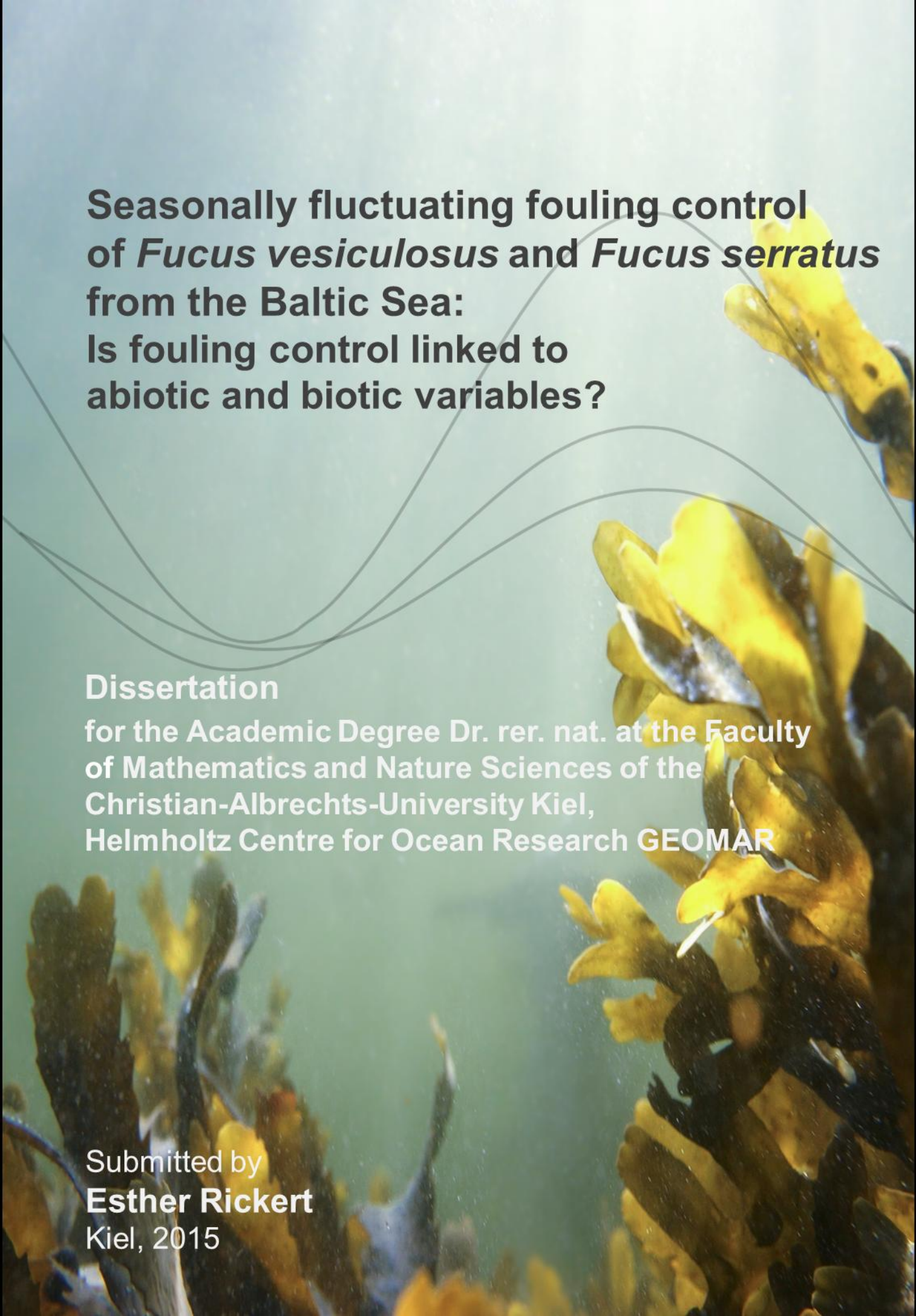
Seasonally fluctuating fouling control of *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea: Is fouling control linked to abiotic and biotic variables?



Dissertation

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

Submitted by
Esther Rickert
Kiel, 2015

The cover photo is taken at the experimental site (Bülk, outer Kiel Fjord) showing the two study organisms *Fucus serratus* (left) and *Fucus vesiculosus* (right). In the background of the photo appears one of the logger/settlement panel stations used during the experiment to record the field parameters.
Cover photo courtesy of Dr Vincent Saderne.

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Abstract

In this doctoral project, I investigated the putative seasonal fluctuating chemical fouling control of the perennial macroalgae *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea under *in situ* conditions as well as potential links between the chemical fouling control and different abiotic and biotic factors.

To investigate these potential relations, it was first necessary to examine if and how abiotic and biotic parameters and the antifouling control of the two *Fucus* species fluctuate with season. To this end, a field experiment running for one year, combined with laboratory work, was designed. During the field experiment, the abiotic parameters light, temperature and salinity were continuously recorded and nutrient concentrations were analysed weekly. The actual micro- and macrofouling pressure was recorded by means of artificial settlement substrate and the fouling pressure was quantified by enumeration of settled foulers via epifluorescence microscopy or stereomicroscopy. Furthermore, 15 *Fucus* individuals per species were collected monthly at the same site to quantify the 'fouling status' via enumeration of associated prokaryotic and diatom cells (microfouler species) as well as of attached *Amphibalanus improvisus* and *Mytilus edulis* (macrofouler species) and the 'energy status' via mannitol analysis. Moreover, associated metabolites were harvested from each Fucus individual surface by means of surface extraction. Surface extracts were investigated for their seasonal fouling control strength by means of an *in situ* settlement bioassay. Fouling control strength was determined by quantification of settled prokaryotic and diatoms cells and the two predominant macrofouler species *A. improvisus* and *M. edulis*. Furthermore, surface extracts were analysed via GC-MS to identify possible seasonal variance in the composition.

The examination of the abiotic and biotic parameters revealed seasonal fluctuations. Light, temperature, salinity and nutrient concentrations exhibited the expected seasonal pattern typical for the temperate Baltic Sea. Fouling pressure also varied with season. Microfouling pressure mainly attributable to prokaryotic and diatom fouling was highest during spring and summer months. Macrofouling pressure was most intense in summer, whereby *A. improvisus* and *M. edulis* were the dominant epizoan fouler species. The fouling status of both *Fucus* species also varied with season. Microfouling on both *Fucus* species was highest in summer. Prokaryotic fouling reached maximal densities in June and August (*F. vesiculosus*) and in June (*F. serratus*). Diatom fouling on *F. vesiculosus* showed peak densities in April and September, whereby diatoms on *F. serratus* were very rare and only present in April

and June. In general, *F. vesiculosus* was more densely fouled than *F. serratus* and prokaryotic fouling quantitatively dominated on both *Fucus* species compared to diatom fouling. Macrofouling on *F. vesiculosus* by epizoans was most intense in July and October and on *F. serratus* between November and February, with a maximum in January. *T*he barnacle *A. improvisus* was found on *F. vesiculosus* in July and August in low numbers. *M. edulis* (juvenile life stages) was maximal in July and August and the most common fouling species on *F. vesiculosus.* On *F. serratus, A. improvisus* was only present in May, while *M. edulis* (juvenile life stages) occurred at lower densities throughout the year. Tissue mannitol of both *Fucus* species showed a clear seasonal variation with increasing concentrations from February to October, followed by a reduction to half the summer values until December. Regarding the microfouling control strength, extracts from both *Fucus* species tended to be less attractive for microfoulers during seasons when field microfouling pressure was highest, suggesting a deployment of defensive metabolites. In contrast to this, *F. vesiculosus* surface extracts revealed a general pro-fouling effect on prokaryotes, compared to solvent blanks. Additionally, microfouling control strength seems to be assisted by periodical cuticula shedding. Macrofouling control strength against the barnacle *A. improvisus* varied with season and matches the seasonal fluctuations in fouling pressure of this species, showing strong control in periods of intensive fouling. For the mussel and transient fouler *M. edulis,* a corresponding pattern was not found. The observed seasonal patterns of micro- and macrofouling control seem not to be linked with the 'energy status' of both *Fucus* species. Surface metabolite analysis revealed differences between spring/summer and autumn/winter months for both Fucus species. The seasonal variance was best explained by the abiotic variables light and temperature. GC-MS analysis showed an up-regulation of mono- and disaccharides and of three hydroxy acids in *F. vesiculosus* summer extracts and an up-regulation of saturated fatty acids and three saccharides in *F. serratus* summer extracts compared to winter extracts.

My thesis highlights the seasonal dynamics of the chemical micro- and macrofouling control of *F. vesiculosus and F. serratus* from the Baltic Sea tested under *in situ* conditions as well as the impact of environmental variables on the fouling control strength. Further, this work demonstrates that both *Fucus* species exhibit pro- and antifouling properties during summer when fouling pressure is highest indicating a complex regulation of biofouling control. The study thus provides new insides into the complex algae-environment-fouler interactions and their seasonal dynamics.

Zusammenfassung

In dieser Doktorarbeit wurde die vermeintlich saisonal fluktuierende chemische Fouling-Kontrolle der mehrjährigen Makroalgen *Fucus vesiculosus* und *Fucus serratus* aus der Ostsee unter *in situ* Bedingungen sowie mögliche Zusammenhänge zwischen der chemischen Foulingkontrolle und verschiedenen abiotischen und biotischen Faktoren untersucht.

Zunächst wurde untersucht, ob und wie die Foulingkontrolle der zwei *Fucus*-Arten sowie die abiotischen und biotischen Parameter saisonal fluktuieren. Zu diesem Zweck wurde ein einjähriges Feldexperiment kombiniert mit Laboranalysen durchgeführt. Während des Feldexperiments wurden die abiotischen Parameter Licht, Temperatur und Salzgehalt kontinuierlich aufgezeichnet und die Nährstoffkonzentrationen wöchentlich analysiert. Der *in situ* Mikro- und Makrofoulingdruck wurde mit Hilfe künstlicher Siedlungssubstrate erfasst und durch direkte Auszählung mittels Epifluoreszenz- bzw. Stereomikroskopie aller gesiedelten Fouler quantifiziert. Darüber hinaus wurden monatlich 15 *Fucus*-Individuen pro Art am selben Standort gesammelt, um den Foulingstatus mittels Auszählung assoziierter Prokaryoten und Diatomeen-Zellen (Mikrofouler-Arten) sowie festsitzender *Amphibalanus improvisus* und *Mytilus edulis* (Makrofouler-Arten) zu beziffern und den Energiestatus mittels Mannitol-Analyse zu quantifizieren. Außerdem wurden von jedem *Fucus*-Individuum oberflächen-assoziierte Metabolite mittels Oberflächenextraktion geerntet. Die Oberflächenextrakte wurden auf ihre saisonale Fouling-Kontrollstärke mit Hilfe von *in situ* Siedlung-Bioassays untersucht. Die Fouling-Kontrollstärke wurde mittels Quantifizierung gesiedelter Prokaryoten und Diatomeen-Zellen sowie der zwei vorherrschenden Makrofouler-Arten *A. improvisus* und *M. edulis* bestimmt. Außerdem wurden die Oberflächenextrakte mittels Gaschromatographie mit Massenspektrometrie-Kopplung (GC-MS) analysiert, um mögliche saisonale Schwankungen in der Zusammensetzung zu identifizieren.

Die abiotischen und biotischen Parameter zeigten saisonale Fluktuationen. Licht, Temperatur, Salinität und Nährstoffkonzentrationen wiesen die erwarteten saisonalen Muster typisch für die temperierte Ostsee auf. Der Fouling-Druck variierte ebenfalls mit der Jahreszeit: Der Mikrofouling-Druck, hauptsächlich geprägt durch Prokaryoten- und Diatomeen, war während der Frühjahrs- und Sommermonate am stärksten. Der Makrofouling-Druck, vor allem durch die dominanten Arten *A. improvisus* und *M. edulis*, war im Sommer am intensivsten. Der Fouling-Status auf beiden *Fucus*-Arten variierte ebenfalls mit der Jahreszeit: Mikrofouling auf beiden *Fucus*-Arten war im Sommer am

höchsten. Prokaryotisches Fouling erreichte im Juni und August maximale Dichten auf *F. vesiculosus* bzw. im Juni auf *F. serratus*. Diatomeen-Fouling auf *F. vesiculosus* zeigte höchste Dichten im April und September, wobei Diatomeen auf *F. serratus* sehr selten waren und nur im April und Juni auftraten. Im Allgemeinen war *F. vesiculosus* dichter besiedelt als *F. serratus* und prokaryotisches Fouling dominierte quantitativ auf beiden *Fucus*-Arten verglichen mit Diatomeen-Fouling. Makrofouling auf *F. vesiculosus* durch Epizoa war im Juli und Oktober am intensivsten und auf *F. serratus* zwischen November und Februar, mit einem Maximum im Januar. Die Seepocke *A. improvisus* wurde auf *F. vesiculosus* im Juli und August in geringer Anzahl gefunden. *M. edulis* (juvenile Lebensstadien) waren am häufigsten im Juli und August und die häufigste Fouler-Art auf *F. vesiculosus*. Auf *F. serratus* war *A. improvisus* nur im Mai anwesend, während *M. edulis* (juvenile Lebensstadien) in geringen Dichten das ganze Jahr hindurch auftraten. Der Mannitolgehalt beider *Fucus*-Arten zeigte eine klare saisonale Variabilität mit ansteigenden Konzentrationen von Februar bis Oktober, gefolgt von einer Reduzierung um die Hälfte der Sommerwerte bis zum Dezember. Bezüglich der Mikrofouling-Kontrollstärke, tendierten die Extrakte beider *Fucus*-Arten dazu, in Jahreszeiten, in denen der Mikrofouling-Druck im Feld am höchsten war weniger attraktiv zu sein. Das deutet den Einsatz von Abwehrmetaboliten an. Im Gegensatz hierzu zeigten die Oberflächenextrakte von *F. vesiculosus* einen allgemeinen profouling Effekt auf Prokaryoten verglichen zu den Lösungsmittel-Blanks. Zusätzlich variierte die Mikrofouling Kontrollstärke gegen die Seepocke *A. improvisus* mit der Jahreszeit und stimmte mit den jahreszeitlichen Fluktuationen im Fouling-Druck dieser Arten überein, ein Hinweis auf starke Kontrolle in Zeiträumen intensivem Foulings. Für die Muschel und vorrübergehenden Fouler *M. edulis* wurde kein entsprechendes Muster gefunden. Die beobachteten jahreszeitlichen Muster von Mikro- und Makrofouling-Kontrollstärke scheinen nicht mit dem Energiestatus beider *Fucus* Arten verbunden zu sein. Die Analyse der Oberflächenmetabolite zeigte Unterschiede zwischen Frühjahr/Sommer und Herbst/Winter für beide *Fucus*- Arten. Die jahreszeitlichen Varianzen erklären sich am besten durch die abiotischen Variablen Licht und Temperatur. GC-MS Analysen zeigten eine Hochregulierung von Mono- und Disacchariden und von drei Hydroxysäuren in *F. vesiculosus* Sommer-Extrakten und eine Hochregulierung von gesättigten Fettsäuren und drei Sacchariden in *F. serratus* Sommer-Extrakten verglichen mit Winter- Extrakten.

Meine Doktorarbeit verdeutlicht die jahreszeitliche Dynamik der chemischen Mikro- und Makrofouling-Kontrolle von *F. vesiculosus* und *F. serratus* aus der Ostsee unter in situ Bedingungen sowie den Einfluss von Umweltvariablen auf die Fouling Kontrollstärke. Zusätzlich zeigt diese Arbeit, dass beide *Fucus* Arten pro- und

antifouling Eigenschaften im Sommer aufweisen, wenn der Fouling-Druck am höchsten ist, was eine komplexe Regulierung der Biofouling-Kontrolle andeutet. Diese Studie liefert somit neue Einsichten in die komplexe Algen-Umwelt-Fouler Interaktion und ihre jahreszeitliche Dynamik.

Content

1. General Introduction

In the euphotic zone of the sea, submerged surfaces in general become densely covered by a variety of different organisms sooner or later, ranging from microscopic prokaryotic cells, diatoms and ciliates to macroscopic barnacles, mussels, small filamentous macroalgae and large macrophyte species. Since many marine organisms reproduce seasonally, their juvenile life forms also occur and many of them strive to settle on free surfaces seasonally. Consequently, the colonisation of submerged surfaces also underlies a seasonal variability. Humans try to prevent these colonisation processes on man-made structures by periodical cleaning or by applying toxic paints. Natural structures, especially the functional outer body surface of many marine organisms, such as ascidians or macroalgae, mostly remain visibly free from macroscopic organisms. The reason for this phenomenon lies in the evolution of a natural fouling control based on physical and chemical mechanisms.

In this doctoral thesis, I investigate the seasonal dynamics of the chemical fouling control of the brown macroalgae *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea as well as possible links to abiotic and biotic factors.

Hereafter, I will use the term ‘fouling control’ instead of the commonly used term ‘antifouling defense’, since previous studies and my thesis have shown that *Fucus* surface extracts have not only deterring but also attracting effects, so-called profouling effects, on fouler species.

1.1 Sessile mode of life and marine epibiosis

In the marine environment, the sessile mode of live is widespread (Harder 2008). The reasons lie in the special physical features of water. First, water is denser than air and has a relatively high viscosity, causing a reduced weight and increased drag on particles. This situation entails the need for some attachment for organisms to avoid drift. Second, the universal solvent and vector character of water supplies ions, nutrients and particles to attached organisms making a permanent sessile mode of life possible (Wahl 2009). Seawater can contain up to 10^7 viruses, 10^6 bacteria cells, 10^3 fungal cells and between 10 to 100 microscopic larvae and

spores per millilitre depending on season (Harder 2008). Most of these organisms strive to settle on hard substrate. For example, alga spores (Fletcher & Callow 1992) and larvae (own observation for *Spirorbis* sp. larvae) need to attach for successful development and bacteria are known to perform best when living on surfaces associated in so-called biofilm communities (Grossart 2010). Considering the amount of potential settlers in one millilitre of seawater and the fact that their preferred sessile life-style requires stable hard substrate, it becomes obvious that available settlement surface in the marine environment, especially in the euphotic zone, is limited (Harder 2008). Established adult sessile organisms and young motile life forms compete for free available settlement substratum, which leads to an immense competition for space in the benthic environment (Wahl 1989, Harder 2008). The emergence of "*epibiosis"* (*epi* "on top" and "*bios"* life), the close association between organisms, is a typical phenomenon in the marine environment and the consequence of limited settlement substratum (Harder 2008). Epibiosis is the mainly facultative association (Wahl & Mark 1999) between one or more organisms growing attached to the outer surface of a substratum organism (Wahl 1989). The overgrown or "*fouled*" organism is termed the "*basibiont*" or host and the organism growing on top of it is termed the "*epibiont*" (Wahl 1989, Harder 2008). The development of such epibiotic communities on living and non-living surfaces is termed "*biofouling*" or "*fouling*" (Wahl 1989, Harder 2008). The colonisation of unoccupied hard substrate in general follows a basic pattern of four colonisation phases: (1) adsorption of organic molecules, such as proteins and polysaccharides, also called molecular biofouling (2) colonisation by prokaryotic cells, (3) colonization of unicellular eukaryotes, such as diatoms, ciliates and (4) colonisation by larvae and spores (Wahl 1989, Dobretsov 2009). The different colonisation phases can occur successively, in parallel or in overlapping order (Cooksey & Wigglesworth-Cooksey 1995, Maki & Mitchell 2002).

1.2 Consequences of epibiosis

Epibiosis can have significant consequences for the host and for the epibiont, entailing benefits and disadvantages for both parties (summarized by Wahl 1989, Harder 2008, Goecke et al. 2010, Wahl et al. 2012).

Microfoulers

Host-microbe associations can have a positive impact on the host. For example, it has been demonstrated that symbiotic bacteria can deliver vitamin B_{12} (cobalamin) as exogenous source to their cobalamin auxotroph algae host (Croft et al. 2005). Further, it has been shown that epi-bacteria that form a dense coverage at the surface of shrimp and lobster embryos have the potential to protect their host from fungal infection by production of an antifungal compound (Gil-Turnes et al. 1989, Gil-Turnes & Fenical 1992). Additionally, marine bacteria are known to mediate further fouling by macrofouler species. Marine bacterial isolates showed inhibitory effects against barnacle larvae (*Balanus amphitrite*) and ascidian larvae (*Ciona intestinalis*) (Holmström et al. 1992). A study on biofilm assemblages revealed that monospecies bacterial biofilms as well as natural microbial assemblages isolated from the macroalgae *Fucus vesiculosus* repel barnacle larvae (Nasrolahi et al. 2012). Additionally, epibiotic bacteria isolated from the surface of different brown macroalgae showed high or moderate antibacterial activity against common fouling bacteria, like *Vibrio* and Photobacterium species (Kanagasabhapathy et al. 2006).

Besides the beneficial impact, microfouling can be detrimental for the host (Wahl 1989, Hollants et al. 2013, Egan et al. 2014). For example, surface associated microfoulers could compete for vital nutrient resources with the host (Hollants et al. 2013). Further, dense microfouling coverages can shade and insulate the host, leading to an inhibited gas exchange and reduced light absorption (for illustration see Figure 1) (Costerton et al. 1987, Gil-Turnes & Fenical 1992, Hollants et al. 2013). In a study by Sand-Jensen (1977), a crust of epiphytic diatoms on eelgrass leaves led to a reduced photosynthesis up to 31 % and a decrease of HCO_3^- diffusion.

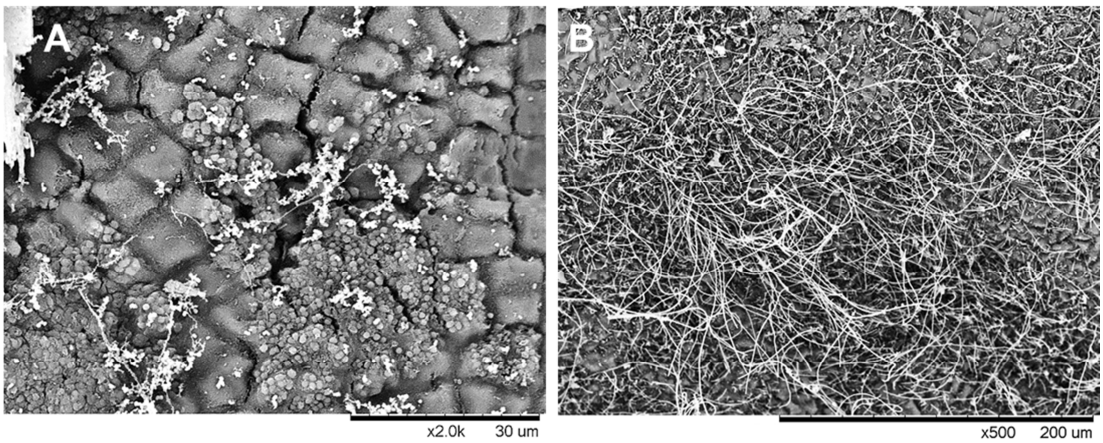

Figure 1. Scanning electron microphotographs of the surface of *Fucus vesiculosus* showing (A) a patchy prokaryotic assemblage consisting of cocci, diplococci and tetrads embedded in a matrix and (B) a dense microfouling cover consisting mainly of filamentous cells. Photos made by Nadja Stärck.

In addition, many bacteria are potential pathogens (Egan et al. 2014). For example, surface associated *Alteromonas* sp. from the Japanese kelp *Laminaria religiosa* have been shown to cause the bleaching disease in this kelp species during spring, when seawater temperature rises (Vairappan et al. 2001). Furthermore, bacterial biofilms have been shown to mediate macrofouling. Bacterial biofilms are recognized by zoospore of the green seaweed Ulva by means of released quorum sensing signal molecules (AHLs, N-acylhomoserine lactones) leading to an increase in zoospore settlement (Joint et al. 2000, Joint et al. 2007, Qian et al. 2007). Bacterial strains isolated from marine biofilms induced larval settlement of the serpulid polychaete *Hydroides elegans*, an early colonist of new substrata (Unabia & Hadfield 1999).

Macrofoulers

Host-macrofouler associations are mainly detrimental for the host (Wahl 1989). Epibiosis leads to a modification of the outer body surface of the host (Wahl 2008). Since for many marine organisms the outer surfaces serve as interface for all interactions with the environment such as light absorption, gas exchange, nutrient uptake, release of gametes or chemical signalling, a functional body surface is essential for the performance and ultimately for the host's survival (Wahl 1989).

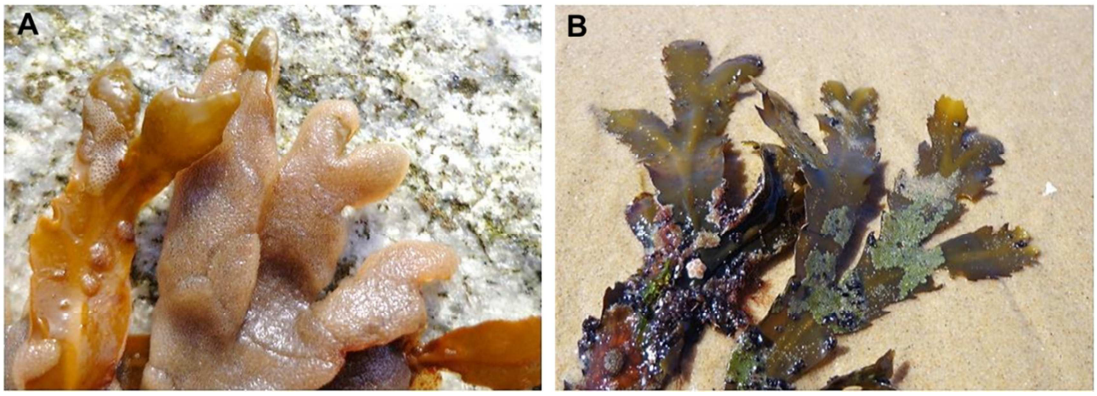

Figure 2. Macrofouling on macroalgae thallus surfaces. (A) A dense bryozoan layer partly blocking the receptacles of *Fucus vesiculosus.* (B) A dense macrofouling assemblage consisting of different macrofouler species associated with the older thallus parts of *Fucus serratus*. Photos made by Esther Rickert.

For example, heavy colonisation on the cephalothorax and rostrum of the prawn *Macrobrachium acanthurus* by barnacles, bryozoans and hydrozoans leads to an increased drag and higher energy costs of movement as well as a disturbed

coordination of movements. Moreover, such a reduced performance makes the prawn more vulnerable to predators (Farrapeira and dos Santos Calado 2010). Furthermore, it could, for instance, be demonstrated that epiphytised *Ascophyllum nodosum* had a reduced reproduction effort due to the physical blockage of receptacles (for illustration see Figure 2A) (Kraberg & Norton 2007). Further, it has been shown that the mortality of the kelp *Saccharina latissima* coincided with heavy epiphytism (80-100 %) during summer (Andersen et al. 2011). The authors argued that accumulated epiphytic load led to light limitation, increased drag and finally to thallus destruction resulting in defoliation and mortality. Typical epiphytised macroalgae are exposed to an increased drag leading to an increased risk of dislodgment (Anderson & Martone 2014) (for illustration see Figure 2). Furthermore, it has been shown that uncontrolled fouling by an epiphytic bryozoan species on kelp could cause a reduced nutrient uptake and photosynthesis leading to a lowered resilience followed loss of the meristematic region (Scheibling & Gagnon 2009) (for illustration see Figure 2 A). Reduced photosynthesis caused by encrusting bryozoans has been also reported form the brown algae *Fucus serratus* (Oswald et al. 1984).

1.3 Macroalgal fouling control mechanism

Thalli of macroalgae consist of relatively undifferentiated tissues representing the interface for vital interactions with the environment such as light absorption, gas exchange and nutrient uptake (da Gama et al. 2014). Moreover, macroalgal fitness and survival inter alia depend on incident light and photosynthesis (da Gama et al. 2014). As photosynthetic organisms, macroalgae grow in the euphotic zone where the density of potential colonisers and competition for settlement space is high (da Gama et al. 2014). Under these circumstances, macroalgae become a settlement substrate for potential colonisers (da Gama et al. 2014). Further, the thallus surface provides nutritious compounds favourable for bacterial settlement and reproduction (Sieburth 1969, Abdullah & Fredriksen 2004, Haas & Wild 2010). The special importance of a functional thallus surface and the constant threat of overgrowth by micro- and macrofoulers have resulted in the evolution of a wide variety of control mechanisms against epibiosis (Pohnert 2004, Harder 2008, Paul & Ritson-Williams 2008, de Nys et al. 2009, da Gama et al. 2014).

The following paragraph gives a few examples of different fouling control strategies found in macroalgae. A physical fouling control mechanism, reported from some macroalgae species, represents the periodically removal of their cuticula, thus eliminating surface associated epiphytes temporarily (Filion-Myklebust & Norton 1981, Sieburth & Tootle 1981, Russell & Veltkamp 1984, Nylund et al. 2005, Yamamoto et al. 2013).

Besides this physical control of fouler species, many macroalgae exhibit chemical fouling control mechanisms (Steinberg et al. 2002, Pohnert 2004, Paul & Ritson-Williams 2008). One fouling control strategy used by green, brown and red macroalgae to prevent or control colonisation by epibacteria is the formation and release of reactive oxygen species (ROS) named “oxidative burst” (Potin 2008, summerised by Goecke et al. 2010 and da Gama et al. 2014). The red macroalga *Gracilaria conferta*, for example, responded with an oxidative burst when oligoagars, analoguous to cell wall degradation products released by degrading bacteria, were added to the algae leading to a reduction of epibacteria (Weinberger & Friedlander 2000).

Another form of chemical fouling control represents the deployment of active secondary metabolites on macroalgal surfaces to control fouler species. This form of chemical fouling control has often been reported from macroalga species belonging to the division Rhodophyta (da Gama et al. 2014). For example, the red macroalga *Delisea pulchra* produces halogenated furanones with antifouling activities (De Nys et al. 1995). Furanones isolated from *D. pulchra* inhibited settlement of *Balanus amphitrite* cyprid larvae, settlement and growth of *Ulva lactuca* algal gametes as well as growth of a marine bacterial strain (De Nys et al. 1995). The red macroalga *Bonnemaisonia hamifera* uses the surface associated secondary metabolite 1,1,3,3-tetrabromo-2-heptanon to inhibit epibacterial growth (Nylund et al. 2005, Nylund et al. 2008). Phlorotannins exuded from the brown alga *Fucus vesiculosus* inhibited the settlement of cyprid larvae from the barnacle *Amphibalanus improvisus* (Brock et al. 2007).

Besides elaborate secondary metabolites, primary metabolites also play a role in chemical fouling control strategies (Pohnert 2012). It has been shown that the pigment fucoxanthin, the amino acid proline and the osmolyte DMSP, isolated from the surface of the brown macroalgae *Fucus vesiculosus*, inhibit bacterial attachment (Saha et al. 2011, Saha et al. 2012). Polyunsaturated fatty acids exuded from the

green alga *Ulva fasciata* exhibited algicidal effects active against different microalgal species (Alamsjah et al. 2008).

1.4 Seasonality of fouling control

One fundamental aspect regarding chemical fouling control is the question of its regulation. It is conceivable that, in analogy to the antifeeding defences in macroalgae (Rohde et al. 2004), chemical fouling control is active when needed, meaning highest fouling control strength during season of high fouling pressure, thus regulated 'on demand'. Seasonal fluctuations in fouling control strength (Hellio et al. 2004, Marechal et al. 2004, Stirk et al. 2007, Wahl et al. 2010, Saha & Wahl 2013) as well as seasonally variable levels of bioactive metabolites (Amade & Lemee 1998, Abdala-Diaz et al. 2006) have been reported in different species of macroalgae. For example, the phenolic phlorotannins in the brown alga *Cystoseira tamariscifolia* (Abdala-Diaz et al. 2006), the antifouling sesquiterpene caulerpenyne from *Caulerpa taxifolia* (Amade & Lemee 1998) showed annual cycles regulated by light intensity. The tissue content of caulerpenyne (Amade & Lemee 1998) and the sesquiterpene elatol from the red alga *Laurencia dendroidea* (Sudatti et al. 2011) also showed a temperature dependency. A seasonal fluctuating microfouling control of *F. vesiculosus* has been demonstrated by testing, via in vitro assays, surface extracts on their activity against different marine bacteria strains in two previous studies (Wahl et al. 2010, Saha & Wahl 2013).

Furthermore, temperate macroalgae, including *F. vesiculosus*, have been shown to exhibit a seasonally fluctuating chemical fouling control synchronised with *in situ* fouling pressure and influenced by the abiotic factors irradiance and seawater temperature (Hellio et al. 2004, Marechal et al. 2004, Wahl et al. 2010).

1.5 Costs of fouling control

Another basic aspect regarding macroalgal fouling and herbivory control is the question of costs. Most hypotheses in plant defence assume that defence causes metabolic costs (Strauss et al. 2002, Stamp 2003) originating from synthesis, transport, and storage of secondary metabolites (Hay & Fenical 1988, Purrington

2000). Further, it has been hypothesised that the required energy has to be allocated and is thus probably missing for other life processes like growth (Cronin 2001). Regarding herbivory control, phlorotannin synthesis, inter alia an herbivory deterrent, has been found to be negatively correlated with growth of the brown macroalga *Fucus vesiculosus* (Jormalainen & Ramsay 2009). However, there are several studies, where no costs of herbivory fouling control were detected (Pansch et al. 2009, Appelhans et al. 2010). One study that analysed the metabolic costs of biofouling control found a significant inverse relationship between fecundity and the level of the bioactive compounds furanones as well as significantly higher growth rates for algae unable to produce furanones, indicating a cost of furanone production (Dworjanyn et al. 2006). In general, however, metabolic costs of chemical fouling control in macroalgae are hardly studied so far (Pavia et al. 2012).

If the deployment of fouling control metabolites is costly, this may not be relevant when the macroalga is not resource limited (Cronin 2001) or they may be reduced when primary metabolites are used as cost effective chemical fouling control metabolites (Pohnert 2012).

1.6 Study organisms

The rockweeds *Fucus vesiculosus* Linnaeus (Phaeophyceae) also known as bladder wrack and *Fucus serratus* Linnaeus (Phaeophyceae) also called serrated wrack, are classified as vegetation determining species of the North Atlantic (Lünning 1985). *F. vesiculosus* has its northern distribution border in the southern Arctic at approx. 73° of northern latitude and its southern borders on the coasts of North Africa (Europe) and North Carolina (North America) at approx. 34° of southern latitude (Lünning 1985). *F. serratus* has its northern distribution limits in the Arctic Ocean (Nowaja Semlja, approx. 73° of northern latitude) and its southern borders on the coasts of Northern Portugal (Europe) and Gulf of Saint Lawrence (North America) at approx. 42° of southern latitude (Lünning 1985).

Both species produce one diploid macrothallus during their life cycle. The thallus is flat and strap-like approx. 2 cm wide and parts of the thalli are pock-marked with cryptostomata (van den Hoek et al. 1995). Thallus growth is apical through meristematic cells (van den Hoek et al. 1995). *F. vesiculosus* and *F. serratus* thalli are flat thalli approx. 2 cm wide. *F. vesiculosus* thalli are characterized by bladders

and rounded thalli borders, whereas *F. serratus* thalli have no bladders and serrated thalli borders (Figure 3). Both *Fucus* species are dioecious. Mature *Fucus* individuals develop conceptacles, depressions located in the distal fertile thallus parts named receptacles. From the conceptacles oogonia and antheridia (depending on the sex of the *Fucus* individual) are released followed by the release of 64 sperm cells from the antheridia and the release of eight eggs from the oogonia. After fertilisation diploid zygotes attach and grow to a new *Fucus* individual (van den Hoek et al. 1995).

Figure 3. The perennial brown macroalgae *Fucus vesiculosus* (left) and *Fucus serratus* (right). Photo made by Esther Rickert.

Both *Fucus* species are two common perennial brown macroalgae species in the Baltic Sea. *F. vesiculosus* is often referred to as keystone species in the Baltic Sea due to its wide distribution and habitat forming characteristic (Kautsky et al. 1992). Today *F. vesiculosus* grows between 0 - 3 m water depth, whereas *F. serratus* occurs from approx. 2 m downwards in the Western Baltic Sea (Malm et al. 2001).In the resent years *F. vesiculosus* showed a reduced depth distribution along with reduced abundance in the Baltic Sea (Rohde et al. 2008, Wahl et al. 2011 and references therein). The shifts in depth distribution have been attributed directly and indirectly to the eutrophication of the Baltic Sea (Wahl et al. 2011 and references therein).

1.7 Thesis outline

At the beginning of my research in 2012, it was known that (*a*) *F. vesiculosus* surface extracts are active against micro- and macrofoulers with the capacity to modulate fouler abundance and composition, that (*b*) the chemical antifouling defence against bacteria seasonally fluctuates, that (*c*) *F. vesiculosus* hosts a characteristic and seasonally variable bacterial community, that (*d*) the *F. vesiculosus* associated bacterial community is affected by abiotic and biotic factors and that (*e*) the associated bacterial community has the potential to affect further fouling by important macrofoulers. Even though former studies investigated the seasonal aspects as well as the effects of abiotic and biotic factors on the antifouling defence of *F. vesiculosus* in parts, possible relations between naturally variable abiotic and biotic factors and the putative synchronised fouling control of *Fucus* was virtually unstudied, especially under *in situ* conditions.

Therefore, the aim of my research was to investigate the putative seasonal variability in fouling control of *F. vesiculosus* and *F. serratus* from the Baltic Sea on micro- and macrofouler species and how the fouling control relates to the natural seasonal variations of abiotic and biotic factors. To this end, the following research questions were formulated and investigated within a 12-months field experiment complemented by laboratory work (work-flow overview Figure 4).

1. Main study questions:

1.1 Does the chemical fouling control strength of **Fucus** *vary with season?*

The putative seasonal variability of the fouling control was investigated on a monthly basis. For this purpose, *Fucus* individuals from both species were sampled at the experimental site from August 2012 to July 2013 and subsequently surface-extracted. For the sake of comparability, all monthly surface extracts were exposed to the natural fouler pool at one time point (Aug/Sep 2013) by means of an *in situ* settlement bioassay to determine their fouling control strength. Fouling control strength was quantified by direct enumeration of the total cell number of settled prokaryotes and diatoms and by quantification of the two dominant macrofouler species *Amphibalanus improvisus* and *Mytilus edulis* (**Paper I** *'Microfouling'* and **Paper II** *'Macrofouling'*).

1.2 Does the surface metabolite composition of **Fucus** *vary with season and which metabolites are up- or down-regulated during seasons of high or low fouling pressure?*

Seasonal variability of *Fucus* surface metabolite composition was investigated by analysing the monthly extracted surface metabolites (sub-samples from extracts tested for fouling control) via GC-MS. Obtained GC-MS data was checked for seasonal variability using multi-dimensional scaling (MDS). Regulated metabolites in summer (high fouling pressure) and winter (low fouling pressure) extracts were detected by Simper analysis *(***Paper III** *'Chemical landscape).*

1.3 Do seasonal fluctuations of **Fucus** *fouling control correlate with (a) fouling pressure and / or (b) the energy status of* **Fucus***?*

To investigate the possible relations between these two seasonally fluctuating variables and the putative seasonally varying fouling control of *Fucus,* correlation tests were performed (**Paper I** *'Microfouling'* and **Paper II** *'Macrofouling'*).

1.4 Does the surface metabolite composition of **Fucus** *correlate with abiotic and biotic variables?*

To investigate possible relationships between light intensity, seawater temperature, nutrient concentration, prokaryotic fouling pressure and the putative seasonally variable composition of metabolites on the surface of both *Fucus* species, a distance based linear model (DistLM) was performed (**Paper III** *'Chemical landscape'*).

2. Minor study questions:

To understand the seasonal patterns and possible links of the seasonal fluctuating fouling control system of *Fucus* it was necessary to investigate the seasonal fluctuations of the following abiotic and biotic factors:

2.1 How do light, temperature, salinity and nutrients vary with season?

The seasonal variability of light, temperature, salinity and nutrient concentrations at the experimental site were continuously recorded via light/temperature and salinity data loggers deployed at the field site of the monthly surveys. Nutrient concentrations were determined from water samples taken weekly (abiotic data

were used in **Paper I** *'Microfouling'*, **Paper II** *'Macrofouling'* and in **Paper III** *'Chemical Landscape'*).

2.2 How does in situ fouling pressure vary with season?

The seasonal variability of the *in situ* fouling pressure at the experimental site was studied by deploying glass slides for microfoulers for seven days each month and PVC panels for macrofoulers for four weeks each month as artificial settlement substrate. Fouling pressure was quantified by direct enumeration of settled prokaryotic and diatom cells (microfouling pressure) and of all settled macrofouler species by means of epifluorescence microscopy and stereomicroscopy, respectively (**Paper I** *'Microfouling'*, **Paper II** *'Macrofouling',* microfouling pressure data was used in **Paper III** *'Chemical Landscape'*).

2.3 Does the fouling status of **Fucus** *vary with season?*

The potential seasonal variations of cumulative micro- and macrofouling densities on both *Fucus* species, termed as 'fouling status', were investigated by direct enumeration of micro- and macrofouling individuals on apical thallus tips via epifluorescence microscopy or on one thallus branch via stereomicroscopy, respectively (**Paper I** *'Microfouling'* and **Paper II** *'Macrofouling'*).

2.4 Does the energy status of **Fucus** *vary with season?*

The potential seasonal variations of the storage compound mannitol, probably an energy reserve and therefore used as general proxy for available energy in both *Fucus* species (termed as 'energy status'), were analysed using a HPLC (**Paper I** *'Microfouling'* and **Paper II** *'Macrofouling'*).

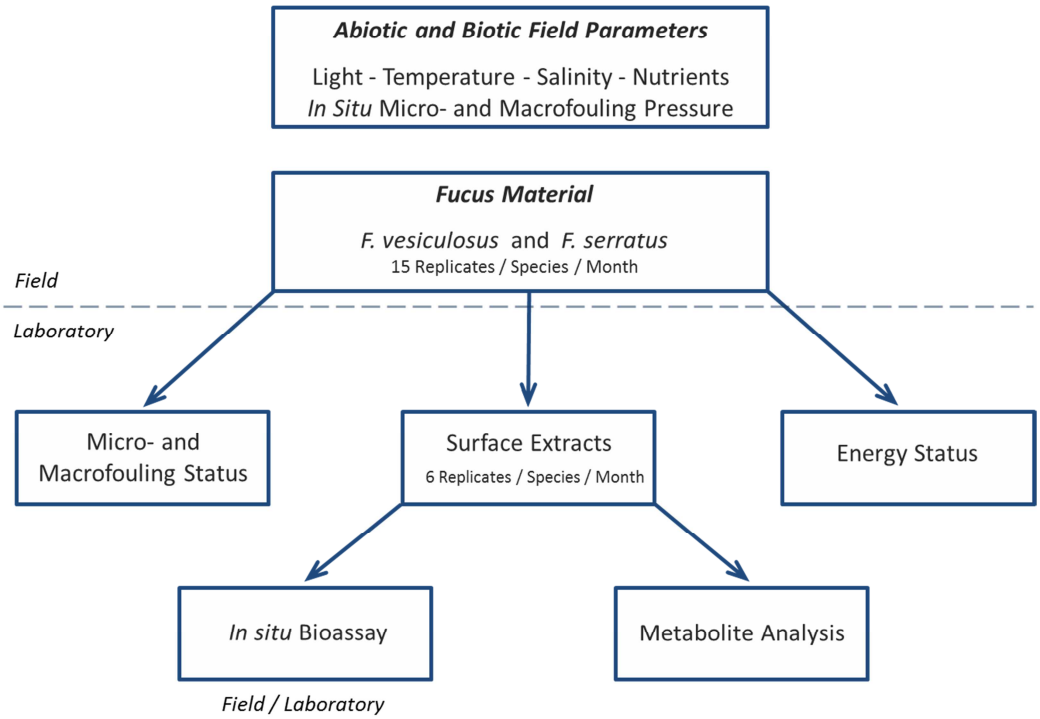

Figure 4. Work flow diagram of the performed field experiment and laboratory analysis to answer the formulated research questions of my doctoral project. The field experiment lasted from August 2012 until July 2013, performed in the outer Kiel Fjord. During the field experiment, light, temperature and salinity were constantly recorded and nutrient concentrations weekly. Further, the actual micro- and macrofouling pressure at the experimental site was recorded in monthly intervals. Also in monthly intervals, 15 *Fucus* individuals per species were collected at the site to harvest surface associated metabolites via surface extraction. The same *Fucus* plants were used to quantify their 'fouling status' (cumulative densities of settled micro- and macrofoulers) and 'energy status' (total mannitol tissue conc.). To assess the putative seasonal variable fouling control of both *Fucus* species, obtained surface extracts were exposed by means of an *in situ* settlement bioassay to the natural fouler pool at one point in time in Aug/Sep 2013. Furthermore, the chemical composition of surface extracts obtained was analysed by GC-MS to identify potential seasonal differences in the metabolite composition.

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2. Publications and Contributions of Authors

Parts of this doctoral thesis have been published or submitted:

Paper I

Esther Rickert, Stanislav N. Gorb, Martin Wahl (**submitted to Marine Biology**) Seasonally fluctuating chemical microfouling control in *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea.

E. Rickert, M. Wahl: experimental design; E. Rickert: field and laboratory work, data analysis, manuscript. S. N. Gorb: access and technical support for scanning electron microscopy. All co-authors commented and corrected the manuscript.

Paper II

Esther Rickert, Ulf Karsten, Georg Pohnert, Martin Wahl (**2015**) Seasonal fluctuations in chemical defenses against macrofouling in *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea. **Biofouling: The Journal of Bioadhesion and Biofilm Research, 31:4, 363-377 DOI: 10.1080 / 08927014.2015.1041020**

E. Rickert, M. Wahl and G. Pohnert: experimental design; E. Rickert: field and laboratory work, data analysis, manuscript. U. Karsten: mannitol quantification. All co-authors commented and corrected the manuscript.

Paper III

Esther Rickert, Martin Wahl, Hannes Richter, Georg Pohnert (**submitted to PLOS ONE**) Seasonal variations in surface metabolite composition of *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea.

E. Rickert, M. Wahl and G. Pohnert: experimental design; E. Rickert: field and laboratory work, data analysis, manuscript. E. Rickert and D. Jacquemoud: GC-MS sample preparation and GC-MS measurements. H. Richter and D. Jacquemoud: GC-MS data analysis and compound identification. All co-authors commented and corrected the manuscript.

Additional publications not part of this doctoral thesis:

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

M. Wahl, V. Jormalainen, J. Kotta, P. Kraufvelin, M. Molis, H. Schubert, H. Pavia, G. Nylund, L. Kautsky, E. Schagerström, M. Saha, **E. Rickert,** S. Freriksen, F. Weinberger (**in preparation**). Shifting biological traits in a large scale environmental gradient: the macroalga *Fucus vesiculosus* along 3000 km of decreasing salinity

Paper I

Submitted to Marine Biology

Seasonally fluctuating chemical microfouling control in *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea

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Abstract

Microfouling in temperate seas underlies seasonal variations and, thus, perennial macroalgae are exposed to fluctuating fouling pressure. Only few studies have examined the link between fouling pressure and algal fouling control. In a one-year field survey, we assessed whether microfouling control of *F. vesiculosus* and *F. serratus* against prokaryotes and pennate diatoms fluctuates with season and correlates with fouling pressure. Monthly microfouler recruitment on glass (reference surface) and on *Fucus*, microfouling control strength of *Fucus* surface metabolites (tested by an *in situ* bioassay approach) and *Fucus* tissue mannitol content (used as proxy for energy availability) were determined. Microfouling pressure and microfouling control of *Fucus* varied seasonally but generally did not correlate with the fouling pressure or with the mannitol content of *Fucus.* Both *Fucus* species exhibited cuticula shedding during all seasons. We conclude that microfouling control in both *Fucus* species was not fine-tuned to microfouling pressure and was not energy-limited.

Keywords *Fucus,* chemical microfouling control, prokaryotic fouling, diatom fouling, cuticula shedding

Introduction

Macroalgae are exposed to an omnipresent, but spatially and temporally variable fouling pressure generated from a wide diversity of pro- and eukaryotes mainly bacteria, microalgae as well as propagules of invertebrates and macroalgae (Harder 2008; Qian et al. 2007; Wahl et al. 2012). Seawater can contain cell densities up to 10^6 bacteria, 10^3 fungi, 10^3 microalgae and 10-100 larvae and spores per ml (Harder 2008). Most of these life forms strive to settle on solid surfaces and especially bacteria perform best when living within biofilms (Grossart 2010). Macroalgal thalli are particularly susceptible to biofilm formations since the algal thallus represents free space for settlement and a rich source of exuded organic matter suitable as nutrients for bacteria (Brylinsky 1977; Khailov and Burlakov 1969; Pregnall 1983). Macroalgae-associated biofilms are usually dominated by bacteria, often the first colonizers (Goecke et al. 2010; Wahl 1989), but can also contain microalgae, fungi, spores and larvae (Goecke et al. 2010; Sieburth and Tootle 1981; Staufenberger et al. 2008). Furthermore, it has been shown that macroalgae-associated bacteria communities can differ between algal species (Lachnit et al. 2009). Alga-associated bacterial strains or communities may mediate further colonization by other bacteria, diatoms or spores (reviewed by Dobretsov et al. (2006)). In some cases macroalgae hosts may be protected from further epiphytic fouling by their epi-bacterial films (Egan et al. 2001; Kumar et al. 2011). However, uncontrolled microfouling of a macroalgae surface can lead to several detrimental effects like hindered trans-epidermal exchange (Wahl 2008; Wahl et al. 2012) or increased shading (Rohde et al. 2008) along with a reduced photosynthesis. Furthermore, pathogenic bacteria in algal-associated biofilms can cause infections followed by tissue loss or even cause mortality (Sawabe et al. 1998; Steinberg et al. 1997). Since the algae outer body surface represents its only interface for all physiological interactions with the environment, an intact thallus surface is essential for growth, reproduction and finally for survival (Wahl et al. 2012).

To control fouling quantitatively and qualitatively, macroalgae have developed chemical antifouling defence systems (da Gama et al. 2014; Nylund et al. 2008). For example the perennial brown alga *Fucus vesiculosus* expresses surface-associated metabolites with antifouling activities (DMSP, proline and fucoxanthin) as well as metabolites with profouling activities (unidentified polar compounds) to control

fouling bacteria (Lachnit et al. 2013; Saha et al. 2012; Saha et al. 2011; Saha et al. 2014).

Additionally, it has been shown that extracts from several different macroalgae species were active against bacteria, fungi, diatoms and macroalgae spores. In some cases, the anti-fouling activity was shown to be seasonally variable (Hellio et al. 2004; Saha and Wahl 2013; Wahl et al. 2010).

If the production of antifouling metabolites is costly and competes with other metabolic functions for limited resources (Coley et al. 1985; Dworjanyn et al. 2006; Strauss et al. 2002) and if the microfouling pressure, in temperate climate zones, varies with season (Hellio et al. 2004; Wahl et al. 2010), a tuning of microfouling control to the *in situ* microfouling pressure would seem to be of selective advantage for the algae.

A few studies have focused on the relationship between the anti-microfouling defence strength and season (Hellio et al. 2004; Saha and Wahl 2013; Wahl et al. 2010). Regarding the chemical anti-microfouling defence of *Fucus,* previous studies have shown that *F. vesiculosus* exhibits seasonal fluctuating defence strength against bacteria (Saha and Wahl 2013; Wahl et al. 2010), but the hypothesis about a real-time correlation to fouling pressure or resource availability was not tested yet.

The aim of this study was to investigate (1) if prokaryotic and diatom fouling pressure in the field varies with season, (2) if *F. vesiculosus* and *F. serratus* fouling control against prokaryotic and diatom fouling varies with season, (3) if the fouling control strength of *F. vesiculosus* and *F. serratus* relates directly to the relative *in situ* fouling pressure (demand driven control) or (4) to the availability of energy chemically stored in the form of mannitol (resource driven control).

Material and methods

Sampling site and collection of alga material

Fucus vesiculosus Linnaeus (1753) and *F. serratus* Linnaeus (1753), two perennial brown algae species, were collected in the outer Kiel Fjord, Germany (54°27'21" N; 10°11'57" E) at depths from 0.5 m und er mid water level from August 2012 to July 2013. At the same site seawater temperature, salinity and light intensity were recorded hourly and nutrients were quantified weekly in water samples. A detailed description of recorded abiotic data can be found in Rickert et al. (2015).

Monthly, 15 individuals of both *Fucus* species were sampled from mixed stands. Collected algae were individually stored in 3 l plastic bags (maintaining a humid atmosphere) and transported to the laboratory in a cooler box. Within 3 to 4 h after sampling, all algae were surface-extracted (see paragraph below). After surface extraction, total tissue mannitol content was measured. Mannitol quantification is described in detail in Rickert et al. (2015). From non-extracted *Fucus* material the apical thallus region (upper 1-2 cm) was used to quantify seasonal variations of *Fucus* associated microfoulers (see paragraph below).

Assessment of microfouler recruitment

Prokaryotic and pennate diatom cells were assessed as the dominant microfoulers on *Fucus* and on glass reference substrate. Cyanobacteria were also detected but not quantified systematically. To quantify the relative *in situ* microfouling pressure in the field, glass microscope slides (n = 9 per month) were vertically exposed (to avoid sedimentation) at the sampling site at a depth of 0.5 m under mid water level for the seven days preceding the monthly sampling of algae. Glass slides were used as settlement substrate to quantify microfoulers directly on the recruitment surface to avoid additional transfer steps along with possible cell losses. The hydrophilic surface of in seawater immersed glass is levelled through molecular fouling after approx. 48 hours (Becker and Wahl 1991). After exposure the slides were fixated in sterile filtered 3.7 % formaldehyde solution at 4 °C overnight. Fixated slides were rinsed with sterile filtered 1x PBS buffer and stored in an ethanol-PBS solution (96 % ethanol:1x PBS, 1:1) at -20 °C until further analysis.

For quantification of prokaryotic and pennate diatom cells approx. 1 cm^2 at the centre of the slides were stained with 10 µl of a ready-to-use DAPI (4'.6-diamidino-2-phenylindole) containing mounting medium (Roti®-Mount FluorCare DAPI, Roth, Karlsruhe, Germany). Subsequently, slip-covered slides were observed using an epifluorescence microscope (Axio Scope.A1, Carl Zeiss Microscopy GmbH, Göttingen, Germany; objective lens EC Plan-NEO FLUAR, 100x/1.3 oil, Carl Zeiss Microscopy GmbH, Göttingen, Germany) and five randomly chosen visual fields of 0.02 mm^2 were photographed (ProgRes® CF, Jenoptik AG, Jena, Germany). On the screen, each captured visual field was overlain with a grid using the image processing free software ImageJ (Schneider et al. 2012) and prokaryotic and

pennate diatom cells in 20 randomly chosen squares (each 50 μm^2) were manually counted.

To quantify the microfouling status of *Fucus* directly on the thallus surface, one thallus piece (approx. 0.2 cm^2) from the young apical thallus region (the distal 1 cm) per alga individual (n = 9 per month and *Fucus* species) was punched out with a cork borer and fixated with sterile filtered 3.7 % formaldehyde solution. It should be mentioned that *F. vesiculosus* exhibits vegetative growth throughout the year with highest growth rate in summer and the lowest one during winter (approx. one third of the summer growth rate) (Lehvo et al. 2001). Therefore, when cut at distance from the meristem *Fucus,* the thalli pieces have a slightly older mean age in winter than in summer. Fixated thallus pieces were rinsed with sterile filtered 1x PBS buffer and stored in an ethanol-PBS solution (96 % ethanol:1x PBS, 1:1) at -20 ℃ until further analysis. Three thallus pieces were put on a microscope slide side-by-side. The up-facing thallus sides were stained with 10 µl of a ready-to-use DAPI (4'.6-diamidino-2-phenylindole) containing mounting medium (Roti®-Mount FluorCare DAPI, Roth, Karlsruhe, Germany) and covered with long glass coverslips. To stabilize both the sample and the coverslip during microscopy the coverslip was fixed laterally with an adhersive tape on the microscope slide. Quantification of *Fucus*-associated microfoulers was carried out by epifluoreszence microscopy (AxioImager.Z1 with a motorized z-axis lifting table, Carl Zeiss Microscopy GmbH, Göttingen, Germany). In order to capture all surface associated cells in the different focal planes, 5 randomly chosen z-stack images per replicate were taken with a focal distance of 0.24 µm (AxioCam MRm, Carl Zeiss Microscopy GmbH, Göttingen, Germany and objective lens Plan-Apochromat / 63x / 1.4 oil DIC, Carl Zeiss Microscopy GmbH, Göttingen, Germany). The use of a monochromatic and highly sensitive camera was essential to obtain a discernible fluorescence dye signal against the bright algal background auto-fluorescence. Recorded z-stack consisted of single image numbers ranging from below 10 up to over 100, depending on the algal surface structure. For cell enumeration each z-stack was combined to a 2D image following the method described by Stratil et al. (2013). Enumeration of cells on 2D images was performed manually as described above (see previous paragraph).

Surface screening

To screen the thallus surface of *Fucus* for cuticula shedding in a scanning electron microscope (SEM), *Fucus* pieces were dehydrated by transferring them serially from ethanol-PBS solution (96 % ethanol:1x PBS, 1:1) across an ascending gradient ethanol series (50%, 70%, 90%, and 100%; v/v). This was followed by critical point drying with carbon dioxide (CPD 030, Optics Balzers, Balzers, Liechtenstein) and sputter-coating with gold-palladium (SCD 004, Optics Balzers, Balzers, Liechtenstein). To analyse the surface condition of *Fucus* for cuticula shedding on a random basis, one to two thallus samples originating from different month throughout all seasons (February, March, June, July, October, November) were examined with a SEM Hitachi TM 3000 (Hitachi High Technologies, Tokyo, Japan).

Surface extraction

For surface extraction, per *Fucus* replicate (*n* = 15 per months and species) approx. 50 g of apparently epiphyte-free thallus tips (distal 5 - 10 cm) were cut off. *Fucus* material was extracted by following the protocol described by de Nys et al. (1998), with minor modifications. Before extraction thallus tips were spin dried in a salad spinner for 30 s to get rid of remaining seawater. Extraction time was reduced to 4 s, since previous extractions tests have shown that after 7 to 8 s of surface extraction the thallus material turned from normal brownish colour to light greenish-brown, indicating the loss of pigments (and other metabolites). During extraction, thallus tips were held with steel tweezers in a 100 ml stirred n-hexane : methanol (1:1) emulsion at room temperature for 4 s. Solvent contact with the cutting edge of the thallus tips was avoided during extraction procedure to prevent a contamination with intercellular compounds. After extraction the extract was filtered through a paper filter (MN 615 ¼, Ø 150 mm, Macherey-Nagel, Düren, Germany) to remove particles. Extracts were reduced under vacuum at 35 ℃ by using a rotation evaporator followed by re-dissolution with 2 ml n-hexane and 2 ml methanol. Extracts were dried under a constant nitrogen flow at 35 ℃ and dry stored at -20 ℃ until further analysis.

Extracted thallus material was rinsed with seawater and scanned for surface area determination with the free analysis software Fiji (Schindelin et al. 2012). The

surface extracted *Fucus* material was also used to analyse the total mannitol contents.

Mannitol analysis

Total mannitol tissue content was determined in surface extracted *Fucus* material. Algal material was freeze-dried and ground into a fine powder. 10 to 20 mg dry weight of alga powder per sample was used for analysis. For detailed method descriptions see Rickert et al. (2015)

In situ bioassays

In situ bioassays were performed to test the seasonal fouling control strength of *F. vesiculosus* and *F. serratus* surface extracts. Tested surface extracts (*n* = 6 per month and *Fucus* species) originated from all month of a year. For bioassay preparation surface extracts were redissolved in 2 ml n-hexane : methanol (1:1) and applied in a two-fold mean boundary layer concentration on 12.56 cm^2 cellulose filter paper (MN 616, Ø 40 mm, Macherey-Nagel, Düren, Germany). Since living algae release metabolites and thus generate and maintain a strong metabolic concentration gradient, the concentrations near the thallus surface (where bacteria settle and grow) are substantially higher than the mean boundary layer concentrations (Grosser et al. 2012). Consequently, the “natural” (i.e. average) concentration calculated for the boundary layer was up-concentrated two-fold to better simulate the conditions in the stratum most relevant for microfoulers (Grosser et al. 2012). Impregnated filter papers were freeze-dried to remove all solvents residues. Cellulose filter papers were covered with 500 µl of a 1 % low melt agarose (Roth, Karlsruhe, Germany) to prolong the residence time of the extracts. Low melt agarose was used to allow working below 35 ℃ which avoids potential thermal degradation of the extracts. To provide a suitable settlement surface for fouling organisms the impregnated and embedded cellulose filter was then covered with a polycarbonate membrane (track-etch membrane, Ø 47 mm, pore size 0.2 µm, Sartorius Stedim Biotech, Göttingen, Germany) which had been aged, to allow molecular fouling and contact angle modification (Becker and Wahl 1991), for 10 days in sterile filtered seawater. Bioassays were assembled in a holder devise as described by Nasrolahi et al. (2012). To facilitate the homogeneous distribution (by

diffusion) of extracts bioassays throughout filter and agarose, the holder device was kept in a humid atmosphere at 4 ℃ for 24 h before exposure to the sea. Always 12 bioassay devices were screw tight on a PVC (polyvinyl chloride) retaining plate and hung in random arrangement at a depth of -0.5 m from a floating dock in the Kiel fjord. In this way they were exposed to the natural fouling organism pool for five days during August/September 2013. Due to the sample size, *F. vesiculosus* bioassays and *F. serratus* bioassays were deployed in succession and thus exposed to different fouler intensities. Therefore a direct comparison of the results was not possible. After five days of exposure, the polycarbonate membranes were removed, rinsed with sterile filtered seawater and fixed with 3.7 % formaldehyde at 4 ℃ overnight. For microfouling analysis a 0.25 cm^2 (0.5 x 0.5 cm) piece of the centre of the polycarbonate membrane was cut out with a scalpel and tagged in the corner with a pencil to identify the side of the membrane which had been exposed to fouling. Membrane pieces were stored in 1x PBS and 96 % ethanol (1:1) at - 20 ℃ until further analysis. For fouling quantification, polycarbonate membrane pieces were carefully rinsed with sterile filtered seawater to remove remaining buffer and non-attached material, then placed with the fouled side upwards on a microscope slide and stained with 10 µml of a ready-to-use DAPI (4'.6-diamidino-2-phenylindole) containing mounting medium (Roti®-Mount FluorCare DAPI, Roth, Karlsruhe, Germany) and covered with a coverslip. Quantification of microfoulers was assessed by epifluorescence microscopy (Axio Scope.A1, Carl Zeiss Microscopy GmbH, Göttingen, Germany; objective lens EC Plan-NEO FLUAR®, 100x/1.3 oil, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Per replicate 10 randomly chosen visual fields were photographed (ProgRes® CF, Jenoptik AG, Jena, Germany) and later analysed with the free software ImageJ (Schneider et al. 2012). For cell enumeration each visual field was overlayed with a grid and in each of 20 randomly chosen squares (each 50 μm^2) prokaryotic and pennate diatom cells were quantified by manual counting.

Data analysis

For each *Fucus* species together with every fouler type a separate one-way analysis of variance (ANOVA, $p \leq 0.05$) was used to assess differences among *Fucus* extracts regarding the fouling densities of the two fouler groups. Data were tested for normality using the Shapiro-Wilks test ($p \leq 0.05$) and histograms, while

homogeneity of variance was verified with residual plots. In addition to ANOVA, Tukey's honest significant difference (HSD) post hoc test ($p \leq 0.05$) identified significant differences in the mean fouling control strength between *Fucus* surface extracts that were collected in the different months of the year (including extract free solvent blanks).

Relative monthly fouling pressure on *Fucus* by the respective microfouler was calculated by dividing the monthly recorded fouling pressure (i.e. microbial foulers per unit area of glass slide per week) by the annual mean fouling pressure. The obtained monthly fouling factors were used to calculate the expected fouling on *Fucus* under the null-hypothesis that the alga did not control in any way the fouling on its thallus. To this end, the annual mean fouling on *Fucus* was multiplied by the monthly fouling factor. Any substantial differences between expected and observed fouling on *Fucus* could be interpreted as a hint for pro- or anti-microfouling activity of the host algae.

Seasonal patterns in fouling control strength were identified by fitting a sinusoidal function (free software CurveExpert 1.4) to the data obtained from the *in situ* bioassays (including all mean data points, $n = 6$) (Hyams 2010).

Correlations between (1) *Fucus* tissue mannitol content and bioassay results and (2) relative field fouling pressure and bioassay results were analysed with Spearman's rank-order correlations for non-normally distributed data. Normality of data was tested by Shapiro-Wilks test ($p \leq 0.05$) and by histograms. A Bonferroni correction was applied for multiple correlated data sets to avoid the increase of the type-I error (Dunn 1961). All statistical analyses, despite the non-linear regression, were performed using the free statistical software R (R 2010).

Results

Environmental parameters

Recorded environmental parameters exhibited a pattern typical for the western Baltic Sea. Surface seawater temperature reached a minimum on 26 January (-0.8 ℃) and maximum on 26 July (23.5 ℃). Average noon photon flux densities increased during spring and summer and reached peak irradiance in August (750 µmol photons m^{-2} s^{-1}) followed by a decrease in autumn and winter (see Rickert et al. 2015). Seawater surface salinity was low during spring and summer with

minimum values at the end of May (around 12) and increased toward autumn and winter with maximum values in October (19.38 on 10 October 2012) along with decreasing seawater temperatures (see Rickert et al. 2015). Dissolved nutrients were elevated in surface sea water during autumn and winter (mean concentration from January to March and from October to December: nitrate + nitrite 5.2 ± 0.8 µmol l−1, ammonium 2.0 ± 0.3 µmol l−1, silicate 23.4 ± 2.0 µmol l−1, and phosphate 0.9 ± 0.1 µmol l−1) and depleted during spring and summer month (mean concentration from April to September: nitrate + nitrite 0.4 ± 0.2 µmol l−1 (mean concentration ± SE), ammonium 1.4 ± 0.3 µmol l−1 (mean concentration ± SE), silicate 13.6 ± 2.4 µmol l−1 (mean concentration ± SE), phosphate 0.6 ± 0.2 µmol l−1 (mean concentration ± SE) (see Rickert et al. 2015). For a detailed description of environmental parameters see Rickert et al. (2015).

Mannitol tissue concentration

Tissue mannitol concentrations of both *Fucus* species increased from February onwards, interrupted by a distinct decrease in April for *F. serratus* and in June for *F. vesiculosus*, reaching maximum concentrations in October (*F. vesiculosus*: 83.30 mg/g dry weight ± SE 2.58; *F. serratus*: 76.19 mg/g dry weight ± SE 4.35). Both *Fucus* species reached minimum tissue mannitol concentrations in December (*F. vesiculosus*: 40.39 mg/g dry weight ± SE 2.00; *F. serratus*: 28.46 mg/g dry weight ± SE 1.32). *F. serratus* revealed all along a slightly lower mannitol concentration compared to *F. vesiculosus*. However, none of the two species showed a fully depletion in mannitol throughout the year (see Rickert et al. 2015). For a detailed description of mannitol concentration see Rickert et al. (2015).

Seasonal fouling in the field

On reference substrate (glass slides) the most intense microfouling, attributable to prokaryotic and diatom fouling, occurred during spring and summer whereby prokaryotic fouling quantitatively dominated (Fig. 1a-d, suppl. Table S1).

Prokaryotic fouling on reference substrate exhibited maximum settlement rates during August (8.02×10^6cells cm^{-2} $week^{-1}$; ±SE 1.37×10^6) and minimum settlement rates during December (2.51×10^5 cells cm^{-2} $week^{-1}$; ±SE 3.85×10^4) with an annual

mean weekly settlement of 1.98 x 10^6 cells cm^{-2} $week^{-1}$ (±SE 3.32 x 10^5, Fig. 1a, b, suppl. Table S1).

Diatom fouling on glass slides exhibited two separate peak densities in April and August and showed an annual mean weekly settlement of 3.66 x 10^4 ± 1.17 x 10^4 cells per cm^2 glass surface (±SE between replicated slides, Fig. 1c, d, suppl. Table S1).

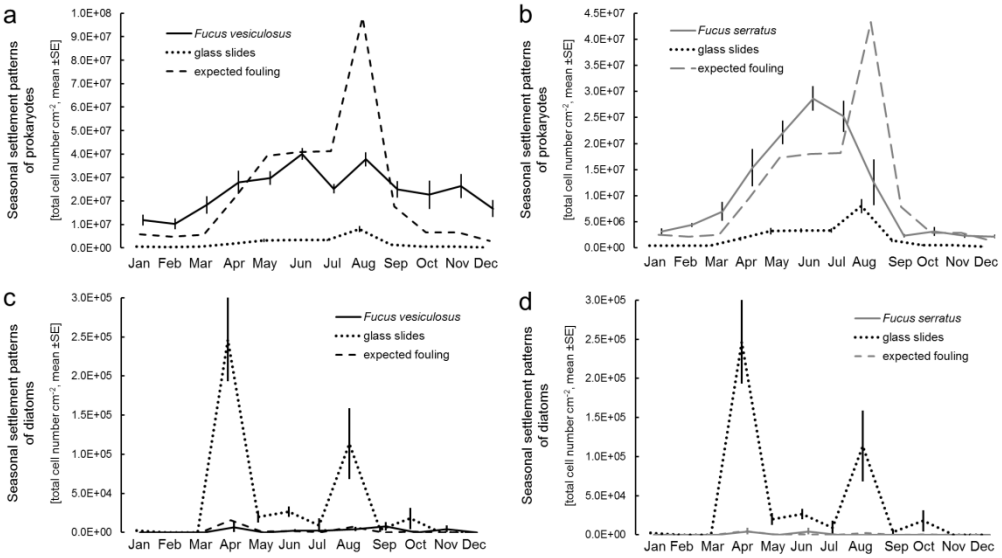

Fig. 1a-d Seasonal settlement patterns in mean total cell and species number (±SE) per cm^2 of *F. vesiculosus* (black solid lines) and *F. serratus* (grey solid lines) thallus surface and glass slides (dotted line) as reference substrates (*n* = 9, for all substrates). Prokaryotes (a, b) and diatoms (c, d) are shown together with the expected fouling (dashed lines) for each *Fucus* species. 'Expected fouling' on *Fucus* was derived from the monthly recorded fouling pressure (glass slides) divided by the annual mean fouling pressure. The resulting microfouling pressure factor was multiplied with the annual mean fouling density of *Fucus* and plotted as 'expected fouling'. Considerable differences between expected and recorded microfouling on *Fucus* could be interpreted as pro- or anti-microfouling activity of *Fucus*.

On *Fucus* spp. microfouling densities by prokaryotes and diatoms reached a maximum in summer and *F. vesiculosus* was more densely fouled than *F. serratus*. *F. serratus* associated prokaryotes showed a sudden decline from July on, whereas *F. vesiculosus* associated prokaryotes decreased gradually from August on. Prokaryotic fouling quantitatively dominated on both *Fucus* species compared to diatom fouling (Fig. 1a-d, suppl. Table S1).

On *F. vesiculosus* prokaryotic fouling increased from March to June with a sudden drop in the cell number in July. Peak cell densities were found in June and

August (4.00 x 10^7± 2.52 x 10^6 and 3.78 x 10^7 ± 2.99 x 10^6 cell number per cm^2 thallus surface ± SE between replicated algae individuals, resp.) (Fig. 1a, suppl. Table S1). Prokaryotic fouling on *F. vesiculosus* reached an annual mean cell density of 2.43 x 10^7 ± 3.52 x 10^6 cells per cm^2 thallus surface (±SE between replicated algae individuals) (suppl. Table S1). Diatoms on *F. vesiculosus* occurred from April to September (except in May) and again in November and reached peak cell densities in April and September (6.67 x 10^3 ± 6.67 x 10^3 and 7.78 x 10^3 ± 5.72 x 10^3 cell numbers per cm^2 thallus surface ±SE between replicated algae individuals, respectively) (Fig. 1c, suppl. Table S1).

On *F. serratus,* microfouling was most intense during spring and summer and quantitatively dominated by prokaryotic fouling. Diatom fouling was scarce. Prokaryotic fouling on *F. serratus* increased from March to June with a maximum cell density in June (2.87 x 10^7 ± 2.32 x 10^6 cells per cm^2 thallus surface ± SE between replicated algae individuals) (Fig. 1b, suppl. Table S1). Prokaryotic fouling on *F. serratus* thalli showed an annual mean cell number of 1.07 x 10^7 ± 1.68 x 10^6 cells per cm^2 thallus surface (± SE between replicated algae individuals) (suppl. Table S1). Diatom fouling on *F. serratus* was very low and occurred only in April and June (4.44 x 10^3 ± 4.44 x 10^3 cells per cm^2 thallus surface ± SE between replicated algae individuals) (Fig. 1d, suppl. Table S1).

In general, prokaryotic fouling occurred in higher cell numbers on living *Fucus* surfaces throughout the year compared to non-living glass surfaces, whereas diatom cells were present in higher number on glass. However, *Fucus* thalli had a longer exposure time than the glass slides (see previous paragraph ‘assessment of microfouler recruitment’).

Expected fouling control strength

The differences between expected and observed microfouling on *Fucus* predicted strongest antifouling control of *F. vesiculosus* against prokaryotic cells during July and August (Fig. 1a) and for *F. serratus* during August and September (Fig. 1b). Strongest antifouling control of both *Fucus* species against diatoms was predicted during April and August (Fig. 1c, d).

Seasonal variation in fouling control strength

Fucus surface extracts exhibited seasonally fluctuating chemical fouling modulation against prokaryotic and diatom settlement as illustrated by the relatively high portion of the variance explained by a sinusoidal fit (see below). It has been described earlier that *Fucus* (*F. vesiculosus*, at least) exerts both pro- and antifouling effects on microfoulers, depending on metabolite and target species considered.

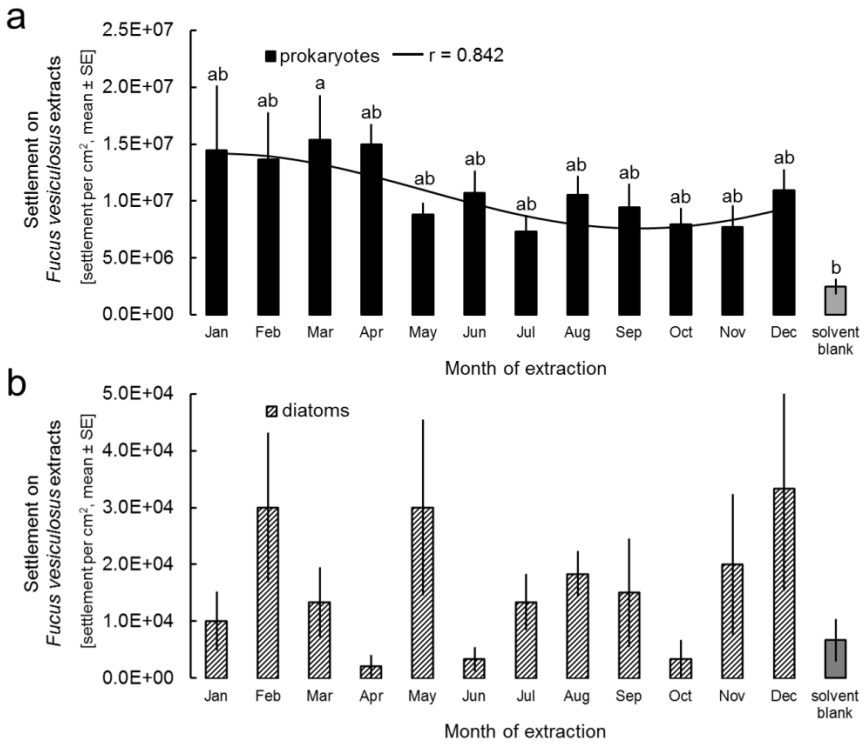

Fig. 2a, b Mean number (±SE) of settled (a) prokaryotes and (b) diatoms on *F. vesiculosus* surface extracts (two-fold average boundary layer conc.) originating from different months (n = 6, in all cases). Solvent blanks are in grey. One-way ANOVA results for prokaryotic settlement F = 1.8, p = 0.06 and for diatom settlement F = 1.4, p= 0.2. Tukey's HSD test ($p \leq 0.05$) revealed only significant differences between group means for prokaryotic settlement (suppl. Table S2). Significant differences are illustrated by letter coding (same letters are not significantly different). Seasonality in microfouling control strength of *F. vesiculosus* against prokaryotic fouling is illustrated by a sinusoidal function fitted to the data. Seasonality in diatom data could not be illustrated by a sinusoidal function.

F. vesiculosus surface extracts showed a net attraction for prokaryotic settlement compared to the solvent blanks in all monthly samples, but these activities were just marginally significant for extracts originating from March (one-way ANOVA, F = 1.8, p = 0.06; Tukey's HSD test $p \leq 0.05$, suppl. Table S2). Lowest settlement (meaning

weak profouling or strong antifouling activities) against prokaryotic cells were found in May, July, October and November (Fig. 2a). The seasonal pattern, modelled as a sinusoidal nonlinear regression, explained 84 % of the variation in activities against prokaryotic cells (Fig. 2a). *F. vesiculosus* surface extracts were generally attractive for diatom settlement, if compared to the solvent blank, and did not significantly deter diatom settlement (one-way ANOVA, $F = 1.4$, $p = 0.2$, Fig. 2b). A sinusoidal nonlinear regression could not be modelled on the diatom data set (Fig. 2b).

F. serratus surface extracts differed significantly in their attractiveness to prokaryotic settlement among months and with regard to the solvent blanks (one-way ANOVA $F = 2.0$, $p = 0.04$). Tukey's HSD test ($p \leq 0.05$) failed to detect any differences between the means (Fig. 3a).

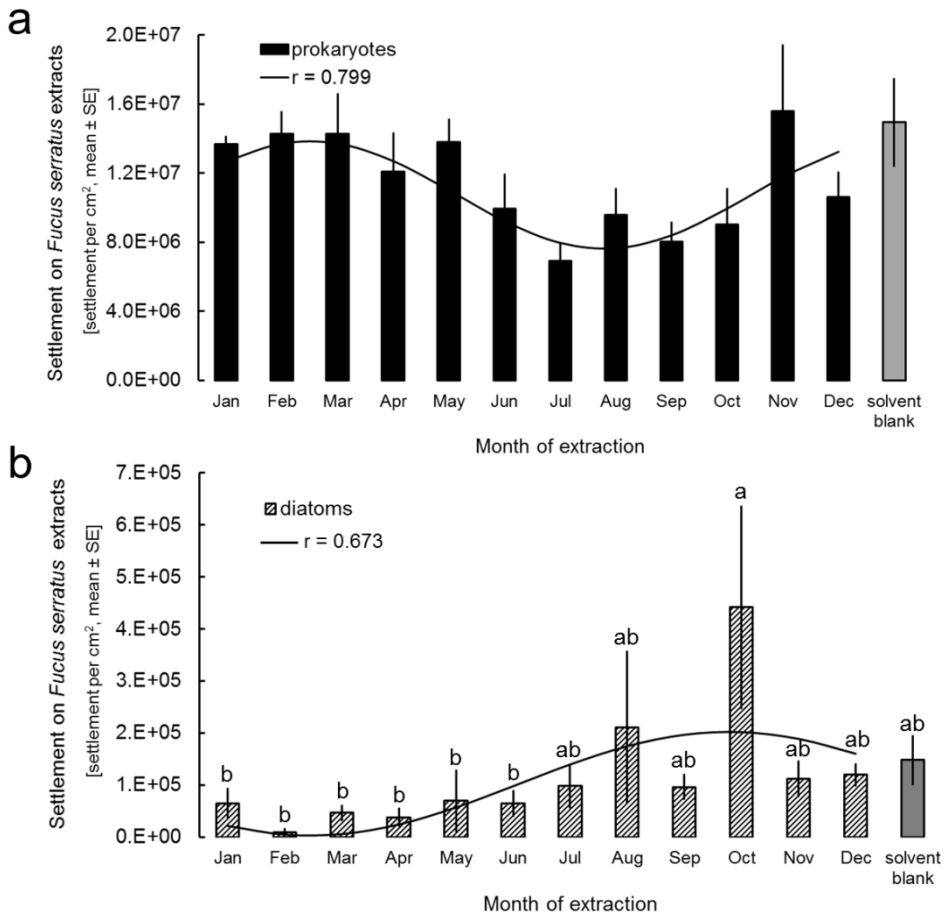

Fig. 3a, b Mean number (±SE) of settled (a) prokaryotes and (b) diatoms on *F. serratus* surface extracts (two-fold average boundary layer conc.) originating from different months ($n = 6$, in all cases). Solvent blanks are in grey. One-way ANOVA results for prokaryotic settlement: $F = 2.0$ $p = 0.04$, whereas for diatom settlement: $F = 2.3$, $p = 0.02$. Tukey's HSD test ($p \leq 0.05$) revealed significant differences between group means for diatom settlement (suppl. Table S2). Significant differences are illustrated by letter coding (same letters indicate not significantly different samples). Seasonality in microfouling control strength of *F. serratus* against prokaryotic and diatom settlement is illustrated by fitting a sinusoidal function to the data.

F. serratus surface extracts tended to be less attractive for prokaryotic settlement in July and September (Fig. 3a). Seasonal variation in activities of *F. serratus* surface extracts against prokaryotic settlement was explained to 80 % by a sinusoidal regression model (Fig. 3a).

The modulatory activities of *F. serratus* surface extracts regarding diatom settlement differed significantly among months and solvent blanks (one-way ANOVA, $F = 2.3$, $p = 0.02$, Fig. 3b). Surface extracts originating from October were significantly more attractive than extracts obtained during January to June (Tukey's HSD test, $p \leq 0.05$; suppl. Table S2). Seasonality of activities can be described by a sinusoidal non-linear regression model, explaining 67 % of the variance (Fig. 3b).

The seasonal fluctuations in the fouling control of *F. vesiculosus and F. serratus* surface extracts were neither correlated with *in situ* fouling pressure nor with the mannitol content of *Fucus* tissue with the sole exception of *F. serratus* prokaryotic fouling control response (bioassay results) and the mannitol content of *F. serratus* (Table 1).

Raw data of *in situ* bioassay results are compiled in suppl. Table S3.

Table 1. Summarized results of correlation tests.

Origin of tested extract	Fouling species in bioassay	Potential driver	rho cor. coefficient	p-value
Fucus vesiculosus	prokaryotes	mannitol [mg/g dry weight]	0.070	0.834
Fucus vesiculosus	diatoms	mannitol [mg/g dry weight]	-0.412	0.184
Fucus serratus	prokaryotes	mannitol [mg/g dry weight]	**-0.664**	**0.022**
Fucus serratus	diatoms	mannitol [mg/g dry weight]	0.308	0.331
Fucus vesiculosus	prokaryotes	*in situ* prokaryotic fouling pressure [cm^2]	-0.270	0.397
Fucus vesiculosus	diatoms	*in situ* diatom fouling pressure [cm^2]	-0.051	0.874
Fucus serratus	prokaryotes	*in situ* prokaryotic fouling pressure [cm^2]	-0.536	0.073
Fucus serratus	diatoms	*in situ* diatom fouling pressure [cm^2]	-0.292	0.357

The table contains correlation coefficients (rho = Spearman's cor. coefficient) and p-values for each correlation test; significance level after Bonferroni correction $p \leq 0.025$; $n = 12$, for each correlation. Significant test results are in bold.

Surface observation in SEM

Both *Fucus* species showed occasional shedding of an exterior cuticula together with microfoulers attached to it. This process led to a (temporarily) microfouler-free epidermis. Example photos are shown in Fig. 4.

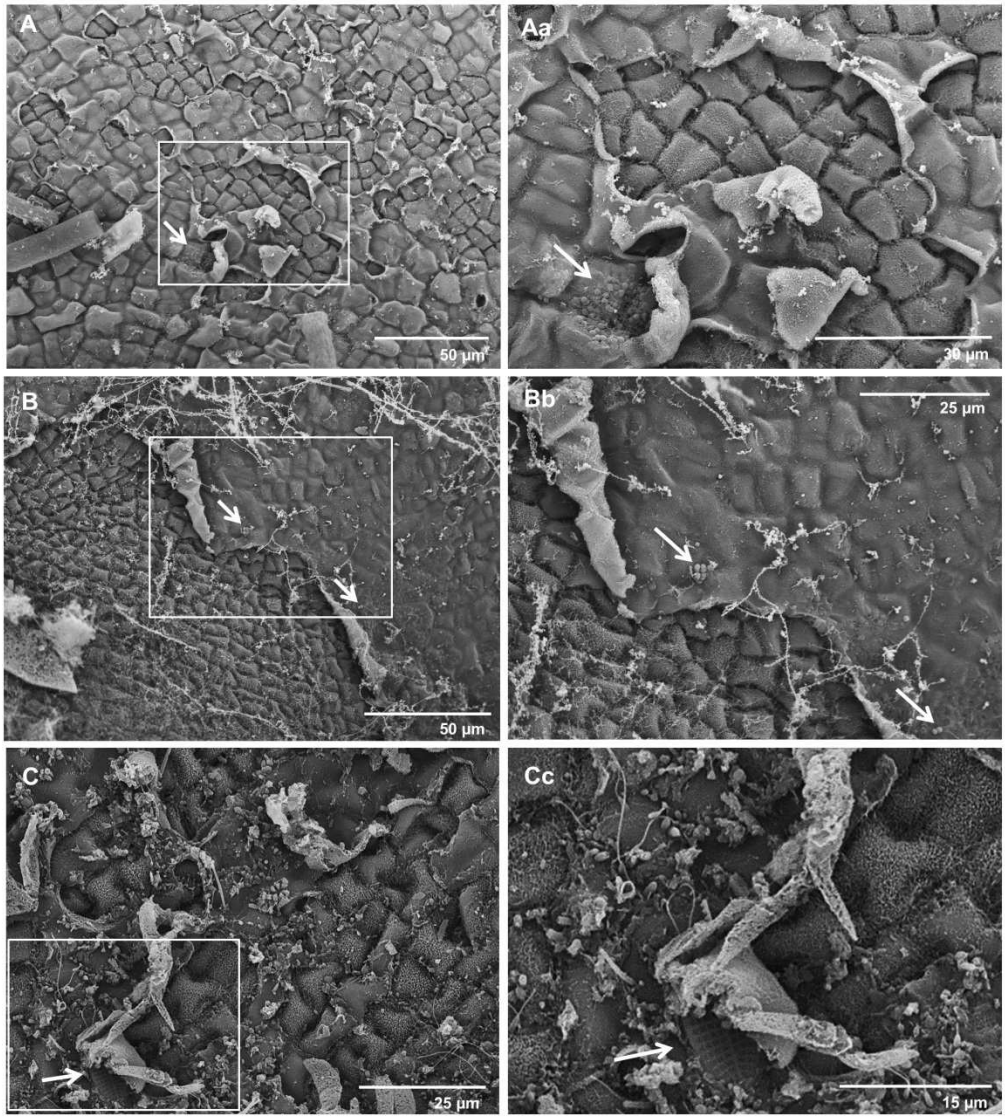

Fig. 4 Scanning electron microscopy (SEM) images of the young apical thallus surface from *F. vesiculosus* (A, C overview and Aa, Cc selected section in detail) and *F. serratus* (B, overview and Bb, selected section in detail) showing shedding of an exterior cuticula layer, overlying epidermis cells, with embedded prokaryotic cells (marked by arrows, see A, Aa), associated prokaryotic cells (marked by arrows, see B, Bb) and associated diatom cells (see C, Cc). Algae samples were taken in March (A, B) and in July (C) 2013.

Discussion

The focus of this study has been on prokaryotes and on pennate diatoms, since they are considered key compounds of algal-associated biofilms (Kiirikki 1996; Martin et al. 2014; Snoeijs 1994). Prokaryotes and diatoms may be particularly important due to their capacity to mediate further biofilm succession (Dobretsov et al. 2006; Steinberg and de Nys 2002). Hereafter we summarize these two fouling groups under the term microfouling.

In the present study we assessed (1) whether microfouling pressure shows seasonal variation in intensity, (2) whether *F. vesiculosus* and *F. serratus* chemically control microfouling, (3) whether the chemical fouling control of *Fucus vesiculosus* and *Fucus serratus* is tuned to *in situ* microfouling pressure and (4) whether the seasonal fluctuations in chemical microfouling controls relate to the relative fouling pressure and/or to energy reserves (mannitol).

Our study revealed that microfouling varied in intensity with season on reference substrate (glass slides) and on the thalli of both *Fucus* species. Further, the chemical fouling control also fluctuated seasonally with regard to prokaryotic and diatom recruitment**,** except for *F. vesiculosus* regarding diatom fouling. Correlation between fouling control strength and the two potential drivers (fouling pressure and energy availability) were generally not found in either *Fucus* species. Only the *F. serratus* bioassay response regarding prokaryotic fouling was negatively correlated with the mannitol content of *F. serratus*.

Additionally, our study could show that both *Fucus* species exhibited shedding of an exterior cuticula layer, removing associated and embedded prokaryotic and diatom cells.

Microfouling in the field

Prokaryotic fouling

Our field experiment showed that prokaryotic fouling exhibited a clear seasonal pattern with substantially increased cell numbers during spring and summer compared to autumn and winter months independently of the substrate (glass slides and *Fucus*). This pattern is explainable with the metabolic promoting conditions during spring and summer. Elevated temperatures stimulate microbial enzymatic activities and microbial metabolisms (Hoppe et al. 2002; Rao 2010; Zaccone et al. 2014). Further, increased light intensities lead to an intensified photosynthesis of

autotrophic prokaryotes. While heterotrophic prokaryotes profit during spring and summer from the utilization of dissolved organic carbon (DOC) and other organic exudates (e. g. dissolved organic nitrogen) (Nalewajk.C and Lean 1972; Tyler and McGlathery 2006; Tyler et al. 2001) released from macrophytes (Barron et al. 2003; Brylinsky 1977; Khailov and Burlakov 1969; Pregnall 1983). All in all this beneficial conditions for prokaryotic metabolism lead to higher cell division rates and elevated cell numbers in surrounding seawater and consequently on neighbouring surfaces, since most planktonic microbes prefer the attached lifestyle (Grossart 2010) during spring and summer month.

However, our findings of increased microbial cells associated with *Fucus* during spring and summer are in contradiction with the results of a previous study from Sieburth and Tootle (1981). The authors found highest microbial fouling on *F. vesiculosus* during April, November and December and lowest fouling during May and October. This mismatch could be addressed to the different thallus sections analysed in the studies (older sections *vs*. young apical sections) since it is known that various thallus regions can host different bacterial assemblages with different quantities (Bengtsson et al. 2010; Lachnit et al. 2013; Staufenberger et al. 2008).

The dominance of prokaryotic fouling, also independently of analysed substrate (slides and *Fucus*), in comparison with diatom fouling is in accordance with findings from Sieburth and Tootle (1981) and not surprising considering that prokaryotes are a ubiquitous majority in aquatic environments with a density of 10^4 - 10^7 cells/ml (Whitman et al. 1998) and as mentioned above that most of these planktonic microbes favour to change the free-living to a surface-associated lifestyle (Grossart 2010). Further, the observed cell densities of *Fucus* associated microbes are in accordance with finding from previous studies. The authors reported for *F. vesiculosus* bacterial cell density from 1.5×10^7 to 1.0×10^8 cells per cm^2 (Stratil et al. 2013) and 7.7×10^6 to 1.9×10^8 cells per cm^2 (Wahl et al. 2010).

The observation that both *Fucus* species hosted higher prokaryotic cell densities as compared to the reference substrate throughout the year is probably due to the fact that the very uneven thallus surface represents a suitable settlement ground. Additionally, exuded algal compounds, e.g. mannitol a suitable energy source for bacteria could have profouling effects and, finally, that the thalli area analysed were exposed to fouling longer than the reference substrata. Differences in exposure time between the reference substrate (one week at a time) and *Fucus* (approx. 4-8

weeks, depending on sampling season) could have led to this elevated cell densities on *Fucus*. As previously mentioned, *F. vesiculosus* from the Baltic Sea exhibits a seasonal variable length growth with increased growth during spring and summer and reduced growth during winter (Lehvo et al. 2001). Thus, it is evident that the analysed apical vegetative thallus tips had a variable higher age, depending on the sampling date. The reported microfouling status of *Fucus* reflects therefore the density of a recently established microfouling community with a slightly variable age. Only the microfouling pressure data obtained from the reference substrate (after one week at a time) reflects the exact fouling pressure *Fucus* is exposed to throughout the year.

The combination of elevated cell numbers on the *Fucus* thalli (relative to the glass slides and the solvent-only bioassay) with the pronounced seasonal fluctuation in chemical control suggests that the proportion of attractive or nutritive metabolites and the repellent metabolites varies throughout the year in a non-random manner. Although not significant, there was a clear trend for the surface extracts of both *Fucus* species to become less attractive (better defended?) in months with elevated fouling pressure. Also a strain-specific chemical defense targeting single prokaryotic strains in different intensities was described from *F. vesiculosus* (Lachnit et al. 2013; Saha and Wahl 2013) while other desired or tolerated strains are spared and may reach elevated cell numbers. Since the present study did not analyse the fouling community compositions on a detailed phylogenetic level, which would have been far beyond the scope of the study considering the sample quantities, only speculations can be made about a strain-specific chemical control.

Diatom fouling

Our field experiment revealed that diatom fouling on glass and on both *Fucus* species exhibited also a seasonal pattern, reaching high cell densities in spring followed by a decline during summer and a second rise in late summer (suppl. Table S1). These findings are in accordance with results from previous studies (Munda 2005; Snoeijs 1994; Wolfstein et al. 2000; Yang et al. 2014). The authors also reported a spring maximum and a summer minimum of diatom fouling on artificial substrate, whereas Munda (2005) also found peak densities of diatom fouling during summer. The observed diatom spring bloom is a common phenomenon among benthic diatoms and is explainable with elevated light and nutrients during spring

along with the tolerance of many diatom species to low temperatures (Snoeijs 1994). Low diatom densities during summer are also often described and probably caused by an increased invertebrate grazing pressure leading to a reduced diatom density despite high diatom growth (Borum 1987; Castenholz 1961). The elevated diatom densities during late summer remain unexplained due to missing nutrient measurements a connection could not be made.

The field experiment revealed lower diatom cell densities on both *Fucus* species than on the reference substrate. One explanation, that would confirm our notion of a chemical fouling control tuned to fouling pressure, could be that *Fucus* is an unattractive settlement surface for diatom cells due to a chemical fouling control of *Fucus* against diatom settlement. The surface extracts of both *Fucus* species were least attractive in the season of most intense diatom settlement (spring), however, only the defense activity of *F. serratus* was significant.

An additional explanation for the low diatom numbers on apical thalli of *Fucus* could be that *Fucus* removes, on young apical thallus sections, epiphytes also via cuticula shedding. Former studies have described such cuticula shedding for several macrophytes as effective antifouling defense mechanism against epiphytic algae (Filion-Myklebust and Norton 1981; Harder 2008; Russell and Veltkamp 1984; Sieburth and Tootle 1981; Yamamoto et al. 2013).

Chemical fouling control strength of Fucus

Comparison between the expected microfouling on *Fucus* and the relative fouling status of *Fucus* suggests a lowering of the general attractiveness of *Fucus* for prokaryotes and diatoms during spring and summer – possibly due to enhanced production of defensive metabolites (Saha et al. 2012; Saha et al. 2011). Our *in situ* bioassay generally showed for both *Fucus* species a reduced fouling of the tested microfouler groups on the extracts from spring and summer. These observations are consistent with our expectation that *Fucus* should more efficiently control fouling in times of intense fouling pressure. A similar seasonal fluctuation of the fouling control strength of *F. vesiculosus* has been shown before (Saha and Wahl 2013).

Why *in situ* tested *F. vesiculosus* surface extracts exhibited a general profouling effect to the natural fouler pool compared to the solvent blanks remains unclear and seems in conflict with earlier results (e.g. Saha and Wahl 2013). One possible explanation could be that the complex extract composition contains profouling

compounds, such as those previously reported from Lachnit et al. (2013) for the polar fractions of *F. vesiculosus* surface extracts. Another possibility is that in contrast to the earlier lab bioassay which used selected bacterial strains from algal surface, in the present case the extract were exposed to the entire natural community of microfoulers.

Finally, prokaryotic and diatom fouling is not exclusively controlled by means of chemical fouling control, but maybe additionally effected by cuticula shedding.

Besides *in situ* fouling pressure as driving force for a tuned fouling control also the resource availability for defense metabolite production could shape the seasonal defense strength pattern of *Fucus* (Wahl et al. 2010). To verify this we used the storage compound mannitol (Lehvo et al. 2001; Michel et al. 2010) as a proxy for energy availability to produce pro- or antifouling metabolites. The fact that we found just one negative correlation between *Fucus* control strength and the tissue mannitol content could not really prove the notion of a resource driven anti-microfouling control since energy reserves were never depleted.

In conclusion, this study revealed that relative microfouling pressure in the field and microfouling control strength of *Fucus* varied with season. Furthermore, monthly collected surface extracts tended to be least attractive for microfoulers during seasons, when in the field microfouling pressure was highest. While this correlation was not significant, the trend is suggestive of a pronounced deployment of defensive metabolites during these periods as shown earlier (Saha and Wahl 2013). Any chemical microfouling control in the two *Fucus* species seems to be assisted by an occasional cuticula shedding that remove attached foulers.

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Suppl. Table S1. Seasonal settlement patterns of prokaryotic and diatom cells associated with *F. vesiculosus*, *F. serratus* and reference substrata (glass slides).

Fouling species / analysed substrata	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Prokaryotes Fv	1.19E+07 ± 2.27E+06	1.02E+07 ± 2.32E+06	1.83E+07 ± 3.72E+06	2.79E+07 ± 4.96E+06	2.97E+07 ± 2.92E+06	4.00E+07 ± 2.52E+06	2.52E+07 ± 2.02E+06	3.78E+07 ± 2.99E+06	2.49E+07 ± 3.59E+06	2.26E+07 ± 6.11E+06	2.63E+07 ± 5.15E+06	1.68E+07 ± 3.71E+06
Prokaryotes Fs	3.19E+06 ± 5.08E+05	4.39E+06 ± 3.91E+05	6.99E+06 ± 1.82E+06	1.54E+07 ± 3.61E+06	2.21E+07 ± 2.28E+06	2.87E+07 ± 2.32E+06	2.52E+07 ± 2.97E+06	1.26E+07 ± 4.36E+06	2.32E+06 ± 2.99E+05	3.13E+06 ± 8.64E+05	2.32E+06 ± 4.33E+05	2.16E+06 ± 3.62E+05
Prokaryotes glass slides	4.73E+05 ± 7.42E+04	3.98E+05 ± 3.89E+4	4.64E+05 ± 5.48E+04	1.77E+06 ± 3.73E+05	3.20E+06 ± 6.72E+05	3.34E+06 ± 3.96E+05	3.37E+06 ± 4.09E+05	8.02E+06 ±1.37E+06	1.45E+06 ± 2.97E+05	5.31E+05 ± 1.31E+05	5.33E+05 ± 1.37E+05	2.51E+05 ± 3.85E+04
Diatoms Fv	0 ± 0	0 ± 0	0 ± 0	6.67E+03 ± 6.67E+03	0 ± 0	2.22E+03 ± 2.22E+03	2.22E+03 ± 2.22E+03	4.44E+03 ± 2.94E+03	7.78E+03 ± 5.72E+03	0 ± 0	4.44E+03 ± 4.44E+03	0 ± 0
Diatoms Fs	0 ± 0	0 ± 0	0 ± 0	4.44E+03 ± 4.44E+03	0 ± 0	4.44E+03 ± 4.44E+03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Diatoms glass slides	2.22E+03 ± 2.22E+03	0 ± 0	0 ± 0	2.47E+05 ± 5.32E+04	2.00E+04 ± 7.45E+03	2.67E+04 ± 6.67E+03	8.89E+03 ± 8.89E+03	1.13E+05 ± 4.53E+04	3.33E+03 ± 3.33E+03	1.78E+04 ± 1.35E+04	0 ± 0	0 ± 0

Fv = *F. vesiculosus*; Fs = *F. serratus;* errors ± SE between replicates; *n* = 9, per month and per *Fucus* species; Jan - Dec = month of sampling.

Suppl. Table S2. Summarized significant results of Tukey's HSD test ($p \leq 0.05$).

Origin of tested extract	Fouling species in bioassay	Significant different data sets	p-value
Fucus vesiculosus	Prokaryotes	solvent blank / Mar	0.036
Fucus serratus	Diatoms	Oct / Jan	0.022
Fucus serratus	Diatoms	Oct / Feb	0.004
Fucus serratus	Diatoms	Oct / Mar	0.013
Fucus serratus	Diatoms	Oct / Apr	0.010
Fucus serratus	Diatoms	Oct / May	0.025
Fucus serratus	Diatoms	Oct / Jun	0.022

Suppl. Table S3. Settlement patterns of prokaryotes and diatoms in settled cells per cm^2 *in situ* bioassay surface containing *F. vesiculosus* and *F. serratus* surface extracts (two-fold average boundary layer conc.) originating from different months of a year.

Fouling species + extract type	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	sol. blank
Prokaryotes Fv extracts	1.45E+07 ± 5.71E+06	1.37E+07 ± 4.11E+06	1.54E+07 ± 3.95E+06	1.50E+07 ± 1.76E+06	8.82E+06 ± 1.04E+06	1.07E+07 ± 1.98E+06	7.31E+06 ± 1.44E+06	1.06E+07 ± 1.64E+06	9.43E+06 ± 2.11E+06	7.93E+06 ± 1.44E+06	7.72E+06 ± 1.90E+06	1.10E+07 ± 1.86E+06	2.50E+06 ± 6.80E+05
Prokaryotes Fs extract	1.37E+07 ± 4.98E+05	1.43E+07 ± 1.33E+06	1.43E+07 ± 2.37E+06	1.21E+07 ± 2.28E+06	1.38E+07 ± 1.35E+06	9.94E+06 ± 2.01E+06	6.90E+06 ± 1.07E+06	9.57E+06 ±1.57E+06	8.01E+06 ± 1.17E+06	9.01E+06 ± 2.11E+06	1.56E+07 ± 3.87E+06	1.06E+07 ± 1.51E+06	1.49E+07 ± 2.56E+06
Diatoms Fv extracts	1.11E+04 ± 5.16E+03	3.00E+04 ± 1.32E+04	1.33E+04 ± 6.15E+03	2.00E+03 ± 2.00E+03	3.00E+04 ± 1.55E+04	3.33E+03 ± 2.11E+03	1.33E+04 ± 4.94E+03	1.83E+04 ± 4.01E+03	1.50E+04 ± 9.57E+03	3.33E+03 ± 3.33E+03	2.00E+04 ± 1.24E+04	3.33E+04 ± 1.80E+04	6.67E+03 ± 3.73E+03
Diatoms Fs extracts	6.50E+04 ± 2.92E+04	1.00E+04 ± 6.83E+03	4.67E+04 ± 1.61E+04	3.83E+04 ± 1.78E+04	7.00E+04 ± 6.01E+04	6.50E+04 ± 2.57E+04	9.83E+04 ± 4.28E+04	2.12E+05 ± 1.46E+05	9.67E+04 ± 2.55E+04	4.42E+05 ± 1.95E+05	1.12E+05 ± 3.53E+04	1.20E+05 ± 2.22E+04	1.48E+05 ± 4.84E+04

Fv = *F. vesiculosus*; Fs = *F. serratus*; errors ± SE; *n* = 6, per month of extraction and per *Fucus* species; Jan - Dec = month of extraction; sol. blank = solvent blank.

Paper II

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Seasonal fluctuations of chemical defenses against macrofouling in *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea

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Abstract

Macroalga, especially perennial species, are exposed to a seasonally variable fouling pressure. We hypothesize that macroalgae regulate their antifouling defense to fouling pressure. During one year, we assessed macrofouling pressure and chemical anti-macrofouling defense strength of the brown algae *Fucus vesiculosus and Fucus serratus* with monthly resolution. Anti-macrofouling defense was assessed by means of surface-extracted *Fucus* metabolites tested at near-natural concentrations in a novel *in situ* bioassay. Additionally, mannitol content of both *Fucus* species was determined to assess resource availability for defense production. Surface chemistry of both *Fucus* species exhibited a seasonal variability in attractiveness to *Amphibalanus improvisus* and *Mytilus edulis*. 50-60% of this variability is explained by a sinusoidal model. Only *Fucus vesiculosus* extracts originating from spring and summer deterred significantly *A. improvisus* settlement. The strength of macroalgal antifouling defense did not correlate with *in situ* macrofouling pressure nor with measured mannitol contents, which, however, were never depleted.

Keywords *Fucus*; seasonal anti-macrofouling defense; chemical defense; *in situ* bioassay; *Amphibalanus improvisus*; *Mytilus edulis*

Introduction

Epibiosis - the facultative association between epibionts (colonizer) and basibionts (colonized host) - is a widespread phenomenon in the marine environment (Wahl 1989, Wahl & Mark 1999, Harder 2008). Epibiosis entails advantages as well as disadvantages for the basibiont. On balance, negative effects of epibiosis on the basibiont often outweigh the beneficial ones (Wahl 1989). Detrimental effects of epibiosis, for example for kelps, comprise increasing weight and drag resulting in blade loss often due to heavy calcareous epibionts (Dixon et al. 1981, Scheibling & Gagnon 2009) or reduced photosynthesis along with disturbance in transcutaneous nutrients and gas exchanges (Wahl 1989). Marine macroalgae, especially slow-growing perennial species such as fucoids, are often covered by a broad spectrum of fouling organisms including micro- and small macroalgae, bacteria, fungi, protozoans and multicellular animals (Korpinen et al. 2007). The epiphytic load on a macroalgal thallus can reach up to 80 to 100 % coverage (Andersen et al. 2011). Epibionts compete with their host for the vital resources of light and nutrients (Korpinen et al. 2007). Epiphytes decreased growth of the brown alga *Fucus vesiculosus* (Jormalainen et al. 2003, Honkanen & Jormalainen 2005) and may cause a growth reduction of over 25 % (Rohde & Wahl 2008). High mortality due to pronounced epiphytisms was reported from kelp species (Scheibling & Gagnon 2009, Andersen et al. 2011). Moreover, a reduced reproductive effort, due to physical blockage of receptacles by an obligate epiphyte was observed in the brown alga *Ascophyllum nodosum* (Kraberg & Norton 2007). Obviously, the consequences of epiphytisms can be dramatic for macroalgae fitness. The detrimental effects on marine macroalgae are possibly one driving factor for the evolution of antifouling defense mechanisms against epibionts in seaweeds (da Gama et al. 2014).

Macroalgae have developed a variety of physical and chemical antifouling defense systems against epibionts (da Gama et al. 2014), such as periodical peripheral cell or meristoderm shedding are effective physical antifouling mechanisms common among red and brown macroalgae (Filion-Myklebust & Norton 1981, Russell & Veltkamp 1984, Davis et al. 1989, Nylund et al. 2005, Yamamoto et al. 2013). Further mechanical defense systems against macrofouling are, for example, the secretion of mucus (reported from *Laminaria solidungula*) is possibly beneficial to inhibit the establishment of algal or animal epibionts (U. Karsten

unpublished results, Coll et al. 1987). Seaweeds are known to be protected against grazing by chemical defense mechanisms based on secondary metabolites (Hay & Fenical 1988, Pohnert 2004, Amsler & Fairhead 2006, Amsler 2008). Studies on the anti-herbivory defense of the perennial brown alga *Fucus vesiculosus* revealed an inducible regulation on demand (Rohde et al. 2004, Rohde & Wahl 2008, Weinberger et al. 2011).

Regarding chemical antifouling defense of *Fucus,* previous studies have focused on microfouling rather than on macrofouling. Investigations on chemical anti-microfouling defense showed that *F. vesiculosus* can inhibit and modulate microfouling by means of surface-associated pro- and antifouling secondary metabolites (Lachnit et al. 2010, Wahl et al. 2010, Saha et al. 2011, Saha et al. 2012, Saha & Wahl 2013). Studies on chemical anti-macrofouling defense revealed that *F. vesiculosus* and *F. evanescens* exude phlorotannins with the potential to deter *A. improvisus* larvae settlement (Wikstrom & Pavia 2004, Brock et al. 2007). Furthermore, it has been shown that *F. vesiculosus* exhibit significant among-genotype variations in tolerance and resistance to fouling (Jormalainen et al. 2003, Honkanen & Jormalainen 2005). Nasrolahi et al. (2012) showed that biofilms isolated from *F. vesiculosus* and *F. serratus* reduced the attachment of *A. improvisus* larvae, whereas a study from Dobretsov (1999) revealed that biofilms and water soluble metabolites from *F. vesiculosus* had no effect on *Mytilus edulis* settlement behavior. These studies demonstrate that *Fucus* and their associated biofilms have the capacity to modulated further fouling but that this capacity may vary among target species. Besides these investigations on anti-macrofouling defense of *Fucus* little is known about the anti-macrofouling defense of *Fucus* and even less about its temporal dynamics.

Macroalgae are exposed to a seasonal fluctuating micro- and macrofouling pressure (Sieburth & Tootle 1981, Arrontes 1990, Lachnit et al. 2010, Wahl et al. 2010) which is defined as substratum colonization per time unit (Wahl et al. 2011). In general micro- and macrofouling in the field is elevated during spring and summer reflecting reproduction cycles, rising temperatures, light and nutrient availability (Thomsen et al. 2010, Wahl et al. 2010, Pansch et al. 2012).

If production of defense metabolites is costly and competes with other metabolic functions for limited resources (Coley et al. 1985, Strauss et al. 2002, Dworjanyn et al. 2006) and that fouling pressure in the field varies with season and with

environmental factors (Borum 1985, Jormalainen et al. 2003, Korpinen et al. 2007, Wahl et al. 2010) it should be of selective advantage to adapt the chemical antifouling defense to the actual fouling pressure. So far, a seasonal variation in antifouling defense has been only reported from *F. vesiculosus* against microfoulers (Wahl et al. 2010, Saha & Wahl 2013) but was never directly related to the current fouling threat. Thus, investigations on *Fucus* anti-macrofouling defense in relation to season and actual macrofouling pressure in the field are lacking.

The perennial brown algae *F. vesiculosus* and *F. serratus* are structurally important belt-forming macroalgal species in the Baltic Sea (Kautsky 1992, Ronnback et al. 2007). *Fucus vesiculosus* mainly occurs on hard substrate in 0 - 3 meter shallow coastal waters (Torn et al. 2006) while *F. serratus* extends to deeper waters. At between 1 and 2 m water depth both species form mixed stands whereas *F. serratus* dominates between 2 and 6 meters (Malm et al. 2001). Both benthic algae inhabit a coastal zone where temperature, light and nutrients availability and consequently fouling underlie a strong seasonal pattern.

The aim of the present study was to investigate (1) how the macrofouling pressure varies seasonally in intensity and community composition, (2) whether *Fucus vesiculosus* and *Fucus serratus* chemically inhibit macrofouling, (3) whether the strength of chemical antifouling defense of *F. vesiculosus* and *F. serratus* relates to prevailing fouling pressure and (4) whether temporal variation in the important energy-storage compound mannitol relates directly to defense strength which might suggest energy limitation as an alternative explanation for fluctuations in chemical antifouling defense of both *Fucus* species.

Material and methods

Study organisms and collection of alga material

This study was carried out with the perennial brown algal species *Fucus vesiculosus* and *Fucus serratus* which occur intermingled in shallow waters in the outer Kiel Fjord, Germany (54°27'21 N; 10°11'57 E) with a tendency for *F. vesiculosus* to occur somewhat shallower (0 - 2 m) than *F. serratus* (0.5 - 3 m). From August 2012 to July 2013, 15 individuals of each species were collected monthly at depths from 0.5 m under mid water level and separately stored in 3 l plastic bags (to obtain a humid atmosphere) and transported in a cooler to minimize

stress during transport to the laboratory and storage. Within 3 to 4 h after sampling all algae were surface extracted and processed (see paragraph 'Surface extraction' below).

The algal material was used to determine seasonal changes in epiphytic coverage and composition, changes in surface associated secondary metabolites and total mannitol content. In order to record the actual fouling pressure at the sampling site PVC plates were exposed monthly and analyzed for their epiphytic coverage and composition. Furthermore, temperature, salinity, and irradiance were continuously recorded, while nutrients concentrations were measured weekly. Surface extracts were tested on their seasonal patterns of antifouling strength performing an *in situ* bioassay.

Surface seawater parameters and nutrients

Temperature, salinity and irradiances were recorded at 0.5 m under mid water level using HOBO U24-002 conductivity loggers and HOBO UA-002-64 pendant temperature/light data loggers (HOBO®, Onset Computer Corporation, Bourne, MA, USA) taking one measurement per hour. Fouling biased irradiances were bypassed by using just the first seven days of recorded light measurements to calculate the daily maximum photon flux rates between noon and 1 pm. Nutrients were analyzed out of weekly collected water samples. The water samples were 0.8 µm pre-filtered with a cellulose acetate filter (Sartorius Stedim Biotech, Göttingen, Germany) and stored until analysis at -20 °C. Nutrient concentrations were measured using a spectrophotometric flow analyzer (SANplus-autoanalyzer-system, Skalar Analytical B.V., Breda, Netherlands) following the suggested standard protocol from Skalar.

Census of epiphytes

To record the actual fouling pressure for each month, roughened (60 grit sand paper) PVC plates of 7x7 cm (n = 15) were horizontally exposed four weeks at the sampling site in 0.5 m under mid water level. The plates were collected in seawater-filled plastic boxes in an upright position to avoid any damage during transport to the laboratory. To record the epiphytic coverage and composition for each month, one thallus branch per *Fucus* replicate was separated and stored in a plastic bag at -20 °C until analysis. Coverage of epiphytes on PVC reference plates and *Fucus* material were estimated on a %-grade scale. PVC plates and *Fucus* material were

first preliminary screened for very small and single fouling organisms with a stereomicroscope (10-fold magnification). Such observations were recorded with the lowest %-value of 0.1 %.The total epiphyte coverage was estimated by naked eye according to following %-grades: 1, 2, 3, 5, 10, 20, 25, and 30 % until 100 % in steps of 10 %. Fouling species were identified to the lowest possible taxonomic level.

Predicted antifouling defense strength

The difference between fouling pressures (fouling recorded on PVC panels) and detected fouling on *F. vesiculosus* and *F. serratus* regarding the major foulers *Amphibalanus improvisus* and *Mytilus edulis* were used to determine the expected defense strength of both *Fucus* species. Recorded seasonal recruitment patterns of *A. improvisus* and *M. edulis* on the three different substrata (PVC and both *Fucus* species) were expressed as mean percentage coverage over one month. Any difference in fouling rates between the PVC plates (reference substrate) and *Fucus* was used to predict antifouling defense strength for both *Fucus* species in the specific month considered.

Surface extraction

For surface extraction approx. 50 g of visibly epiphyte-free and non-fertile thallus tips (upper 5-10 cm) per replicate (n = 15 per months, for both species) were cut off. Before extraction the thallus tips were spin-dried for 30 s to remove most of the attached seawater. The extraction procedure was carried out following the protocol described by de Nys et al. (1998) with minor modifications. For surface extraction thallus tips were held at the cut edge with a long steel tweezers and dipped for 4 s into a constantly stirred emulsion of n-hexane and methanol (1:1 v/v). During extraction procedure any contact with the cut edge was avoided to prevent leaching of intracellular compounds. Former studies from Lachnit et al. (2010) and Saha et al. (2011) showed an epidermal cell lysis when extraction time exceeded 10 s. Since in preliminary surface extraction tests some *Fucus* thallus tips turned from normal brown-green to bright green color indicating a loss of pigments already after 7 to 8 s of extraction, the extraction time to 4 s to avoid any risk of cell lysis. After extraction the resulting solution was filtered through a paper filter (Macherey-Nagel 615 1/4, Ø 150 mm, Düren, Germany) to remove particles. The solvent volume was reduced under vacuum at 35 °C using a rotary evaporator. After solvent evaporation the

resulting extracts were re-dissolved in 2 ml n-hexane and 2 ml methanol and quantitatively halved for *in situ* bioassays and chemical analytical studies (data not shown). Re-dissolved extracts were placed on a heating block at 35 °C and evaporated under a constant nitrogen flow. Dried extracts were stored until further analysis in 2 ml glass vials at -20 °C.

The extracted algae were scanned for surface area quantification using the biological-image analysis software “Fiji” (Schindelin et al. 2012). Surface extracted and scanned alga material was further used for the mannitol analysis.

Mannitol analysis

Surface extracted algal material was freeze-dried and ground to a fine powder. 10 - 20 mg dry weight per sample was used for the mannitol analysis according to the ethanol based extraction method described by Karsten et al. (1991) and the analytical protocol of Nitschke et al. (2010) using an Agilent HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) system with isocratic elution equipped with a differential refractive index detector. Mannitol contents are expressed as mg mannitol per gram dry weight.

In situ bioassays

To quantify the seasonal variability in antifouling strength of *F. vesiculosus* and *F. serratus* surface extracts originating from different months of the year had to be tested synchronously to warrant an identical colonizer pool for all monthly samples. To this end a novel *in situ* bioassay was developed in our laboratory (Nasrolahi et al. 2012). Surface extracts (n = 6 per months and species) were re-dissolved in *n*-hexane and methanol (1:1 v/v) and used to repeatedly impregnate a cellulose filter paper (MN 616, Ø 40 mm) until a two-fold mean boundary layer concentration (referring to mg extracted compounds cm^{-2} *Fucus* thallus surface) was obtained. Since a living alga actively release and thus maintain a strong metabolite gradient with near surface concentrations much higher than the average boundary layer concentrations (Grosser et al. 2012) we tried to designed accordingly the bioassay approach by applying the two-fold mean boundary layer concentration and thus generating an approximated metabolite concentration on the filter paper. The impregnated filter paper was freeze-dried to eliminate the solvents. To stabilize the extracts on the filter and reduce the leaching of metabolites the extract-loaded filter

was covered with 500 µl 1 % low melt agarose (Roth, Germany) at 28 °C. To provide a suitable settlement surface for foulers the embedded extracts were then covered with a polycarbonate filter membrane, (Ø 47 mm, pore size 0.2 µm, track-etch membrane, Sartorius Stedim Biotech, Göttingen, Germany). Before application the membranes were incubated in sterile filtered seawater to age the surface for 10 days. Assembling the bioassays took place in PVC holders described by Nasrolahi et al. (2012) (see Fig. 1). Approx. 9.60 cm^2 of bioassay surface were available for settlement after closing the screw-on lid of the PVC holder.

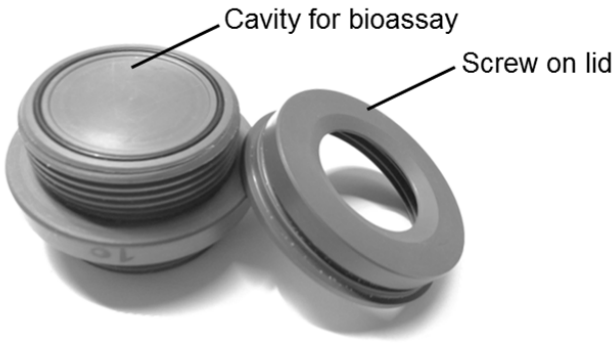

Fig. 1. *In situ* bioassay holder developed by Nasrolahi and co-workers (2012). Extract-loaded and low melt agarose impregnated cellulose filter were placed in the cavity for bioassays and covered with a polycarbonate membrane serving as a suitable settlement surface for foulers. Each bioassay holder represented a single replicate.

The assembled bioassays were stored in a water-saturated atmosphere at 4 °C in the dark for 24 h to allow an equally distribution by diffusion of the impregnated molecules within the cellulose membranes. Bioassays with *F. vesiculosus* and *F. serratus* extracts were successively exposed to the natural fouler pool in 0.5 m deep water at the institute pier, Kiel Fjord for five days during August/September 2013. Due to the time-shifted exposure of *F. vesiculosus* and *F. serratus* bioassays a comparison of the antifouling strength between the two species was not possible. After five days the polycarbonate membranes were removed and fixed in sterile filtered 3.7 % formaldehyde. The settlement by the most important local macrofouling species, *Amphibalanus improvisus* and *Mytilus edulis,* was quantified under a stereomicroscope.

Data analysis

To test for differences in the mean number of settlement events of *A. improvisus* and *M. edulis* on *Fucus* surface extracts between experimental groups a one-way ANOVA was conducted. Homogeneity of variance was tested on the base of a residual plot, while normality of errors was assessed by Shapiro-Wilk tests and by histograms. Significant differences in antifouling activities between *Fucus* surface extracts obtained in different months of the year were identified by Tukey's honest significant difference (HSD) *post hoc* test ($p \leq 0.05$). These comparisons also included the blanks, i.e. bioassays only treated with solvents.

Assuming that a seasonal fluctuating defense should follow a sinus curve, seasonality in antifouling defense strength against *A. improvisus* and *M. edulis* was identified with a sinusoidal model (nonlinear regression model) that included mean data points ($n = 6$) from the *in situ* bioassay results. Sinusoidal models were applied with the curve fitting free software CurveExpert 1.4 (Hyams 2010).

The amount of covariance between (1) *Fucus* mannitol content and the *A. improvisus* and *M. edulis* settlement results of *in situ* bioassays and (2) fouling pressures monitored in the field and *in situ* bioassay results was assessed with Pearson's correlations (in case of normal data) or with Spearman's rank correlations (in case of non-normal data). Normality of data was tested by Shapiro-Wilk tests and with histograms. If data sets were repeatedly used for different correlations we lowered the level of significance by applying the Bonferroni correction to avoid an increase in the type-I error rate (Dunn 1961).

All statistical analyses were performed with the free statistical computing software R (R Development Core Team 2010).

Results

Surface seawater conditions

Recorded environmental parameters and nutrients concentrations followed a seasonal cycle typical for Northern Germany (suppl. Fig. S1a, b and Fig. S2). Minimum surface water temperatures were reached on 26 January (- 0.8 ℃) and maximum mean surface water temperatures were reached on 26 July (23.5 ℃). Average noon photon flux densities increased from March onwards and reached peak irradiances in August (750 µmol photons m^{-2} s^{-1}). Increased surface seawater

temperatures correlated with lower salinities. Low nutrient concentrations were measured during spring and summer (mean concentration from April to September: nitrate + nitrite 0.4 ± 0.2 µmol/l (mean conc. ± SE), ammonium 1.4 ± 0.3 µmol/l (mean conc. ± SE), silicate 13.6 ± 2.4 µmol/l (mean conc. ± SE), phosphate 0.6 ± 0.2 µmol/l (mean conc. ± SE) followed by increasing concentrations during autumn and winter (mean concentration from January to March and from October to December: nitrate + nitrite 5.2 ± 0.8 µmol/l, ammonium 2.0 ± 0.3 µmol/l, silicate 23.4 ± 2.0 µmol/l, phosphate 0.9 ± 0.1 µmol/l).

Seasonal mannitol content of vegetative thallus apices

Mannitol content of vegetative thallus apices in both species showed an increase from February to October, with intermittent decreases in May (*F. serratus*) and July (*F. vesiculosus*). The October maximum was followed by a reduction to half the summer values until December (Fig. 2). Except in July and August, *F. serratus* always showed a lower mannitol content than *F. vesiculosus* with a mean difference of 17 %.

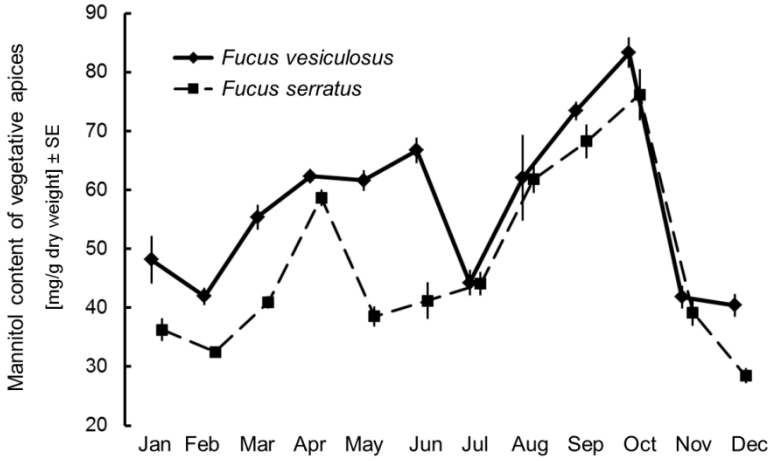

Fig. 2. Seasonal variations in the mean mannitol content of vegetative *Fucus vesiculosus* and *Fucus serratus* thallus apices (*n* = 15 per month). Error bars are ± SE.

Seasonal fouling in the field

On reference substrata (PVC panels), most intense fouling by macrofauna occurred in June and July (4.5 ± 1.0 % and 4.1 ± 1.2 % cover ± SE between replicated PVC panels) (Fig. 3 and Table 1). *Amphibalanus improvisus* and *Mytilus*

edulis were the most common epizoans with a maximum coverage in July (*A. improvisus* 2.2 ± 0. 9 % mean coverage ± SE between replicated PVC panels) and in June and July (*M. edulis* 1.5 ± 0.4 % and 1.9 ± 0.3 % mean coverage ± SE between replicated PVC panels) (suppl. Fig. S3a, b and Table 1). Compared to animal fouling, the epiphytic fouling dominated in general. Epiphyte coverage showed two distinct peaks in March and July (43.7 ± 6.5 % and 31.6 ± 1.9 % mean coverage ± SE between replicated PVC panels) (Fig. 3 and Table 1).

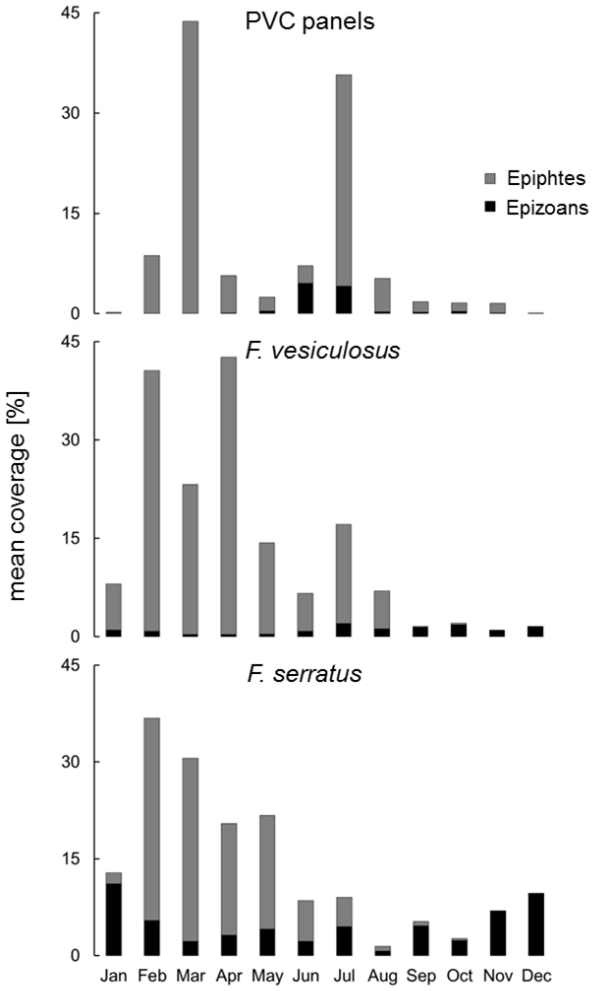

Fig. 3. Seasonal variability in the mean coverage [%] of epiphytes (gray bars) and epizoans (black bars) recorded on PVC, *Fucus vesiculosus and Fucus serratus* at the sampling site (Bülk, outer Kiel Fjord, Germany) over a period of one year (*n* = 15 per month).

Table 1. Mean coverage [%] of epizoans and epiphytes recorded on PVC plates at the sampling site (Bülk, outer Kiel Fjord, Germany) over one year.

Epizoan and epiphyte species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Amphibalanus improvisus (ad.)							0.4 ± 0.2					
Amphibalanus improvisus (juv.)						0.1 ± 0.1	1.8 ± 0.7					
Amphibalanus improvisus (cypris)							+					
Folliculina sp.					0.1 ± 0.1	0.9 ± 0.1	+					
Foraminifera					+	0.1 ± 0.1						
Laomedea sp.						1.9 ± 0.3				+		
Mytilus edulis (ad., < 20 mm)							0.1 ± 0.1					
Mytilus edulis (juv., 1-2 mm)	+			0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	1.8 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.04	
Mytilus edulis (l.)						1.2 ± 0.3						
Callithamnion sp.										0.1 ± 0.1		
Ceramium sp.					0.2 ± 0.1	0.3 ± 0.1			+	+		
Chaetomorpha sp.							0.4 ± 0.1					
Cladophora sp.						+			0.1 ± 0.05	0.1 ± 0.05		
Cyanobacteria		0.1 ± 0.00				0.8 ± 0.2	29.2 ±1.3	+	0.4 ± 0.2	0.1 ± 0.01	0.7 ± 0.1	
Diatoms								4.9 ± 0.9	1.1 ± 0.5	1.0 ± 0.3	0.8 ± 0.2	0.1 ± 0.05
Diatoms (tube dwelling)	0.2 ± 0.04	8.6 ± 0.5		4.9 ± 1.2	0.8 ± 0.1		0.1 ± 0.1					
Fucus sp.					0.9 ± 0.2	0.1 ± 0.1						
Monostroma grevellei				+								
Pilaiella sp.*or Ectocarpus* sp.				+		0.2 ± 0.1						
Polysiphonia sp.					0.1 ± 0.1	1.2 ± 0.1	1.9 ± 0.4					
Red algae germling								+				
Ulothrix sp.			43.7 ±6.5									
Ulva sp.	+			0.7 ± 0.3								
Urospora sp.		+										

15 replicates per month; errors are ± SE; values are rounded; + = values < 0.1; empty field = species not recorded; ad. = adult, juv. = juvenile, l. = larvae.

On *F. vesiculosus* epizoans fouling peaked in July and October (2.2 ± 1.1 % and 1.9 ± 0.8 % mean coverage ± SE between replicated algae individuals, resp.) (Fig. 3 and Table 2). *Amphibalanus improvisus* (early recruits) was found on *F. vesiculosus* in July and August in low numbers (July 0.1 ± 0.1 % and August 0.01 ± 0.01 % mean coverage ± SE between replicated algae individuals) (suppl. Fig. S3a, b and Table 2). *Mytilus edulis* (juvenile life stages) was the most common fouling species on *F. vesiculosus* with a maximum in July and August (1.4 ± 0.4 % and 1.1 ± 0.3 % mean coverage ± SE between replicated algae individuals, resp.) (suppl. Fig. S3a, b, Table 2). Epiphytic fouling on *F. vesiculosus* generally dominated compared to animal fouling with abundance peaks in February and April (39.8 ± 4.1 % and 42.4 ± 8.7 % mean coverage ± SE between replicated algae individuals, resp.) (Fig. 3 and Table 2).

Fucus serratus showed the most intense animal fouling between November and February with a maximum in January (11.1 ± 5.4 % mean coverage ± SE between replicated algae individuals) (Fig. 3 and Table 3). Most common foulers on *F. serratus* were the bryozoan species *Alcyonidium gelatinosum* and *Electra pilosa*. *Amphibalanus improvisus* (early recruits) was only present on *F. serratus* in May (0.1 ± 0.1 % ± SE between replicated algae individuals) while *Mytilus edulis* (juvenile life stages) occurred in lower densities throughout the year (suppl. Fig. S3a, b and Table 3). Epiphytic fouling on *F. serratus* dominated in general compared to epizoic fouling and was highest in spring with coverage peaks in February (31.3 ± 6.7 % ± SE between algae individuals) and in March (28.4 ± 6.8 % ± SE between algae individuals) (Fig. 3 and Table 3).

Table 2. Mean coverage [%] of epizoans and epiphytes recorded on *Fucus vesiculosus* at the sampling site (Bülk, outer Kiel Fjord, Germany) over one year.

Epizoan and epiphyte species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Alcyonidium diaphanum		+										
Alcyonidium gelatinosum						0.1 ± 0.1	0.1 ± 0.1		0.9 ± 0.7	0.1 ± 0.1		
Amphibalanus improvisus (juv.)							0.1 ± 0.1	+				
Aurelia sp. (polyp)							0.1 ± 0.1	0.1 ± 0.1				
Clava multicornis									+	0.1 ± 0.05		
Dynamena pumila										0.1 ± 0.1		
Electra crustalenta							0.1 ± 0.1					
Electra pilosa	0.9 ± 0.4	0.6 ± 0.2	0.2 ± 0.1	0.1 ± 0.1			0.1 ± 0.1		0.1 ± 0.1	1.1 ± 0.3	0.1 ± 0.1	0.6 ± 0.1
Hydrobia sp.									0.1 ± 0.1			
Laomedea sp.						+	0.1 ± 0.1					
Mytilus edulis (ad., < 20 mm)							0.2 ± 0.1		0.3 ± 0.1			0.3 ± 0.2
Mytilus edulis (juv.)	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	1.4 ± 0.4	1.1 ± 0.3	0.1 ± 0.1	0.4 ± 0.1	0.8 ± 0.4	0.6 ± 0.3
Spirorbis sp.										0.1 ± 0.1		
Ceramium sp.								0.1 ± 0.1				
Ceramium teniconis				0.1 ± 0.1			0.2 ± 0.1		+			
Ceramium virgatum					0.3 ± 0.3		0.1 ± 0.1					
Cladophora sp.						0.1 ± 0.1	0.1 ± 0.1	0.6 ± 0.3				
Corda filum				0.1 ± 0.1								
Cyanobacteria	+	0.1 ±0.01		0.1 ±0.01	3.2 ± 0.8		0.3 ± 0.2	0.1 ± 0.1			0.1 ± 0.1	
Diatoms (tube dwelling)	7.0 ± 2.0	39.7 ±4.1	22.3 ±2.6	15.9 ±2.4								
Elachista fuciola			0.5 ± 0.3	0.1 ± 0.1	4.5 ± 1.8	5.0 ± 2.0	13.7 ±3.5	4.0 ± 1.5	0.1 ± 0.1	0.2 ± 0.1		
Fucus sp. (juv.)								1.0 ± 0.4		0.1 ± 0.05		
Fucus sp.(germling)					+	0.1 ± 0.1						
Pilaiella sp. or *Ectocarpus* sp.				26.0 ±5.9	5.8 ± 2.8	0.5 ± 0.4						
Polysiphonia fucoides				0.1 ± 0.1		+	0.5 ± 0.2					
Rhizoclonium riparium							0.1 ± 0.1					
Spongonema tomentosus						0.1 ± 0.1	0.1 ± 0.1					

15 replicates per month; errors are ± SE; values are rounded; + = values < 0.1; empty field = species not recorded; ad. = adult, juv. = juvenile.

Table 3. Mean coverage [%] of epizoans and epiphytes recorded on *Fucus serratus* at the sampling site (Bülk, outer Kiel Fjord, Germany) over one year.

Epizoan and epiphyte species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Alcyonidium diaphanum	3.4 ± 1.9											
Alcyonidium gelatinosum	6.1 ± 2.6	2.8 ± 1.2	1.3 ± 0.5	2.0 ± 0.8	3.4 ± 1.5	1.7 ± 0.9	2.9 ± 1.5	0.1 ± 0.1	3.9 ± 1.2	1.9 ± 0.7	5.8 ± 1.8	8.6 ± 2.3
Alcyonidium hirsutum							0.1 ± 0.1					
Amphibalanus improvisus (juv.)					0.1 ± 0.1							
Clava multicornis		0.9 ± 0.4					0.1 ± 0.05					
Dynamena pumila	0.8 ± 0.7	0.7 ± 0.4		0.7 ± 0.4	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1		0.1 ± 0.1			0.1 ± 0.1
Electra pilosa	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.3 ± 0.1	0.2 ± 0.1		0.3 ± 0.3	+	0.4 ± 0.2	0.4 ± 0.2	1.0 ± 0.3	1.0 ± 0.2
Laomedea sp.						0.1 ± 0.0						
Mytilus edulis (ad., < 20 mm)		0.1 ± 0.1						0.1 ± 0.1	0.2 ± 0.1			
Mytilus edulis (juv.)		0.1 ± 0.1		0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	1.0 ± 0.3	0.4 ± 0.1	+	+	0.1 ± 0.05	
Ceramium teniconis		0.1 ± 0.1					0.1 ± 0.1					
Cyanobacteria	+	+	+	0.1 ± 0.01	0.1 ± 0.1							
Cystoclonium sp.		0.5 ± 0.3							0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	
Diatoms (tube dwelling)	1.7 ± 0.9	30.7 ±6.3	26.7±6.1	7.7 ± 1.3								
Elachista fuciola			1.7 ± 0.7	0.5 ± 0.1	5.7 ± 2.2	6.3 ± 2.2	4.5 ± 1.1	0.8 ± 0.7	0.7 ± 0.3	0.2 ± 0.1		
Fucus sp. (juv.)						+						
Pilaiella sp. or *Ectocarpus* sp.				9.0 ± 1.9	11,7 ±5.0							
Spongonema tomentosus					0.1 ± 0.1							
Ulva sp.				0.1 ± 0.1								

15 replicates per month; errors are ± SE; values are rounded; + = values < 0.1; empty field = species not recorded; ad. = adult, juv. = juvenile.

Predicted antifouling defense strength

The difference between fouling pressure (PVC plates) and detected fouling on *F. vesiculosus* and *F. serratus* predicted strongest antifouling defense against *A. improvisus* for both *Fucus* species during July (Fig. 4a). Strongest antifouling defense against *M. edulis* of both *Fucus* species were predicted for June and July according the seasonal recruitment comparison (Fig. 4b).

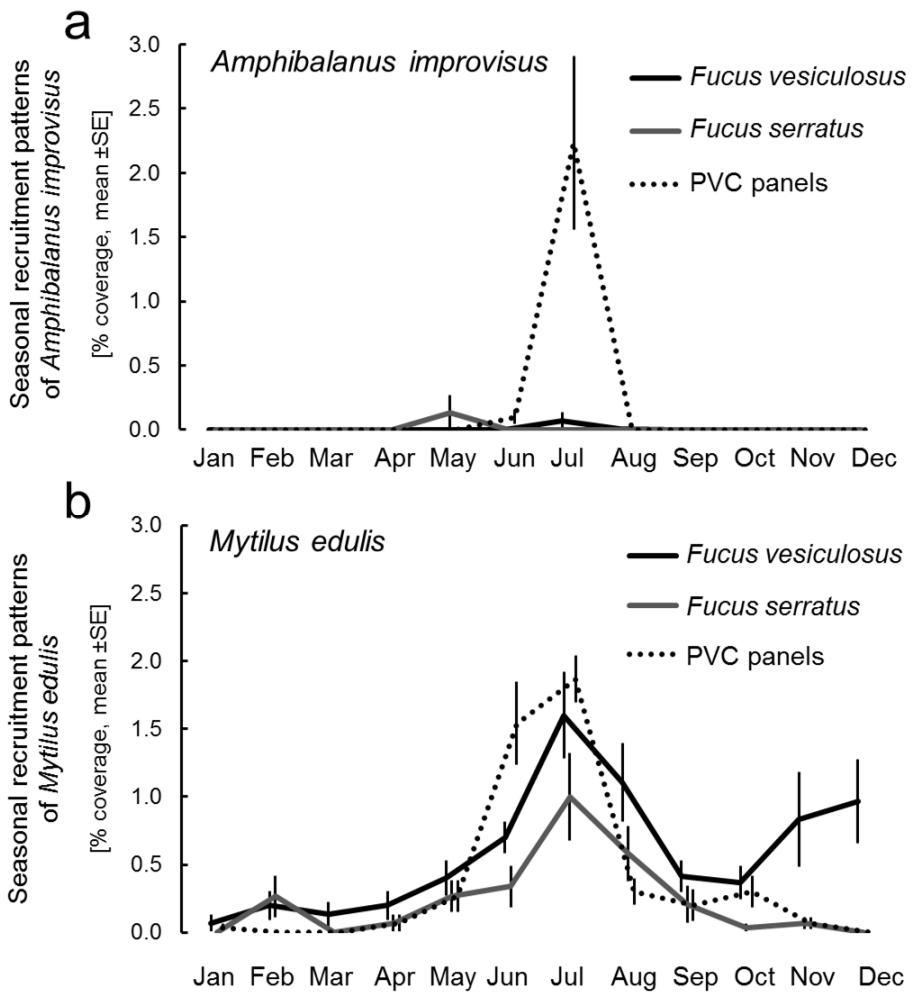

Fig. 4a, b. Seasonal variability in the mean coverage [%] of the epizoan foulers *Amphibalanus improvisus* (a) and *Mytilus edulis* (b) recorded on PVC plates (PVC), *Fucus vesiculosus* (Fv) and *Fucus serratus* (Fs) at the sampling site (Bülk, outer Kiel Fjord, Germany) over the period of one year (*n* = 15 per month). Error bars are ± SE.

Seasonal variation in antifouling defense strength

Surface extracts of *F. vesiculosus* and *F. serratus* showed seasonality in their antifouling activity against *A. improvisus*. Extract activity was highest during the spring and summer months. The seasonal pattern, modeled as a sinusoidal nonlinear regression, explained more than 60 % of the variation in antifouling activities for *F. vesiculosus* and *F. serratus*. (Fig. 5a, b and Table S1).

F. vesiculosus surface extracts reduced settlement of *A. improvisus* relative to the solvent blank in all monthly samples (Fig. 5a), but this effect was not significant on extracts originating from February, March, October and November. Anti-macrofouling defense against *A. improvisus* was strongest in January, May and August (one-way ANOVA, $F = 4.6$, $p < 0.001$, Tukey's HSD test, $p \leq 0.05$). The anti-macrofouling activities of *F. vesiculosus* surface extracts from January, April, May, June, July and August were significantly stronger than activities from November extracts (Tukey's HSD test, $p \leq 0.05$) (Fig. 5a and Table S2).

F. vesiculosus extracts did not significantly repel *M. edulis* larvae at any time of the year (one-way ANOVA, $F = 1.3$, $p = 0.26$). A sinusoidal nonlinear regression explained 61 % of the seasonal variations in the monthly extracts modulation of non-significant *M. edulis* settlement (Fig. 5a).

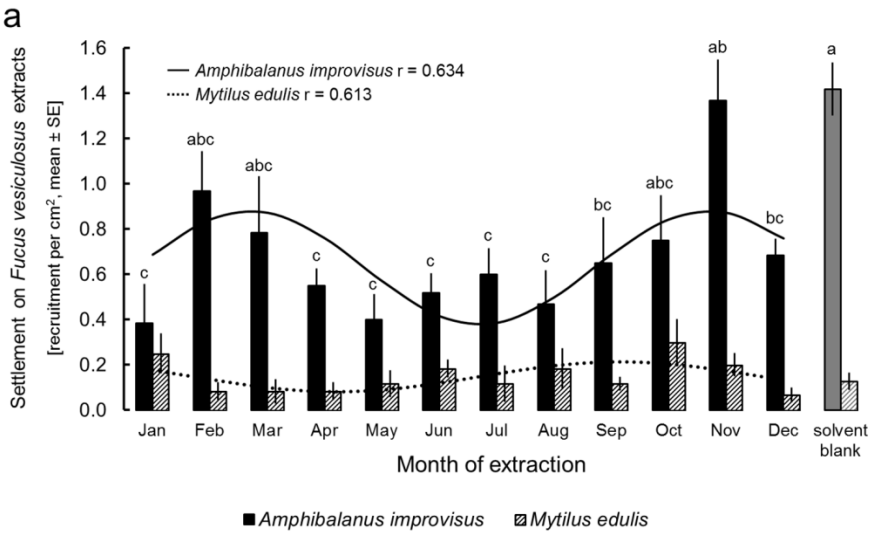

Fig. 5 a. Mean number of settlement events ±SE of *Amphibalanus improvisus* (black bars) and *Mytilus edulis* (striped bars) on *Fucus vesiculosus* surface extracts (twofold mean boundary layer conc.) obtained in different months. Corresponding solvent blanks are grey (*A. improvisus*) and grey striped (*M. edulis*). Results from one-way ANOVA ($n = 6$, in all cases) for *A. improvisus* $F = 4.6$, $p < 0.001$ and for *M. edulis* $F = 1.3$, $p > 0.1$. Significant differences between group means are indicated by letter coding (for *A. improvisus* only), groups with the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$). For *M. edulis* no significant differences between groups were observed. Seasonality in antifouling defense strength of *F. vesiculosus* against *A. improvisus* (solid line) and *M. edulis* (dashed line) is illustrated by fitting a nonlinear regression model to the data.

F. serratus surface extracts did not significantly repel *A. improvisus* in any month of the year (one-way ANOVA, F = 1.0, p = 0.42, Fig. 5b). A sinusoidal nonlinear regression explained 63 % of the seasonal variations in modulated non-significant fouling effects of *F. serratus* surface extract activities against *A. improvisus* (Fig. 5b). The settlement of *M. edulis* onto *F. serratus* surface extracts varied among months of extraction (one-way ANOVA, F = 2.1, p = < 0.05) but were generally not significantly different from the solvent blank. July surface extracts were significantly more attractive than extracts originating from December and October (Tukey's HSD test, $p \leq 0.05$). The seasonal variation was explained to 52 %, by a sinusoidal nonlinear regression, (Fig. 5b and Table S2).

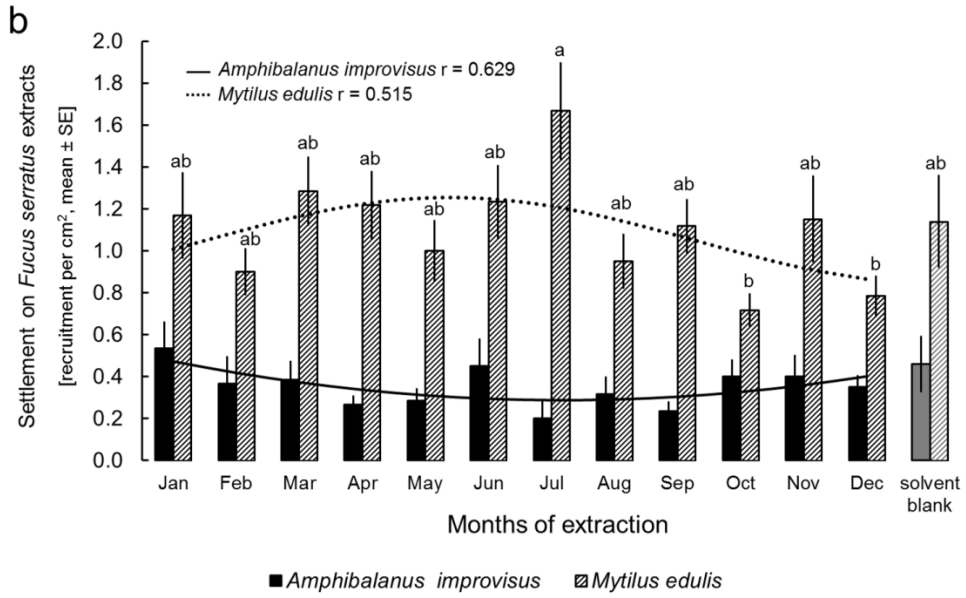

Fig. 5 b. Mean number of settlement events ±SE of *Amphibalanus improvisus* (black bars) and *Mytilus edulis* (striped bars) on *Fucus serratus* surface extracts (twofold mean boundary layer conc.) obtained in different months. Corresponding solvent blanks are grey (*A. improvisus*) and grey striped (*M. edulis*). Results from one-way ANOVA (n = 6, in all cases) for *A. improvisus* F = 1.0, $p > 0.1$ and for *M. edulis* F = 2.1, $p < 0.05$. Significant differences between group means are indicated by letter coding (for *M. edulis* only), groups with the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$). In case of *A. improvisus* no significant differences between groups were observed. Seasonality in antifouling defense strength of *F. serratus* against *A. improvisus* (solid line) and *M. edulis* (dashed line) are illustrated by fitting a nonlinear regression model to the data.

Anti-macrofouling activities in *F. vesiculosus* and *F. serratus* against *A. improvisus* and *M. edulis* were neither correlated with the mannitol content of the algae nor with *in situ* fouling pressures as recorded on PVC plates (Table 4).

Table 4. Summarized results of different correlation tests.

Algal species	Fouling species in bioassay [cm^2]	Potential driver	Cor. coefficient	*p*-value
Fucus vesiculosus	*Amphibalanus improvisus*	Mannitol [mg/g]	r = -0.347	0.269
F. vesiculosus	*Mytilus edulis*	Mannitol [mg/g]	r = 0.409	0.187
Fucus serratus	*A. improvisus*	Mannitol [mg/g]	r = -0.305	0.334
F. serratus	*M. edulis*	Mannitol [mg/g]	r = -0.152	0.637
Fucus vesiculosus	*Amphibalanus improvisus*	*In situ* fouling pressure [% cover]	rho = -0.044	0.893
F. vesiculosus	*Mytilus edulis*	*In situ* fouling pressure [% cover]	rho = 0.085	0.794
Fucus serratus	*A. improvisus*	*In situ* fouling pressure [% cover]	rho = -0.481	0.113
F. serratus	*M.edulis*	*In situ* fouling pressure [% cover]	rho = 0.591	0.043

The table contains correlation coefficients (r = Pearson's and rho = Spearman's cor. coefficient) and *p*-values for each correlation test. $n = 12$, for each correlation. Significance level after Bonferroni correction $p \leq 0.025$.

Discussion

The main purpose of the present study was to test (1) if macrofouling pressure in the field varies seasonally in intensity and composition, (2) whether *F. vesiculosus* and/or *F. serratus* chemically inhibit macrofouling, (3) whether the strength of chemical antifouling defense of *F. vesiculosus* and *F. serratus* is related to prevailing fouling pressure and (4) whether there are hints for a resource dependency of chemical antifouling defense.

This study revealed that environmental parameters as well as macrofouling in the field followed a pronounced seasonal pattern. Furthermore, *in situ* bioassays, loaded with a two-fold mean boundary layer concentration of *Fucus* surface extracts, showed a seasonally fluctuating and species dependent chemical antifouling defense of *F. vesiculosus* and *F. serratus* against the barnacle *A. improvisus* and the mussel *M. edulis*. However, chemical antifouling defense strength (*in situ* bioassays) of both *Fucus* species did neither correlate with the storage compound mannitol, a proxy for stored energy availability (Yamaguchi et al. 1966, Schmitz & Lobban 1976), nor with the general fouling pressure.

Macrofouling

Our field experiment revealed a seasonal pattern of epiphytic and epizoic macrofouling as well as of environmental parameters, as previously reported (Hagermann 1966, Rindi & Guiry 2004, Wahl et al. 2010, Hammann et al. 2013). Autotrophic fouling dominated on all substrates (PVC and *Fucus*) and exhibited a clear seasonal pattern, increasing with irradiation and temperature during spring and summer along with the depletion of surface water nutrients due to increasing primary production. The decrease of epiphytic fouling during autumn and winter is most likely because of cessation of the vegetation period of annual algae. The dominance of the autotrophic relative to the heterotrophic components of fouling is probably attributable to the shallow depth (0.5 - 1.5 m, depending on water level) and ample light supply (in spring and summer). *Fucus serratus* occurs slightly deeper than *F. vesiculosus* (Malm et al. 2001) and hosts less epiphytes and more epizoans than PVC plates and *F. vesiculosus*. Disregarding agile species (with exception of *M. edulis*) may have contributed to the apparent dominance of autotrophic epibionts.

Heterotrophic fouling showed a less pronounced seasonal pattern as compared to phototrophic fouling. Epizoans on PVC plates exhibited the clearest seasonality with a maximum in June and July, when recruitment of many species is generally high. In contrast to the PVC panels, *F. vesiculosus* revealed high animal fouling densities not only in summer, but also in autumn and winter. *Fucus serratus* associated epizoans showed peak densities during winter. These differences between PVC and *Fucus* associated epizoans can be explained by the short exposure time (four weeks at a time) of the PVC substratum. Considering that both *Fucus* species are perennial with a life time of three to five years or even longer, depending on environmental conditions (Rees 1932, Knight & Parke 1950), it is reasonable to assume that perennial foulers which settled during spring and summer would persist over winter producing a seasonal fouling pattern less “pure” than on the panels. Only the fouling data obtained from the PVC plates reflect the actual fouling pressure *Fucus* is exposed to in the field in the course of a given month.

Fouling of Amphibalanus improvisus and Mytilus edulis

The barnacle *A. improvisus* and the mussel *M. edulis* are considered as two common biofouling species (Berntsson & Jonsson 2003, Railkin 2004, Wikstrom & Pavia 2004, Bloecher et al. 2013). While the first species can heavily colonize *Fucus* thalli during peak settlement seasons (June, July) (Rohde et al. 2008, Nasrolahi et al. 2012, Pansch et al. 2012), *M. edulis* can also heavily colonize macroalgae thalli by forming dense mussel layers (80 to 100 % coverage) on the kelp *Saccharina latissima* or on the seagrass *Zostera marina* (own observations).

The field experiment revealed higher densities of *A. improvisus* on PVC plates as compared to *F. vesiculosus* and *F. serratus*, suggesting a settlement-deterrent effect for both *Fucus* species against the barnacle. Our findings corroborate results from Wikstrom & Pavia (2004) and Brock et al. (2007), the latter ones observed in a field study significant higher barnacle densities on rocks compared to adjacent *F. vesiculosus* as well as the avoidance for algal fronds during the peak settlement season. Wikstrom and Pavia (2004) reported higher barnacle recruitment on Perspex panels compared to *F. vesiculosus*. The presumed settlement-deterrent effect of *F. vesiculosus* and *F. serratus* against *A. improvisus* may be due to released algal secondary metabolites (Koivikko et al. 2005).

It should be mentioned that our observations of *A. improvisus* settlement events on PVC reflect a reduced pattern of what Thomsen et al. (2010) described, namely *A. improvisus* settlement from January until October on PVC settlement panels located in the inner Kiel Fjord. The fact that we found *A. improvisus* on PVC just during the main recruitment phase in June and July is possibly due to differences of the experimental sites (inner Kiel Fjord *vs*. outer Kiel Fjord). In our study the experimental site was wave exposed and located in the outer Kiel Fjord while the experimental site of Thomsen and co-workers was a sheltered site in the inner Fjord at the institute's pier. These differences could have led to very different settlement conditions for cyprid larvae. Our results probably reflect successful settlement events during the peak recruitment season when many cyprid larvae tried to settle. A further explanation for the relatively weak and temporally compressed *A. improvisus* settlement could be that our PVC plates were deployed in a *Fucus* bed presumably surrounded by an elevated level of exuded algal secondary metabolites such as phlorotannins (Koivikko et al. 2005), creating a deterrent environment for cyprid larvae. A previous laboratory study by Wikstrom & Pavia

(2004) showed that phlorotannins decreased the settlement of cyprid larvae and Brock et al. (2007) demonstrated that phlorotannins *in situ* can reach concentrations inhibitory for cyprid settlement. Therefore, it seems possible that algal secondary metabolites released from the *Fucus* canopy had led to a reduced cyprid settlement on our PVC plates.

With regard to the second important fouler, *Mytilus edulis*, our field experiment found no significant differences in recruitment on PVC, *F. vesiculosus* and *F. serratus*, suggesting that both *Fucus* species did not repel juvenile and adult *M. edulis*. One explanation could be that *M. edulis* is a mobile fouling species with several attachment and detachment phases during its early life. In fact, young larvae prefer to settle onto filamentous structures, while post-larval *M. edulis* preferentially settle into mussel beds (Bayne 1964, Bayne 1976). Therefore we assume that *M. edulis* is not a real threat for *Fucus*. Interestingly, we found no larvae of *M. edulis* on either *Fucus* species. This observation matches with findings from a former study (Dobretsov 1999), which was explained with the flat thallus of *Fucus*. Other possible explanations could be (1) the settlement preference of *M. edulis* larvae on artificial substrata (Dobretsov & Wahl 2001) and (2) a possible repellent effect through excretion of molecular signals by *F. vesiculosus* and *F. serratus* as well as by their associated biofilm communities (Dobretsov 1999).

Antifouling defense strength

The extraction of alga surface metabolites using the dipping method gives average concentrations over the entire concentration gradient within the boundary layer surrounding the producer surface (Grosser et al. 2012). Grosser and co-workers (2012) showed that inside the diffusion boundary layer, 0 -150 µm above the thallus surface of *Fucus*, the spatial distribution of non-polar algae metabolites forms a strong gradient declining with distance from thallus surface. Consequently, real concentrations as encountered by a settler approaching the thallus surface increase towards the surface dramatically. To at least partially mimic the concentration in the thallus-near part of the boundary layer (where attachment happens) we used a concentration of twice the boundary layer mean.

Our *in situ* bioassays revealed that both *Fucus* species show a seasonal pattern of chemical antifouling defense strength against *A. improvisus.* We had, indeed, hypothesized that the chemical antifouling defense of *F. vesiculosus and F. serratus*

would be induced and regulated by the seasonally fluctuating fouling pressure. Such fine-tuned defense pattern could be demand-driven. Increasing fouling during spring/summer generated by reproduction and larvae recruitment would entail a rising demand of antifouling defense, as postulated by Wahl et al. (2010). In contrast, antifouling defense of *Fucus* against *M. edulis* were weak or non-existent and consequently did not show any seasonal pattern in either *Fucus* species (Fig. 5a, b). As mentioned above, *M. edulis* could be just a transient guest and, thus, not a real threat for *Fucus*, soliciting no demand for defense.

Alternatively or additionally to the hypothesis that antifouling defense in *Fucus* is demand driven seasonal fluctuations in these defenses could also reflect the availability of resources (Coley et al. 1985, Strauss et al. 2002, Wahl et al. 2010). In our study we used the concentration of the storage compound mannitol (Lehvo et al. 2001) as a general proxy for (stored) energy, *inter alia*, for defense metabolite production. The tissue mannitol content of vegetative thallus tips varied considerably between seasons but was never depleted. A correlation between antifouling defense (*in situ* bioassays) and the tissue mannitol content was not found. Seasonal mannitol fluctuations were reported for *Xiphophora gladiate* (Fucales) and for *F. vesiculosus* from the Finland Baltic Sea (Gillanders & Brown 1994, Lehvo et al. 2001). The authors assumed that mannitol may represent a carbon reserve and is used for receptacle formation (Gillanders & Brown 1994) and/or is mobilized for growth during unfavorable light conditions such as winter (Lehvo et al. 2001).

In conclusion, this study has shown that in *F. vesiculosus* and *F. serratus* the chemical antifouling defense against the barnacle *A. improvisus* varies with season and matches the seasonal fluctuations in fouling pressure of this barnacle, i.e. strong defense in phases of strong fouling. Interestingly, a corresponding pattern was not detected with regard to the -transient- fouler *M. edulis*. The observed seasonal fluctuations of anti-barnacle defense do not seem to reflect the availability of resources.

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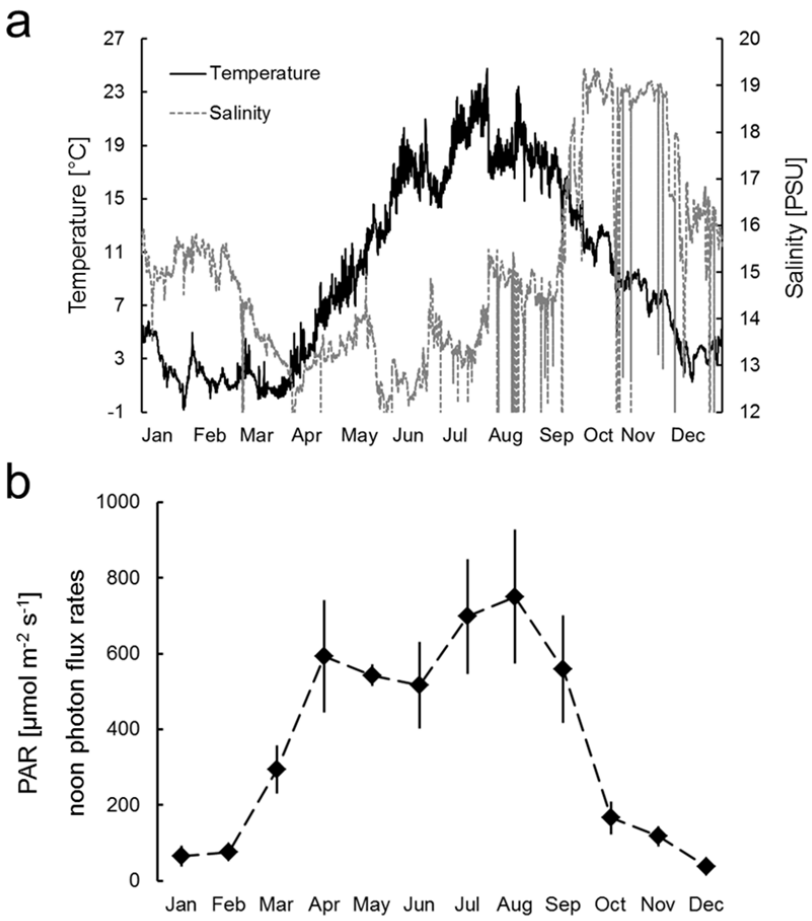
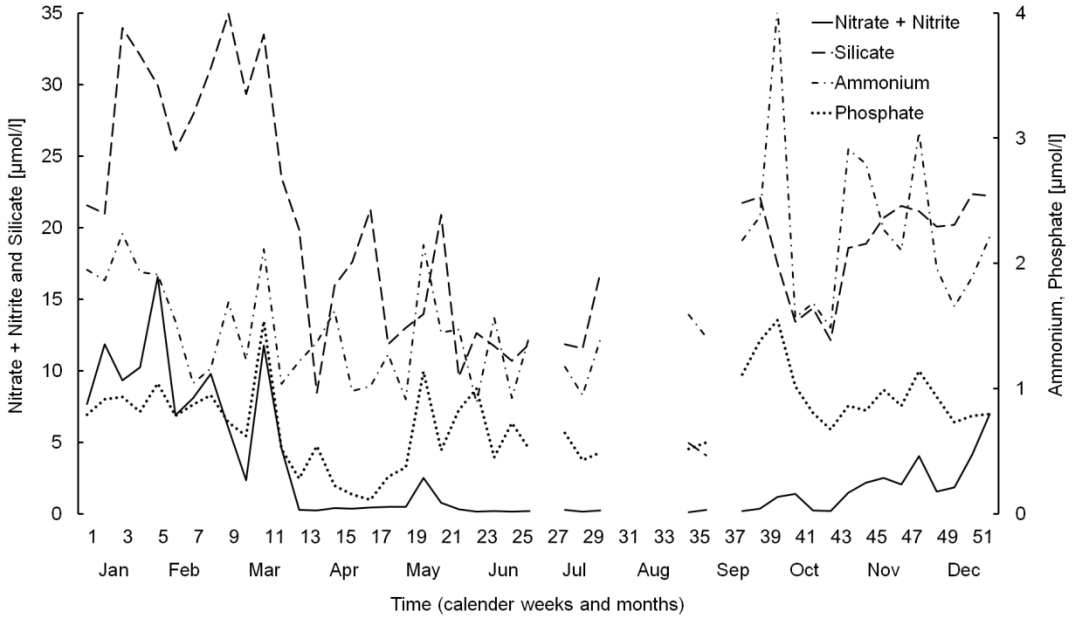
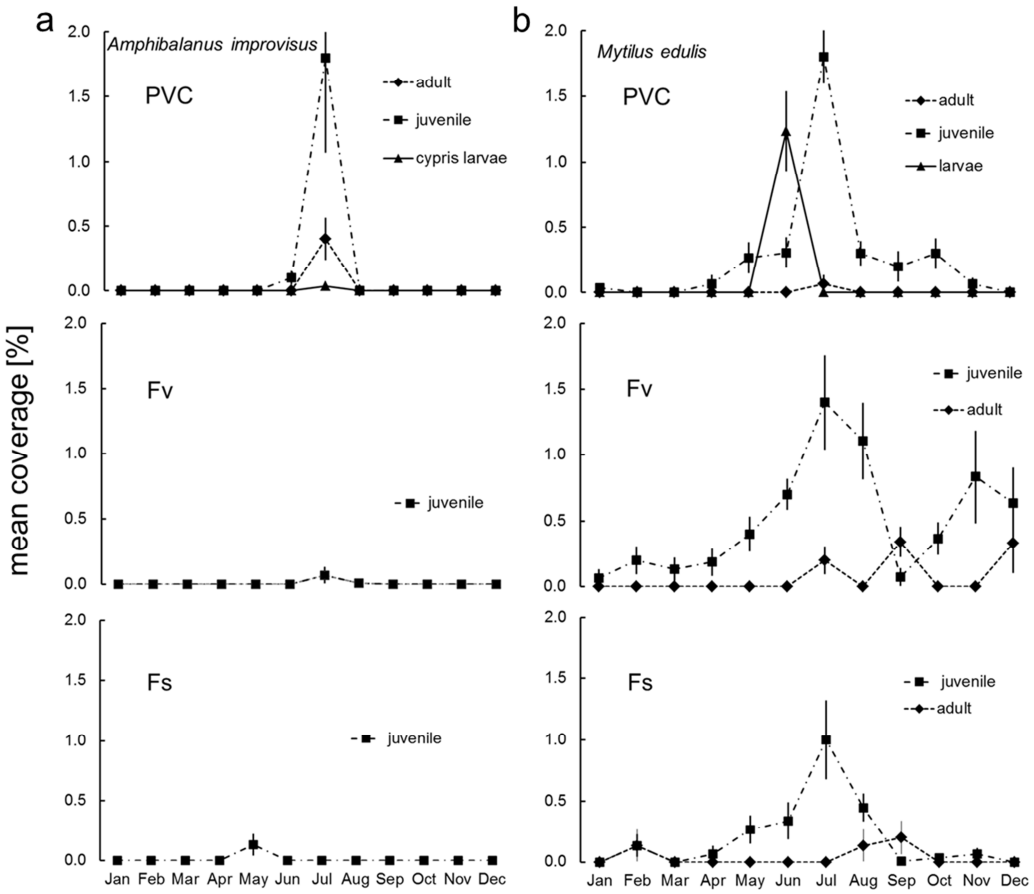
Supplementary Information



Supplementary Fig. S1a, b. Seasonal variation of mean surface seawater temperature, salinity and photosynthetic photon flux rate (PAR) recorded at the sampling site (Bülk, outer Kiel Fjord, Germany) in 0.5 m water depth under mid water level. Temperature, salinity and PAR photon flux rates were continuously measured by loggers (U24-002 conductivity logger and pendant temperature/light logger, HOBO®, Onset Computer Corporation) taking one measurement per hour (n = 3 per month). Error bars are ± SE. "Noon photon flux rates" represent the average dose registered between noon and 1 pm on the first seven days of each month.



Supplementary Fig. S2. Seasonal variations of mean surface seawater nutrient concentrations measured weekly (*n* = 3 per week) at the sampling site (Bülk, outer Kiel Fjord, Germany) in 1 - 1.5 m water depth (depending on water level). Gaps between the lines are due to missing measurements.



Supplementary Fig. S3a, b. Seasonal variability in the mean coverage [%] of the epizoan foulers *Amphibalanus improvisus* (a) and *Mytilus edulis* (b) recorded on PVC plates (PVC), *Fucus vesiculosus* (Fv) and *Fucus serratus* (Fs) at the sampling site (Bülk, outer Kiel Fjord, Germany) over the period of one year (*n* = 15 per month). Error bars are ± SE.

Table S1. Mean settlement events of *Amphibalanus improvisus* and *Mytilus edulis* on *Fucus vesiculosus* and *Fucus serratus* surface extracts (2-fold mean boundary layer conc.) per cm^2 bioassay surface.

Fouling species on extracts	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Blank
Amphibalanus improvisus on Fv extracts	0.4 ±0.2	1.0 ±0.2	0.8 ±0.3	0.6 ±0.1	0.4 ±0.1	0.5 ±0.1	0.6 ±0.1	0.5 ±0.2	0.7 ±0.2	0.8 ±0.2	1.4 ±0.2	0.7 ±0.1	1.4 ±0.1
Mytilus edulis on Fv extracts	0.3 ±0.1	0.1 ±0.0	0.1 ±0.1	0.1 ±0.0	0.1 ±0.1	0.2 ±0.0	0.1 ±0.1	0.2 ±0.1	0.1 ±0.0	0.3 ±0.1	0.2 ±0.1	0.1 ±0.0	0.1 ±0.0
Amphibalanus improvisus on Fs extracts	0.5 ±0.1	0.4 ±0.1	0.4 ±0.1	0.3 ±0.0	0.3 ±0.1	0.5 ±0.1	0.2 ±0.1	0.3 ±0.1	0.2 ±0.0	0.4 ±0.1	0.4 ±0.1	0.4 ±0.1	0.5 ±0.1
Mytilus edulis on Fs extracts	1.2 ±0.2	0.9 ±0.1	1.3 ±0.2	1.2 ±0.2	1.0 ±0.1	1.2 ±0.2	1.7 ±0.2	1.0 ±0.1	1.1 ±0.1	0.7 ±0.1	1.2 ±0.2	0.8 ±0.1	1.1 ±0.2

n = 6, per month; errors are ±SE; values are rounded; Fv = *F. vesiculosus*; Fs = *F. serratus*.

Table S2. Summarized significant results of Tukey's HSD test ($p \leq 0.05$).

Alga species	Fouling species in bioassay	Significant different data sets	P-value
Fucus vesiculosus	*Amphibalanus improvisus*	Nov / Jan	0.003
Fucus vesiculosus	*Amphibalanus improvisus*	Nov / Apr	0.029
Fucus vesiculosus	*Amphibalanus improvisus*	Nov / May	0.004
Fucus vesiculosus	*Amphibalanus improvisus*	Nov / Jun	0.019
Fucus vesiculosus	*Amphibalanus improvisus*	Nov / Jul	0.053
Fucus vesiculosus	*Amphibalanus improvisus*	Nov / Aug	0.009
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Sep	0.035
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Jan	0.001
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Apr	0.009
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / May	0.001
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Jun	0.006
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Jul	0.019
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Aug	0.003
Fucus vesiculosus	*Amphibalanus improvisus*	solvent blank / Dec	0.053
Fucus serratus	*Mytilus edulis*	Dec / Jul	0.025
Fucus serratus	*Mytilus edulis*	Oct / Jul	0.011

Paper III

Submitted to PLOS ONE

Seasonal variations in surface metabolite composition of *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea

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Abstract

The perennial macroalgae *Fucus* is known to exude metabolites through its outer thallus surface some of which have pro- and antifouling properties. Seasonal fluctuations of natural fouling pressure and of *Fucus* chemical fouling control strength regarding micro- and macrofoulers have been observed suggesting that control strength varies with threat. So far, a study on the seasonal composition of surface associated metabolites, responsible for much of the fouling control, is missing. In a one-year field survey, we sampled monthly the two co-occurring species *F. vesiculosus* and *F. serratus* individuals (six per species and month). We analysed by means of gas chromatography-mass spectrometry (GC-MS) the chemical composition of surface associated metabolites of both *Fucus* species to describe temporal patterns in chemical surface composition. Subsequently we search for correlation between the up- and downregulation of metabolites and prokaryotic fouling control strength. Additionally, we correlated monthly recorded abiotic parameters from the sampling site with the variation in the chemical surface landscape of *Fucus.* Our study revealed that the chemical surface composition of both *Fucus* species exhibit substantial seasonal differences between spring/summer and autumn/winter months. Light and temperature explained most of the seasonal variability in surface metabolite composition of both *Fucus* species. A strong summerly upregulation of saccharides and hydroxy acids in *F. vesiculosus* and of fatty acids and two saccharides in *F. serratus* was observed. It is conceivable that these upregulated molecules have an antagonistic effect on associated microfoulers caused by the profouling effect of sugars and the antifouling effect of organic acids.

Keywords: *Fucus*, macroalgae surface metabolites, carbohydrates, fatty acids, hydroxy acids, chemical pro- and antifouling control, seasonal fluctuation patterns

Introduction

Macroalgae surfaces function as an interface with the aquatic environments. All essential physiological processes like light absorption, gas exchange, nutrients uptake or the disposal of metabolic products happen via this outer interface (Wahl et al. 2012). Being most active interfaces macroalgal thallus surfaces are often enriched with photosynthesis products like oxygen and carbohydrates (Abdullah & Fredriksen 2004, Goecke et al. 2010, Haas & Wild 2010). At the same time macroalgae surfaces are exposed to a diverse and seasonally variable prokaryotic fouling pressure and are typically colonized by up to 10^7 - 10^8 bacteria cells per cm^2 of algal surface, depending on the algal species (Bengtsson et al. 2010, Saha & Wahl 2013, Stratil et al. 2013). Uncontrolled microbial fouling would entail a reduction of incoming light (Wahl et al. 2010) as well as reduced gas and nutrient exchange resulting in lower photosynthesis efficiency (as described for epiphytes on seagrass in Sand-Jensen 1977, Wahl 1989). Further, uncontrolled bacterial pathogens can cause algal diseases (Egan et al. 2014). Thus macroalgae fitness and, ultimately, survival should depend on an efficient fouling control admitting the beneficial and repelling the detrimental bacteria while keeping the overall abundance of epibacteria at a tolerable level. It is therefore to be expected that the evolutionary pressure for macroalgae to evolve mechanisms against uncontrolled bacterial colonization is high. Several studies have demonstrated that macroalgae developed chemical defense mechanisms against potential bacterial foulers by means of exuded metabolites to prevent or regulate bacterial attachment, growth and hence the density of associated bacteria (Nylund et al. 2005, Dworjanyn et al. 2006, Saha et al. 2011, Saha et al. 2012). Furthermore, it has been shown that different macroalgae metabolites can have pro- and antifouling effects with the power to shape the composition of the bacterial community composition (Persson et al. 2011, Sneed & Pohnert 2011a, Sneed & Pohnert 2011b, Lachnit et al. 2013).

Since macroalgae are photosynthetic organisms their metabolism strongly depends on environmental parameters like light and temperature but also on the availability of nutrients (Davison 1991, Raikar et al. 2001, Nygard & Dring 2008, Nejrup et al. 2013). It has been shown for some brown algal species that the tissue content as well as exudation rates of polyphenols and carbohydrates varies in response to environmental parameters (Sieburth 1969, Ragan & Jensen 1978, Pavia & Toth 2000, Abdullah & Fredriksen 2004). Additionally, it has been reported for different macroalgae species that the chemical defense strength or even specific antifouling metabolites against bacteria exhibit seasonal variations, showing a general up-regulation during summer months when metabolic rates and fouling pressure are high (Amade & Lemee 1998, Culioli et al. 2002, Hellio et al. 2004, Marechal et al. 2004). As fouling pressure as well as resource availability varies during the year especially in temperate regions, it could be expected that macroalgae in such a fluctuating environment exhibit also synchronized anti-bacterial defence strength. A simultaneous assessment of the temporal patterns of fouling pressure, fouling control strength and the chemical landscape at the thallus surface through all seasons has not been undertaken before.

The present study focused on the perennial brown macroalgae *F. vesiculosus* and *F. serratus* from the temperate Baltic Sea. Previous studies showed that *F. vesiculosus* chemically control bacterial attachment via exuded metabolites (Wahl et al. 2010, Saha et al. 2011, Saha et al. 2012, Lachnit et al. 2013). The metabolites fucoxanthin, dimethylsulphopropionate (DMSP) and proline were found to be present at the immediate vicinity of *F. vesiculosus* surfaces with the capacity to inhibit bacterial attachment and to modulate the bacterial community composition (Saha et al. 2011, Grosser et al. 2012, Saha et al. 2012, Lachnit et al. 2013). Additionally, recent studies revealed that *F. vesiculosus* surface extracts also exhibit profouling effects on bacterial attachment (Lachnit et al. 2013, Letschert 2014, Rickert et al. submitted). Furthermore, several studies with focus on the seasonality of the bacterial antifouling defense strength of *F. vesiculosus* revealed seasonal fluctuating antifouling activities along with *in situ* bacterial fouling pressure intensities (Wahl et al. 2010, Saha & Wahl 2013, Rickert et al. submitted).

However, to date only little is known about the seasonal composition of *Fucus* surface metabolites and how environmental parameters like light, temperature, nutrients and prokaryotic fouling pressure influences the metabolic response of

Fucus. In-depth knowledge regarding the chemical composition of *Fucus* surface metabolites and their seasonal patterns is essential to gain a better understanding of the chemical fouling control of *Fucus*.

The aim of the present study was to investigate the seasonal variation in surface metabolite composition of *F. vesiculosus* and *F. serratus* and how the metabolite composition relates to the seasonal variations in the environmental factors light, temperature, nutrients and fouling pressure. The following questions structured the project: (1) Are there significant differences in the surface chemistry composition of *Fucus* between different seasons? (2) Which metabolites contribute most to the seasonal differences in surface chemistry? (3) Which abiotic parameters correlate significantly with the metabolite composition of *Fucus*?

Material and Methods

Algae material

The two perennial brown macroalgae *Fucus vesiculosus* Linnaeus (1753) *and Fucus serratus* Linnaeus (1753) were monthly sampled over an entire year (August 2012 - July 2013) at Bülk, outer Kiel Fjord, Germany (54°27'21 N / 10°11'57 E). Per month six non-fertile algal individuals per species were collected at depths of 0.5 m . Transportation to the laboratory took place in 3 l plastic bags and a cooler box to avoid desiccation and temperature stress.

Environmental parameters and fouling pressure

At the algae sampling site data loggers (HOBO UA-002-64, Onset Computer Corporation, Bourne, Massachusetts, USA) were deployed at 0.5 m depth and temperature and light were recorded hourly From the same depths weekly water samples were analysed for nitrogen (nitrate + nitrite, ammonium) and phosphate concentrations. For detailed method descriptions see Rickert et al. (2015).

To assess the relative seasonal variation in prokaryotic fouling pressure at the sampling site horizontal oriented microscope slides (n = 9) were exposed for seven days each month. After retrieval, slides were fixed in 3.7 % formaldehyde solution at 4 °C overnight followed by rinsing with sterile filtered 1x PBS solution and storage in

a PBS-ethanol solution (1:1 v/v of 1x PBS and 96% ethanol) at -20 ℃ until further sample preparation. Approx. 1 cm^2 of the microscopy slides was stained with 10 µl of a ready-to-use DAPI (4'.6-diamidino-2-phenylindole) containing mounting medium (Roti®-Mount FluorCare DAPI, Roth, Karlsruhe, Germany) and covered with a cover glass. For prokaryotic cell enumeration five randomly selected visual fields per replicate were photographed (epifluorescence microscope: Axio Scope.A1, Carl Zeiss Microscopy GmbH, Göttingen, Germany; camera: ProgRes® CF, Jenoptik, Jena, Germany). Photos were manually analysed by counting all prokaryotic cells in 20 randomly selected squares (each 50 $µm^2$). For detailed method descriptions see Rickert et al. (2015).

Surface extraction

Six *Fucus* individuals per species and month were surface-extracted. Per algae individual approx. 50 g (approx. 1500 cm^2) of the upper 5-10 cm apical thalli tips, devoid of macrofoulers, were cut off. The surface extraction of *Fucus* was performed according to the protocol of de Nys et al. (1998) with minor modifications. Before extraction, thalli tips were spin dried in a salad spinner for 30 s to remove excess seawater from the algae material. Extraction time was set to 4 s, to minimize the risk of epidermis damage and extraction of internal metabolites (for details see Rickert et al. 2015). For extraction 3-6 thallus tips (depending on size and branching) were dipped into 100 ml of a constantly stirred n-hexane and methanol (1:1 v/v) emulsion for 4 s. Careful attention was paid to ensure that the cut surface had no contact with the solvents to avoid any leaching of internal metabolites. Surface extractions were performed within 3 to 4 hours after algae sampling. Extracts were filtered with a paper filter (MN 615 ¼, Ø 150 mm, Macherey-Nagel, Düren, Germany) to remove particles and reduced at 35 ℃ under vacuum with a rotation evaporator. Reduced extracts were redissolved respectively with 2 ml n-hexane and methanol. Under constant nitrogen flow extracts were dried at 35℃ and stored until GC-MS sample preparation at -20 ℃.

Solvent blanks (n = 4) for GC-MS analysis were prepared by performing the whole extraction procedure without algae material.

GC-MS sample preparation and analysis

For GC-MS dry *Fucus* extracts were redissolved first with 2x 800 µl of heptane (≥ 99.9 % GC grade, Sigma-Aldrich Chemie Gmbh, Munich, Germany) followed by 1 min of vortexing and transfer to a new vial. Remaining undissolved extracts were treated with 2x 800 µl of methanol (≥ 99.9 % GC grade, Sigma-Aldrich Chemie Gmbh, Munich, Germany) and 1 min of vortexing to complete the resolving process. Respectively 40 µl of heptane and methanol solved extracts were mixed and 2 µl of ribitol internal standard solution (0.4 mmol in water, Sigma-Aldrich, Germany) were added followed by evaporation to dryness under vacuum for ~ 3 h.

Sample derivatisation was performed according to the protocol by Vidoudez & Pohnert (2012). For derivatisation 50 µl of a fresh prepared methoxymation solution (20 mg methoxyamine hydrochloride, Sigma-Aldrich Chemie Gmbh, Munich, Germany, dissolved in 1 ml of pyridine) were added to the sample followed by 1 min of vortexing. Prepared samples were first incubated at 60 °C for 1 h followed by a second incubation step at room temperature for 9 h. Silylation solution was fresh prepared by adding with a glass syringe 40 µl of retention time index mix (Sigma-Aldrich Chemie Gmbh, Munich, Germany) into a new vial of *N*-methyl-*N*-trifluoroacetamide (MSTFA, 1 ml aliquots, Macherey-Nagel, Düren, Germany). Of this silylation solution 50 µl were added with a glass syring to the sample and incubated at 40 °C for 1 h. Solvent blank samples were prepared for GC-MS analysis in the same way like extract samples. After incubation samples were transferred into vials with glass inserts and analysis with a GCT Premier TOF mass spectrometer (Waters / Micromass, Manchester, UK).

Immediate GC-MS analysis and further data processing were performed as described by Vidoudez & Pohnert (2012). The DB-5ms column had a length of 30 m attached to a 5.7 m pre-column, source temperature was set to 250°C, and the split to 4. The oven temperature was hold for 3 min at 75°C, increased with 12°C/min to 315°C and hold at that temperature for 7min. Mass spectra were obtained with 10scans/sec.

GC-MS data processing

Chromatogram deconvolution was performed using AMDIS 2.71 with a smoothing window of 5 scans and peak integration using MET-IDEA 2.08 with a lower mass limit of 50.

Each GC-MS extract measured data were corrected to the internal standard ribitol with dividing each extracts readings by the respectively ribitol measured value. Further ribitol-corrected measured data were corrected by the measured data of the solvent blanks. For blank correction each measured data was subtracted by the mean (n = 4) of solvent blanks. After ribitol and solvent blank correction all negative values were converted to zero.

Identification of metabolites

Peaks were tentatively identified with the spectral library NIST 2011.

Statistical Analysis

All multivariate analyses were performed using the software package Plymouth Routines in Multivariate Ecological Research (PRIMER) version 6 and PERMANOVA+ add-on (Clarke & Gorley 2006, Anderson et al. 2008).

In order to test for significant differences in the metabolite composition of *Fucus* surface extracts originating from different seasons an analysis of similarity (ANOSIM) was performed and a metric multi-dimensional scaling (MDS) plot was generated to visualize the resulting similarity/dissimilarity patterns. These analyses were based on square root transformed GC-MS data (masses). On the basis of these data the related resemblance matrix (Bray-Curtis similarity) was calculated for all samples (in all cases n = 6 per month; exceptions in the *F. vesiculosus* data set: May and July n = 5 and August n = 4). Global-R statistic was used to test for significant differences between groups (factor season). Classification of the factor 'season' was performed according to the meteorological seasons for the northern hemisphere (Dec., Jan., Feb. = winter; Mar., Apr., May = spring; Jun., Jul., Aug. = summer; Sep., Oct., Nov. = autumn). R-values range from 0 to 1, where high values indicating a high variety among seasons. R-values of > 0.25 show that the patterns are not random.

To assess the relationship between the variation of *Fucus* surface chemistry and the environmental variables (temperature, light, nutrients and prokaryotic fouling pressure) a distance-based linear model (DistLM) was performed. With this procedure it was first tested if there are significant correlations between the

multivariate *Fucus* surface chemistry and each of the environmental variables (marginal tests). In the next steps the DistLM procedure runs through all variable combinations to identify which set best explains the patterns in the *Fucus* surface chemistry data (sequential tests).

Prior running DistLM, data sets were prepared as followed: the data resemblance matrix containing the square root transformed *Fucus* surface chemistry data (GC-MS data) were converted to a centroid resemblance matrix (based on Bray-Curtis similarities). The environmental variable data were normalized and selected as predictor variables. The conversion of the *Fucus* chemistry data into a centroid resemblance matrix was necessary to match the chemistry matrix with the environmental variable matrix, both matrices had a similar sample size (n = 12, month). The following DistLM settings were used: stepwise selection, adjusted R^2 criterion and 9999 permutations.

To analyse which masses or rather molecules are most strongly up- or down regulated in winter and summer surface extracts a Simper routine (similarity percentage analysis) was performed by comparing the winter and summer GC-MS measured values (masses) based on square root transformed values. From the Simper result table all masses cumulative contributing to 75 % of the observed differences were selected. For further analysis first from each mass, the log of the ratio between the GC-MS masses in summer and winter extracts was calculated. Second, the detected masses were ranked according to their log ratio values with a cut off at 0.7 corresponding to a five-fold increase in summer relative to winter (see ration summer/winter, Table 5 and 6).

Results

Seasonal variability of Fucus surface chemistry

The chemical composition of *Fucus vesiculosus* surface extracts differed significant among seasons (ANOSIM global test: global R = 0.342, p = 0.0001). The composition of *F. vesiculosus* surface extracts sampled in winter differed significantly from surface extracts sampled in spring (ANOSIM pairwise tests:

winter/spring R statistic = 0.399, p = 0.0001) and summer (ANOSIM pairwise tests: winter/summer R statistic = 0.72, p = 0.0001). Summer extracts differed significantly from autumn extracts (ANOSIM pairwise tests: summer/autumn R statistic = 0.346, p = 0.0001) (Table 1 and Figure 1).

Table 1. Pairwise test results (ANOSIM) for *Fucus vesiculosus* chemical composition of surface extracts.

Groups	R statistic	*p*-value	Significance level %
Winter, Spring	**0.399**	**0.0001**	**0.01**
Winter, Summer	**0.72**	**0.0001**	**0.01**
Winter, Autumn	0.239	0.0006	0.06
Spring, Summer	0.161	0.004	0.4
Spring, Autumn	0.231	0.0002	0.02
Summer, Autumn	**0.346**	**0.0001**	**0.01**

R-values > 0.25 indicating statistical significant discrimination among groups (highlighted in bold).

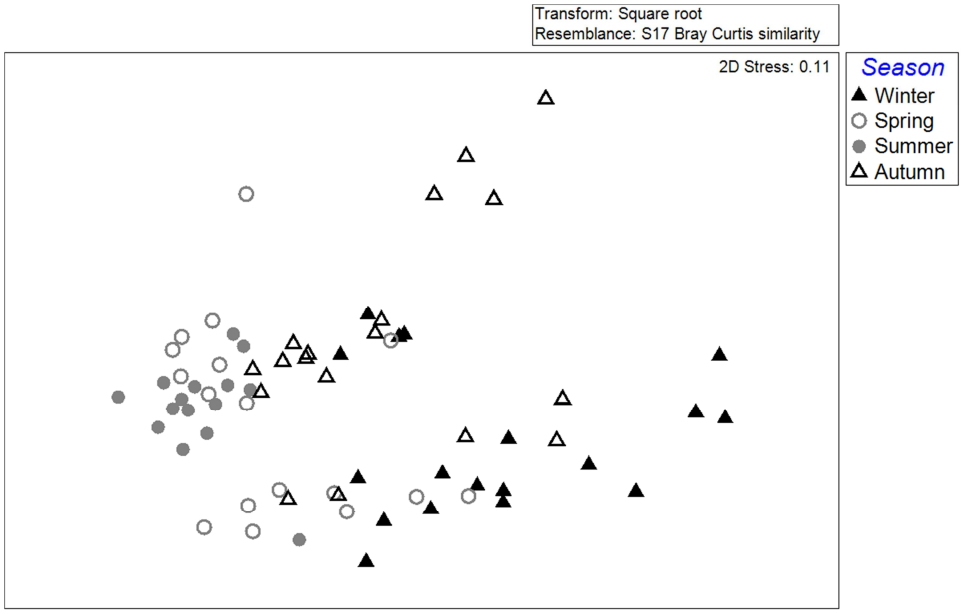

Fig. 1. MDS (multi-dimensional scaling) plot of the variance in *Fucus vesiculosus* surface extract composition originating from different seasons. Symbols representing single monthly samples of *F. vesiculosus* individuals within the four seasons (n = 6 per month; exceptions: May (spring) and July (summer) n = 5, August (summer) n = 4).

The chemical composition of *Fucus serratus* surface extracts differed significantly among seasons (ANOSIM global test: global R = 0.293, p = 0.0001). The composition of winter extracts differed significantly from that of spring extracts (ANOSIM pairwise tests: winter/spring R statistic = 0.472, p = 0.0001) and summer extracts (ANOSIM pairwise tests: winter/summer R statistic = 0.338, p = 0.0001). Spring extracts differed significantly from autumn surface extracts (ANOSIM pairwise tests: spring/autumn R statistic = 0.425, p = 0.0001) (Table 2 and Figure 2).

These statistical differences are represented well in the MDS representation (Fig. 1 and 2).

Table 2. Pairwise test results (ANOSIM) for *Fucus serratus* chemical composition of surface extracts.

Groups	*R statistic*	*p-value*	*Significance level %*
Winter, Spring	**0.472**	**0.0001**	**0.01**
Winter, Summer	**0.338**	**0.0001**	**0.01**
Winter, Autumn	0.129	0.007	0.7
Spring, Summer	0.198	0.0006	0.06
Spring, Autumn	**0.425**	**0.0001**	**0.01**
Summer, Autumn	0.208	0.0007	0.07

R-values > 0.25 indicating statistical significant discrimination among groups (highlighted in bold).

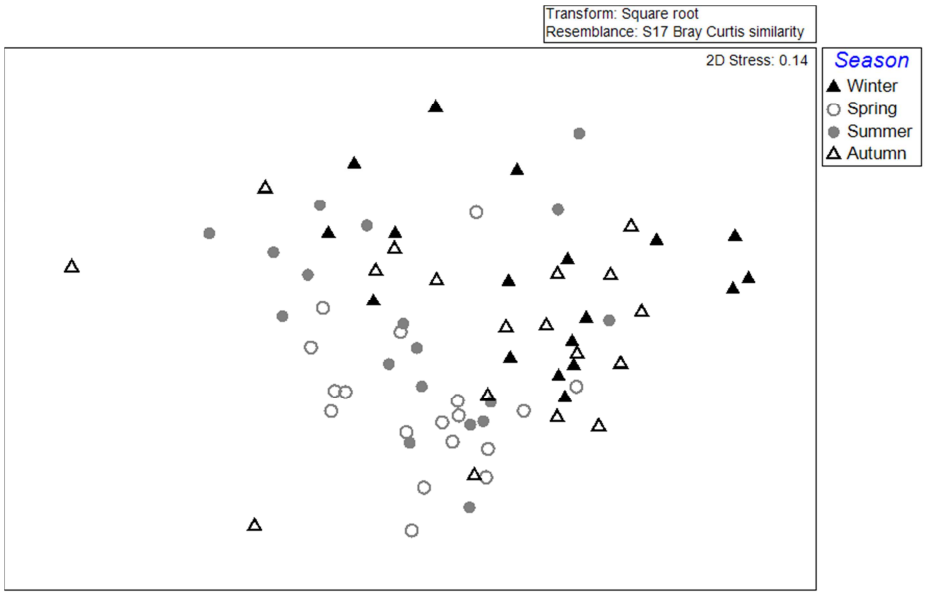

Fig. 2. MDS (multi-dimensional scaling) plot of the variance in *Fucus serratus* surface extract composition originating from different seasons. Symbols representing single monthly *F. serratus* individuals within the four seasons (in all cases n = 6 per month).

Relationship between surface chemistry composition and environmental variables

The distance-based linear model (DistLM) analysis detected significant correlations between the surface chemistry composition of *Fucus* and the environmental variables (Table 3 and Table 4).

For *Fucus vesiculosus*, the sequential tests of the distance-based linear model revealed that the combination of light and temperature has the highest explanatory power, together explaining 56.7 % of the variance of *F. vesiculosus* surface chemistry (Table 3).

Table 3. Results of distance-based linear model (DistLM). Relationship between *Fucus vesiculosus* surface chemistry composition and the predictor variables (temperature, light, phosphate, prokaryotic fouling pressure). Model output contains only the best fitting variables.

Variable	*Adj. R^2*	*SS(trac)*	*Pseudo-F*	*P*	*Prop.*	*Cumul*	*res.df*
+ Light	**0.4475**	1607.5	9.9115	**0.0005**	0.4977	0.4977	10
+Temperature	0.4708	223.81	1.4408	0.2397	6.9306E-2	0.5670	9

The distance-based redundancy (dbRDA) plot illustrates the separation of the surface chemistry samples along the first and second axis correlating with the most important variable light on the first axis and with the variable temperature on the second axis. The variation on the first axis mainly separates spring and summer extract samples from autumn and winter samples (Figure 3). Light correlates with the first axis which explains 49.8 % of the variation in chemical composition. Temperature correlates with the second axis which explains 6.9 % of the variation in chemical composition (Figure 3).

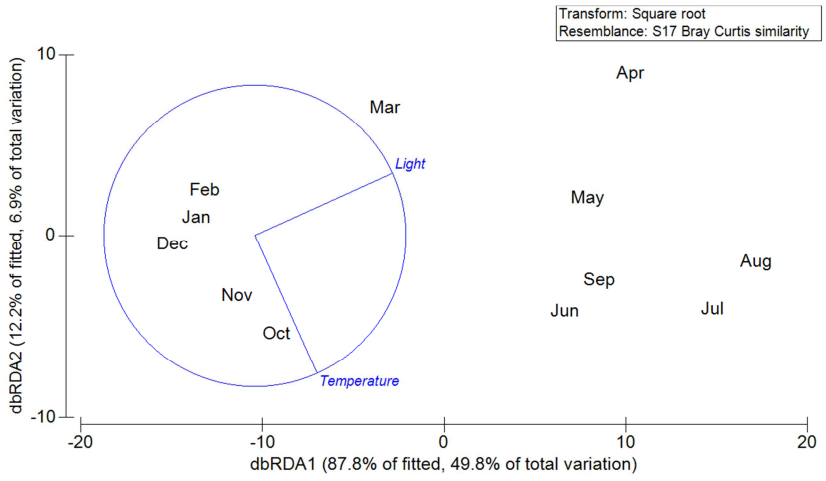

Fig. 3. dbRDA plot (distance-based redundancy analysis) of the distLM model based on the two predictor variables (temperature and light) fitted to the variance in *Fucus vesiculosus* surface chemistry composition.

For *Fucus serratus*, the sequential tests of the distance-based linear model exhibited that the combination of all four environmental variables (light, temperature, phosphate and fouling) has the highest relevance, together explaining 61.01 % of the variance of *F. serratus* surface chemistry (Table 4).

Table 4. Results of distance-based linear model (DistLM). Relationship between *Fucus serratus* surface chemistry composition and the predictor variables (temperature, light, phosphate, prokaryotic fouling pressure). Model output contains only the best fitting variables.

Variable	*Adj. R^2*	*SS(trace)*	*Pseudo-F*	*P*	*Prop.*	*Cumul.*	*res.df*
+ Light	**0.2167**	770.99	4.0435	**0.0097**	0.2879	0.2879	10
+ Temperature	**0.3244**	426.66	2.5944	**0.0419**	0.1593	0.4472	9
+ Phosphate	0.3582	230.25	1.4738	0.2207	8.5987E-2	0.53325	8
+ Fouling	0.3874	205.96	1.3811	0.2378	7.6915E-2	0.61016	7

The dbRDA ordination plot shows that the two most important variables light and phosphate correlate with the first axis which explains 33.8 % to the variation in chemical composition. Along the first axis light and phosphate are negatively correlated to each other resulting in a distinct grouping of mainly winter and autumn extract samples from summer and spring samples (Fig. 4). Temperature and prokaryotic fouling correlate with the second axis which explains 14.9 % to the variation in chemical composition (Fig. 4).

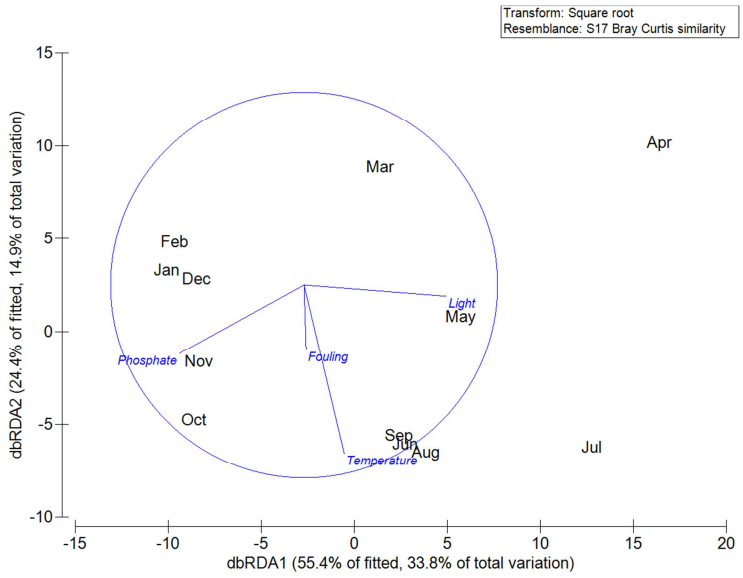

Fig. 4. dbRDA plot (distance-based redundancy analysis) of the distLM model based on the four predictor variables (temperature, light, phosphor and fouling) fitted to the variance in *Fucus serratus* surface chemistry composition.

Regulation of surface associated metabolites

Comparison of *F. vesiculosus* winter and summer surface extract composition revealed that two main signal groups (retention time 13-14 and 20-23 min, resp.) dominated by carbohydrates exhibited a pronounced up-regulation in summer extracts. Mono- and disaccharides were the prevalent up-regulated molecules in summer surface extracts. Furthermore, three different hydroxy acids were found up-regulated: citric, hydroxypropanoic and maleic acid. Citric acid was found to be only present in summer surface extracts, whereas hydroxypropanoic acid and maleic acid were present during both seasons with a 7.7 and 5-fold up-regulation, respectively, in summer extracts compared to winter extracts (Table 5).

Comparing *F. serratus* winter and summer surface extract composition showed that also two main signal groups (retention time 13-17 and 20-28 min) dominated by saturated fatty acids were up-regulated in summer surface extracts. Hexadecanoic acid (or palmitic acid) and octadecanoic acid (or stearic acid) were only present in summer surface extracts. Pentadecanoic and docosanoic acids were present in both seasons with a 4.5 and 5-fold up-regulation, respectively, in summer extracts compared to winter extracts (Table 6). Further, two carbohydrate molecules were found to be up-regulated in summer extracts compared to winter extracts, whereby the detected disaccharide was 18-fold up-regulated comparing winter and summer extracts (Table 6).

Table 5. Regulated metabolites in summer and winter surface extracts of *F. vesiculosus* from simper analysis. Metabolites are ranked by regulation strength (log ratio).

Metabolite or class	GC-MS mass	Rt (*min*)	Winter (*av. abund.*)	Summer (*av. abund.*)	Ratio *(summer/winter)*	Log ratio *(summer/winter)*	Contrib. (%)
Citric acid	273.1	14.29	< 0.0001*	0.24	2400	**3.38**	0.81
Monosacch.	117.1	13.60	0.19	< 0.0001*	0.0005	**-3.28**	0.63
Disacch.	205.1	21.90	< 0.0001*	0.14	1400	**3.15**	0.46
unknown	97.1	23.54	< 0.0001*	0.11	1100	**3.04**	0.36
Disacch.	204.1	21.89	0.01	0.23	23	**1.36**	0.74
Disacch.	117.0	20.43	0.01	0.22	22	**1.34**	0.69
Monosach.	319.2	14.95	0.01	0.16	16	**1.20**	0.49
Disacch.	217.1	21.88	0.01	0.15	15	**1.18**	0.48
Monosacch.	245.1	14.31	0.01	0.12	12	**1.08**	0.37
Disacch.	75.0	20.89	0.01	0.11	11	**1.04**	0.35
Disacch.	103.1	21.89	0.01	0.11	11	**1.04**	0.34
Monosacch.	205.1	14.95	0.02	0.17	8.50	**0.93**	0.51
Disacch.	204.1	21.13	0.02	0.17	8.50	**0.93**	0.48
Hydroxy-propanoic acid	117.1	5.90	0.03	0.23	7.67	**0.88**	0.75
Disacch.	273.0	20.44	0.02	0.15	7.50	**0.88**	0.45
Monosacch.	205.1	13.03	0.05	0.36	7.20	**0.86**	1.04
Disacch.	363.2	20.51	0.07	0.44	6.29	**0.80**	1.21
Disacch.	361.2	20.45	0.19	1.16	6.11	**0.79**	3.19
Disacch.	217.1	20.46	0.14	0.85	6.07	**0.78**	2.37
Disacch.	361.2	21.13	0.06	0.35	5.83	**0.77**	0.98
Monosacch.	103.1	14.74	0.03	0.17	5.67	**0.75**	0.46
Disacch.	231.1	20.49	0.03	0.16	5.33	**0.73**	0.46
Maleic acid**	131.1	7.57	0.04	0.2	5.00	**0.70**	0.54

GC-MS mass = gas chromatography–mass spectrometry mass output; Rt = retention time; av. abund. = average abundance derived from the relative peak area; Contrib. %= contribution in % to the dissimilarity between winter and summer group; < 0.0001* = original value was 0, transformed to calculate the ratio and log ratio; Monosacch. = Monosaccharide; Disacch. = Disaccharide; Maleic acid** = determined only very vaguely.

Table 6. Regulated metabolites in winter and summer surface extracts of *F. serratus* from simper analysis. Metabolites are ranked by regulation strength (log ratio).

Metabolite or class	Mass	Rt (min)	Winter (av. abund.)	Summer (av. abund.)	Ratio *(Summer/Winter)*	Log ratio *(Summer/Winter)*	Contrib. (%)
Hexadecanoic acid / FA	129.0	16.15	< 0.0001*	0.09	900.0	**2.95**	0.48
Octadecanoic acid / FA	341.2	17.66	< 0.0001*	0.06	600.0	**2.78**	0.35
unknown	204.1	28.41	< 0.0001*	0.05	5.0	**2.70**	0.28
Disacch.	204.1	21.86	0.02	0.36	18.0	**1.26**	2.06
unkown	149.0	19.69	0.01	0.13	13.0	**1.11**	0.74
Pentadecanoic acid / FA	299.2	15.05	0.02	0.1	5.0	**0.7**	0.48
Docosanoic acid /FA	129.0	20.36	0.02	0.09	4.5	**0.65**	0.50
Sugar derivate/ Sacch.	263.1	13.03	0.02	0.09	4.5	**0.65**	0.42

Mass = gas chromatography–mass spectrometry mass output; Rt = retention time; av. abund. = average abundance derived from the relative peak area; Contrib. %= contribution in % to the dissimilarity between winter and summer group; < 0.0001* = original value was 0, transformed to calculate the ratio and log ratio; FA = Fatty acid; Disacch. = Disaccharide; Sacch. = Saccharide.

Discussion

The perennial macroalgae *Fucus vesiculosus and F, serratus* are known to exhibit a seasonal variable chemical control of micro- and macrofoulers with a tendency of stronger fouling control strength during seasons of high fouling pressure (Wahl et al. 2010, Saha & Wahl 2013, Rickert et al. 2015). Therefore it seems reasonable to assume that the chemical metabolite composition at the interface, which approaching foulers are first confronted with, is also not static but rather seasonal variable. To investigate this issue, the main focus of the present study was on the seasonal composition of *F. vesiculosus* and *F. serratus* associated surface metabolites.

Our study revealed that both *Fucus* species exhibited significant differences in surface chemistry composition between the seasons. Striking differences in surface metabolite composition were found between the two season group's summer/spring and winter/autumn. For both *Fucus* species light was identified as the environmental variable with the highest explanatory power regarding the seasonal variance of the

surface metabolite composition. Additionally, in summer surface extracts compared to winter extracts for *F. vesiculosus* a pronounced up-regulation of mono- and disaccharides and hydroxy acids and for *F. serratus* up-regulated saccharides and fatty acids were found.

Seasonal variation and the relationship to environmental variables

Light was the most important variable contributing to the seasonal variance in surface metabolite composition, but temperature also contributed to the variance. Phosphate -as nutrient proxy- and prokaryotic fouling pressure had less explanatory power (DistLM analysis, sequential test).

The strong relationship between light and surface metabolite composition is not surprising considering the fact that *Fucus* is a photosynthetic organism. Increasing light intensities -up to light saturation- during spring and summer months in macroalgae leads to higher photosynthetic rates (King & Schramm 1976, Brinkhuis 1977, Raven & Hurd 2012) and consequently to elevated levels of photosynthates (Chapman & Craigie 1978, Pavia & Toth 2000, Lehvo et al. 2001). Former studies observed that the phenolic compound phlorotannin in the brown alga *Cystoseira tamariscifolia* (Abdala-Diaz et al. 2006) and the antifouling metabolite caulerpenyne from *Caulerpa taxifolia* (Amade & Lemee 1998) exhibit annual cycles regulated by solar radiation, showing higher compound concentrations in moths with greatest irradiance. Such light-dependent metabolite production in macroalgae and their partial exudation in *Fucus* (actively or passively by leaking) through its outer thallus surface as described for phlorotannins (Koivikko et al. 2005, Brock et al. 2007), the pigment fucoxanthin (Saha et al. 2011, Grosser et al. 2012) or dissolved organic carbon (Sieburth 1969) could be the drivers for the observed seasonal variance in surface metabolite composition significantly correlated to light. Our results of up-regulated saccharides in summer surface extracts is in accordance with the findings from Sieburth (1969) who found that *F. vesiculosus* exude as much as 30 % of photosynthetically fixed carbon.

Beside light, temperature was the second most important variable contributing to the seasonal variation in surface metabolite composition. *F. serratus* surface metabolite composition was significantly influenced by temperature, whereby *F. vesiculosus* surface metabolites showed a less strong and non-significant relationship with temperature. The found relation could be indirect since

photosynthesis is also controlled by temperature (Davison 1991). Since temperature influence the activities of several key enzymes of the carbon metabolism such as the ribulose-1.5-bisphosphate carboxylase oxygenase (RuBisCO) (Davison & Davison 1987, Sukenik et al. 1987) as well as physical processes like diffusion, carbon fixation and thus indirectly photosynthetic rates are strongly and positively influenced by temperature (Davison 1991) as long as temperature does not reach stressful levels. Typically photosynthetic performance increase with increasing temperature up to an temperature maximum (Davison 1991, Terrados & Ros 1992, Masini et al. 1995, Colvard et al. 2014). In this context it should be mentioned that *F. vesiculosus* from the Baltic Sea, a temperate sea with a pronounced temperature range between summer and winter months (Hammann et al. 2013, Rickert et al. 2015), exhibit typically higher photosynthetic rates at low temperatures and a decreasing maximal ETR (electron transport rates) beyond approx. 25 ℃ (Nygard & Dring 2008, Graiff et al. 2015). Thus rising temperatures during spring and summer may have a double effect by accelerating metabolism, including photosynthesis, and by facilitating the diffusive transport of nutrients and CO_2 through the viscous boundary layer to the thallus surface. The consequence apparently is higher release of metabolites like organic acids or carbohydrates into the diffusive boundary layer on the thallus surface (from where we extracted them).

The differences between *F. vesiculosus* and *F. serratus* regarding the explanatory power of light and temperature for the seasonal surface metabolite variation could be attributable to the slightly shallower habitat of *F. vesiculosus.* This depth difference between should affect irradiation more than temperature or nutrients. In this context it should be mentioned that *F. vesiculosus* is more frequent exposed to air during low tides due to wind conditions. Such air exposure can lead to desiccation stress along with photoinhibition, but generally *F. vesiculosus* recovers rapidly when exposed to seawater (Kawamitsu et al. 2000).

Nutrient availability or, rather phosphate as proxy showed no significant influence on the chemical surface composition of both *Fucus* species. This lack of strong relationship between nutrient availability and the surface metabolite composition is surprising considering the fact that nutrients are known to modify the metabolism of plants (Longstreth & Nobel 1980). Previous studies on macroalgae have shown that a sufficient mineral nutrient supply leads to a more effective metabolism with higher photosynthetic capacities and elevated growth or biomass (Lapointe 1987,

Menendez et al. 2002, Nygard & Dring 2008). Especially dissolved nitrogen is known to favour photosynthesis since nitrogen is essential for protein synthesis, many key carbon assimilatory enzymes like ribulose-1.5 bisphosphate carboxylase oxygenase (rubisco) as well as chlorophyll (Menendez et al. 2002) and hence photosynthetic rates are dependent on nitrogen availability (Wheeler & Weidner 1983). For *F. vesiculosus* it has been demonstrated that elevated nutrient concentrations (NH_4, NO_3, PO_4) enhance the photosynthetic efficiency (Nygard & Dring 2008) and that accumulated tissue nitrogen could be the primary factor for the concentration of phenolic compounds in *F. vesiculosus* (Ilvessalo & Tuomi 1989). The lack of significant relationship between nutrient availability and surface metabolite composition in the present study may be attributable to the fact that many macroalgae including *F. vesiculosus* have the ability to use internal nitrogen reserves for metabolic performance like growth during seasons of nitrogen deficiency (Mizuta et al. 1992, Lehvo et al. 2001, Fong et al. 2004). Therefore it seems reasonable that during our survey the metabolism of both *Fucus* species was probably not nitrogen or nutrient-limited. This could be explained by the fact that *F. vesiculosus* has the ability to store nitrogen and to utilize stored nitrogen during conditions were inorganic nitrogen in seawater is depleted (Lehvo et al. 2001). Additionally, our findings regarding light and nutrients show similarities with the results from Pavia & Toth (2000). The authors reported that nitrogen availability has low explanatory power regarding the variation in tissue phlorotannin content of *F. vesiculosus*, whereby light exhibited greater importance in predicting the phlorotannin variability.

The seasonal variation on prokaryotic fouling pressure neither related to the surface metabolite variability in both *Fucus* species. This result is surprising and in partial contradiction with regard to results of several previous studies (Hellio et al. 2004, Maréchal et al. 2004, Saha et al. 2011, Rickert et al. submitted) showing that different macroalgae including *F. vesiculosus* exhibit a chemical antifouling defense tuned to microbial fouling pressure. Referring to these former findings and considering that the outer thallus surface represents the alga interface for all interactions with the environment (Wahl et al. 2012) and that in many macroalgae defense metabolites are often concentrated in the outer meristoderm layers (Tugwell & Branch 1989) or in specialized cells located on the thallus surface (Nylund et al. 2009) suggest a response relationship between the *in situ* microbial pressure and

the composition of surface associated metabolites. One possible explanation for this discrepancy could be that surface associated anti-microfouling compounds of *F. vesiculosus* has been found to be present in very small concentrations, within the lower nanogram to microgram-range (e.g. proline 0.09 - 0.59 ng cm^{-2}; dimethylsulphopropionate (DMSP) 0.12 - 1.08 ng cm^{-2}; fucoxanthin 0.7 - 9 µg cm^{-2}) (Saha et al. 2011, Saha et al. 2012). Therefore, it seems reasonable that these fine chemical signals become lost in the remaining mixture of surface associated metabolites and thus cannot be related to the seasonal variability in *Fucus* metabolite composition.

Furthermore, in this context it should be mentioned that *Fucus* and macroalgae in general do not exist in an axenic state in nature, but rather in a holobiont-like system tightly associated with a diverse community comprising mainly prokaryotes, fungi and diatoms (Lachnit et al. 2011, Wahl et al. 2012, Egan et al. 2013). Consequently, the analysed *Fucus* surface extracts obtained by the dipping extraction technique represents the surface metabolome of *Fucus* and its associated micro-epibionts.

Regulation of surface associated metabolites

F. vesiculosus summer and winter surface extract analysis revealed an up-regulation of mono- and disaccharides, citric acid, hydroxypropanoic acid as well as maleic acid in summer extracts compared to winter surface extracts.

Our findings of up-regulated mono- and disaccharides match with previous results which showed that many macroalgae, including fucoids exude large amounts of photosynthates (up to 30 % of total fixed carbon) as dissolved organic carbon (DOC) mainly consisting of carbohydrates like the monosaccharide glucose (Sieburth 1969, Pregnall 1983, Carlson & Carlson 1984, Haas & Wild 2010, Wyatt et al. 2014). Sieburth (1969) showed that the exudation of organic matter in *F. vesiculosus* is directly coupled to photosynthesis, increasing with solar radiation. Additionally, it has been shown that the DOC release by many different macroalgae species (from kelp to green algae) exhibit also a seasonal variation correlated to light availability and temperature as well as synchronized with growth and photosynthetic rates (Abdullah & Fredriksen 2004, Wada et al. 2007, Haas & Wild 2010). This presumption is also supported by our findings that light followed by temperature has the strongest exploratory power regarding the seasonal variability of *Fucus* surface metabolite composition (as discussed earlier). Since mono- and

disaccharides, especially the monosaccharide glucose functions as ubiquitous energy source from bacteria to human, the observed up-regulation of mono- and disaccharides on *Fucus* surface should entail a profouling effect on the generally elevated microbial foulers pool during summer months (Saha & Wahl 2013).

Beside saccharides, the hydroxy acids: citric, hydroxypropanoic and maleic acid were found up-regulated in *F. vesiculosus* summer surface extracts compared to winter extracts.

Citric acid or citrate, the conjugated base of citric acid, is in all aerobe organisms the first intermediate product of the citric acid cycle, the oxidative breakdown of organic molecules for energy generation and provision of intermediate products for biosynthesis. Therefore it seems reasonable to assume that the pronounced up-regulation of citric acid could be connected to higher metabolic turn overs of *Fucus* during summer months. Hydroxypropanoic acids have been found in most brown and red algae as well as in low concentrations in green algae (De Rosa et al. 2001, Kamenarska et al. 2002, Kamenarska et al. 2004). To our knowledge maleic acids have not been reported so far from macroalgae. Since from all three detected hydroxy acids antimicrobial activities has been reported, mainly from surveys with a medicinal or food technology background, (Daly 1982, Ferrer-Luque et al. 2010, Sebastianes et al. 2012, In et al. 2013) as well as an enhanced antimicrobial effect by mixing citric and maleic acids (Ferrer-Luque et al. 2010) it is conceivable that these organic acids could function as antibacterial agents on the thallus surface reducing and regulating microbial densities. An antifouling effect obviously depends on the *in situ* surface concentrations of the respective acids and on the individually sensitivity of the respective bacterial strains.

F. serratus summer surface extracts analysis showed an up-regulation of two saccharides as well as of different fatty acids (FA). The dominant presence of FA among up-regulated metabolites in summer extracts is not exceptional, since marine macroalgae are rich in FA (Fleurence et al. 1994, Khotimchenko 1998, Khotimchenko et al. 2002), with hexadecanoic acid or palmitic acid the most common saturated fatty acid in many macroalgae, with 21-42 % of all fatty acid (Nelson et al. 2002). Many FA have antimicrobial effects (Kabara et al. 1972, Ouattara et al. 1997, McGaw et al. 2002). Palmitic acid, for instance, has antibacterial activity against different bacterial strains including mycobacteria (Yff et al. 2002, Seidel & Taylor 2004). The up-regulation of saccharides in *F. serratus*

surface extracts is in accordance with the findings from *F. vesiculosus* and can be similarly interpreted (see previous paragraph).

Concluding, our findings of up-regulated summer surface metabolites in *F. vesiculosus* and *F. serratus* indicates that primary metabolites with potential pro- and antifouling properties are present on both *Fucus* surfaces, originating from *Fucus* itself and, presumably, from its associated biofilm community. It is reasonably possible that these metabolites exhibit an antagonistic interaction regulating the composition of associated microbial surface community of *Fucus* which is different from other co-occurring living and dead surfaces and varies among seasons (Lachnit et al. 2011). *Fucus*-specific biofilms have in their turn the capacity to affect further fouling (Nasrolahi et al. 2012).

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3. General Discussion

The aim of the present thesis was to investigate the putative seasonal variability in fouling control of *Fucus vesiculosus* and *Fucus serratus* from the Baltic Sea under *in situ* conditions and how the fouling control relates to the natural seasonal variations in abiotic and biotic factors.

I have demonstrated that both *Fucus* species exhibit a seasonal fluctuating fouling control against prokaryotes and diatoms as well as against the barnacle *Amphibalanus improvisus*. Further, I could show that the seasonal fluctuating fouling control roughly matched the seasonal fluctuations in fouling pressure of these species (Paper I and II). The strength of prokaryotic fouling control of *Fucus* could not be correlated with the energy status of *Fucus,* with the sole exception of *F. serratus'* prokaryotic fouling control strength (Paper I and II). Furthermore, I found that *F. vesiculosus'* surface extracts in general attracted prokaryotes, whereas a reduced profouling effect was detected during summer months (Paper I). Surface metabolite analysis showed an up-regulation of primary metabolites with profouling and potential antifouling properties during summer (Paper III). Seasonality of surface metabolite composition was best explained by the abiotic factors light and temperature (Paper III). Finally, I could show that both *Fucus* species assist chemical microfouling control via cuticula shedding (Paper I).

Main study questions (see Thesis outline) and received answers.

1.1 Does the chemical fouling control strength of Fucus *vary with season?*	Yes. (Paper I and II)
1.2 Does the surface metabolite composition of Fucus *vary with season and which metabolites are up- or down-regulated during seasons of high or low fouling pressure?*	Yes, spring/summer and autumn/winter surface metabolite composition varied significantly. During summer, season of high fouling pressure, carbohydrates and organic acids were upregulated metabolites. (Paper III)
1.3 Do seasonal fluctuations of Fucus *fouling control correlate with (a) fouling pressure and / or (b) the energy status of* Fucus*?*	(*a*) Yes, there is a trend. (Paper I and II) (*b*) No, with the sole exception of *F. serratus* prokaryotic fouling control strength. (Paper I)
1.4 Does the surface metabolite composition of Fucus *correlate with abiotic and biotic variables?*	Yes, seasonal variability in surface chemistry composition was best explained by light and temperature. (Paper III)

3.1 Seasonal fluctuations of the fouling control system of *Fucus*

My thesis revealed that both *Fucus* species exhibited a seasonally fluctuating chemical fouling control on prokaryotic and diatom cells as well as on the barnacle *Amphibalanus improvisus* and the mussel *Mytilus edulis* under in situ conditions for the first time, with the sole exception of diatom cells on *Fucus vesiculosus* surface extracts (Paper I and Paper II). In general, both *Fucus* species possessed the highest micro- and macrofouling control strength from spring to late summer months (Paper I and II). This finding is in accordance with previous studies which demonstrated seasonal variations in the chemical micro- and macrofouling control of different temperate macroalgae species, including *F. vesiculosus* under laboratory conditions (Hellio et al. 2004, Marechal et al. 2004, Stirk et al. 2007, Wahl et al. 2010, Saha & Wahl 2013).

Besides demonstrating the previously known chemical fouling control, my study revealed that both *Fucus* species also have a mechanical fouling control in the form of cuticula peeling, which removes surface-associated microfoulers (see Paper I). The tissue removal occurred in both *Fucus* species periodically throughout all seasons. Such mechanical fouling control by means of tissue sloughing has been previously reported from many different macroalgae species (Filion-Myklebust & Norton 1981, Sieburth & Tootle 1981, Russell & Veltkamp 1984, Nylund et al. 2005, Harder 2008, Yamamoto et al. 2013). To my knowledge, this thesis is the first study to demonstrate that *F. vesiculosus* and *F. serratus* control fouling on their apical thallus regions by cuticula sloughing. Since this phenomenon was not quantified, further investigations are needed to understand the contribution and ecological relevance of this mechanism with regard to the fouling control of *Fucus*.

3.2 Possible causes for seasonal fluctuations of the fouling control system of *Fucus*

A differentiation between proximate and ultimate causes for an observed phenomenon is often made in biological/ecological studies. The **proximate causes** or ‘effective causes’ refer to the mechanisms or immediate relations of a biological

phenomenon (*how?*), whereas the higher-level **ultimate causes** refer to the biological function or fundamental relations of a biological circumstance (*why?* or *what for?*).

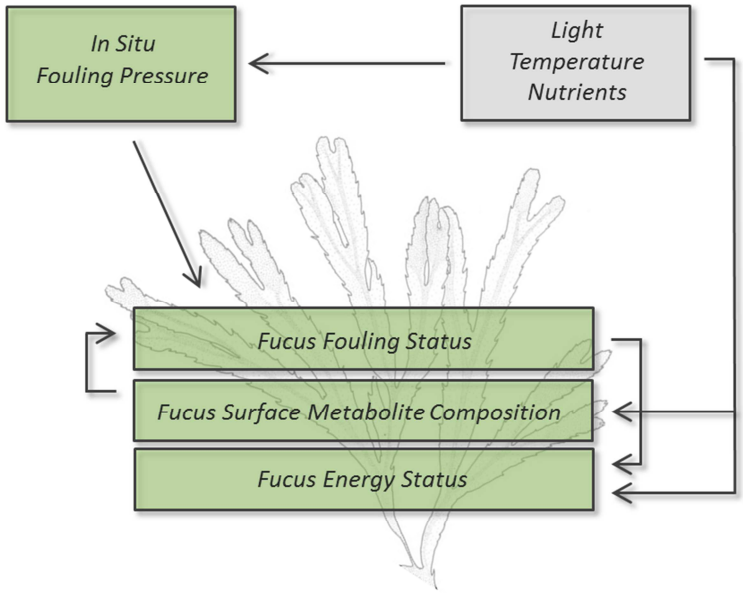

Figure 5. Conceptual model of different abiotic (grey) and biotic (green) factors influencing the fouling control system of *Fucus***.** The proximate causes include the abiotic factors (light, temperature and nutrient concentrations), the surface metabolite composition of *Fucus* as well as the fouling status of *Fucus*, whereas ultimate causes include the energy status of *Fucus* and the in situ fouling pressure. *Alga* drawing from Per Arvid Åsen (1980) slightly modified.

3.2.1 Proximate causes for seasonal fluctuations of the fouling control system of *Fucus*

Previous studies showed seasonally variable levels of bioactive fouling control metabolites for different species of macroalgae (see introduction, Amade & Lemee 1998, Abdala-Diaz et al. 2006). However, previous studies did not investigate the underlying chemical landscape of the seasonally fluctuating fouling control for *Fucus.* The analysis of the surface metabolite composition of *Fucus*, covering all seasons, was therefore of crucial importance for the further understanding of the observed seasonal chemical fouling control and possible causes. It should be mentioned here that each surface extract sample used for *in situ* bioassays (Paper I and Paper II) and for GC-MS analysis (Paper III) represented a quantitatively halved sample. This procedure allows a direct comparison between the detected fouling control strength by means of *in situ* bioassays (Paper I and Paper II) and the surface

metabolite composition analysed via gas chromatography / mass spectrometry analysis (GC-MS) (Paper III).

GC-MS analysis of the surface metabolite composition of both *Fucus* species revealed a pronounced seasonal variability with significant differences between autumn/winter and spring/summer months (Paper III). Moreover, the comparison of winter and summer surface metabolites revealed that mono- and disaccharides as well as organic acids were up-regulated during summer months on both *Fucus* species (Paper III). This finding is in agreement with former studies, which showed that macroalgae exude (actively or passively by leaching) photosynthetic metabolites, e.g. low molecular weight monosaccharides (Sieburth 1969, Haas & Wild 2010, Wyatt et al. 2014). The presence of carbohydrates on *Fucus* surfaces could explain the observed profouling effect on prokaryotic cells on *F. vesiculosus* extracts, whereas the trend of a lower attractiveness despite up-regulated carbohydrates during summer months could indicate a fouling control. This fouling reduction could possibly originate from low concentrated deterring compounds as reported by Saha and co-workers (2011, 2012) (this aspect was briefly discussed in Paper III). Such a combination of attracting and repelling effects has been demonstrated in former studies reporting that macroalgae, including *F. vesiculosus,* exhibit an attracting or stimulating effects on prokaryotes besides their repelling features (Seshadri & Sieburth 1975, Goecke et al. 2010, Lachnit et al. 2013). Further, the observed profouling effect of *F. vesiculosus* surface extracts on prokaryotes match with the finding that *F. vesiculosus* exhibited higher prokaryotic fouling compared to *F. serratus* (see Paper I) or to the kelp species *Laminaria hyperborean* (Bengtsson et al. 2010) in the field throughout the year (Paper I). The reason why only *F. vesiculosus* surface extracts exhibited a profouling effect on prokaryotic settlement may be explained by the fact that, compared to *F. serratus* summer surface extracts, *F. vesiculosus* summer extracts showed a higher up-regulation of primary metabolites during summer months (Paper III).

Distance-based linear modelling (DistLM) revealed that the seasonal variability in the surface metabolite composition of both *Fucus* species was best explained by the abiotic factors light and temperature (Paper III). Since *Fucus* is a photosynthetic organism, this finding is not surprising. Several studies have highlighted the impact of light (Rohde et al. 2008, Wahl et al. 2010) and temperature (Graiff et al. 2015) on *Fucus* photosynthesis. Additionally, previous studies showed that the seasonally

variable metabolite levels are influenced by irradiance and seawater temperature (Amade & Lemee 1998, Sudatti et al. 2011).

The detected seasonal variations in *Fucus* metabolite composition (Paper III) could be seen as one possible proximate cause for the observed seasonal fluctuations in the fouling control of *Fucus* (Paper I and Paper II).

The observed seasonal chemical fouling control (Paper I and Paper II) as well as the seasonal variable surface metabolite composition (Paper III) cannot be exclusively attributed to *Fucus*, since the applied 'dipping-method' (modified after de Nys et al. 1998) provides surface extracts consisting of metabolites derived from *Fucus* and its surface associated microfoulers. Saha and co-workers (2011) could show that the antibacterial fucoxanthin originates from *Fucus* exudation and not from associated diatoms via prevention of diatom growth. However, in my experiments and in all studies applying surface extraction techniques, the analysed surface extracts were an inseparable mixture of chemical compounds originating from the holobiont *Fucus* (see below). This fact should not be interpreted as a limitation but rather as the normal state. From an ecological point of view, macroalgae should be seen as a functional entity, also termed as 'holobiont', consisting of the host algae and the associated microbiota (Egan et al. 2013). Several studies regarding macroalgae-bacteria interactions have revealed the close relationship between macroalgae and their associated bacteria, often with beneficial functions related to host defence or host health (see Introduction) (reviewed by Egan et al. 2013, Hollants et al. 2013, Singh & Reddy 2014). Regarding macroalgae fouling control, it has been demonstrated that the marine epiphytic bacterium *Pseudoalteromonas tunicate* has the potential to protect its hosts, the green alga *Ulva australis*, against common fouling organisms like algal spores and marine fungi (Rao et al. 2007). Bacterial biofilms consisting of natural assemblages obtained from *F. vesiculosus* as well as of mono-species biofilms have been shown to hinder the settlement of *Amphibalanus improvisus* cypris larvae (Nasrolahi et al. 2012). Beneficial services relating to the fouling control of the macroalga host, as described above, indicate the vital role of some associated microorganisms and could be seen as a hint for the reason why *Fucus* is not better defended against microorganisms or even axenic. It would be possible that the seasonally fluctuating fouling control of *Fucus* benefits also from symbionts or associated microfoulers.

Further investigations are necessary to answer the question which role the natural assemblage of associated bacteria or even fungi paly in the observed chemical fouling control of *Fucus*.

Besides having a chemical and mechanical fouling control (see 6.1), the thallus surface of macroalgae per se represents a selective microenvironment for approaching microorganisms and small epiphytic fouler species. When small foulers reach the surface of *F. vesiculosus*, they first encounter the overlying topmost water layer termed “diffusion boundary layer”, which has a thickness of approx. 150-250 µm, depending on velocity and temperature (personal communication Yvonne Sawall). The diffusion boundary layer is characterized by strong gradients in O_2, CO_2 and pH and is highly dynamic depending on *Fucus’* photosynthetic activity and respiration (Spilling et al. 2010). Spilling and co-workers (2010), for example, showed that O_2 concentrations and pH at the thallus surface of *F. vesiculosus* increased with light intensities reaching highest O_2 concentrations of >1 mM and a pH of 9.5. Furthermore, they demonstrated that the O_2 concentrations increased from approx. 90 % to 290 % saturation and pH increased from approx. 8.0 to approx. 8.8. at the thallus surface after six minutes of illumination (~500 µmol photons * m^{-2} *s^{-1}) of a dark-acclimated *F. vesiculosus*.

The oxygen concentrations within the layer can reach hyperoxic levels possibly along with the formation of reactive oxygen species (ROS) during daytime (Irwin & Davenport 2002). Moreover, the uneven thallus surface of *Fucus* provides cavities, termed cryptostomata, with hypoxic conditions during darkness (Spilling et al. 2010). In addition, it is conceivable that metabolites exuded from the ‘holobiont’ also form gradients within the diffusion boundary layer. Obviously, microorganisms and small epiphytic organisms settling within the boundary layer of *Fucus* have to resist this harsh and highly dynamic environment or even prefer it. Thus, it seems likely that this special microenvironment leads to a selection of potential fouler species from the *in situ* microfouler pool and therefore may function as an additional secondary fouling control. Moreover, considering the pronounced day/night fluctuations within the boundary layer, driven by photosynthetic activity, it would be conceivable that the boundary layer conditions are seasonally variable, influenced by the photosynthetic performance of *Fucus* (Graiff et al. 2015), possibly leading to a seasonally variable preselection of potential microfoulers.

3.2.2 Ultimate causes for seasonal fluctuations of the fouling control system of *Fucus*

In my studies, I demonstrated that the *in situ* micro- and macrofouling pressure is seasonally variable, reaching peak intensities during spring and summer months along with increasing irradiance, seawater temperature and nutrient availability (Paper I and Paper II). Further, the recorded fouling control strength of both *Fucus* species tended to be synchronised with the *in situ* fouling pressure of prokaryotic cells as well as that of the barnacle *A. improvisus* (Paper I and Paper II). This result is in accordance with previous studies which showed the seasonally variable chemical fouling control of different temperate macroalgae to be tuned to the *in situ* fouling pressure (Hellio et al. 2004, Marechal et al. 2004, Wahl et al. 2010). Such fine-tuned fouling control should in general be beneficial for macroalgae, especially when considering the often discussed aspects of potential metabolic costs of chemical fouling control (see Introduction). One study that analysed the metabolic costs of defence, including biofouling, found a significant inverse relationship between fecundity and the level of the bioactive compounds furanones as well as significant higher growth rates for algae unable to produce furanones, indicating a cost of furanone production (Dworjanyn et al. 2006).

In my studies, I demonstrated that both *Fucus* species were not energy limited throughout the year. For this purpose we used the intracellular mannitol concentration of both *Fucus* species as a general proxy for energy available for defense metabolite production (Paper II) or other metabolic demands. Tissue mannitol concentrations in both *Fucus* species showed a clear seasonal fluctuation with increasing concentrations from February to October, followed by a reduction in late autumn and winter. However, no complete mannitol utilization was found during the seasonal cycle, indicating that the fouling control of both *Fucus* species was not energy or carbon limited during my experiment. This finding is in accordance with a previous study, which demonstrated that the mannitol reserve of *F. vesiculosus* from the Northern Baltic Sea was never fully depleted during an annual cycle (Lehvo et al. 2001). Further, it has previously been shown that *F. vesiculosus* can store nitrogen (Lehvo et al. 2001), which would also rule out a possible nitrogen limitation of the fouling control.

The GC-MS analysis of the surface metabolite composition showed that mainly primary metabolites are up-regulated on *Fucus* surfaces during summer months

when the thallus surface is least attractive. This could indicate that these metabolites are somehow involved in the fouling control. If this is the case, then possible metabolic costs of chemical fouling control could be reduced with the deployment of bioactive primary metabolites. Recent studies have shown that primary metabolites can function as cost effective and suitable metabolites in the chemical defence of macroalgae (summerized by Pohnert 2012). Regarding the fouling control of *F. vesiculosus,* it has already been shown that metabolites fulfilling tasks in primary metabolism, like the amino acid proline or the osmolyte dimethylsulfoniopropionate (DMSP) as well as the pigment fucoxanthin are active against bacterial settlement and growth (Saha et al. 2011, Saha et al. 2012, Lachnit et al. 2013). Since a fouling control based on simple primary metabolites would not cause extra costs for synthesis or storage in specialized cells, e.g. gland cells in the red alga *Bonnemaisonia hamifera* (Nylund et al. 2008), such fouling control would be a cost-saving strategy (Pohnert 2012).

Besides the recorded quantitative seasonal variations in prokaryotic fouling pressure, it is conceivable that the density of potential pathogens also varies with season, since it has been demonstrated that virulence gene expression of opportunistic pathogens can be influenced by environmental conditions (Egan et al. 2014). Increasing temperature leading to warming of seawater is one environmental key factor inducing virulence (Case et al. 2011, Kimes et al. 2012, Guijarro et al. 2015). A study on epibacterial communities attached to the surface of Baltic *F. vesiculosus* suggested increasing relative abundances of potential pathogenic bacterial families (including *Pseudoalteromonadaceae*, *Vibrionaceae*, *Alteromonadaceae*) during summer, reaching highest abundances in autumn (personal communication with Birte Mensch). Therefore it is conceivable that the number of potential pathogens increase during summer months due to elevated seawater temperatures leading to an elevated pathogenic threat entailed with an increased need of fouling control for *Fucus*.

3.4 Consequences for population dynamics

One underinvestigated aspect of the chemical fouling control of macroalgae is the influence of exuded macroalgal fouling control metabolites on benthic

community dynamics. Since available settlement surface in the marine environment is limited (Harder 2008), competition for space in the benthic environment is immense (Wahl 1989). It is therefore conceivable that a chemical fouling control originating from the Baltic Sea's keystone species *Fucus vesiculosus* (Kautsky et al. 1992) as well as from *Fucus serratus* affects the formation and development of the natural fouling community in the proximity of *Fucus*. A previous study showed that in the immediate neighbourhood of different benthic organisms, including *F. serratus*, the natural fouling community on hard substrata was modulated in its community development, probably impacted by the exudation of metabolites influencing settlement or advection success and/or post-settlement survival (Wahl 2001). Furthermore, it has been demonstrated that cypris larvae of the barnacle *Amphibalanus improvisus* preferred stones as settlement substrate over *F. vesiculosus* thalli and that the low larval preference for *Fucus* was due to waterborne metabolites, probably exuded phlorotannins from *F. vesiculosus* (Brock et al. 2007). These examples illustrate the potential influence of macroalgae exuded metabolites on the settlement success and thus the survival and establishment of neighbouring benthic communities. Considering that my thesis reveals the highest fouling control of *Fucus* and the highest *in situ* fouling pressure to be synchronised for prokaryotic foulers and the barnacle *A. improvisus*, it is possible that these two fouler species are influenced. This would mean that *A. improvisus* has less available settlement substrate during settlement seasons leading to a higher settlement pressure on other available surfaces. For prokaryotes it could mean that a specific community develops on *Fucus* thalli (Lachnit et al. 2009) and adjacent surfaces, probably leading to a subsequent and specific colonization (Hadfield 2011).

Thus, *Fucus* chemical fouling control more than likely affects the community development and the structure of the surrounding environment.

3.5 Consequences of changing environmental parameters

F. vesiculosus, the key stone species of the Baltic Sea (Kautsky et al. 1992), and its relative *F. serratus* inhabit an environment that is influenced by eutrophication (HELCOM 2014). The Baltic Sea is especially susceptible to eutrophication due to several combined reasons. First the bordering countries with high populations and

human activities result in large nutrient loads (mainly nitrogen and phosphorous). Second its inland sea characteristics lead to a limited water exchange and a longer residence time of water (HELCOM 2009). Eutrophication is known to modify biotic interactions in macroalgae communities by causing enhanced growth of fast-growing opportunistic macroalgae entailing increasing epibiotism and grazing (Korpinen et al. 2007). Slow-growing perennial macroalgae such as *Fucus* are negatively affected by higher epibiotism (Korpinen et al. 2007). A previous study showed that epiphytic load decreases the growth of *F. vesiculosus* (Rohde et al. 2008). In addition, eutrophication leads to turbidity of the seawater due to phytoplankton growth (HELCOM 2009). Turbid seawater reduces the depth penetration of sunlight, leading to a reduced light availability for benthic macroalgae species, including *Fucus*. *F. vesiculosus* declined in the past decades from deeper waters shifting its vertical depth distribution upwards (Kautsky et al. 1986). This depth decline of *F. vesiculosus* from deeper waters has been attributed indirectly to eutrophication and directly to a lack of light supply caused by epibiotism and turbid water (Kautsky et al. 1986, Voigt & Schramm 1991).

Eutrophication in combination with the predicted future sea surface temperature in the Baltic Sea (increase by 0.5 ℃ to 5 ℃ by th e end of the century) (BACC 2008, Neumann & Friedland 2010) will probably increase the fouling pressure for *Fucus* in the Baltic Sea. However, regarding the chemical microfouling control of *F. vesiculosus* a previous study demonstrated that surface associated active metabolites, such as fucoxanthin and DMSP, were influenced in their natural surface concentrations by the abiotic factors light and temperature. The study also showed that under all tested light and temperature conditions at least one of the fouling control metabolites were concentrated high enough to reduce bacterial settlement, indicating that *F. vesiculosus* is capable to deal with shading and warming (Saha et al. 2014). Therefore, it seems conceivable that at least with respect to the microfouling control *F. vesiculosus* is capable to control a putative increasing fouling threat.

3.6 Conclusion

This thesis shows that the micro- and macrofouling pressure in the field and the micro- and macrofouling control strength of *Fucus vesiculosus* and *Fucus serratus*

tested under natural conditions varies with season. Surface extracts tended to be least attractive for micro- and macrofoulers during seasons when the respective field fouling pressure was highest. While this correlation was not significant, the trend is suggestive of a pronounced deployment of fouling control metabolites during periods of high fouling pressure as shown earlier (Saha & Wahl 2013). Surprisingly, a similar pattern was not detected for the transient fouler *M. edulis* and only restricted for diatoms, indicating a species specific macrofouling control like it has been demonstrated for bacteria (Saha & Wahl 2013). The observed seasonal fluctuations of micro- and macrofouling control do not seem to reflect the availability of resources. Furthermore, this thesis exhibits a clear seasonal variation in surface metabolite composition of both *Fucus* species, with significant differences between spring/summer and autumn/winter extracts. Variability in surface metabolite composition was best explained by the abiotic factors light and temperature. Additionally, a pronounced up-regulation of mono- and disaccharides as well as hydroxy acids was detected in *F. vesiculosus* and *F. serratus* surface extracts during summer months compared to winter months. This up-regulation indicates that primary metabolites with potential fouling control properties are present on both *Fucus* surfaces, originating from *Fucus* itself or from its associated microfoulers.

It is conceivable that these primary metabolites, maybe together with undetected lower concentrated *Fucus* compounds such as fucoxanthin or DMSP (Saha et al. 2011, Saha et al. 2012), exhibit an antagonistic interaction regulating the specific composition of surface associated microbes of *Fucus*. In turn, the *Fucus*-specific biofilms could modify subsequent macrofouling (Nasrolahi et al. 2012).

3.7 References

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5. Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Ich habe diese Arbeit weder ganz noch teilweise im Rahmen eines anderen Prüfungsverfahrens vorgelegt. Bei der Erstellung dieser Arbeit habe ich mich an die Regeln guter wissenschaftlicher Praxis gehalten.

Kiel, den

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