary functions, energy limitation might not affect their production. If energy limitation is severe then these metabolites might be affected less when their primary function is of higher priority.

My thesis highlights the evidence of a multicomponent anti-settlement chemical defence strategy of *F. vesiculosus* against bacteria. The combined use of multiple protection mechanisms has been reported surprisingly seldom (Targett et al. 1983; Wahl & Banaigs 1991; Thomason et al. 1996; Wahl et al. 1998), but the results of my study (Chapters I and II) strongly support the concept of multi-component defence. These multiple repellents may interact and have a stronger combined efficiency. They could also more easily compensate when resources are limited. This thesis reflects the necessity to study all possible defence modes of an organism in detail, in order to do justice to the complex ecological truth.

Spatial and temporal variability of defences

Investigating the seasonal and spatial variability of anti-settlement defence of *Fucus* against bacteria in Chapter IV has enabled me to study the dynamics of antifouling defence of the alga in an ecological perspective. Until now relatively few studies have investigated seasonal and geographical variation of antifouling defence in marine algae (Hellio et al. 2004; Plouguerné et al. 2010). *Bifurcaria bifurcata* has been found to show a seasonal variability in the chemical composition of nonpolar compounds (Culioli et al. 2002). A temporal variation of antifouling activity has been reported from *B. bifurcata* when the extracts were tested

against two marine bacteria- *Cobetia marina* and *Pseudoalteromonas haloplanktis*, and against cyprid larvae of the barnacle, *Balanus amphitrite*. A spatial variation of antifouling activity was also observed for the brown alga *Sargassum vulgare* when the crude extracts of different polarity scales were tested against marine fouling bacteria, microphytobenthic strains and the mussel *Perna perna* (Plouguerné 2010). However, none of these studies were based on surface bound metabolites. Thus they were unable to differentiate between surface compounds that are relevant for the antifouling defence and compounds stored in the tissue but not affecting potential foulers. In this study (Chapter IV) the pattern of surface concentrations of defence compounds detected in *F. vesiculosus* (with peak activity in late summer/ autumn) corresponded well to the seasonally fluctuating fouling pressure by bacteria in the Western Baltic. The antifouling defence was apparently demand driven, as higher defence was detected in parallel to higher cell numbers of bacteria in the water column. At a spatial scale, algae collected from Poel consistently had a higher activity strength than algae collected at Gelting (ca. 140 km apart), which might be explained by variations in the local environmental conditions at these two distant sites. Factors such as salinity, light, UV, grazing, temperature, hydrodynamics or nutrients are known to influence the production of chemical defence compounds in brown seaweeds (Hemmi et al. 2004; Macaya et al. 2005; Wiencke et al. 2007; Yun et al. 2007). Gelting and Poel differ in several environmental factors, with Gelting having a higher salinity and lower water temperature than Poel, and the Poel population experiencing more frequent transient exposure to air, UV and temperature stress, as it dwells just a few centimetres below the water surface (K. Maczassek and S. Stratil pers. comm.). All these factors may have contributed to the variation in defence between both populations (for details see Chaper IV). Although the biotic and abiotic factors involved remain subject to speculation, a clear and consistent spatial and temporal anti-fouling defence variability was identified for the first time for an alga in an ecological

context. This variability requires a detailed investigation of the underlying specific mechanisms responsible for the defence dynamics.

Conclusions

In a nutshell, the work reported in this thesis gives new insights into the multilevel antifouling mechanisms of marine organisms. It identifies a novel secondary role as defensive metabolite for the photopigment fucoxanthin in *Fucus vesiculosus*. It further reports on DMSP as an anti-foulant, together with the amino acid proline. Antifouling defence at the algal surface is shown to vary with season and location (presumably in response to fouling pressure) and to be moderately affected by environmental stress.

My study also pinpoints the need of studying the possible existence of multiple defence strategies of organisms, in order to allow for a better understanding of basibiont-epibiont interactions and to predict future responses of organisms to single or multiple environmental factors/ changes.

Looking ahead

In future the anti-bacterial metabolites of *F. vesiculosus* should also be tested against macrofoulers, in order to verify whether any possible multiple actions of these chemicals against both micro- and macrofoulers exist.

Since defence chemistry varies within organisms (Cronin and Hay 1996), one more interesting aspect could be to understand how the surface content of these deterrents varies at a given time and whether there is interplay among these polar and non-polar inhibitors at different parts of the alga, as suggested in the discussion.

F. vesiculosus has been found to induce or reduce its anti-feeding defence in response to grazing pressure (Rohde and Wahl 2008). No such information is available until now in relation to anti-fouling chemical resistance for *Fucus* or any other algae (Wahl et al. 2010).

Defence has been found to be apparently demand driven with season (Chapter IV). However, the driving factors (for e.g. influence of fouling pressure) of such variation were not identified. Therefore it would be interesting to see whether the defence metabolites are up or down regulated in relation to the presence of potential foulers around.

DMSP and its cleavage products (DMS and acrylic acid) have been reported earlier to have anti-grazing effects in other marine algae (Van Alstyne et al. 2001; Lyons et al. 2007). Thus a possible dual use of this compound by *Fucus* as an antifoulant and an antiherbivory agent would not be surprising and may be tested in the future. It is not known whether or how antifouling and antifeeding defences in algae interact (are they independent / antagonistic / linked by shared biosynthetic pathways?) (Wahl et al. 2010). Thus further investigation might help us in answering these questions and help us in better understanding the chemical defence ecology of *Fucus*.

Lastly, environmental change involves parallel shifts of many other factors, excluding the marginal habitats which are mainly characterised by single stresses or drastic change of one abiotic factor (Wahl et al. 2011). Thus, along with studies on single biotic or abiotic factors, an interactive effect of these factors should be more often examined, in order to better comprehend the fate of *Fucus* or any other marine study organism in association with the proposed regional/ global climate change.

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Erklärung

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

Kiel, den

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