

**Ökologie von Makroalgen-assoziierten Mikroben:
Effekte von Umweltbedingungen auf die
Gemeinschaftsstruktur von Epibakterien und abwehrende
Effekte von mikrobiellen Epibionten auf Seepockenlarven**

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Table of Contents

Abstract	v
Abstract in German/Zusammenfassung	vii
List of Abbreviations	ix
1 Introduction	1
1.1 The Microenvironment on Macroalgal Surfaces	4
1.2 The Role of Microbes in Health and Development of their Hosts	6
1.2.1 The Role of Bacteria Associated with Macroalgae	7
1.2.2 Microbial Biofilms Produce Cues for Macrofoulers	9
1.3 <i>Fucus vesiculosus</i> in the Baltic Sea and its Abiotic Environment	10
1.4 Interactions between <i>Fucus vesiculosus</i> and Associated Bacteria	11
1.5 Pyrosequencing for Analysis of Bacterial Communities	12
1.6 Research Questions	14
2 Publications and Contribution of Authors	17
3 Paper I: Temperature-Driven Shifts in the Epibiotic Bacterial Community Composition of the Brown Macroalga <i>Fucus vesiculosus</i>	19
4 Paper II: Do Irradiance and Salinity Affect Compositional Traits of Epibacterial Communities on the Brown Macroalga <i>Fucus vesiculosus</i> ?	33
5 Paper III: A Protective Coat of Microorganisms on Macroalgae: Inhibitory Effects of Bacterial Biofilms and Epibiotic Microbial Assemblages on Barnacle Attachment	51
6 Discussion and Conclusion	67
6.1 Which Factors Structure Epibacterial Communities?	69
6.1.1 Abiotic Factors	69
6.1.2 Host Defense	71
6.1.3 Interactions within the Epibiotic Biofilm	71
6.1.4 Grazers and Macrofoulers	72
6.1.5 Random Processes	73
6.2 The Role of Epibacteria in a Changing Environment	73
6.2.1 Pathogens	74
6.2.2 Mutualistic Role of Microbial Assemblages	75
Conclusion	75
References	77
Supporting Information	87
Paper I	88
Paper II	90
Paper III	91
Acknowledgements	93
Eidesstattliche Erklärung	95

Abstract

In this doctoral project, I investigated the effects of abiotic factors on the composition and cell density of epibacterial communities associated with the ecologically important brown macroalga *Fucus vesiculosus* in laboratory and outdoor experiments. During the experiments, specimens of the macroalgal host and their microbial biofilm were exposed to different levels of either temperature, irradiance, or salinity. Subsequently, bacteria were harvested from the macroalga, and bacterial community composition was analyzed by culture-independent analyses of 16S rRNA gene sequences. Cell densities were enumerated via epifluorescence microscopy directly on the algal surface. Furthermore, I studied the effects of natural microbial assemblages on the attachment rates of barnacle larvae. For this purpose, the microbial biofilm community was harvested from the macroalgal surface and exposed to barnacle larvae during their peak settlement season in the Kiel Fjord in a specially constructed bioassay apparatus.

As revealed by redundancy analyses, epibacterial communities significantly clustered along the temperature (5, 10, 15, 20, 25°C) and salinity (5, 19, 25) gradients but not along the gradient of irradiance (100%, 44%, 23%, 12%, 5%, and 0% of sunlight reaching the algal surface). Temperature and salinity are important factors in structuring epibacterial communities since they each accounted for about 20% of the variation in the bacterial community composition. In general, <10% of bacterial OTUs (operational taxonomic units grouped at the 97% sequence similarity level) significantly correlated with the abiotic factors. The differences in community composition due to temperature were largely attributed to the Rhodobacteraceae, the bacterial family whose mean relative abundance was twice as high at the high temperatures than at the low temperatures. Members of the subphylum Betaproteobacteria made up about a third of the bacterial community at the low salinity level (5) on average and were nearly absent at the higher salinity levels. Members of the Gammaproteobacteria and of the Actinobacteria were on average at least twice as abundant at the higher salinities than at the low salinity. Furthermore, the type of substrate (stones were included in the salinity experiment as non-living reference substrate) had a significant effect on the composition of epibacteria. Epiphytic and epilithic bacterial communities differed significantly due to a higher mean relative percentage of members of the phylum Cyanobacteria. They made up about 8% on average in the epilithic community, while they were nearly absent on *F. vesiculosus*. Furthermore, members of the subphylum Gammaproteobacteria were at least twice as abundant in epiphytic than in epilithic bacterial communities. Epibacterial richness (i.e., the number of OTUs) significantly differed between treatment levels, although variability between algal individuals of one treatment level was generally high. OUT richness was highest at 15°C, lowest at a salinity of 5, and lowest at 44% irradiance reaching the algal surface. The most even distribution of epibacterial groups (evenness expressed with the Inverse Simpson

Index) showed similar patterns as richness, although in the temperature experiment this was a non-significant trend. Epibacterial densities did not significantly differ between levels of temperature but decreased with shading. However, variability between individuals of one treatment level was often larger than between replicates of one treatment level. Whether direct or indirect effects via altered biotic interactions may have caused the observed shifts is discussed in the appropriate sections. In bioassays conducted in the Kiel Fjord, barnacle cyprids attached significantly less to filters treated with microbial assemblages harvested from *F. vesiculosus* than to non-treated seawater filters. This indicates a repellent effect of the microbes. The most pronounced repellent effect was found with microbial assemblages that had been kept at 15°C for 14 days (54% fewer attached cyprids than on filters without microbes).

My study contributes to the basic understanding of the ecology of host-associated bacterial communities and the interactions between host, epibacteria, and macrofoulers: with the tool of high-throughput sequencing, high bacterial diversity for this macroalgal host was confirmed and a detailed description of community composition under different abiotic conditions was achieved. The impact that abiotic factors can have on this highly complex epibacterial community has been elucidated and may become more important in the future taking into consideration predicted climate change scenarios. The suspected potential of the epibacterial biofilm of playing a mutualistic role for the macroalgal host by assisting the host in its own defense against macrofouling was confirmed, adding to the notion that microbial communities should be considered when studying macroalgae and other marine hosts.

Abstract in German/Zusammenfassung

In dieser Doktorarbeit habe ich in Laborexperimenten den Einfluss von abiotischen Faktoren auf die Struktur und Zelldichte der epibakteriellen Gemeinschaften, die mit der ökologisch wertvollen braunen Makroalge *Fucus vesiculosus* assoziiert sind, untersucht. Individuen der Makroalge mit ihrem mikrobiellen Biofilm wurden verschiedenen Temperatur-, Licht-, oder Salinitätsbedingungen ausgesetzt. Daraufhin wurden die Bakterien von der Oberfläche der Algen beprobt und ihre Gemeinschaftsstruktur mittels kulturunabhängiger Analyse der 16S rRNA Gensequenzen untersucht. Bakterielle Zelldichten wurden durch Epifluoreszenzmikroskopie bestimmt. Des Weiteren habe ich den Einfluss der natürlichen mikrobiellen Gemeinschaft auf die Besiedlungsrate von Seepockenlarven untersucht. Zu diesem Zweck wurde die mikrobielle Gemeinschaft von der Algenoberfläche genommen. In einem speziell hierfür konstruiertem Apparat wurde dann die mikrobielle Gemeinschaft Seepockenlarven während ihrer Siedlungsperiode in der Kieler Förde präsentiert.

Redundanzanalysen zeigten, dass sich die Bakteriengemeinschaften signifikant entlang des Temperaturgradienten (5, 10, 15, 20, 25°C) und des Salinitätsgradienten (5, 19, 25) aber nicht entlang des Lichtgradienten (100%, 44%, 23%, 12%, 5%, und 0% des Sonnenlichts an der Algenoberfläche) gruppiert hat. Temperatur und Salinität sind beides wichtige Faktoren, die die Struktur der Bakteriengemeinschaft beeinflussen, da sie beide ca. 20% der Variabilität in der Struktur erklärt haben. Im Allgemeinen haben <10% der bakteriellen OTUs (d.h. operational taxonomic units (taxonomische Einheiten) die bei 97% Sequenzähnlichkeit gruppiert wurden) signifikant mit dem abiotischen Faktor korreliert. Die Unterschiede in der bakteriellen Gemeinschaftsstruktur auf Grund der Temperaturbehandlung waren hauptsächlich auf die Familie der Rhodobacteraceae zurückzuführen. Ihre relative Abundanz war bei der höchsten Temperaturstufe doppelt so hoch als bei der niedrigsten. Mitglieder des Subphylum Betaproteobacteria machten ca. ein Drittel der Gemeinschaft bei Salinität 5 aus und waren bei den höheren Salinitäten (19, 25) kaum vorhanden. Ferner waren Mitglieder der Gammaproteobacteria und der Actinobacteria im Mittel doppelt so häufig bei den höheren Salinitäten als bei Salinität 5. Außerdem hatte der Substrattyp (Steine dienten als Referenzsubstrat im Salinitätsexperiment) einen signifikanten Effekt auf die Zusammensetzung der epibakteriellen Gemeinschaft. Epiphytische und epilithische Bakteriengemeinschaften unterschieden sich signifikant, da Mitglieder des Phylum Cyanobacteria im Mittel ca. 8% der Gemeinschaft auf Steinen darstellten, aber auf *F. vesiculosus* nahezu abwesend waren. Ferner waren Mitglieder des Subphylum Gammaproteobacteria mindestens doppelt so häufig in epiphytischen als in epilithischen Gemeinschaften vertreten. Die Anzahl der OTUs unterschied sich signifikant zwischen den Behandlungsstufen, wobei die Variabilität bei Algenindividuen einer Behandlungsstufe generell hoch war. Alphadiversität

(Anzahl der unterschiedlichen OTUs) war bei 15°C am höchsten, und bei Salinität 5 und bei 44% Sonnenlicht am niedrigsten. Ebenso waren die Häufigkeiten der OTUs (beschrieben mit dem Inverse Simpson Index) bei diesen Behandlungsstufen am gleichmäßigsten verteilt, wobei die Beobachtung im Temperaturexperiment ein nicht signifikanter Trend war. Zelldichten waren nicht abhängig von der Temperatur aber nahmen mit zunehmender Beschattung ab. Allerdings war die Variabilität zwischen Gemeinschaften auf Algenindividuen einer Behandlungsstufe oft größer als zwischen Replikaten einer Behandlungsstufe. Ob die abiotischen Faktoren direkte oder indirekte Einflüsse über veränderte biotische Interaktionen haben, wird an entsprechender Stelle diskutiert. In Versuchen, die in der Kieler Förde durchgeführt wurden, siedelten signifikant weniger Seepockenlarven (Cyprislarven) auf Filtern, die mit den mikrobiellen Gemeinschaften behandelt wurden als auf Filtern ohne Mikroben. Dies weist darauf hin, dass die Gemeinschaften einen abwehrenden Effekt haben. Der Effekt war am größten bei mikrobiellen Gemeinschaften, die 14 Tage bei 15°C gehältert wurden (54% weniger gesiedelte Larven als bei Filtern ohne mikrobielle Gemeinschaft) .

Meine Forschungsarbeit trägt dazu bei, die Ökologie von wirtsassoziierten bakteriellen Gemeinschaften und die Interaktionen von Wirtsalge, Epibakterien und Makrofoulern besser zu verstehen. Mit Hilfe von modernen Sequenzierungsmethoden wurde die zuvor vermutete hohe bakterielle Diversität beschrieben und die Gemeinschaftsstruktur bei verschiedenen abiotischen Bedingungen detailliert charakterisiert. Der Einfluss den Temperatur, Licht und Salinität auf die komplexe Struktur der epibakteriellen Gemeinschaft haben kann, wurde erklärt. In Zukunft werden diese Effekte womöglich eine noch größere Rolle spielen, wenn man Vorhersagen des Klimawandels betrachtet. Das vermutete Potenzial des epibakteriellen Biofilms der Wirtsalge zu nutzen, indem er ihre Verteidigung gegen Makrofouler unterstützt, wurde bestätigt. Dies trägt zum Verständnis bei, dass mikrobielle Gemeinschaften bei Untersuchungen von Makroalgen und anderen Meeresorganismen einbezogen werden sollten.

List of Abbreviations

ANOVA = analysis of variance

ANCOVA = analysis of covariance

DAPI = 4',6-diamidino-2-phenylindole

DBL = diffusive boundary layer

DGGE = denaturing gradient gel electrophoresis

DNA = deoxyribonucleic acid

EPS = extracellular polymeric substances

EU = experimental unit

FS = *Fucus serratus*

FSW = filtered seawater

FV = *Fucus vesiculosus*

OD = optical density

OTU = operational taxonomic unit

PCR = polymerase chain reaction

PS = *Polysiphonia stricta*

PVC = polyvinyl chloride

rRNA = ribosomal ribonucleic acid

TAE = tris-acetate-EDTA

Introduction



1. Introduction

The term “phyllosphere” was independently coined by two microbial ecologists, J. Ruinen and F.T. Last, in the 1950s and refers to the environment for microorganisms on the surface of plant leaves (Last 1955, Ruinen 1956). Ruinen (1961) deemed it an “ecologically neglected milieu”. Today, there are still many unanswered questions about the ecology of surface-associated microbial communities (and other microbiomes, e.g. in the gut, in soil, and in water). In recent years however, microbes on the surface of host organisms have been the focus of many studies and their ecological importance is becoming more and more apparent.

My doctoral project focuses on the microbial community associated with the surface of *Fucus vesiculosus*, an abundant and ecologically important brown macroalga species along the Baltic Sea Coast (Figure 1). Specifically, I have investigated how abiotic factors (temperature, irradiance, and salinity) affect the structure of epibacterial communities (Paper I and II) and I have highlighted the role of the epimicrobial assemblage in repelling barnacle larvae from attaching to substrate, suggesting a beneficial role for its host *F. vesiculosus* (Paper III).

In analogy to the term ‘phyllosphere’ stated above, I will use the term ‘thallosphere’ to describe the microenvironment for microbes found on the thallus i.e., the plantlike body of a macroalga. In the following, I will introduce the physico-chemical properties of the ‘thallosphere’ as the microhabitat for microbes, particularly bacteria (section 1.1). Then, the ecological role of



Figure 1 Dense *Fucus* meadow in the Western Baltic Sea. Photo courtesy of Uli Kunz

bacteria for eukaryote hosts in general will be introduced before focusing on macroalgal hosts (section 1.2). Subsequently, the macroalgal species that served as experimental host organism in my study, *F. vesiculosus*, and its current and predicted abiotic environment in the Baltic Sea will be described (section 1.3). Furthermore, I will address studies on the bacterial communities inhabiting this macroalga (section 1.4) that led to my research questions (section 1.6). Finally, I will briefly address the advantages and limitations of the pyrosequencing method that I applied for taxonomic community analysis of epibacteria associated with *F. vesiculosus* and its importance for studies on microbial diversity (section 1.5).

1.1 The Microenvironment on Macroalgal Surfaces

Available space on nearly every surface in marine systems is generally rapidly fouled by organisms of different taxa, the first colonizers usually being bacteria (reviewed in Wahl, 1989). Eventually, a biofilm forms, which is an aggregation of microbial cells adhered to a surface encased in a slimy matrix of extracellular polymeric substances (EPS) i.e., polysaccharides, proteins, nucleic acids, lipids and other macromolecules (Costerton *et al.*, 1995, Battin *et al.*, 2007). A mature biofilm is viewed as a complex, three dimensional “microbial landscape” (e.g., single species films in lung epithelia or complex stream biofilms) (Battin *et al.*, 2007) or “a city of microbes” (Watnick and Kolter, 2000). However, this kind of biofilm is less likely to be found on living host organisms, in part due to effective host defense mechanisms against micro- and macrofouling (reviewed in Railkin, 2004). On macroalgae, the biofilm inhabitants are organisms of different domains of life that are united by their small size and can comprise bacteria (e.g., Sieburth and Tootle, 1981), archaea (e.g., Trias *et al.*, 2012), fungi (e.g., Zuccaro *et al.*, 2003, Loque *et al.*, 2010), fungus-like Oomycota (Küpper *et al.*, 2006), and microalgae (mainly diatoms) (e.g., Karsten *et al.*, 2006, Lam *et al.*, 2008) upon which other microscopic organisms graze and settle (e.g., protozoans, spores, larvae).

The microenvironment surrounding an algal surface, the “thallosphere”, is delimited by a boundary layer i.e., a diffusion gradient of 35 μm to 700 μm thickness depending on water flow velocity and macroalgal morphology (reviewed in Hurd, 2000). It is the space that a microbe first encounters when it reaches the vicinity of an algal surface from the planktonic environment. Metabolites and gases, e.g., exuded carbohydrates, phenolic compounds, halocarbons, oxygen, carbondioxide, are released from the algal thallus (e.g., Nightingale *et al.*, 1995, Abdullah and Fredriksen, 2004, Haas and Wild, 2010) and pass through the boundary layer. Under laboratory conditions, the physico-chemical properties in the “thallosphere” of *Fucus vesiculosus* can be investigated on a micrometer scale due to an advancement in technology: Microsensors that measure with a spatial resolution of $<100 \mu\text{m}$ have yielded insight into the pH and oxygen concentrations in the algal tissue and in the diffusive boundary layer (DBL) surrounding the thallus (Spilling *et*

al., 2010, Matthias Fischer unpublished data). The microenvironment (pH and O₂) varies due to the dynamic photosynthetic activity of the host, and different concentrations of O₂ were found above different tissue types (Spilling *et al.*, 2010). Oxygen measurements during darkness at the cryptostomata, hairy cavities of fucoid thalli (Figure 2), revealed that its concentration was very low reaching less than 7 μg L⁻¹ versus > 60 μg L⁻¹ above smooth parts of the thallus. Although these are non-replicated results, they hint at considerable variation along very small spatial scales with possible implications for the microbes inhabiting the thallus. The authors suggested that the cryptostomata could provide a habitat for anaerobic bacteria (Spilling *et al.*, 2010). Further characterization of the milieu has been achieved by confocal resonance Raman microspectroscopy, a method which allows visualization of the distribution of carotenoids, organic pigments, within the DBL of two macroalgal species, *F. vesiculosus* and *Ulva* sp. (Grosser *et al.*, 2012).

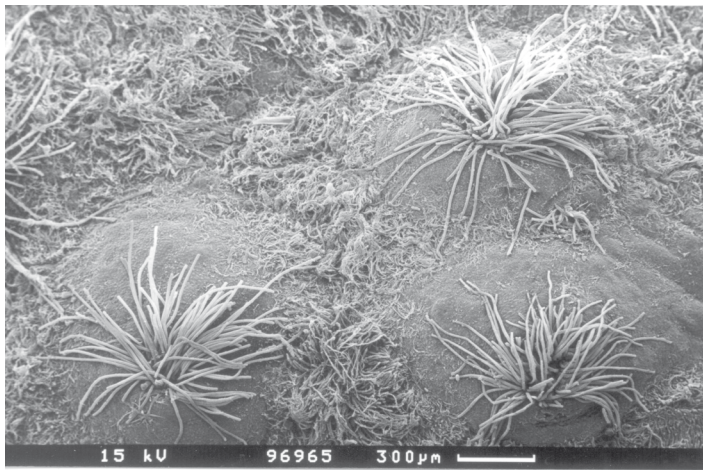


Figure 2 Topography of the microenvironment on the surface of *Fucus vesiculosus*. Scanning electron micrograph of cryptostomata, hairy cavities of fucoid thalli.

A steep decline in the concentration of carotenoids was detected in the DBL with distance from the algal thalli (150 μm), regardless of thallus region. One of the investigated carotenoids, the xanthophyll fucoxanthin, is involved in the antibacterial defense of *F. vesiculosus* (Saha *et al.*, 2011, Lachnit *et al.*, 2013). Thus, the possibility of measuring the gradients of metabolites involved in the microfouling defense may improve the understanding of

ecologically relevant processes within the DBL (Grosser *et al.*, 2012). The two above mentioned studies provide evidence that there are microenvironments within the “thallosphere” of *F. vesiculosus* reflected in the high variability and steep gradients of physico-chemical parameters. This variability is reflected in the abundance of epibacteria and in the composition of epibacterial communities. Bacteria can exhibit a patchy distribution on macroalgal surfaces (e.g., Fig. 1 in Tujula *et al.*, 2006, Fig. 2 in Bengtsson *et al.*, 2010, Figure 3) and the composition of an epibacterial community can differ along the algal thallus. The latter was exemplified by Staufenberger *et al.*, (2008), who observed differences in the composition of bacterial phylotypes on the rhizoid, cauloid, meristem, and old phylloid of a kelp species. At this time, we do not yet know to what extent the above mentioned variability in the microenvironment determines the distribution of bacteria and their community composition on macroalgal surfaces at a microscale. Which factors, in general, determine the distribution of host-associated microorganisms and their community composition are discussed in section 6.1.

1.2 The Role of Microbes in Health and Development of their Hosts

The term “holobiont” refers to an association of one or more members of different species living together (“a symbiotic complex”) (Margulis, 1991) and is now widely used to describe the close association of animal and plant hosts with microorganisms (e.g., Rosenberg *et al.*, 2007, Bourne *et al.*, 2009, Fraune *et al.*, 2009, Feldhaar, 2011, Barott and Rohwer, 2012, Egan *et al.*, 2012, Fan *et al.*, 2013). This reflects the scientific consensus that microbes are essential in the development and health of their hosts and cannot be ignored in the effort to understand the ecology and even evolution of host organisms (Fraune and Bosch, 2010, Bosch and McFall-Ngai, 2011, Rosenberg and Zilber-Rosenberg, 2011, Gilbert *et al.*, 2012). Zilber-Rosenberg and Rosenberg (2008) proposed the “hologenome theory of evolution“, which suggests, that the host and its associated microbes or, more precisely, the sum of the genetic information of the host and its microbiota, are the unit of selection. There are four generalizations that underlie this theory: (1) All animals and plants live in association with microbes, which are (2) transmitted between generations, (3) the symbiosis affects the fitness of the holobiont within its environment, and (4) changes (e.g., driven by environmental stress) in either the host or the microbiota genomes can lead to variation in the hologenome (the metagenome of the microbes can change more rapidly than the host’s genome) (Rosenberg *et al.*, 2007, Zilber-Rosenberg and Rosenberg, 2008). While generalizations (1) and (4) of the theory hold true for epimicrobial communities associated with macroalgae, to my knowledge, there is no evidence yet for (2) and (3). It is true that macroalgae are symbiotic with microorganisms (e.g., reviewed in Goecke *et al.*, 2010) and that the relative abundances of bacterial phylotypes associated with the host (and thus their metagenome) can change rapidly under changing environmental conditions (Paper I and II of the thesis at hand). However, whether this shift in community composition leads to more or less variation in the hologenome, increasing or decreasing the fitness of the host, and whether these microorganisms are reliably transmitted between generations, has not, to my knowledge, been investigated or proven. [This excludes the endosymbiotic procaryotes that have evolved into chloroplasts and mitochondria and are transmitted from one generation to the next via cytoplasmic inheritance. The holobiont concept goes beyond this ancient tight relationship since it includes the diversity of entire microbial communities (Zilber-Rosenberg and Rosenberg, 2008)]. Regardless of this, macroalgae and associated microbes are also referred to as “holobionts” (Barott *et al.*, 2011, de Oliveira *et al.*, 2012, Egan *et al.*, 2012, Harder *et al.*, 2012), and they have been subject of many investigations that prove that microbial communities are not only important in the decomposition and thus in the cycling of algae-derived carbon, but also play a significant role in health and development of the living host. Some of these will be exemplified in the following section.

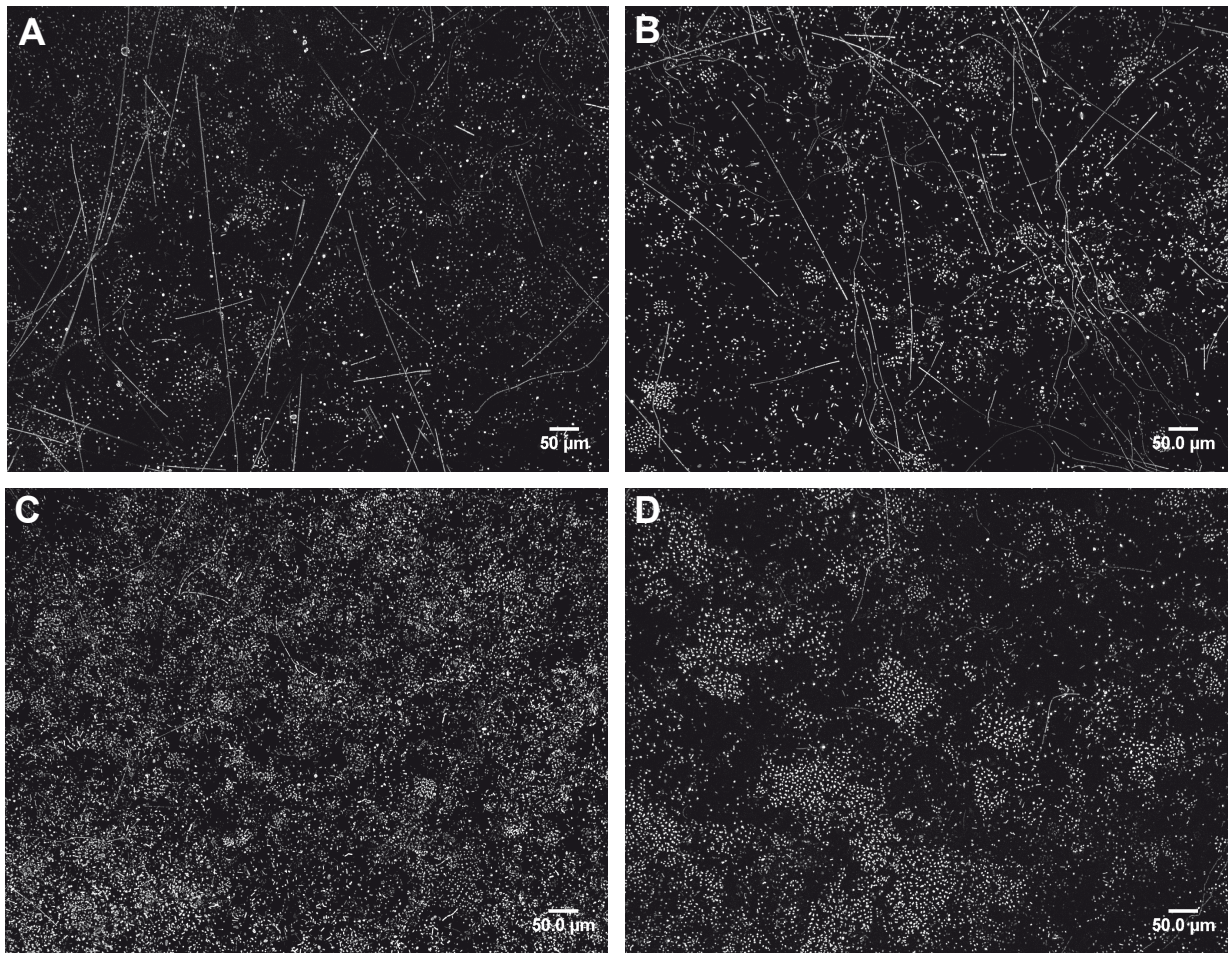


Figure 3 A-D Epibacteria on the surface of *Fucus vesiculosus*. Bacterial cells were stained with DAPI and visualized by epifluorescence microscopy at a magnification of 630. Background noise from host algal autofluorescence was removed according to the protocol in paper I. Variability in bacterial cell density at a micrometer scale was representative for most algal specimens under investigation.

1.2.1 The Role of Bacteria Associated with Macroalgae

While microbial biofilm communities on macroalgae can comprise several taxa from different domains of life as stated above, it is the bacteria that have been most intensely studied and were the focus of my research project. In recent years, several reviews on the bacteria associated with macroalgae have been published (Goecke *et al.*, 2010, Egan *et al.*, 2012, Friedrich 2012, Hollants *et al.*, 2013). While the scientific focus on bacteria may be somewhat of a research bias neglecting other functionally important microbes, such as microalgae and fungi (but see Lam *et al.*, 2008, Gleason *et al.*, 2011 and references therein), they often are the dominant and active members of the microbial host-associated communities (e.g., Fierer and Lennon 2011, de Oliveira *et al.*, 2012). The number of bacterial cells can reach up to 90 million on one square centimeter of algal surface on *F. vesiculosus* (Figure 3C). They produce a number of enzymes and bioactive compounds that have the potential to directly influence the host and alter interactions between

hosts and the environment. There are numerous case studies that highlight these antagonistic and mutualistic chemical processes.

Bacteria isolated from macroalgae often have enzymes that allow them to degrade polysaccharides that make up algal cell walls (e.g., agar, alginate, laminaran, fucoidan, porphyran, carrageen, fucan, mannan, cellulase) (for a list of bacteria producing these special enzymes, see Goecke *et al.*, 2010). Bacteria that have the ability to degrade macroalgal polysaccharides have not only been isolated from seaweed surfaces but have also been found in, at first glance, rather conspicuous places: in the guts of terrestrial hosts. At second glance, this observation makes sense because these hosts savor seaweeds. The breed of North Ronaldsay sheep on the Orkney Islands in Scotland that feed on brown seaweeds (Williams *et al.*, 2013) and Japanese people who eat the red alga *Porphyra sp.* in sushi (Hehemann *et al.*, 2010) are hosts to bacteria with algal cell wall degrading properties. In the latter study, the gut bacterium, that has the ability to degrade porphyran, acquired the genes responsible for this unique enzymatic ability from a marine bacterium via horizontal gene transfer. These are two interesting examples of how bacteria can benefit the host by aiding in the digestion of a very specialized diet. For macroalgae, however, these cell wall-degrading bacteria can have detrimental effects. Due to their enzymatic equipment they play a role in the decomposition process of algal detritus or senescencing fronds (Laycock, 1974), but it has been shown that they can also cause disease symptoms (bleaching) by agarolytic activity in previously healthy individuals of a red alga (Jaffray and Coyne, 1996). Certain bacterial taxa (e.g., *Pseudoalteromonas sp.*, *Halomonas sp.*, *Vibrio sp.*) have been correlated with rot symptoms in many cases, however, could not always be confirmed as the causative agent. This suggests that they are opportunistic pathogens, and/or the bacteria causing symptoms were others that cannot be readily cultured (Wang *et al.*, 2008 and references therein). There are a number of other pathogenic microbes (e.g., oomycetes) that have been identified on macroalgae that cause damage to the hosts in form of rot symptoms and galls and cause major economic losses in seaweed aquaculture (Gachon *et al.*, 2010). The emerging issue of bacterial pathogens increasing in the oceans due to climate change and the resulting implications for macroalgal hosts is briefly discussed in Paper I and is further detailed in the general discussion in the light of my findings (section 6.2.1). Aside from the detrimental effects on their hosts, bacteria act as mutualistic epiphytes, providing the algae with beneficial substances. Some green, brown, and red macroalgal species have been shown to grow abnormally under axenic conditions (Fries 1970, Fries, 1975, Provasoli *et al.*, 1977, Tatewaki *et al.*, 1983, Fries, 1988). In many cases, bacteria have been identified as inducers of healthy morphogenesis and in some cases germination (e.g., Provasoli and Pintner, 1980, Nakanishi *et al.*, 1996, Matsuo *et al.*, 2003, Spoerner *et al.*, 2012). For example, bacteria isolated from a green algal surface produce a substance, termed thallusin, that induces germination and healthy morphogenesis in several green alga species (Matsuo *et al.*,

2005). Furthermore, nitrogen-fixing bacteria provide their hosts with bioavailable nitrogen, others reduce toxic stress (reviewed in Goecke *et al.*, 2010). Many seaweed-associated bacteria produce bioactive compounds that are active against other microbes (e.g., Buck *et al.*, 1962, Lemos *et al.*, 1985, Boyd *et al.*, 1999, Egan *et al.*, 2000, Wiese *et al.*, 2009, Sugathan *et al.*, 2012). These were suggested and shown in some cases to also provide an advantage for the host by inhibiting macrofouling (e.g., Holmström *et al.*, 1996, Armstrong *et al.*, 2001).

1.2.2 Microbial Biofilms Produce Cues for Macrofoulers

Marine organisms produce and receive chemical cues that form the “language” which regulates their behavior and thus structures populations, communities, and ecosystems (Hay, 2009). Many marine invertebrate larvae e.g., polychaetes, barnacles, sponges, corals, echinoderms, bryozoans, molluscs, ascidians, and others, detect chemical signals from the environment, and based on this, can choose their attachment sites (Hadfield and Paul, 2001). Chemical signals emitted by biofilms, mostly by the bacterial members, have been shown to induce settlement and metamorphosis of larvae but they can also act as inhibitors of settlement, thus directing the larvae to sites suitable for further development (reviewed in Wieczorek and Todd, 1998, Hadfield, 2011). From the point of view of a macroalgal host, intense fouling by larvae can reduce light reaching the algal thallus and possibly increase stressful drag (Wahl, 2008). It may seem that a macroalgal thallus is not the best substrate of choice for a fouling larva due to the macroalgal’s ability to produce biogenic chemicals that can directly affect fouling organisms (reviewed in Bhadury and Wright, 2004, Paul *et al.*, 2011). However, we have observed that at times when fouling pressure is high, barnacle larvae do settle in high numbers on *F. vesiculosus* (Figure S2, page 91). Bacteria isolated from living marine plant surfaces were found to inhibit larvae of one barnacle species and of green algal spores indicating their potential protective role for the hosts (Holmström *et al.*, 1996). However, the question remained as to what extent these observations were relevant in natural settings. This was the motivation to test whether complex microbial assemblages, as they commonly occur in the wild, harvested from the surface of *F. vesiculosus* influence the settlement rates of barnacle larvae (cyprids) in the field. Most of the studies before had focused on the effect of single strain bacterial films or had investigated the effects of complex communities grown on non-living substrata (they are introduced in paper III). An exception to this is the study by Dobretsov (1999) where the effect of biofilm harvested from living specimens of *F. vesiculosus* on veliger larvae of the mussel *Mytilus edulis* were tested in a chemotactic test chamber. However, in this study the colonizing process was investigated in the laboratory not in the field. In my study, the bioassays with microbial assemblages harvested from *F. vesiculosus* were conducted in the field with barnacle larvae of *Amphibalanus improvisus*.

For evidence that the bacterial members of the biofilm community elicit responses, these assays were also done with single bacterial strains originally isolated from macroalgae. *Amphibalanus improvisus* is the dominant macrofouler in the Kiel Fjord that readily settles on hard substrata in July-August. The cyprid larva develops into the first benthic stage after six planktonic naupliar stages. It reversibly attaches to the substratum, actively explores the chemical and mechanical properties of the surface with its sensory antennules, and, if suitable, attaches permanently and metamorphoses into an adult (Anil *et al.*, 2010, Thiyagarajan, 2010).

1.3 *Fucus vesiculosus* in the Baltic Sea and its Abiotic Environment

Fucus vesiculosus (L), common name bladder wrack, belongs to the class of the brown algae (Phaeophyceae). It occurs along the Atlantic East and West coasts with its northernmost distribution in the Subarctic and its southernmost occurrence at the Moroccan coast; it is also found in abundance in the brackish Baltic Sea and extends into the brackish White Sea (Lüning, 1985, Coyer *et al.*, 2011). In the Baltic Sea, the dioecious perennial macroalga is the most important canopy forming foundation species (Kautsky *et al.*, 1992). It has declined at several locations in the past decades, shifting the vertical distribution limit upward (Kautsky *et al.*, 1986, Vogt and Schramm, 1991, reviewed in Schramm, 1996, Torn *et al.*, 2006), with some transitional regional signs of recovery (Nilsson *et al.*, 2005). The decline was suggested to be linked to an indirect effect of eutrophication via light attenuation by phytoplankton and epiphytes causing low light-stress for the photosynthetic organism (Kautsky *et al.*, 1986, Vogt and Schramm, 1991, Torn *et al.*, 2006), the latter was experimentally proven by Rohde *et al.*, (2008). However, locally, other factors may have contributed to the decline. These are e.g., direct detrimental effects of high nutrient concentrations on germling survival (Bergström *et al.*, 2003), sedimentation (Eriksson and Johansson, 2003), grazing (Nilsson *et al.*, 2005, Korpinen and Jormalainen 2008), toxic substances from pulp mill effluents (Kautsky, 1992) and hard substrate removal (stone fishing) (Karez and Schories, 2005).

In Lüning (1985), *F. vesiculosus* is listed as a eurytherm species tolerating a wide range of temperatures with a temperature optimum at 15°C. In the atidal Baltic Sea, it grows submerged and is only occasionally and locally air-exposed due to wind driven fluctuations in water levels. Submerged, it can endure seasonal fluctuations in water temperatures around freezing in winter to about 20°C in summer, the seasonal temperature range in the Western Baltic (Kerstin Maczassek personal communication, personal observation, Figure S1, page 88). Sublethal indirect stress effects of temperatures deviating in both directions from the ambient temperature at the time of specimen collection have been shown to reduce antifeeding defenses (Weinberger *et al.*, 2011), whereas antifouling resistance against bacterial isolates in the laboratory was not temperature-

dependant (Wahl *et al.*, 2010). *F. vesiculosus* is euryhaline and thus not prone to osmotic stress in a wide range of salinity which explains its occurrence in the brackish Baltic Sea up to the Gulf of Bothnia and Gulf of Finland, where salinity is about 4 (Kautsky *et al.*, 1992, Serrão *et al.*, 1999). Gametes of *F. vesiculosus*, unlike those of marine congeners, do not suffer as much from osmotic stress at low salinities and can reproduce successfully at a salinity of 4 (Serrão *et al.*, 1996). However, egg release, sperm motility, and fertilization decreases at low osmolality (Serrão *et al.*, 1996). Reproductive success seems to be much more a matter of chance in low salinity habitats than in marine environments, depending on many favorable abiotic and biotic conditions occurring together (Serrão *et al.*, 1999). An alternative reproductive strategy, i.e., asexual reproduction, in populations of *F. vesiculosus* living at the low salinity margin has been observed (Tatarenkov *et al.*, 2005).

In the future, climate is predicted to change due to the continuous emission of greenhouse gases (IPCC, 2007). In the Western Baltic Sea this may lead to an increase in sea surface temperature by 0.5°C to 5°C and a decrease in salinity of up to 2 g per kg due to increased precipitation and river inflow projected from regional climate models by the end of the century (BACC Author Team, 2008, Neumann and Friedland, 2011). The possible implications for the host, especially of temperature stress, with respect to antifouling defenses, epiphytic bacterial communities, and their role in the defense against macrofouling will be discussed in section 6.2.2 linking the results of Paper I and III.

1.4 Interactions between *Fucus vesiculosus* and Associated Bacteria

Bacterial communities are more similar on populations of *F. vesiculosus* originating from two different habitats (North and Baltic Sea) than between different algal species from the same habitat (Lachnit *et al.*, 2009). The more related the host algal species are, the more similar their communities are (Lachnit *et al.*, 2009). At the beginning of my project, this was the first indication that algal hosts have species-specific communities that may be controlled by some aspect of host physiology (e.g., morphology, physiological status, defense). There was also some basic knowledge about the seasonal fluctuations in the abundance and morphotypes of bacteria on *F. vesiculosus* from West Atlantic populations based on scanning electron microscopic observation (Sieburth and Tootle, 1981). The authors noted a denser biofilm in spring (April) and winter (November, December) and suggested that this may be related to an increased production of toxic phenolic substances of the host algae in summer previously observed in populations of different algal species from the coasts of Britain (Hornsey and Hide, 1974). At the time, there was only limited knowledge on other types of defense compounds and their effects on complex epibiotic microbial communities. The scientific community furthermore did not know to what

extent abiotic factors structure these complex epibacterial communities and affect their diversity. The importance of the abiotic factors temperature, salinity, and irradiance for *F. vesiculosus*, as exemplified in the previous paragraph, led me to choose these as experimental factors in my project. In the past years, about a dozen studies (including those of my doctoral project) have answered some of the questions about epiphytic bacteria and their interactions with the host *F. vesiculosus*. In order to reduce redundancy, these studies will not be introduced any further now, since they are cited in the discussions of paper I, II, III and in the general discussion.

1.5 Pyrosequencing for Analysis of Bacterial Communities

At the beginning of my doctoral project in 2009, knowledge on the complexity and diversity in community structure of epibacteria associated with macroalgae was scarce. To my knowledge, there were no studies that had applied high-throughput sequencing methods in order to describe the structure and diversity of algae-associated bacterial communities. Most studies before that time had used culture dependent methods, DNA fingerprinting methods, and in some cases cloning and Sanger sequencing (reviewed in Goecke *et al.*, 2010). These studies have gained considerable insight into many interactions between macroalgae and certain bacterial strains and have also been able to characterize a part of the community members. Based on the divergence of the number of bacteria that could be cultured on nutrient media and the number of cells and species observed under the microscope, it became clear early on in microbiological studies that bacterial diversity is likely underestimated and that the microbes should be investigated in their natural environment (Henrici, 1933). The bacteria that are difficult to culture because of special nutrient requirements or because they live in consortia (Burmølle *et al.*, 2006) and depend on other microbes for growth were overlooked. Due to these methodological constraints, researchers could never study the structure and ecological functions of entire complex environmental bacterial communities. In recent years, due to the establishment of methods such as high-throughput sequencing, studies on the diversity of complex microbial communities have become possible. In several large scale projects the microbes on and in hosts and in the environment have been and are being studied. The ‘Human Microbiome Project’ has characterized the human microbiota at several body sites (Huttenhower *et al.*, 2012, Methé *et al.*, 2012). Soil microbes are the target of the ‘Terragenome Project’¹, and the largest scale analysis of microbial communities yet is being undertaken in the ‘Earth Microbiome Project’² with the aim of sequencing over 200 000 samples for characterization of the microbial communities in different ecosystems across the globe. The application of high-throughput sequencing in the analysis of host-associated bacteria has allowed a broader view of the entire community and has resulted in several publications on the composition

¹ Project webpage: <http://www.terrigenome.org/> date accessed: 15. January 2013

² Project webpage: <http://www.earthmicrobiome.org/> date accessed: 15. January 2013

of bacterial communities associated with marine hosts e.g., corals (Cárdenas *et al.*, 2012, Lee *et al.*, 2012), sponges (Webster *et al.*, 2010, Lee *et al.*, 2011, Jackson *et al.*, 2012, Schmitt *et al.*, 2012, Arellano *et al.*, 2013), a polychaete (Neave *et al.*, 2012), an ascidian (Behrendt *et al.*, 2012), and macroalgae (Barott *et al.*, 2011, Burke *et al.*, 2011, Bengtsson *et al.*, 2012, Lachnit *et al.*, 2013, Stratil *et al.*, 2013). Most of these studies inferred taxonomic information based on the analysis of the hypervariable region(s) of the gene coding for the 16S rRNA and, with the aid of computing techniques, have analyzed bacterial community composition (i.e., relative abundances of different bacterial groups) and diversity. Some general patterns that have been observed in these studies on epibacterial communities are discussed in Paper I and II. As with most methods, there are some disadvantages that have to be taken into consideration when preparing samples, analyzing, and interpreting the data. The method relies on PCR for amplification of genetic material with all the associated biases, such as chimeric sequence formation, heterogeneity in the numbers of 16S rRNA gene regions on prokaryotic chromosomes resulting in potential bias when estimating the abundances of phylotypes, and differential PCR amplification (reviewed in Wintzingerode *et al.*, 1997). Also, 454 pyrosequencing can lead to an overestimation of diversity due to sequencing errors (Kunin *et al.*, 2010). Databases that the sequences are matched to in order to assign taxonomy are not yet complete for many understudied environments. Therefore, a part of the community may be labelled as unclassified at the current state. Furthermore, there is limited inference about the function of the communities based on the analysis of 16S rRNA genes. It is not always possible to predict the physiological capacities of a bacterium based on its phylogenetic relationship to other bacteria (in Harwood and Buckley, 2008). Two organisms that are very similar in the genetic make-up of their 16S rRNA gene do not necessarily share the same function. In order to reduce bias, the sequence data is computationally preprocessed before statistical analysis removing erroneous sequences (“denoising”). The sequences are usually grouped at a 97% similarity level, which, at the current state of knowledge, is accepted as a valid method reducing artificial inflation of diversity estimates (Kunin *et al.*, 2010 and above-mentioned marine case studies). The function of the bacteria, however, remains a matter of speculation if no other genes besides the 16S rRNA gene are sequenced. Despite these limitations, the main advantage of pyrosequencing is that microbial diversity not captured by enrichment techniques is now much more deeply accessible, including the rare biosphere (Huse *et al.*, 2008), at lower relative costs compared to cloning and Sanger sequencing (Hudson, 2008). Only if the entire microbial community can be captured, can patterns in their distribution, abundance, and diversity under different experimental and environmental scenarios be accurately studied.

1.6 Research Questions

I formulated my research questions equipped with the background knowledge that a) *Fucus vesiculosus* harbors a species-specific bacterial community (varying to a certain degree on individuals) b) that the community is likely involved in the healthy functioning of the host c) that it is structured to a certain degree by the abiotic environment and d) that this environment is variable and affected by climate change. In this context, I investigated how the abiotic factors temperature, irradiance, and salinity affect the epibacterial community with respect to composition, diversity, and cell density (the latter was not investigated in the salinity experiment) and how the epiphytic microorganisms affect further settlement by an important macrofouler species. These questions were investigated in four separate experiments, the results of which have been compiled in three papers and are the content of the next three chapters.

1. *Does temperature affect the composition of epibacterial communities and their cell density?*

The effect of temperature was studied in the laboratory by exposing the host algae and associated microbes to five different temperatures, harvesting the bacterial community at different time points, and analyzing it via DGGE fingerprinting and via 454 pyrosequencing for one sampling point. Cell densities were assessed by enumeration of cells visualized by epifluorescence microscopy (Paper I).

2. *Does irradiance affect the composition of epibacterial communities and their cell density?*

The influence of irradiance on community composition and cell density was investigated in an outdoor experiment with six treatment levels focusing on low irradiance levels. Cell densities were assessed by enumeration of cells visualized by epifluorescence microscopy (Paper II).

3. *Does salinity affect the composition of epibacterial communities? Are there differences in community composition between epibacterial communities on algae and on non-living reference substrata?* Salinity as a driving force in structuring epibacterial communities was investigated in an outdoor experiment exposing algae and epibacteria to three salinity levels. In this experiment non-living reference substrata (stones collected in the vicinity of algal specimens) were included for comparison of epiphytic and epilithic bacterial communities (Paper II).

4. *Does the natural microbial community harvested from *F. vesiculosus* affect the attachment of barnacle larvae? Is the effect different for biofilm reared at different temperatures?* In field bioassays, the effect of microbial assemblages and single bacterial strains isolated from macroalgae on fouling by barnacle larvae was assessed. Microbial assemblages were previously exposed to three different temperatures in order to find out whether temperature-driven changes in the DNA fingerprint of the bacterial community affected the number of attached cyprids (Paper III).

Publications and Contributions of Authors

2 Publications and Contributions of Authors

Paper I

Stratil SB, Neulinger SC, Knecht H, Friedrichs AK, Wahl M (2013) Temperature-Driven Shifts in the Epibiotic Bacterial Community Composition of the Brown Macroalga *Fucus vesiculosus*. *Microbiology Open* 2: 338-349

SBS, MW designed the experiments. SBS conducted the experiments and laboratory work. SBS, SCN, HK analyzed the data. HK preprocessed the pyrosequencing data, SCN performed the redundancy analysis and produced the RDA plots and Fig. 5, SBS wrote the paper. SCN, HK, AKF, MW commented on and made corrections to manuscript drafts.

Paper II

Stratil SB, Neulinger SC, Knecht H, Wahl M (**submitted manuscript**) Do Salinity and Irradiation Affect Compositional Traits of Epibacterial Communities on the Brown Macroalga *Fucus vesiculosus*?

SBS, MW designed the experiments. SBS conducted the experiments and laboratory work. SBS, SCN, HK analyzed the data. HK preprocessed the pyrosequencing data, SCN performed the redundancy analysis and produced the RDA plots and Fig.1. SBS wrote the paper. SCN, HK, AKF, MW commented on and made corrections to manuscript drafts.

Paper III

Nasrolahi A*, **Stratil SB***, Jacob KJ, Wahl M (2012) A Protective Coat of Microbes on Macroalgae: Inhibitory Effects of Bacterial Biofilms and Epibiotic Microbial Assemblages on Barnacle Attachment. *FEMS Microbiology Ecology* 81: 583-595

*Both authors contributed equally

SBS, AN, MW designed the experiments. SBS, KJJ, AN conducted the experiments and laboratory work. SBS, AN, KJJ analyzed the data. SBS, AN wrote the paper. MW commented on and made corrections to the manuscript drafts.

Paper I

**Temperature-Driven Shifts in the Epibiotic Bacterial
Community of the Brown Macroalga *Fucus vesiculosus***

ORIGINAL RESEARCH

Temperature-driven shifts in the epibiotic bacterial community composition of the brown macroalga *Fucus vesiculosus*

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Abstract

The thallus surface of the brown macroalga *Fucus vesiculosus* is covered by a specific biofilm community. This biofilm supposedly plays an important role in the interaction between host and environment. So far, we know little about compositional or functional shifts of this epibiotic bacterial community under changing environmental conditions. In this study, the response of the microbiota to different temperatures with respect to cell density and community composition was analyzed by nonculture-based methods (denaturing gradient gel electrophoresis and 454 pyrosequencing of the 16S rRNA gene). Redundancy analysis showed that despite high variability among host individuals temperature accounted for 20% of the variation in the bacterial community composition, whereas cell density did not differ between groups. Across all samples, 4341 bacterial operational taxonomic units (OTUs) at a 97% similarity level were identified. Eight percent of OTUs were significantly correlated with low, medium, and high temperatures. Notably, the family Rhodobacteraceae increased in relative abundance from 20% to 50% with increasing temperature. OTU diversity (evenness and richness) was higher at 15°C than at the lower and higher temperatures. Considering their known and presumed ecological functions for the host, change in the epibacterial community may entail shifts in the performance of the host alga.

Introduction

The important roles of surface-associated biofilms in transepidermal exchange processes and other interactions between host and environment have recently been described (Wahl et al. 2012). In the past years, studies of the "microbiome," that is, entire microbial communities in environmental samples, have become possible with the aid of high-throughput sequencing methods (Margulies et al. 2005). These studies have highlighted the diversity, specificity, and dynamics of these communities, for example, in air (Bowers et al. 2011), in water (Gilbert et al. 2012), in soil (Roesch et al. 2007), in sands (Gobet et al. 2012), and associated with hosts. Terrestrial host organisms such as humans (e.g., Costello et al. 2009) and trees (Redford et al. 2010) have been investigated. In the field

of biological oceanography, the number of studies which investigate the diversity and ecological role of complex bacterial communities associated with corals (Cárdenas et al. 2012), sponges (Webster et al. 2010; Lee et al. 2011; Jackson et al. 2012; Schmitt et al. 2012), polychaetes (Neave et al. 2012), ascidians (Behrendt et al. 2012), and macroalgae (Bengtsson et al. 2012) are steadily emerging. Marine macroalgae are known to carry diverse bacterial communities (reviewed in Hollants et al. 2013) which chemically interact with their hosts in harmful and beneficial ways (reviewed in Goecke et al. 2010). The hosts provide a rich source of carbons, for example, fucoidan, agar, and alginate that bacteria enzymatically degrade (reviewed in Goecke et al. 2010). The bacterial community on several species of brown, green, and red algae has been previously studied using fingerprinting, cloning, and

hybridization methods. These studies have shown that the community is quite species-specific (Lachnit *et al.* 2009), albeit with a high variability between individuals of one species (Tujula *et al.* 2010). Furthermore, the community is tissue-specific to some degree (Staufenberger *et al.* 2008) and significantly differs from the bacterioplankton in the surrounding water column (Bengtsson *et al.* 2010; Burke *et al.* 2011a).

The brown macroalga, *Fucus vesiculosus*, a foundation species in the Western Baltic Sea, has been subject of several studies focusing on its interactions with epibacteria. These studies have shown that the epibacterial community, while being species specific (Lachnit *et al.* 2009), varies seasonally (Lachnit *et al.* 2011). The intricate biofilm community has been shown to affect further settlement by bacteria and diatoms (K. Laufer and S. Alpert, unpubl. data) and to be involved in the repulsion of barnacle larvae (Nasrolahi *et al.* 2012), exemplifying possible beneficial functions these epibacteria may have for their host. The host alga itself has also been shown to chemically repel certain bacteria while attracting others (Saha *et al.* 2011), suggesting that certain members of the bacterial community may be detrimental to the host.

However, information on the detailed structure of the bacterial community on this ecologically important macroalga and how it responds to and possibly mediates environmental shifts is still lacking. Equipped with the tools of high-throughput sequencing, it is possible to take an in-depth look at the structure of microbial communities on marine macroalgae and investigate how they respond to abiotic factors. Temperature is an important abiotic factor in determining the growth of *F. vesiculosus* and possibly its associated bacteria. At elevated temperatures, the alga shows signs of stress (as indicated by weakened antifeeding defenses) (Weinberger *et al.* 2011) and microfouling pressure becomes more severe (Wahl *et al.* 2010; M. Fischer, pers. comm.). Considering these findings, we expect an increase of epibacteria on the thallus surface at higher temperatures. Temperature also deserves attention in experiments because at the sea surface it is predicted to increase in the Western Baltic by 0.5–2.5°C (Neumann and Friedland 2011) or even 4°C (BACC Author Team 2008) within the next century, respectively, depending on the model. This will possibly cause stress for the alga species and may restructure its epibacterial community.

In this study, we conducted a laboratory experiment in which *F. vesiculosus* was cultured at different temperatures. We sampled the alga's associated epibacterial community and sequenced the V1–V2 region of the 16S rRNA gene in order to analyze the bacterial community composition. We predicted that temperature will lead to shifts in the bacterial community composition and would increase the overall number of bacterial cells on the thallus.

Experimental Procedures

Sampling, experimental design, and setup

Twenty-five individuals of the brown macroalgal species *F. vesiculosus* were collected in the Kiel Bight, Western Baltic (54°27'N, 10°11'E), at the end of October 2009. They were transported to the laboratory in plastic bags within 2 hours of collection. Subsequently, each algal individual was placed in one 25-L aquarium. Algal individuals were incubated for 28 days at five different temperatures: 5, 10, 15, 20, and 25°C ($\pm 0.5^\circ\text{C}$). Replication per temperature level was five, resulting in 25 aquaria with independent bodies of water. In order to assure constant temperature levels, the five tanks of one temperature level were placed in larger water baths which were filled with water that was heated or cooled to the respective temperature. Water was circulated with water pumps (Aqua EL Circulator 350; Aquael GmbH, Erkrath, Germany) in the water baths and in the individual tanks in order to ensure homogeneously distributed temperatures. The experiment was run in a constant temperature chamber set at 15°C. We adjusted the higher temperatures (i.e., 20°C and 25°C) with three heating rods (Schego 600; Schemel & Goetz GmbH & Co KG, Offenbach am Main, Germany) per water bath. The lower temperatures (i.e., 5°C and 10°C) were achieved by circulating the water of the water bath through a cooler (Aqua Medic Titanium 1500; Aqua Medic GmbH, Bissendorf, Germany). The temperature levels were maintained constant throughout the experiment by temperature sensors (Aqua Medic T2001HC, Aqua Medic GmbH) that electronically controlled the heating rods; the water coolers had integrated temperature sensors. Water temperatures in the 25 experimental units were furthermore measured at least once a day with a standard digital thermometer to ensure controlled temperature levels, never deviating by more than 0.5°C. The temperature range in the experiment was chosen based on temperatures experienced by *F. vesiculosus* in its natural habitat and based on the prediction for warming of the Baltic Sea due to global change. Temperatures of up to 20°C are experienced by *F. vesiculosus* in its natural habitat during the course of the seasons (Figure S1). Water temperatures of 25°C are rarely reached in the Western Baltic, but can occasionally occur during the summer months in shallow waters (<50 cm) (K. Maczassek, unpubl. data). Light was supplied by metal halide lamps (250 W; 10,000–12,000 K) delivering $100 \pm 5 \mu\text{mol}/\text{m}^2/\text{s}$ at the thallus surface and a dark/light regime of 16:8. Water in the tanks had a salinity of 16 and was obtained by mixing 1/3 fjord water and 2/3 artificial seawater (Instant Ocean®, Blacksburg, VA) in order to dilute nutrients and thus reduce diatom growth. Artificial seawater was aged for 48 h before it was distributed to the

tanks. Water in the tanks was exchanged weekly. After 0, 7, 14, and 21 days of incubation, the bacteria on the algal surfaces were sampled (always on the day before the water in the tanks was exchanged) for analysis of community composition and after 28 days for quantification of bacterial cells. Two algal fronds per individual were softly rinsed for 10 s with sterile seawater in order to remove loosely attached particles including unattached biofilm components. Then the bacteria on approx. 15 cm² (visual estimation) per branch of young tissue were sampled with a sterile cotton swab (one swab per branch). The swab was placed in a 1.5-mL Eppendorf tube and frozen at -80°C until DNA extraction. Sampled algal fronds were marked with a colored thread in order to prevent resampling.

DNA extraction

DNA was extracted with the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol for buccal cotton swabs with an additional incubation step at 95°C for 8 min after the second lysis step of the protocol with buffer AL. DNA was eluted with nuclease-free water and stored at -20°C until further analysis.

Bacterial community composition via 16S rRNA gene sequence analysis

For a first rough overview of the bacterial community composition at different temperature levels and sampling times, we used the fingerprinting method "Denaturing Gradient Gel Electrophoresis" (DGGE) (Muyzer et al. 1993). We sampled the bacterial community at day 0, 7, 14, and 21 according to the procedure described above. Based on DGGE band patterns, the samples of day 0 (before temperature incubation) did not significantly differ. At day 7, 14, and 21 of temperature incubation, we detected significant differences in the epibacterial community composition by redundancy analysis (RDA, see below) (Figure S2A and B; Table S2; Figure 2). As the shifts were more pronounced after 14 days, indicating that the temperature-driven change was not finalized after 7 days, we chose the samples of day 14 for an in-depth look of the epibacterial community via barcoding and 454 pyrosequencing.

PCR-DGGE

The V3 region of 16S rRNA gene sequences of bacteria was amplified using the primer pair 341F- GC (5'-[CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G] CCT ACG GGA GGC AGC AG-3') and 534R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al. 1993) (Eurofins MWG Synthesis GmbH, Ebersberg, Germany).

Polymerase chain reaction (PCR) amplifications were prepared and subsequent DGGE was performed following the protocol in Nasrolahi et al. (2012) and references therein. Bands (presence or absence) of the DGGE fingerprint were called by the bare eye because we have found that this method is less error prone than band calling by computer software. DGGE band counts were treated as operational taxonomic units (OTUs) in subsequent RDA (see below).

454 pyrosequencing

Samples were prepared for pyrosequencing following the procedure described in Rehman et al. (2011). Briefly, fragments of ~450 base pairs (bp) of the V1-V2 hypervariable region of the 16S rRNA gene were amplified using the primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') with 454 Life Sciences (Roche, Penzberg, Germany) adapter B (not shown) and 338R (5'-TGCTGCCTCCCGTAG GAGT-3') with 454 Life Sciences adapter A (not shown). The reverse primer contained a multiplex barcode identifier sequence (10 bp) which allowed identification of individual samples. After PCR amplification in duplicates per sample, the DNA was purified and quantified (for details see Rehman et al. 2011). Equimolar amounts of DNA from the 25 samples were pooled and amplicon libraries were sequenced with a 454 GS-FLX pyrosequencer using the Titanium Sequencing Kit (Roche, Penzberg, Germany) at the Institute of Clinical Molecular Biology (ICMB), Kiel, Germany.

Denosing of sequence data

With the software PANGEA (Giongo et al. 2010), individual samples were sorted according to their barcode (Perl script command *barcode.pl*) and low-quality reads were removed from the data set using a Phred quality score of 20. Further denosing steps were done with the program mothur (Schloss et al. 2009). Sequences with homopolymers longer than 8 bp, with ambiguous bases, and with errors in the primer and barcode sequences were removed (*trim.seqs*). Barcodes and primers were removed before sequences were aligned (*align.seqs*) with the SILVA rRNA database (Pruesse et al. 2007) and screened for a minimum length of 200 nucleotides (*screen.seqs*). Vertical gaps in aligned sequences were removed (*filter.seqs*). Chimeric sequences were identified and removed using the *chimera.uchime* command. Sequences were binned at the 97% similarity level to form OTUs by average neighbor clustering.

Taxonomic assignment of OTUs

Reads were classified using the Greengenes 16S rRNA reference database (DeSantis et al. 2006) (*classify.seqs*) with

the following parameters: method = Bayesian, ksize = 8, iters = 1000, bootstrap cutoff = 60. Sequences identified as chloroplast sequences (44 reads) were removed from the data set before further analysis as these may have originated from the host algae and/or microalgae and do not represent bacteria. OTUs were linked with the results of the classification using the command (*classify.otu*). For comparison to the Greengenes classification, the 15 OTUs listed in Table 2 were also classified using the RDP (Ribosomal Database Project) Bayesian classifier (Wang et al. 2007).

1352 high-quality sequences per sample were randomly subsampled for calculation of diversity indices and for redundancy analysis.

These sequence data have been submitted to the NCBI Sequence Read Archive under accession No. SRX195663-SRX195687.

Redundancy analysis

RDA was used to analyze the OTU abundance data along the temperature gradient. RDA is a constrained linear ordination method. As opposed to unconstrained ordination methods (e.g., principal component analysis, PCA), which are used for exploratory analysis, constrained ordination explains the relationship between response variables (e.g., species; in our study: OTUs) and explanatory variables (environmental variables; in our study: temperature). Conceptually, RDA is a multivariate linear regression analysis followed by PCA (Borcard et al. 2011). RDA, which is based on Euclidean distances between samples, has been deemed inappropriate for analysis of community count data with many zeros. This is because the Euclidean distance between samples which do not have any species in common (as indicated by the zeros in data tables) is smaller than between samples with some species in common. However, transformations for use on community count data have been introduced which make the method suitable for RDA analysis (Legendre and Gallagher 2001). In the study at hand, OTU abundances were Hellinger transformed. This transformation down-weights highly abundant OTUs while avoiding overweighing of rare OTUs (as opposed to, e.g., the chi-square metric; see formula and discussion in Legendre and Gallagher (2001)). RDA was thus done on Hellinger distances between samples (i.e., Euclidean distance of Hellinger-transformed data). As RDA is a linear method, nonlinear OTU responses to temperature (T) may not be accurately represented by the analysis. An elegant solution to this problem is to use a second-degree explanatory variable (in our case, T^2) which models unimodal responses (Borcard et al. 2011). In order to assess the individual contribution of T and T^2 to the total variance variation

partitioning was done (Borcard et al. 2011). In this way, we were able to detect OTUs linearly correlated with temperature, as well as those showing a unimodal response to temperature. The latter are negatively correlated with the squared temperature term (cf. Borcard et al. 2011). OTUs were divided into three categories according to Pearson's correlation coefficient: positive correlation with T , negative correlation with T , and negative correlation with T^2 . All correlations within the same category with a coefficient of determination (R^2) of at least 0.15 were tested for significance, applying Benjamini–Hochberg correction to account for multiple testing. As an additional criterion to prioritize significant OTUs in the analysis, we took their vector lengths in the RDA correlation biplot: the longer the vector of an OTU, the higher its contribution to the set of constrained axes (i.e., the visualized variance) underlying the plot. Analyses were done with R (R Development Core Team 2012) using the *vegan* package for multivariate analysis of ecological communities (Oksanen et al. 2012).

Bacterial diversity

In order to assess to which degree bacterial diversity was captured, Good's coverage was calculated by dividing the number of OTUs that had been observed once by the total number of OTUs in the sample. OTU richness was plotted individually for all samples. Evenness, a measure of the homogeneity in relative abundance of the different OTUs making up the richness, is expressed by the Inverse Simpson Index. For statistical analysis of diversity measures, regressions were done in R. The Akaike information criterion (AIC) was used to test the relative goodness of fit for the regressions on temperature. It showed that the binomial model improved the fit over the linear model for the regression of OTU richness on temperature, whereas this was not the case for the regression of the Inverse Simpson Index on temperature (Table S3).

Quantification of epibacterial cells

In order to assess the effect of temperature on bacterial cell density, direct cell counts on the surface of the thalli at a magnification of 630 \times were conducted by epifluorescence microscopy (AxioImager.Z1, with a motorized Z-axis lifting table, Zeiss, Jena, Germany) from samples that had been incubated at the experimental temperatures for 4 weeks. We followed the protocol by Bengtsson et al. (2010), adjusting it to the needs of our algal material. 0.5 cm² of algal tissue (younger parts below the meristematic tips) per sample was stained with 0.2% (v/v) 4,6-diamidino-2-phenylindole (DAPI, Life Technologies

GmbH, Darmstadt, Germany) for 10 min ($n = 3$ per temperature level, except for 5°C where $n = 2$). Tissue samples were placed on glass slides, a drop of mounting medium (Roti®-Mount FluorCare DAPI, Roth, Germany) was applied, and cover slips were fixed with small pieces of adhesive poster tack. Due to the uneven microtopography of the thallus surface of *F. vesiculosus*, bacterial cells were present in several focal planes. Therefore, stack images (between 5 and 180 single layers of 0.2 μm distance, depending on the individual tissue) of 7–10 visual fields per sample were taken with a monochromatic camera (AxioCam MRm, Zeiss). Before hand counting the bacteria captured on the images, background noise, mostly due to autofluorescence of the host algae cells, was removed. The convolve filter (settings: [-1 -1 -1 -1 -1\ -1 -1 -1 -1 -1\ -1 -1 24 -1 -1\ -1 -1 -1 -1 -1 -1 -1 -1 -1 -1], max. intensity) in the software ImageJ (Schneider et al. 2012) removed most of the background noise. Denoised stacks were converted into Z-projects resulting in all layers combined in one single image. The image was overlaid with a digital counting grid of 50 μm^2 grid size (ImageJ plugin “grid”). As the number of cells on one single picture exceeded a reliably countable cell density, cells in 20 randomly predefined and evenly distributed squares (each 50 μm^2) per picture were counted. The mean number of cells per cm^2 was calculated.

Results

In a laboratory setup, we investigated the effect of temperature (5, 10, 15, 20, and 25°C) on the bacterial community composition ($n = 5$ per temperature level) after 14 days of incubation and on cell density ($n = 3$ per temperature level, except for $n = 2$ at 5°C) after 28 days of incubation. Since the time points of sampling differed for logistical reasons, we will not relate the bacterial cell densities to the pyrosequencing data and discuss them separately.

Pyrosequencing yielded 133,343 reads across all 25 samples. After the quality check 26% of reads were discarded; that is, 99,142 sequences with an average read length of 300 bp remained in the data set. A total of 4341 OTUs across all samples grouped at the 97% similarity level were identified. Good’s coverage ranged from 78% to 85% (Table S1) indicating that the entire diversity was not captured.

Effect of temperature on community composition

A significant effect of temperature on the bacterial community composition was found by RDA of the DGGE data based on the band pattern in the gel (Fig. 1) and the

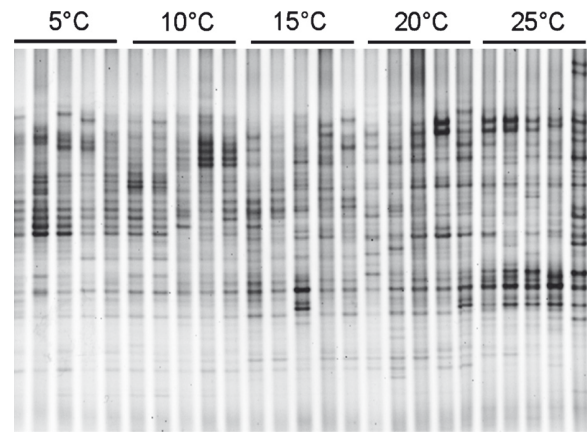


Figure 1. Denaturing gradient gel electrophoresis (DGGE) fingerprint of 16S rRNA gene sequences (V3 region) of epibacteria from *Fucus vesiculosus* cultured at different temperatures for 14 days (5, 10, 15, 20, and 25°C; $n = 5$ per temperature level).

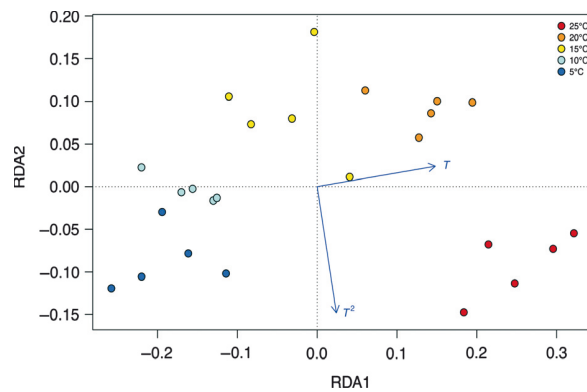


Figure 2. Redundancy analysis of the Hellinger-transformed presence absence data matrix based on bands of the denaturing gradient gel electrophoresis (DGGE) fingerprint of epibacteria from *Fucus vesiculosus* cultured at different temperatures for 14 days (5, 10, 15, 20, and 25°C; $n = 5$ per temperature level). Distance biplot showing relationships between samples (scaling 1) as depicted by the sample points and the temperature gradient vectors (T for linear relations and T^2 for unimodal relations).

454 pyrosequencing reads. Samples separated along the temperature gradient (Figs 2, 3A) as indicated by the distances between sample points which represent 2-D approximations of their Hellinger distances. The explanatory variable temperature (modeled by T and T^2) accounted for approx. 25% of variation in the DGGE data and 20% in the pyrosequencing data. Adding T^2 improved the fit of the model of the pyrosequencing data by explaining 4% of the variation (RDA Axis 2, Table 1). Some OTUs were significantly correlated with temperature as indicated by the angles between the lines of OTUs and the explanatory variable (Fig. 3B). One hundred and

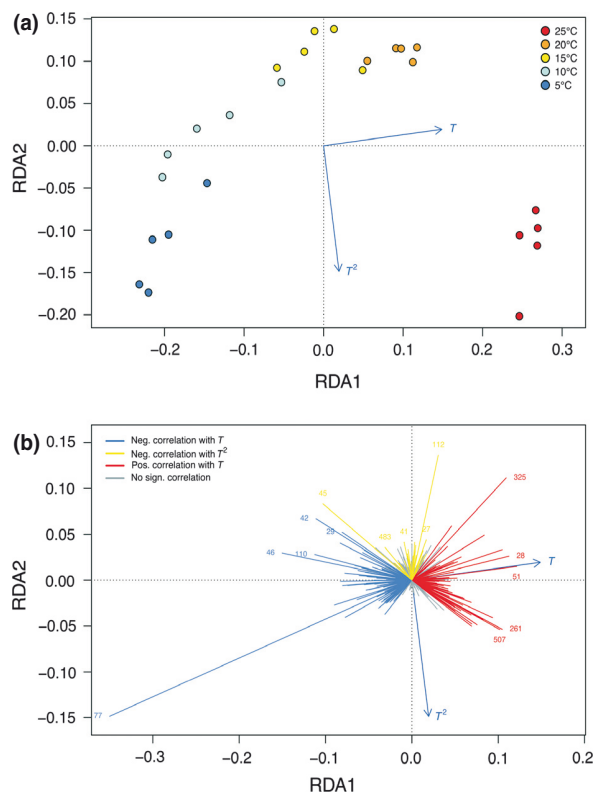


Figure 3. Redundancy analysis of the abundances of different bacterial groups (i.e., operational taxonomic units [OTUs]) associated with the macroalga *Fucus vesiculosus* incubated at different temperatures for 14 days (5, 10, 15, 20, and 25°C; $n = 5$ per temperature level). Data was obtained by 454 pyrosequencing. (a) Distance biplot showing relationships between samples (scaling 1) as depicted by the sample points and the temperature gradient vectors (T for linear relations and T^2 for unimodal relations). (b) Correlation biplot (scaling 2) showing lines for OTUs with highest species scores. OTU vectors pointing in the direction of the temperature vectors (T) correlate positively with the variable. OTU vectors pointing in the opposite direction of the T vector correlate negatively with the variable. OTUs correlating with T^2 point in the opposite direction of the respective T^2 vector (due to the negative quadratic term in the unimodal model). OTUs with a larger influence on the model are indicated by longer vectors, whereas OTUs with vector tips near the origin of the plot are of lesser importance.

Table 1. Amount of variation explained by temperature (T) and the squared temperature term (T^2) in the redundancy analysis of the Hellinger-transformed presence absence data matrix based on bands of the denaturing gradient gel electrophoresis (DGGE) fingerprint and of the pyrosequencing data from epibacteria associated with *Fucus vesiculosus* cultured at different temperatures for 14 days.

Data set	% Variation explained	
	T	T^2
Pyrosequencing	16	4
DGGE	22	3

eighty-four OTUs increased in abundance with temperature, 151 OTUs decreased in abundance with increasing temperature, and 30 OTUs showed a unimodal response to temperature indicating that they were most abundant at the intermediate temperature (15°C). The 15 OTUs with the strongest correlations, as indicated by vector length, are listed in Table 2 and shown in Fig. 3B. Of these, three were nearly absent (<5 reads) at the lower temperature levels (OTU # 325, 261, 507) and two were nearly absent at the high temperature levels (OTU # 77, 46) (Table S4). Correlated OTUs made up 8% of the total number of OTUs and ~61% of reads (averaged for the five samples of each temperature level). The cumulative relative abundances of OTUs that were negatively correlated with temperature decreased from 65% to 5% with increasing temperature. The ones that were positively correlated with temperature increased in abundance from 2% to 50% with increasing temperature. OTUs that were negatively correlated with T^2 (i.e., had the highest abundance at the intermediate temperature) increased from 5% to 12% and then decreased again to <5% (Fig. 4).

Diversity of the epibacterial communities

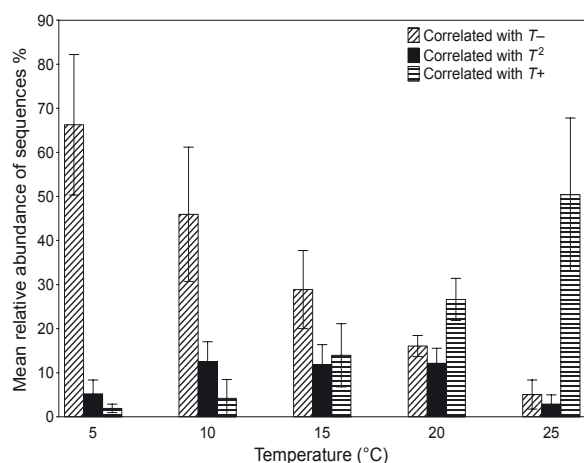
OTU richness was significantly higher at 15°C but variation in OTU richness between replicates of one temperature level was high resulting in a relatively low explanatory quality of the model (Fig. 5). The Inverse Simpson Index is an evenness index which takes OTU abundance into account; the higher the value the more balanced are the abundances of OTUs in the community. The lowest values were found in two of the 5°C samples indicating that they were dominated by a few taxa. Overall, the trend of higher evenness in the 15°C samples is also seen here, however, variation between the samples is high and the single highest evenness is found in a 25°C sample (Fig. 6).

Taxonomic classification

Ninety-two percent of the OTUs were classified into 21 phyla while ~8% of the OTUs could not be classified. At the phylum level, most of the OTUs were classified as Proteobacteria (~68%) and Bacteroidetes (~18%) across all samples. The other 19 phyla together made up ~6% of the reads. The class Alphaproteobacteria was the most abundant at all temperatures (65% at 5°C to 53% at 25°C, Fig. 7). At the family level, Rhodobacteraceae were the most abundant in all samples (at least 20%) and they more than doubled in abundance from 5°C to 25°C (Figure S3). The 15 OTUs that had the most influence on the compositional shifts driven by temperature were classified in the Greengenes and RDP databases. In the latter,

Table 2. The top 15 operational taxonomic units (OTUs) that were negatively correlated with T (abundant at low temperatures), negatively correlated with T^2 (abundant at medium temperatures), and positively correlated with T (abundant at high temperatures).

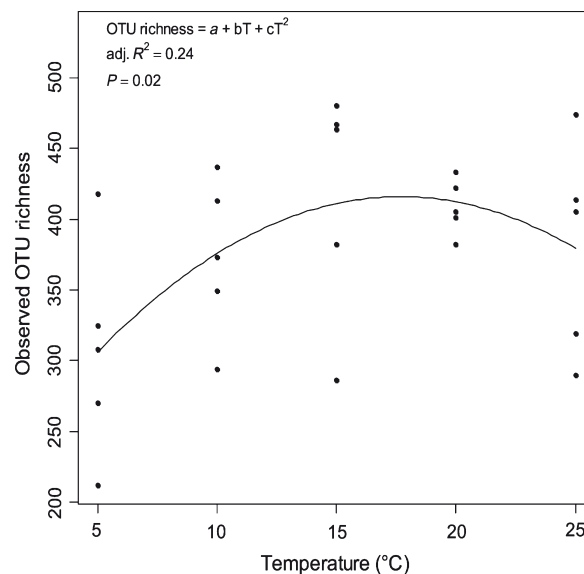
# OTU	Classification		Correlation with temperature			
	Greengenes	Ribosomal database project (sab score)	Neg.	Neg. T^2	Pos.	Vector length
77	Unclass. α -Proteobacteria	<i>Hellea balneolensis</i> (0.8)	x			0.54
46	Unclass. Proteobacteria	<i>Loktanela rosea</i> (0.94)	x			0.21
42	Unclass. Rhodobacteraceae	<i>Loktanela maricola</i> (0.95)	x			0.18
110	Unclass. α -Proteobacteria	Unclass. α -Proteobacteria	x			0.16
29	<i>Erythrobacter longus</i>	<i>Erythrobacter longus</i> (0.92)	x			0.14
112	Unclass. Myxococcales	Unclass. Myxococcales		x		0.20
45	Unclass. γ -Proteobacteria	<i>Granulosicoccus antarcticus</i> (0.96)		x		0.19
483	Unclass. Rhodobacteraceae	<i>Loktanela koreensis</i> (0.91)		x		0.07
27	Unclass. Loktanela	<i>Loktanela rosea</i> (0.94)		x		0.07
41	Unclass. δ -Proteobacteria	Unclass. Proteobacteria		x		0.06
325	Unclass. α -Proteobacteria	Unclass. α -Proteobacteria			x	0.22
51	Unclass. Rhodobacteraceae	Unclass. Rhodobacteraceae			x	0.17
261	Unclass. Rhodobacteraceae	Unclass. Rhodobacteraceae			x	0.17
28	Unclass. Rhodobacteraceae	Unclass. Rhodobacteraceae			x	0.16
507	Unclass. Rhodobacteraceae	Unclass. Rhodobacteraceae			x	0.16

**Figure 4.** Cumulative relative abundances of operational taxonomic units (OTUs) that were negatively correlated with temperature (correlated with T^-), positively correlated with temperature (correlated with T^+), and negatively correlated with the squared temperature term (correlated with T^2), that is, showing a unimodal response to temperature. Bars represent the mean of five replicates per temperature level and error bars represent $\pm 95\%$ CI.

classification at lower taxonomic levels (i.e., genera or species) could be obtained in some cases (Table 2).

Effect of temperature on cell density

The number of bacterial cells ranged from 1.5×10^7 to 1×10^8 cells per cm^2 of algal surface with an average cell density of 5×10^7 per cm^2 . Variability between replicates of one temperature level was high (Fig. 8).

**Figure 5.** Operational taxonomic unit (OTU) richness at different temperatures ($n = 5$ per temperature level).

Discussion

In order to assess the effect of temperature on the bacterial community composition associated with the thallus surface of the brown macroalga, *F. vesiculosus*, we chose the fingerprinting method DGGE as a way to give a first overview of potential community shifts. Even though DGGE has limitations (such as comigration of different phylotypes into one band (Sekiguchi et al. 2001), the method was suitable as a first and

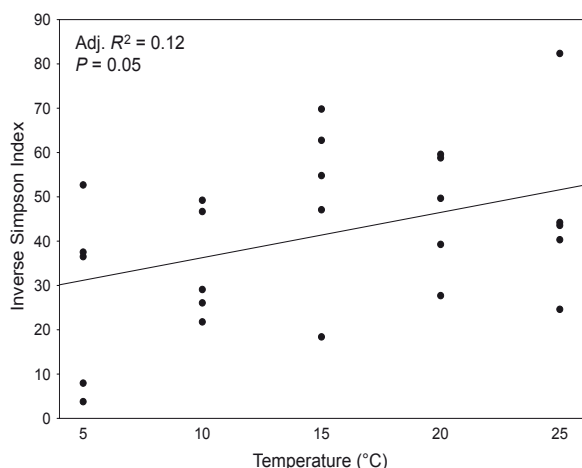


Figure 6. Evenness of operational taxonomic units (OTUs) at different temperatures: inverse Simpson index ($n = 5$ per temperature level).

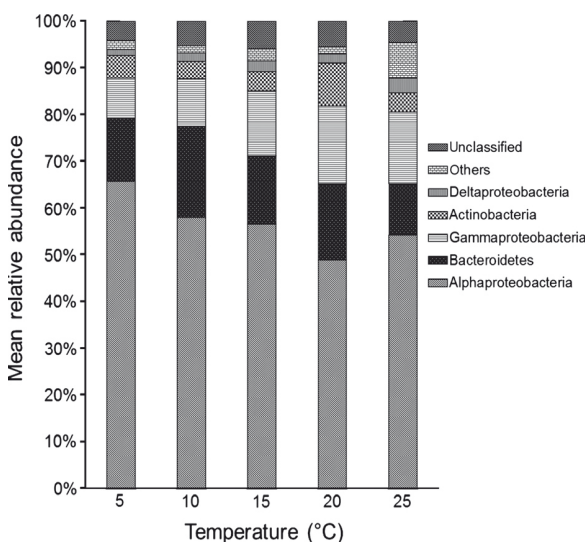


Figure 7. The top five phyla: relative abundances of operational taxonomic units (OTUs) (97% similarity). The phylum Proteobacteria is subclassified into Alpha-, Gamma-, and Delta proteobacteria. The rest of the phyla are compiled in "others."

conservative exploration of shifts that were due to the temperature treatment (as shown by redundancy analysis (RDA) explaining 25% of the variation). The more sophisticated method of 454 pyrosequencing confirmed the results based on DGGE by explaining a similar amount of variation (20%) in the data set by RDA. Despite the fact that different regions of the 16S rRNA gene were amplified (V3 and V1/V2, respectively) the results of both methods were strikingly similar. Based on these results, we find the combination of inexpensive DGGE fingerprinting for exploratory purposes followed by pyrosequencing for a more detailed look at the bac-

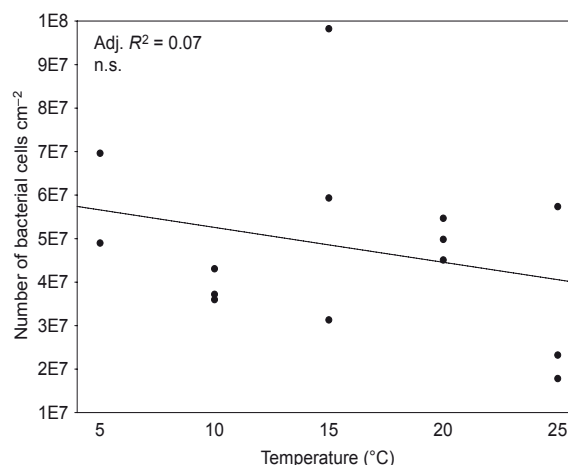


Figure 8. Number of 4,6-diamidino-2-phenylindole (DAPI) stained bacterial cells on the surface of *Fucus vesiculosus* after 28 days of temperature treatment. $N = 3$ except for $n = 2$ at 5°C.

terial community a suitable method for identifying the effects of environmental factors on the bacterial community composition.

Temperature accounted for 20% of the compositional differences in epibiotic bacterial communities on the host groups exposed to different temperatures. This leaves a large proportion of variation to be explained by factors not controlled in our experiment. As all other abiotic factors such as light and salinity were kept identical among treatment groups (as far as possible), biotic factors such as host alga physiology, as well as diverse interactions between the bacteria, host, and other members of the biofilm community may have covaried among treatment groups and somewhat blurred the direct temperature effect. It is likely that random processes also contributed to the variability in the community composition observed in our study. In studies on epibacterial communities on a green macroalga species, the authors observed high taxonomic diversity and variability of epibacteria (Tujula *et al.* 2010; Burke *et al.* 2011a) with a set of core functional genes (Burke *et al.* 2011b) on different algal individuals of the same species. The lottery hypothesis (Sale 1976) serves as an explanation for these observations, asserting that any bacterial taxon from within a functional guild can occupy space on the host alga if it gets there first (Burke *et al.* 2011a,b). Thus, it combines random and deterministic processes in explaining epibacterial community assembly.

At the higher taxonomic levels, Proteobacteria (namely Alpha-, Gamma-, Delta proteobacteria), Bacteroidetes, and Actinobacteria were the most abundant taxa found on *F. vesiculosus* in all temperature groups. These are very diverse groups and they are found in many different environments and in/on host organisms (Lachnit *et al.* 2011; Cárdenas *et al.* 2012; Jackson *et al.* 2012; Schmitt *et al.*

2012). Taxa that were positively correlated with temperature could not be classified any further than to family level and mostly belonged to the Rhodobacteraceae. While temperature had an influence on community composition, it did not significantly affect the density of epibiotic bacterial cells on the surface of *F. vesiculosus*. The observed high interindividual variability corresponds to findings from the brown alga species *Laminaria hyperborea* (Bengtsson *et al.* 2010). If *F. vesiculosus* regulates the absolute number of bacteria on its surface, the alga's capacity for control was not impaired by the temperature treatment. We had expected a direct and indirect influence of temperature on epibacterial density. Direct, because settlement rates, metabolism, and division rates of planktonic and epibiotic bacteria should increase with warming; indirect, because the host alga appears to perform best at intermediate temperature (Wahl *et al.* 2010; Weinberger *et al.* 2011). The indirect temperature effect might alter the microhabitat at the thallus surface as well as the host's capacity to chemically control microfouling. Interestingly, this was not the case. Either temperature does not affect these traits of host and epibacteria, or direct and indirect effects neutralize each other. These aspects await further investigations.

Barcoded pyrosequencing allows production of 16S rRNA gene sequence libraries much larger than those produced with Sanger sequencing (Margulies *et al.* 2005) for many samples in one run (Hamady *et al.* 2008). Despite the sophistication of this method there has been some concern that it overestimates diversity (Kunin *et al.* 2010). In order to minimize this effect, we used a stringent quality check and then binned the OTUs at a 97% similarity level (rather than at 98 or 99%). This is commonly done in studies (Lee *et al.* 2011; Schmitt *et al.* 2012) and has been suggested as an acceptable threshold by Kunin *et al.* (2010). Even though the quality check was rigorous, 4341 OTUs across all samples, that is, on 25 individuals of one alga species, were identified. This number is high when compared with the number of OTUs (also binned at a 97% similarity level) found on leaves of 56 terrestrial tree species (5476 OTUs), on 32 species of marine sponges (2567 OTUs) (Redford *et al.* 2010; Schmitt *et al.* 2012), and also on a kelp species (1108 OTUs) (Bengtsson *et al.* 2012). A direct comparison of OTU richness to most other studies is not easy, due to differences in the quality check of the sequence data, in sequence length, or in the different variable regions of the targeted 16S rRNA gene. Differences in these experimental factors can lead to differing diversity estimates (Engelbrektson *et al.* 2010). Despite this limitation, bacterial richness seems to be exceptionally high on *F. vesiculosus* (keeping in mind that the entire diversity may not even have been sampled as indicated by Good's

coverage, which did not reach 100%). The high OTU richness in our study is due to the large number of rare reads indicating that the "rare biosphere" (Sogin *et al.* 2006) makes up a large part of the epibacterial community on *F. vesiculosus*. It has been shown that members of the rare biosphere can become very abundant after disturbance (Sjoestedt *et al.* 2012). In our study, only 8% of the 4341 OTUs were significantly correlated with temperature, indicating that a large part of the bacterial community was unaffected by temperature, as far as their abundances are concerned. However, these 8% of OTUs made up 60% of reads on average. In a study by Bengtsson *et al.* (2012) on the diversity of kelp-associated bacteria, the community was also dominated by a few abundant OTUs. As evenness was low, the kelp blades were suggested to be "low-diversity habitats." While we observed the same pattern (dominance of a few OTUs), we conclude that algae surfaces can also be viewed as "conditional high-diversity habitats" as OTUs that are rare under certain conditions may become very abundant when conditions change, thus possibly contributing to interactions between host and microbiota. In our study, there were three OTUs (# 325, 261, 507; see Table S4) that were rare (defined as <5 reads) or absent at lower temperatures and more abundant in the higher temperature treatments. Inversely, two OTUs were abundant at lower temperatures and were rare at higher temperatures (# 77, 46; see Table S4). When a bacterial group practically disappears from the algal surface a potential ecological function may no longer be maintained – unless the vanishing species is replaced by a functionally similar one. If the replacing strain is functionally different, new interactions may arise. Despite substantial variation between replicates, the observation of maximum richness and evenness at 15°C is interesting. In this context, it is noteworthy that Nasrolahi *et al.* (2012) found that microbial assemblages (dominated by bacteria) harvested from the surface of *F. vesiculosus* had the highest repellent effect on barnacle larvae when the host algae were cultured at 15°C (as compared to 5 and 20°C). Epibacterial diversity was not assessed in the Nasrolahi study. Although our results unequivocally show that the epibiotic biofilms change in composition and diversity in response to warming, we do not know as yet how this reorganization affects ecological functions of the biofilm. Taxonomically different bacteria may form guilds whose members are functionally equivalent as has been shown before for the epibacterial community on a green macroalga (Burke *et al.* 2011b). The risk, however, exists that they do not.

High summer temperatures, that are expected to increase in the Western Baltic in the course of climate change (BACC Author Team 2008; Neumann and Friedland 2011), will likely cause stress for the macroalga whose

conspecifics in the North Sea grow best at 15°C (Lüning 1985) and whose chemical antifeeding defenses may be jeopardized (Weinberger et al. 2011). With increasing temperatures an increasing risk of disease associated with benthic organisms has been observed, for example, in corals (Bally and Garrabou 2007) and in the red alga *Delisea pulchra* (Case et al. 2011). In the latter study, a bacterium from the Roseobacter clade was identified as the pathogen that causes bleaching in the red alga *D. pulchra* (Case et al. 2011; Fernandes et al. 2011). The infections by this pathogen are observed more during the summer time and were shown to be more severe at a high temperature. The pathogen belongs to the family Rhodobacteraceae, a group that correlated positively with temperature in our experiment (Table 2). While this result is noteworthy, not all bacteria from the Roseobacter clade or from other members of the Rhodobacteraceae family are pathogens, so the risk of disease for *F. vesiculosus* at elevated temperatures cannot be discussed further at present. Numbers of other potentially pathogenic bacteria, such as Vibrionaceae and Alteromonadaceae, have also been correlated with increasing temperatures in corals and in seawater (reviewed in Wahl et al. 2012). In our study, members of the Vibrionaceae and Alteromonadaceae were present on the alga surface, albeit in extremely low numbers. Only one of over 4000 OTUs, an unclassified Alteromonadaceae, increased in abundance with increasing temperature but still was present only in very low number.

In this study, we have shown that temperature has a distinct effect on the community structure of epibacteria on the macroalga *F. vesiculosus*, but that there are still more factors that account for the observed variability between host individuals. Some of these factors that are deemed most important for the ecology of *F. vesiculosus* and its epibacterial community apart from temperature (such as salinity and irradiance) will be addressed in a subsequent study. The temporal dynamics of temperature-driven shifts will have to be investigated in long-term studies. Changing temperatures, as predicted for the future, may alter the bacterial communities and possibly their function, unless these communities are resilient and return to their original composition or are functionally redundant. Our study adds evidence that host interindividual variability in bacterial community composition is a widespread pattern, observed in humans, terrestrial plants, and several marine organisms. If the dominant members of the community that are affected by temperature also are the active and functionally important members of the biofilm community, a shift due to temperature may alter the interactions between bacteria and their macroalgal host and therefore possibly their interactions with macrofoulers and grazers. In the future, metagenomic and metatranscriptomic studies could shed light on these questions.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

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

Figure S1. Mean (circle in the center) water temperature at approx. 1.5 m water depth at the sampling site of *Fucus vesiculosus* during the course of 1 year (May 2009 to May 2010) with standard deviation (thick lines) and minimum and maximum values (thin lines).

Figure S2. Redundancy Analysis of the Hellinger-transformed presence absence data matrix based on bands of the DGGE fingerprint of epibacteria from *Fucus vesiculosus* cultured at different temperatures for 7 days (A) and 21 days (B) (5, 10, 15, 20, and 25°C; $n = 5$ per temperature level, except for $n = 4$ at 25°C). Distance biplot showing relationships between samples (scaling 1) as depicted by the sample points and the temperature gradient vectors (T for linear relations and T^2 for unimodal relations).

Figure S3. Relative abundances of OTUs at the family level. For clarity, only the ten most abundant families are individually depicted in the bars; the rest of the classified families are compiled in “others.”

Table S1. Good’s coverage in %. Mean of five replicates of each temperature level with standard deviation.

Table S2. Amount of variation explained by temperature (T) and the squared temperature term (T^2) in the redundancy analysis of the Hellinger-transformed presence absence data matrix based on bands of the DGGE fingerprint of epibacteria from *Fucus vesiculosus* cultured at different temperatures for 7 days and 21 days (see also Figure S2A and B).

Table S3. Statistical analysis of community diversity: Comparison of goodness of fit of regressions (AIC, Akaike Information Criterion) and statistical output.

Table S4. The top 15 OTUs: Number of cumulative reads of the respective OTU across all replicates ($n = 5$) of one temperature level (the number of total reads per temperature level was 6740).

Paper II

**Do Irradiation and Salinity Affect Compositional Traits of
Epibacterial Communities on the Brown
Macroalga *Fucus vesiculosus*?**

SUBMITTED MANUSCRIPT

Do Irradiation and Salinity Affect Compositional Traits of Epibacterial Communities on the Brown Macroalga *Fucus vesiculosus*?

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Abstract

Epibiotic biofilms have the potential to control major aspects of the biology and ecology of their hosts. Their composition and function may thus be essential for the health of the host. In two outdoor mesocosm experiments, we tested the influence of irradiance and salinity on the composition and irradiation effects on cell density of epibacterial communities associated with the brown macroalga *Fucus vesiculosus*. In the salinity experiment, non-living reference substrata (stones) were included. Alga individuals were incubated at six different irradiance levels (100% sunlight reaching the algal surface, 44%, 23%, 13%, 5%, 0%) and at three salinities (5, 19, 25) for ten and 14 days, respectively. Subsequently, the composition of their surface-associated bacterial communities was analyzed by 454 pyrosequencing of 16S rRNA gene sequences. Redundancy analysis revealed that the composition of epiphytic and epilithic communities significantly differed and were both affected by salinity, whereas epiphytic communities did not cluster according to irradiance. Epibacterial α -diversity was lowest at half-maximum irradiance and was significantly lower at salinity 5 but did not differ between substrata. The results indicate that irradiance and salinity are important in the structuring of alga-associated epibacterial communities with respect to composition and/or diversity. Whether direct effects or indirect effects via altered biotic interactions may have been responsible for the observed shifts is discussed.

Introduction

Irradiance and salinity are two among several major environmental factors determining the healthy state of the ecologically important brown macroalga *Fucus vesiculosus* (reviewed in Wahl *et al.*, 2011). They have been correlated with the vertical and horizontal distribution limits of one or several life stages of this macroalgal species in the Baltic Sea (Kautsky *et al.*, 1986, Torn *et al.*, 2006, Rohde *et al.*, 2008, reviewed in Wahl *et al.*, 2011, Alexandridis *et al.*, 2012.). Their influence on the complex epibacterial community associated with this macroalgal host (Lachnit *et al.*, 2009, Lachnit *et al.*, 2011) has to date not been explored by pyrosequencing. Since the surface of benthic organisms is the functional interface between host and environment (both biotic and abiotic), the bacteria-dominated microbial community inhabiting the surface can modulate interactions between them (reviewed in Wahl, 2008, Harder, 2009). The ecological role of epibiotic biofilms for marine organisms has been highlighted in Wahl *et al.*, (2012): Epibiotic bacteria have the potential to modulate the host's access to resources (light, nutrients, micronutrients, vitamins), to process waste products released by the host, to modify the impact of pollutants, to interfere with the host's communication via allelochemicals, to promote or inhibit infections by pathogens and parasites, or to modulate the micro- and macro-fouling pressure on the host's surface. Other roles, like the modulation of consumption pressure by grazers, are presumed but not yet proven. Given this important ecological role of epibiotic biofilms, it is crucial to know how composition (and function) of these biofilms on *F. vesiculosus* are controlled by the host and the environment. The former has recently been described to some extent, showing that *F. vesiculosus* produces antibiotic and probiotic metabolites (Saha *et al.*, 2011, Goecke *et al.*, 2012, Saha *et al.*, 2012, Lachnit *et al.*, 2013). In contrast, the effect of abiotic factors, which are highly variable in coastal ecosystems and are shifting in the course of climate change (BACC Author Team, 2008, Neumann and Friedland, 2011), on the epibacterial community is understudied. It has been shown before that a reduction of irradiance did not affect epibacterial densities at lower temperatures, but significantly decreased the number of epibacterial cells under higher temperatures and that shading furthermore affected the epibacterial community composition (Wahl *et al.*, 2010). However, the DNA fingerprinting technique applied in that study captured only a small part of the epibacterial community. Thus, our knowledge about the effect of different irradiance levels on community composition and diversity is incomplete at best. Our intention is to deepen our understanding of interactions between epibacterial communities and their environment along several lanes. First, we substantially improve the taxonomic resolution by using high-throughput sequencing (Margulies *et al.*, 2005). This allows the detection of a larger part of complex bacterial communities in environmental samples without laborious cloning steps (Huse *et al.*, 2008). Second, we will focus on the effects of low irradiance levels, since a reduction of irradiance has been shown to affect the epibacterial community (see above), to reduce the antimicrobial defense capacity of *F. vesiculosus* (Wahl *et al.*,

2010), and may be linked to past, present and future distributional shifts of this macroalga (Torn *et al.*, 2006, Rohde *et al.*, 2008). Finally, we will include salinity as a potential driver for structural change in epibiotic communities, since it has been identified as an important factor in structuring microbial communities (Lozupone and Knight, 2007), it determines the geographical distribution of most species in the Baltic, and it is expected to decrease over the coming decades in the Baltic Sea (BACC Author Team, 2008). Recently, a study on the effects of salinity on the bacterial community associated with the decomposition dynamics of dead specimens of *F. vesiculosus* in an estuarine ecosystem at the northwestern coast of Portugal using DNA fingerprinting methods has been published (Martins *et al.*, 2012). The authors found that the bacterial communities significantly differed along the estuarine salinity gradient (euhaline, mesohaline, and limnetic). To our knowledge, the effect of salinity on the entire epibacterial community of living *F. vesiculosus* attached to substratum has not been studied before using pyrosequencing methods.

In the study at hand, we conducted two outdoor mesocosm experiments in order to assess the impact of the abiotic factors irradiance and salinity on the epibacterial community associated with *F. vesiculosus*. We hypothesize that irradiance and salinity will drive shifts in the composition of epibacterial communities and that the number of bacterial cells will change when the host alga and associated biofilm are shaded.

Material and Methods

Individuals of the brown macroalgal species *Fucus vesiculosus* were collected in the Kiel Fjord, Western Baltic at 54°27'16"N, 10°11'54"E in March 2011 (for irradiance treatment) and at 54°23'53"N, 10°12'47"E in November 2011 (for salinity treatment). They were transported to the lab within two hours after collection and stored in outdoor tanks (25 L per replicate in the light and 1200 L per replicate in the salinity experiment, respectively) until commencement of the experiments. These were conducted in the vicinity of the GEOMAR institute in Kiel, Germany. Temperature was monitored in both experiments and was constant at 5°C and 8°C, respectively, throughout the experiments. Water in the tanks was exchanged after seven days in the salinity experiment, whereas we had a flow-through set-up in the irradiance experiment where water was exchanged at a flow rate of ca. 40 L per hour.

Irradiance Treatment

Altogether 30 algal individuals were used for experimentation in March 2011. They were distributed to 25 L tanks each and exposed to six levels of irradiance - 100%, 44%, 23%, 12%, 5%, and 0% sunlight reaching the algal surfaces - resulting in five replicates per irradiance level. Irradiation treatments were done by covering the tank with one to four layers of black mosquito gauze. Tanks with ambient conditions were not covered, and tanks with complete darkness were

covered with black plastic bags. Irradiance was monitored with HOBO® data loggers (Onset, USA).

Salinity Treatment

Altogether 12 algal individuals were used for experimentation in November 2011. They were distributed between three salinity levels (5, 19, 25) within the distributional range of *F. vesiculosus* in the Baltic Sea (Kalvas and Kautsky, 1993, Pereyra *et al.*, 2009) resulting in four replicates per salinity level. Salinity in the Kiel Fjord at the time of experimentation was about 19. A salinity of 25 is reached in the Western Baltic in the Kattegat area and 5 is near the lower salinity level of 4 that determines the distribution range of *F. vesiculosus* (Kalvas and Kautsky, 1993). The high salinity level was obtained by mixing fjord water and salt (Instant Ocean®, Blacksburg, VA); the low salinity level was obtained by diluting fjord water with sterile-filtered fresh water. Even though the number of bacterial colonizers was reduced by this process, approx. 1/4 of the volume of the tank (i.e., 300 L in the 1200 L tanks) constituted fjord water with the natural community composition of the colonizer pool present. Since composition of the bacterioplankton community is not altered by the dilution, we do not expect the dilution to affect the composition of the epibacterial biofilm as far as colonizing effects are concerned. Artificial seawater was prepared 48 hours before it was distributed to the tanks. Additionally, 12 stones (similar to the ones the algae attach to and about the size of a hand) were collected at the same sampling site as the alga individuals and were included in the experiment as non-living reference substrata. One stone was placed in one tank along with one algal individual in order to expose them to the same colonizer pool. Since they were placed together in one tank (1200 L), the samples were not independent in principle. We dealt with this limitation by checking if there were significant differences in the bacterial communities attributed to the pairing of algae and stones (i.e., being placed together in the same tank). We were able to eliminate the influence of salinity on the data by regressing the abundances of OTUs on the salinity terms (Salinity and Salinity²) and using the residuals of this regression instead of the OTU abundances for comparison of community composition. (Regressing OTU abundances on the Salinity term, models linear relationships and regressing on the Salinity² term models a unimodal relationship. For a more detailed explanation of OTUs and the Salinity terms, see redundancy analysis (RDA) below and references therein). The remaining influence of alga-stone pairs was tested as follows: We calculated pairwise Hellinger distances of epibacterial communities from the 121 alga-stone combinations and determined the point-biserial correlation with the pairing status of these substrates (1 for 'paired', 11 instances; 0 for 'unpaired', 110 instances). Significance *p* of the correlation coefficient *r* was determined in a permutation tail probability test by randomly assigning pairing status to substrate combinations and calculating the randomized correlation coefficient *r** 999 999 times (The overall number

possible permutations is 5×10^{22}). P was calculated as $(\sum(|r^*| \geq |r|) + 1) / 1\,000\,000$ (numerator, number of absolute r^* values equal to larger than the original absolute r value, including the latter; denominator, total number of values). This two-sided test resulted in $p = 0.451$, meaning that the correlation coefficient was not larger or smaller than one obtained by chance. Thus, differences between the communities from paired alga and stone were not larger or smaller than pairs from different tanks, and the samples were treated as independent in the statistical analysis.

Sampling of Epibacterial Community

Two algal fronds per individual were rinsed for 10 s with sterile seawater in order to remove loosely attached particles. The bacteria on approx. 15 cm^2 (visual estimation) per branch of young tissue were sampled with a sterile cotton swab (one swab per branch). We used a stencil of 15 cm^2 to swab the surface of each stone. Each swab was placed in a 1.5 ml Eppendorf tube and kept on ice until transportation to the lab, where it was frozen at -80°C until DNA extraction. Sampled algal fronds were coded with small cable ties in order to prevent resampling.

454 Pyrosequencing, Preprocessing of Reads, and Classification

DNA extraction and 454 pyrosequencing was done following the protocol in Stratil *et al.*, (2013) and references therein, differing only in the number of cycles in the PCR (30 cycles for the samples of the irradiance treatment and 35 cycles for samples of the salinity treatment). Briefly, fragments of ~ 450 base pairs (bp) of the V1-V2 hypervariable region of the 16S rRNA gene were amplified. After amplification equimolar amounts of DNA from the 30 samples of the light experiment and 24 samples of the salinity experiment (12 algae and 12 stones), respectively, were pooled and amplicon libraries were sequenced with a 454 GS-FLX pyrosequencer using the Titanium Sequencing Kit (Roche, Penzberg, Germany) at the Institute of Clinical Molecular Biology (ICMB), Kiel, Germany. Denoising of sequence data, grouping of OTUs (operational taxonomic units) at 97% sequence similarity, and taxonomic assignment of OTUs in the Greengenes 16S rRNA reference database (DeSantis *et al.*, 2006) was done following the steps in Stratil *et al.*, (2013). In the irradiance experiment, 69 OTUs were classified as microalgal chloroplasts (mainly Stramenopiles) and three OTUs were unclassified chloroplasts. In the salinity experiment, 113 OTUs were classified as microalgal chloroplasts (mainly Stramenopiles) and 46 OTUs were unclassified chloroplasts. We included these OTUs in the analysis because there were interesting differences in the relative abundances of microalgal OTUs on *F. vesiculosus* and on stones in the salinity experiment. Most of the reads classified as microalgal chloroplasts were found in the epilithic microbial communities, excluding the possibility that they originated from host algal chloroplasts. While there was no comparison with reference substrata in the irradiance experiment, we still included these microalgal OTUs since they made up only a small fraction of

the total number of reads (<5% independently of irradiance treatment).

We randomly subsampled 1 024 (irradiance treatment) and 2 024 (salinity treatment) high-quality sequences per sample for calculation of diversity indices and for redundancy analysis. Sequences were submitted to the sequence read archive of NCBI under accession numbers SRX204294-SRX204322 (irradiance) and SRX206342-SRX206364 (salinity).

Redundancy Analysis (RDA)

We applied RDA to analyze OTU abundances along the irradiance and salinity gradients. RDA is a linear constrained (canonical) ordination method. As opposed to unconstrained ordination methods (e.g., PCA), which are used for exploratory analysis, constrained ordination explains the relationship between response variables (e.g., species; in our study: OTUs) and explanatory variables (in our experiments: irradiance, salinity, and substrate i.e., alga vs. stone, respectively). For details on RDA for the analysis of epibacterial communities on *F. vesiculosus* and for its utility to analyze OTUs with a nonlinear distribution along the environmental gradient, see Stratil *et al.* (2013) and references therein. We performed variation partitioning to assess the individual contribution of salinity, the squared salinity term, and substrate to total variance. Adjusted R^2 (variance proportions) of individual descriptors controlled for the effects of all other terms were determined (Borcard *et al.*, 2011). Since RDA did not reveal any significant differences between samples of the irradiance treatment, we refrained from analyzing OTU data of this experiment in greater depth. OTUs of the salinity experiment were divided into five categories according to Pearson's correlation coefficient: positive correlation with salinity, negative correlation with salinity, negative correlation with Salinity², positive correlation with algae as substrata and positive correlation with stones as substrata. Correlations within the same category with a coefficient of determination (R^2) of at least 0.15 were tested for significance applying Benjamini-Hochberg correction to account for multiple testing. As an additional criterion to prioritize significant OTUs in the analysis, we used their vector lengths in the RDA correlation biplot: the longer an OTU's vector, the higher its contribution to the set of constrained axes underlying the plot. Analyses were done with R (R Development Core Team, 2012) using the vegan package for multivariate analysis of ecological communities (Oksanen *et al.*, 2012).

Bacterial Diversity

For statistical analysis of diversity measures in the irradiance experiment, a regression was done. OTU richness was plotted individually for all samples. Evenness, a measure of the relative abundance of the different OTUs making up the richness, is expressed by the Inverse Simpson index, a dominance index that is sensitive to the most common OTUs. The values for the Inverse Simpson Index were square root-transformed for normally distributed residuals. The Akaike

Information Criterion (AIC) was used to assess the relative goodness of fit for the regressions on irradiance. The lowest value (indicating a better fit) was found with the binomial model (Table S1, page 90). The effect of the continuous predictor salinity on epiphytic and on epilithic bacterial diversity could not be modeled at the same time in an ANCOVA because the requirement for the same relationship between epiphytic and epilithic bacterial diversity along the continuous predictor salinity were not given. Therefore, in order to statistically compare diversity between salinity levels and substrata a two-factorial ANOVA was done in R with salinity as a categorical factor. OTU richness and evenness were log-transformed for normal distribution according to the Shapiro-Wilk test, and homogeneity of variances was confirmed with the Fligner-Killeen test.

Epibacterial Cell Density

In order to assess the effect of irradiance on bacterial cell density, direct cell counts on the surface of the thalli were conducted by epifluorescence microscopy. We followed the protocol in Stratil *et al.*, 2013 and references therein. We analyzed samples from four of the six irradiance levels (100%, 44%, 23%, 0% sunlight reaching the algal surfaces). Replication was 5 per irradiance level, except for $n = 4$ at 23% irradiance where one replicate was lost due to insufficient image quality. The relationship between the mean number of cells per cm^2 and irradiance was assessed by linear regression.

Results

Effects of Irradiance

We identified 3 251 OTUs grouped at the 97% sequence similarity level. RDA revealed that samples did not significantly cluster along the gradient of irradiance (data not shown).

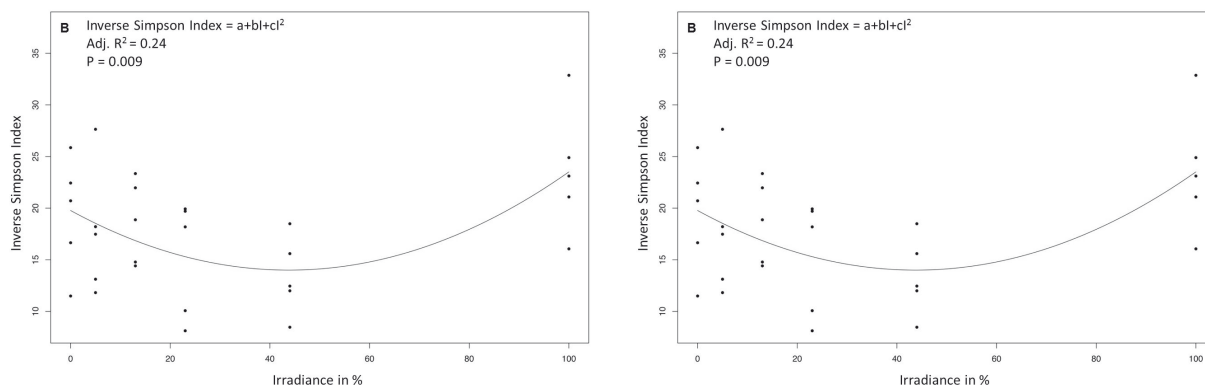


Fig. 1 Diversity of epibacterial communities associated with the macroalgal host *Fucus vesiculosus* cultured at different irradiance intensities for ten days. ($n = 5$, except for $n = 4$ at 44% sunlight). **A** OTU richness (operational taxonomic units grouped at 97% sequence similarity). **B** Evenness (Altogether, 72 OTUs were classified as chloroplasts of a total of 3 251 OTUs).

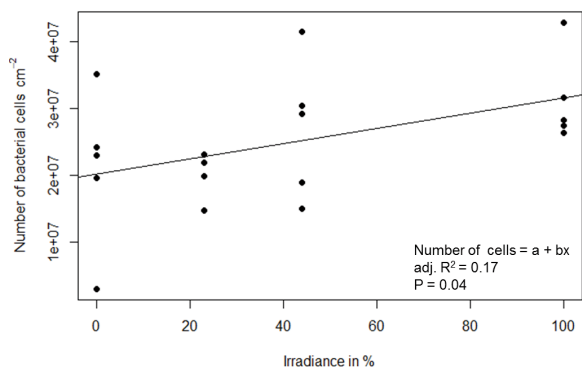


Fig. 2 Number of DAPI-stained bacterial cells on the surface of *Fucus vesiculosus* incubated at different irradiance levels for ten days ($n = 5$, except for $n = 4$ at 23% sunlight).

Furthermore, microalgal chloroplasts were detected at all irradiance levels making up $< 5\%$ of the sampled communities (Fig S1, page 90). Cell density ranged from approx. 3×10^6 to 4×10^7 per cm^2 and, on average, shading by 77% decreased cell density by about 36%, however variability between individuals of one treatment level was high (Fig. 2). The regression shows a weak linear decline in cell density with shading, however, the model is largely driven by one sample at 0% sunlight, where cell densities were one magnitude smaller than in the other samples ($R^2 = 0.17$, $p = 0.04$) (Fig. 2).

Effects of Salinity

OTUs clustered along the salinity gradient and by substrate (distances between sample points are 2-D approximations of their Hellinger distances (Fig. 3)). RDA revealed that the explanatory variable salinity and the factor substrate (alga vs. stone) accounted for 18.8% and 17.5% of

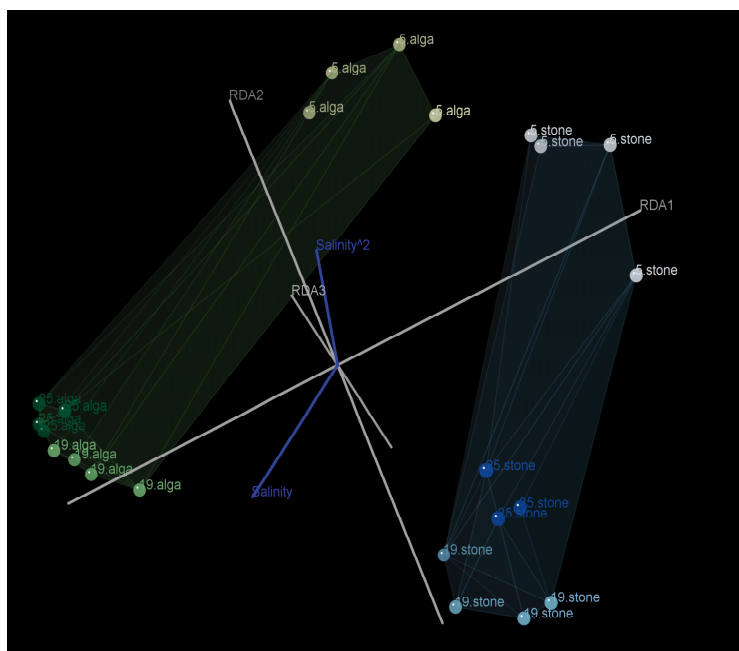


Fig. 3 Redundancy analysis of the abundances of different bacterial groups (operational taxonomic units at 97% sequence similarity) associated with the macroalga *Fucus vesiculosus* and with non-living reference substrata (stones collected in the vicinity of the algal specimens) incubated at different salinities for 14 days ($n = 4$, except for $n = 3$ on stones at salinity 25). Data were obtained from 454 pyrosequencing of the V1/V2 region of 16S rRNA gene sequences. Distance biplot (Euclidean distances of Hellinger-transformed OTU abundances) illustrating relationships between sample points as depicted by the sample points and the salinity gradient vectors (Salinity for linear relations and Salinity^2 for unimodal relations).

OTU richness and evenness were lowest at 44% ambient sunlight and significantly increased towards lower and higher irradiance levels ($R^2 = 0.26$, $p = 0.007$; $R^2 = 0.24$, $p = 0.009$, respectively) despite a wide scattering between host individuals (Fig. 1A and B). Overall, OTUs with the highest relative abundances were classified as Bacteroidetes (38-45%) and Alphaproteobacteria (49%), followed by Gammaproteobacteria and Actinobacteria, (each $\leq 5\%$) (Fig S1, page 90).

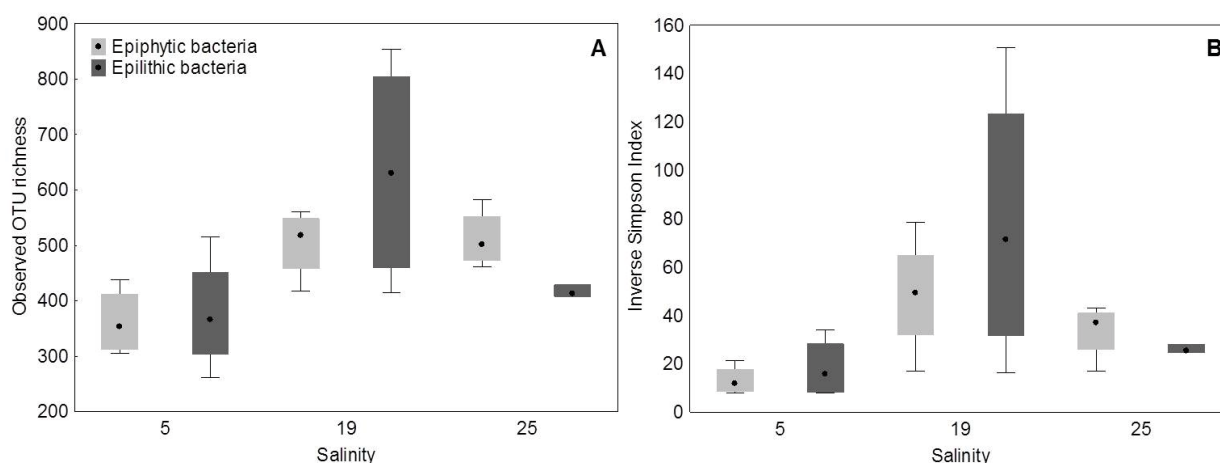


Fig. 4 Diversity of epiphytic (from *Fucus vesiculosus*) and epilithic bacterial communities cultured at different salinities for 14 days. (n = 4, except for n = 3 for epiphytic microbial communities at salinity 25), **A** OTU richness. **B** Evenness. Box and whiskers depict median, 25%-75% percentiles, and min max. (Altogether, 159 OTUs were classified as chloroplasts of a total of 2 494 epiphytic OTUs and 2 885 epilithic OTUs).

the variation in the bacterial community composition, respectively (Fig. 3). Adding Salinity² allowed detection of OTUs that showed a unimodal response to salinity and improved the model fit by explaining 2.5% of the total variation. Salinity significantly affected OTU richness and evenness. Bacterial α -diversity was lowest at salinity 5 despite substantial variability among replicates, regardless of substrata (Fig. 4 A and B, for results of two-factorial ANOVA see Table 1). The most striking differences due to salinity in epibacterial community composition were found in the low salinity samples on algae and stones alike: here, members of the subphylum Betaproteobacteria made up 30% and 15% of the community, respectively, whereas they were nearly absent (<1%) at the other salinities (Fig. 5). Furthermore, on both substrata, members of the Gammaproteobacteria and of the Actinobacteria were on average at least twice as abundant at the higher salinities than at the low salinity (Fig. 5). The main differences between OTU abundances on algae and stones were due to members of the phylum Cyanobacteria. They were nearly absent in the epiphytic bacterial community but were present on stones where they made up at least 8%

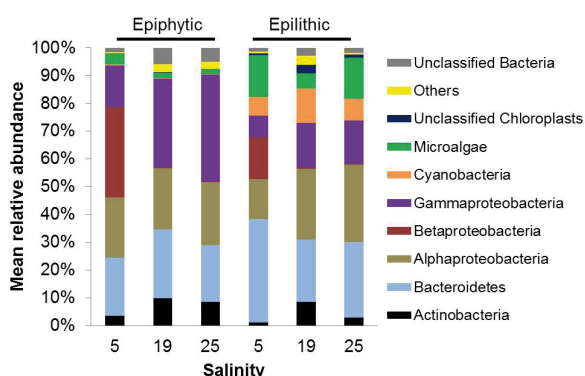


Fig. 5 OTUs (operational taxonomic units grouped at 97% sequence similarity) with the highest mean relative abundances at the phylum and subphylum-level, respectively. Microalgal chloroplast OTUs are included. OTUs with abundances < 1% are compiled in „others“ (n = 4 per salinity level, except for n = 3 for epiphytic microbial communities at salinity 25).

(Fig. 5) of the community. Furthermore, mean relative abundances of members of the subphylum Gammaproteobacteria were at least twice as high on macroalgal hosts than on stones. On average, more microalgal chloroplasts were detected in the epilithic microbial community compared to the epiphytic microbial community (Fig. 5). Altogether, we identified 2 494 OTUs on algae and 2 885 on stones. Of these, 852 (18.8%) were shared among substrata (i.e., detected on at least one

replicate of each substratum) and five (0.1%) bacterial OTUs were found in all samples (i.e., the “core microbiome” in its strictest sense). Altogether, the abundances of 136 (approx. 5% of total) OTUs on algae and 106 (approx. 4% of total) OTUs on stones correlated significantly with salinity (positively, negatively, and negatively with the squared salinity term). Furthermore, OTUs that significantly correlated with substrate type were identified. Of these, those with the strongest influence on the observed patterns determined by correlation strength and vector length are listed in Table 2.

Table 1 Results of the two-factorial ANOVA of epibacterial diversity (operational taxonomic units were grouped at 97% sequence similarity) after 14 days of salinity treatment

ANOVA	OTU richness					Evenness				
	Df	Sum Sq	Mean Sq	F	p	Df	Sum Sq	Mean Sq	F	p
Substrate	1	0.001	0.001	0.016	0.902	1	0.079	0.079	0.199	0.661
Salinity	2	0.709	0.355	8.086	0.003	2	6.723	3.361	8.480	0.003
Substrate*Salinity	2	0.143	0.072	1.631	0.225	2	0.259	0.130	0.327	0.725
Residuals	17	0.746	0.044			17	6.739	0.396		

Table 2 OTUs (operational taxonomic units grouped at 97% sequence similarity) that had the most influence in the redundancy analysis and were negatively correlated with salinity (abundant at low salinity), positively correlated with salinity (abundant at high salinity), negatively correlated with the squared salinity term ($Salinity^2$) (unimodal distribution i.e., highest abundances at the intermediate salinity), and positively correlated with substrate after 14 days of salinity treatment.

OTU #	Substrate	Correlated with	Classification (Greengenes)	R^2 ($p < 0.05$)	Vector length
394	alga	S+	unclass. Gammaproteobacteria	0.80	0.298
22	alga	S-	unclass. Hydrogenophaga	0.88	0.524
34	alga	S-	unclass. Alphaproteobacteria	0.82	0.235
86	alga	S-	unclass. Hydrogenophaga	0.78	0.208
31	alga	S-	unclass. Maribacter	0.74	0.143
670	alga	S ² -	unclass. Lacinutrix	0.79	0.028
161	stone	S+	unclass. Rhodobacteraceae	0.82	0.129
2763	stone	S+	unclass. Gammaproteobacteria	0.6	0.248
584	stone	S+	unclass. Saprospiraceae	0.46	0.133
86	stone	S-	unclass. Hydrogenophaga	0.90	0.156
22	stone	S-	unclas. Hydrogenophaga	0.76	0.318
31	stone	S-	unclass. Maribacter	0.51	0.274
455	stone	S ² -	unclass. Gammaproteobacteria	0.62	0.039
394		alga	unclass. Gammaproteobacteria	0.62	0.24
39		alga	unclass. Gammaproteobacteria	0.77	0.212
38		alga	unclass. Gammaproteobacteria	0.82	0.176
77		alga	unclass. Acidimicrobiales	0.83	0.145
1116		stone	unclassified Chroococcales	0.45	0.203
31		stone	unclas. Maribacter	0.41	0.149
1577		stone	unclass. Stramenopiles	0.43	0.119
1552		stone	unclass. Leptolyngbya	0.42	0.119

Discussion

In this study, we assessed the influence of irradiance and salinity on host-associated bacterial microbiota in two separate experiments. While both experiments aim at revealing structural shifts in the epibacterial community on *Fucus vesiculosus* using similar methods and response variables (community composition and diversity), they differ in some parts of their experimental design and set-up. We will thus discuss the results of these two experiments without making direct comparisons between the datasets.

Surprisingly, the composition of bacterial communities from different algal individuals

did not significantly group by irradiance, i.e., beta diversity (OTU turnover) between treatment levels was not larger than between host individuals. However, we detected significant differences between communities exposed to different levels of irradiance in terms of OTU richness as well as evenness (expressed as the Inverse Simpson Index). OTU richness differed on average by ca. 5% and 15% between half-maximum vs. lower and maximum sunlight intensity, respectively. Yet, this shift was too inconsistent among host individuals to allow recognition of significant compositional differences between treatment levels. While we detected no significant difference in β -diversity due to irradiance effects, there were pronounced compositional differences due to salinity. In both experiments we observed a highly diverse algae-associated bacterial community with a total of 3 179 (irradiance treatment in spring) and 2 424 (salinity treatment in fall) OTUs at 97% sequence similarity (excluding OTUs that were classified as microalgal chloroplasts). Variability of the bacterial microbiome between replicate host individuals was high, confirming previous results from epibacterial communities on macroalgae (Tujula *et al.*, 2010, Lachnit *et al.*, 2011, Bengtsson *et al.*, 2012, Stratil *et al.*, 2013). Another recurrent pattern in host-associated bacterial communities is the presence of many rare OTUs and only few highly abundant OTUs (e.g., Webster *et al.*, 2010, Bengtsson *et al.*, 2012, Jackson *et al.*, 2012, Schmitt *et al.*, 2012, Stratil *et al.*, 2013). In our experiment we have shown that irradiance and salinity affect community composition and/or bacterial richness. There are two general pathways of how epibacteria can be affected by environmental abiotic factors – a) directly by environmentally induced physiological changes and b) indirectly via altered biotic interactions. The latter could be environmentally induced differences in host physiology e.g., the amount or kind of exuded carbons and/or host anti- and profouling metabolites which in turn can affect epibacteria (reviewed in Goecke *et al.*, 2010, Wahl *et al.*, 2012). Furthermore, altered interactions between members of the epibacterial community, such as communication -typically by quorum sensing-, metabolic cooperation or competition (Elias and Banin, 2012) could cause compositional shifts. It is possible that grazers (micro- and macroscopic) and macrofoulers also play a role in the interaction shifts. The effect of macroscopic grazing and fouling organisms can be ruled out because they were not present on the algae specimens during experimentation. Indications for direct or indirect mechanisms leading to shifts in community composition will be discussed in the following.

We had expected to see that shading would in a direct way lead to clear shifts in community composition as has been shown before for the bacterial community of this alga species (Wahl *et al.*, 2010). There, the relative abundance of phototrophs (Cyanobacteria), which are a group likely to be directly affected by irradiance since they obtain energy from light, was significantly reduced, and the proportion of Gammaproteobacteria increased with shading. This pattern was not confirmed in our study, where the relative abundances of phyla were very similar between irradiance levels, only slightly differing in the amount of Bacteroidetes which were slightly less

abundant (by 5%) at the half-maximum irradiance level. Comparison with the above cited study is limited due to different DNA extraction protocols and analyses. A biological explanation, however, for the different observations could be seasonal variation in the bacterial community composition present on the alga surface (Bengtsson *et al.*, 2010, Tujula *et al.*, 2010, Lachnit *et al.*, 2011). The sensitivity to irradiance of OTUs making up a community in spring may differ from those in a community in fall. Indeed, in a survey conducted in two consecutive years, Cyanobacteria were more abundant on *F. vesiculosus* in summer than in winter and largely contributed to the seasonal shifts observed in bacterial community composition (Lachnit *et al.*, 2011). Thus, we may have observed a change in the abundance of phototrophs when conducting the experiment during a different season with many Cyanobacteria present. In our study, bacterial cell density decreased with shading, however, the linear decline in cell densities with shading is largely due to very low cell densities in one sample at 100% shading. Overall, average bacterial cell density was highest at 100% sunlight, however, the variability between cell densities of individuals from one treatment level was high. In a previous study on bacterial cell density on *F. vesiculosus*, shading did not affect epibacterial densities at lower temperatures; however, under warmer conditions, shading significantly decreased their numbers (Wahl *et al.*, 2010). Thus, a single stressor (in this case low irradiance) can be without effect under certain conditions but may act differently coupled with another abiotic stressor (e.g., elevated temperature) (Wahl *et al.*, 2010). These findings make a case for multifactorial experiments investigating the host-associated microbiome. The generally very high bacterial numbers on *F. vesiculous* in comparison to many other algal species (see examples listed in Wahl *et al.*, 2012) let us assume that this alga is a particularly rich habitat for bacteria.

The observed pattern in epibacterial diversity may be in part explained by host defense. Antimicrobial defense of *F. vesiculosus* relies on at least three different compounds (Saha *et al.*, 2012, Lachnit *et al.*, 2013) and is strain-specific in many cases (Saha *et al.*, 2011, Goecke *et al.*, 2012, Saha *et al.*, 2012). Therefore, it is conceivable that shifts in the defense cocktail may have affected strains differently resulting in lower taxonomic diversity at about the half-maximum sunlight level and also in lower cell numbers at 23% sunlight. Specific bacterial taxa may have been up- or down-regulated by host-exuded metabolites accumulating in the thallus boundary layer (Grosser *et al.*, 2012). However, a final answer to this question cannot be given at the moment, since our results do not confirm causation between host defense and epibacterial community composition and cell densities.

In the salinity experiment, we included non-living reference substrata in the study. The comparison between bacterial communities on living hosts (potentially chemically defended) and non-living substrata (undefended) can separate the direct effects of abiotic factors vs. indirect effects via biotic interactions. On both substrata, salinity significantly affected epibacterial community composition as revealed by RDA. On both substrata the bacterial taxon that on average was more prevalent at the low salinity was Betaproteobacteria and the taxon that was less abundant

was Gammaproteobacteria, respectively. Furthermore, epibacterial diversity was lowest at the low salinity level. The OTUs that were most abundant at the low salinity level were the same ones on algae and on stones. These results indicate that both, epiphytic and epilithic bacterial communities, were directly affected by salinity rather than indirectly via salinity-driven shifts in algal physiology. However, OTUs with the highest relative abundances at salinity 19 and 25 were different ones on algae than on stones and different OTUs correlated with either algae or stones (Table 1). These observed differences could be due to the specificity of epiphytic and epilithic bacterial communities which shared only about one fifth of OTUs. Salinity could directly influence bacterial taxa that are present on one substrate but not on the other. Alternatively, the differences could be explained by indirect salinity effects via alterations in the biotic interactions between host alga and/or within the biofilm community. The bacterial communities on two stone samples had conspicuously more OTUs at salinity 19 that were also less evenly distributed than in the other samples, which is reflected in the greater distances between these samples in the RDA. This was not observed in the epiphytic bacterial community. Possibly, at salinity 19, the salinity in the Kiel Fjord at the time of experimentation, the alga may have a stabilizing effect on the epibacterial community composition. Interestingly, we also observed a lower proportion of Cyanobacteria and microalgal chloroplasts on *F. vesiculosus* than on stones. The microalgal chloroplasts were mainly classified as Stramenopiles. Based on microscopic examination, these microalgal chloroplasts are likely from diatoms, a microalgal taxon within the Stramenopiles. This indicates that the host algae are well defended against Cyanobacteria and microalgal foulers, regardless of salinity. Possibly, this defense is assisted by the specific epibacterial community on *F. vesiculosus*. Considering the high taxonomic diversity, there are multiple biotic interactions within the highly complex epibacterial community that could in turn have effects on community composition. We cannot say anything about the absolute quantitative impact of the regulation of Cyanobacteria and microalgae, since cell densities (bacterial and microalgal) on stones and algae were not quantified.

In conclusion, we can state that irradiance influences α -diversity and, to a certain degree, cell densities of the bacterial community associated with *F. vesiculosus* but does not significantly affect the overall community composition, at least not over short time spans. Salinity was confirmed as one major factor shaping bacterial community composition. The observed compositional differences due to salinity were attributed to just a few members of the community (5% of OTUs) whose abundance correlated with the abiotic factor. Since a large portion of the observed variation could not be explained, multi-factorial studies are required in order to better understand the observed patterns. The mechanisms behind the observed shifts are not yet fully understood. We have indications for direct salinity effects by a comparison with non-living reference substrata. Host defense and interactions within the biofilm community are likely to contribute to the observed variation but are presently not understood

within this highly complex host-associated bacterial community. Considering the known role of epibacteria as modulating force between host and environment, the complex shifts in the epibacterial community brought on by irradiance and salinity may change these interactions.

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Paper III

**A Protective Coat of Microbes on Macroalgae:
Inhibitory Effects of Bacterial Biofilms and Epibiotic
Microbial Assemblages on Barnacle Attachment**



RESEARCH ARTICLE

A protective coat of microorganisms on macroalgae: inhibitory effects of bacterial biofilms and epibiotic microbial assemblages on barnacle attachment

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bacterial biofilms; *Fucus*; larval attachment; *Amphibalanus improvisus*; DGGE; temperature effects.

Abstract

Effects of epibiotic bacteria associated with macroalgae on barnacle larval attachment were investigated. Eight bacterial isolates obtained from samples of three macroalga species were cultured as monospecies bacterial films and tested for their activity against barnacle (*Amphibalanus improvisus*) attachment in field experiments (Western Baltic Sea). Furthermore, natural biofilm communities associated with the surface of the local brown alga, *Fucus vesiculosus*, which were exposed to different temperatures (5, 15 and 20 °C), were harvested and subsequently tested. Generally, monospecies bacterial biofilms, as well as natural microbial assemblages, inhibited barnacle attachment by 20–67%. Denaturing gradient gel electrophoresis fingerprints showed that temperature treatment shifted the bacterial community composition and weakened the repellent effects at 20 °C. Repellent effects were absent when settlement pressure of cyprids was high. Nonviable bacteria tended to repel cyprids when compared to the unfilmed surfaces. We conclude that biofilms can have a repellent effect benefiting the host by preventing heavy fouling on its surface. However, severe settlement pressure, as well as stressful temperature, may reduce the protective effects of the alga's biofilm. Our results add to the notion that the performance of *F. vesiculosus* may be reduced by multiple stressors in the course of global warming.

Introduction

In marine habitats, nearly all submerged natural and artificial surfaces become covered by biofilm. Microorganisms, such as bacteria and diatoms, are among the first colonizers (Wahl, 1989) that can substantially change the physical and chemical properties of the substratum (Characklis & Cooksey, 1983), making it suitable or unsuitable for colonization by invertebrate larvae (reviewed by Qian *et al.*, 2007). Inhibitory effects of biofilms on larval settlement have been documented in many studies (e.g. Olivier *et al.*, 2000; Lau *et al.*, 2003; Dobretsov & Qian, 2006; Rao *et al.*, 2007; Ganesan *et al.*, 2010). However, inductive or neutral effects have also been reported (e.g. Wiczeorek *et al.*, 1995; Harder *et al.*, 2002; Ganesan *et al.*, 2010). The inhibitory effect of biofilms has been mainly attributed to their bacterial components (Maki *et al.*, 1988; Holmström *et al.*, 1992; Avelin Mary *et al.*, 1993; Anil & Khandeparker, 1998; Lau & Qian, 2000; Khandeparker *et al.*, 2002, 2003;

Dobretsov & Qian, 2006). Previous studies have shown that bacterial surface chemistry, microtopography and a range of bacterial products from small-molecule metabolites to high-molecular-weight extracellular polymers mediate cyprid settlement (reviewed by Qian *et al.*, 2007). It is further known that biofilm community composition, which can be a determinant for larval attachment, can be affected by environmental factors, such as temperature, and that these altered biofilm communities can change the settlement behaviour of benthic larvae (Lau *et al.*, 2005). Structural and chemical cues (e.g. 6, 9-heptadecadiene and 12-octadecenoic acid, Hung *et al.*, 2007) from the natural biofilm community play an important ecological role in the natural environment of barnacle larvae by aiding in the search for suitable habitat. For instance, more barnacle larvae attach to substrate with biofilms from environments that are favourable to barnacle recruitment than to substrate with biofilms from less favourable environments (Hung *et al.*, 2007). *Amphibalanus amphitrite* cyprids from

the mid-intertidal can also distinguish between biofilms from different tidal ranges and settle preferentially on intertidal biofilms compared to subtidal biofilms and unfiled surfaces (Qian *et al.*, 2003). Furthermore, the attachment behaviour of the barnacle species *A. amphitrite* and *Balanus trigonus* was shown to depend on the temperature at which biofilms were grown, with more cyprids attaching to biofilms formed at higher temperatures (> 23 °C) than to ones formed at 16 °C (Lau *et al.*, 2005).

Most studies on interactions between biofilms and invertebrate colonizers have focused their experiments on biofilms grown on nonliving substrata (e.g. Qian *et al.*, 2003; Hung *et al.*, 2007). In the marine system, however, also living surfaces such as macroalgae thalli are potentially suitable substratum for invertebrate larvae in competition for space. Uncontrolled colonization of epibiotic microorganisms on the macroalga usually has detrimental effects, for example, by shading its surface and thus reducing its growth rate (Rohde *et al.*, 2008). To counteract this, the brown macroalga, *Fucus vesiculosus*, can produce secondary metabolites that directly repel invertebrate larvae (Brock *et al.*, 2007; Lachnit *et al.*, 2010). Lachnit *et al.* (2010) suggested that *F. vesiculosus* can also indirectly protect its surface from unwanted colonizers via the regulation of a beneficial epibiotic bacterial biofilm community that produces repellent substances. If a potentially beneficial epibiotic bacterial community undergoes a shift in its composition (via direct stress and/or via indirect stress on the alga), the attachment of macrofouler larvae could in turn be affected. The immensely important function of biofilms in attachment processes of barnacles (and other invertebrate larvae) and the potential interaction with their macroalgal host led us to investigate the effect of biofilms composed of bacteria epibiotic on macroalgae on the number of attached *Amphibalanus improvisus* cyprids in field assays. Specifically, our objectives were (1) to test the effect of monospecies bacterial biofilms originally isolated from macroalgal hosts on the attachment of barnacle larvae, (2) to investigate how effective these monospecies biofilms are under natural settlement pressures, (3) to test the effect of water-soluble chemical compounds from a natural assemblage of microorganisms detached from their brown macroalgal host, *F. vesiculosus*, on the attachment of barnacle larvae and (4) to test the indirect effects of environmental abiotic stress (i.e. temperature) via a shift in the epibiotic bacterial community composition on the attachment of cyprids.

Materials and methods

Monospecies bacterial films

Monospecies bacterial films were developed from eight bacterial strains, which had been isolated from three locally

(Kiel Bight, Western Baltic Sea) common macroalgal species (Schories *et al.*, 2009), and were tested for their effects on the attachment of cyprids in field assays. According to 16S rRNA gene sequences, bacterial isolates affiliated to *Pseudoalteromonas mariniglutinosa* (JQ867499), *Pseudoalteromonas tunicata* (JQ867503) and *Shewanella baltica* (JQ867502); *Bacillus foraminis* (JQ867498) isolated from the brown macroalga *F. vesiculosus* (Linnaeus 1753); *Ulvibacter litoralis* (JQ867505) and *Photobacterium halotolerans* (JQ867504) isolated from *Fucus serratus* (Linnaeus 1753); *Pseudoalteromonas arctica* (JQ867501) and *Shewanella basaltis* (JQ867500) isolated from the red alga *Polysiphonia stricta* (Dillwyn) Greville 1824. The 16S rRNA gene sequences of the bacterial isolates were deposited in GenBank under the accession numbers JQ867498–JQ867505.

Bacterial cultures were grown in sterile nutrient broth [5 g peptone + 3 g yeast in 1 L of filtered seawater (FSW)]. Each strain was inoculated to individual culture flasks containing 50 mL of sterile nutrient broth prepared with 0.22- μ m FSW and incubated for 45–48 h at 20 °C. Prior to the assays, the optical density (OD) of the bacterial cultures was determined with a Beckman Du 650 spectrophotometer at a wavelength of 600 nm, using pure medium as blank. All working cultures were adjusted to an OD of 1.5. Cultures were diluted to the mentioned OD with sterile medium when necessary.

Formation of monospecies bacterial films

Bacteria were harvested from cultures and adjusted to around 10^8 cells mL⁻¹ by turbidimetry. About 0.2- μ m polycarbonate filters (Sartorius, Germany) with a diameter of 47 mm served as settlement substratum for the bacteria. The sterile filters were placed onto a specially constructed sterile apparatus and fixed tightly with a screw-on lid that partially covered the filter (Fig. 1). This reduced the filter diameter to 35 mm and resulted in an effective attachment area of 9.62 cm² onto which 2 mL of bacterial suspension was pipetted. After 4 h of incubation under a sterile hood at room temperature (ca. 20 °C) under continuous light, the filters were gently rinsed with autoclaved FSW to remove unattached bacteria. Cells attached to the filter surface will be referred to as monospecies bacterial films hereafter and were formed as described previously for all experiments.

Preparation of nonviable bacterial films

Monospecies bacterial films were pretreated with formaldehyde to generate nonviable films. Bacterial films were covered with 2 mL of formaldehyde (3.7% in FSW) for 30 min and were subsequently rinsed five times with autoclaved FSW (Lau *et al.*, 2003). Control dishes did not

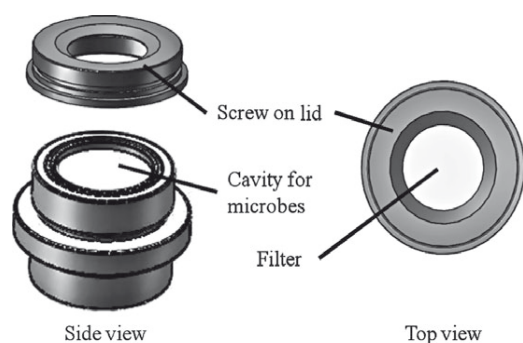


Fig. 1. Apparatus used in the cyprid attachment field assays. Natural biofilms were placed in the cavity underneath the filter during assays, monospecies bacteria formed a film on top of the filter, which in all cases was used as attachment substratum for cyprids. The area available for attachment was 9.62 cm². The screw-on lid tightly sealed the filter cavity and held the filter in place. One apparatus was used for one replicate. Individual apparatuses were fixed to a PVC frame and deployed in the Kiel Fjord. Courtesy of Ralf Schwarz.

contain any bacterial film but were treated with formaldehyde following the same procedure as for the bacterial films.

Enumeration of bacterial cells

Before initiating the attachment bioassay, the density of bacteria on reference filters was enumerated using epifluorescence microscopy (Zeiss Axio Scope A1 fluorescence microscope). After 4 h of incubation, the bacterial films on the filters were fixed with 3.7% formaldehyde in FSW for 15 min and subsequently stained with 0.2% (v/v) 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 15 min at room temperature. For bacterial enumeration, cell numbers in five randomly chosen fields of view were counted at $\times 1000$ magnification. In the fifth experiment, we did not enumerate bacteria. Microscopic examination of the filters, however, showed a similar coverage of the filters by bacterial cells as in the other experiments, indicating that settlement of bacteria was successful. The relationship between bacterial density and attached cyprids was analysed. Data from experiment II were excluded from analysis because there was no effect of bacterial films on cyprid attachment owing to high settlement pressure.

Barnacle attachment assays

The barnacle life cycle progresses through six naupliar stages followed by a nonfeeding cyprid larva, which is specialized for settlement. Peak settlement of *A. improvisus* in the Kiel Fjord usually occurs in July and August (Thomsen *et al.*, 2010; Nasrolahi *et al.*, 2012). During this period, barnacles commonly settle in high numbers within a short period of time. However, settlement

pressure may fluctuate drastically between days. Daily observation of settlement panels allowed us to visually estimate settlement pressure during the experiments. We ran a series of field experiments during July–August 2011 as follows:

At the beginning of the cyprid settlement period, panels with monospecies bacterial films were deployed for 7–24 h, depending on settlement pressure, subsequently retrieved, and the number of attached cyprids (irreversible attachment) was counted using a binocular microscope. Five individual experiments were conducted (I–V). Experiment I was carried out before the high settlement pressure period (for 24 h). Experiment II was conducted at the very peak settlement of cyprids (for 24 h). Experiment III was carried out during high settlement pressure but exposure time was reduced to 7 h in order to make it comparable to experiment I in terms of cumulative attachment and in order to examine the role of settlement pressure (comparing experiments II and III) in masking antisettlement effects. Experiment IV was carried out to test the effect of nonviable bacterial films on cyprid attachment (for 7 h). Because several studies have shown the repellent effects of *Pseudoalteromonas* species on marine invertebrate larvae (e.g. Holmström *et al.*, 2002 and references therein), experiment V was run to test the effect of *Pseudoalteromonas* species isolated from *F. vesiculosus* and *P. stricta* on cyprid attachment. Five to 7 replicates per bacterial film were used for each experiment depending on the number of bacterial strains used in the respective experiment. The single apparatus was fixed on a PVC frame that was deployed at a water depth of 1.5 m. The filmed surfaces served as attachment substratum for cyprids and faced downward to avoid sinking particles from the water column to accumulate on the filters.

Natural assemblage of microorganisms associated with *F. vesiculosus*

Sampling, experimental design and setup

Thirty *F. vesiculosus* thalli, each attached to one small rock (defined as one *Fucus* individual), were collected in the Baltic Sea, Kiel Bight (54°27'N, 10°11'E), at a water depth of approximately 0.5 m in July 2011. Within 2 h of collection, they were brought to the laboratory in individual plastic bags and were transferred to aquaria of the experimental setup in a constant temperature chamber at the IFM-GEOMAR in Kiel. The experimental units (EU) were 20-L plastic aquaria – one for each algal thallus resulting in 30 EUs. Aquaria were filled with FSW from the Kiel Fjord, which was maintained at three different temperature levels: 5, 15 and 20 °C using a water cooler

(Titan 2000, Aqua medic) or 300- and 600-W heating rods (SCHEGO, Germany), respectively. All three temperature levels are experienced by *F. vesiculosus* in its natural habitat during the course of the season. Temperatures above 15 °C enhance biotic stress in Baltic Sea *Fucus* (Wahl *et al.*, 2011). Thus, the 20 °C treatment can be considered stressful for the brown alga and its biofilm. Light was supplied by neon tubes with an intensity of $100 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a light/dark regime of 16 : 8. Bubbling stones supplied the tanks with air, and water in the aquaria was exchanged once a week.

Biofilms associated with the surface of *F. vesiculosus* were harvested after 14 days of incubation at the different temperatures for use in the cyprid settlement assays. One large thallus branch of each individual alga was rinsed with sterile seawater for 5 s to reduce loosely attached particles and was swabbed with three sterile cotton swabs. We know from microscopic analysis that a biofilm on *F. vesiculosus* is, on average, 11 μm thick (M. Fischer, E. Rickert, unpublished data). To harvest enough biofilm material to obtain a natural concentration of cells in an experimental volume of 1 mL, we swabbed a thallus surface of 900 cm^2 ($= 1 \text{ mL} / 11 \mu\text{m} = 1 \text{ cm}^3 / 0.0011 \text{ cm}$) and resuspended the microbial cells (after washing, see below) in 1 mL of sterile SW. By removing a natural biofilm from the alga, the original biofilm state is no longer intact. We, therefore, will refer to the suspension of microorganisms as 'microbial assemblage' and not as biofilm. Each swab was placed in a 2-mL Eppendorf tube containing 1 mL of sterile seawater, which was vortexed for 15 s to remove the biofilms from the swab. After discarding the cotton swab, the microbial assemblage was centrifuged for 5 min at 1677 *g*. The resulting pellet was washed in two steps to remove residual metabolites originating from the host alga. First, the pellet was washed with 1 mL of sterile seawater, the supernatant was discarded, and the centrifugation step was repeated. Again 1 mL of seawater was added. Then, 666 μL of the supernatant was pipetted off. The remaining suspensions of microorganisms from three swabs (each 334 μL) originating from one algal individual were combined (resulting volume = 1002 μL) in the cavity of the attachment apparatus (Fig. 1). The filter was placed on top of the cavity containing the microbial assemblage and sealed tightly with a screw-on lid. This construction allowed water-soluble metabolites produced by the natural assemblage of microorganisms to diffuse through the filter, but retained all organisms $< 0.2 \mu\text{m}$. The top of the filter served as settlement substratum for the cyprid larvae. The settlement panels were suspended for 16 h in the Kiel Fjord at a water depth of 1.5 m facing downward, after which the number of attached cyprids was counted

at $\times 10$ magnification. The assays were repeated in two separate runs owing to limited space on the settlement panels. Both repeats were conducted within 5 days during which experimental conditions were similar. Therefore, we pooled the data of both repeats (resulting in 10 replicates for statistical analysis).

Enumeration of microbial cells

The numbers of microbial cells (bacteria and cells that were not bacteria, that is, mostly diatoms) in the wells of the attachment apparatus at the beginning of the assays were enumerated in a subsample (100 μL) of the suspensions of five replicates per temperature level. The samples were prepared and counted using an epifluorescence microscope following the procedure mentioned previously with the exception that cells in 20 visual fields per sample were counted.

Epibacterial community composition

Sampling

To analyse the epibacterial community composition at the different temperatures via denaturing gradient gel electrophoresis (DGGE), each algal individual was sampled for its associated bacteria. Prior to sampling, approximately 20 cm^2 (visual estimation) of algal thallus was rinsed with sterile seawater for 5 s to reduce loosely attached particles. Subsequently, the bacteria were harvested with one sterile cotton swab per sample and stored in 2-mL Eppendorf tubes at $-80 \text{ }^\circ\text{C}$ until DNA extraction.

DNA extraction

DNA was extracted with the QIAamp DNA Mini Kit (Qiagen GmbH) following the manufacturers' protocol for buccal cotton swabs with an additional incubation step at 95 °C for 8 min after the protocol's second lysis step with buffer AL. DNA was eluted with nuclease-free water (Qiagen GmbH) and stored at $-20 \text{ }^\circ\text{C}$ until PCR-DGGE.

PCR-DGGE

The V3 region of 16S rRNA gene sequences of bacteria was amplified using the primer pair 341F-GC (5'-[CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G] CCT ACG GGA GGC AGC AG-3') and 534R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1993) (Eurofins MWG Synthesis GmbH). PCR amplifications were prepared with puReTaqTMReady

-To-Go™ Beads (GE Healthcare Europe GmbH), 1 µL of each primer [10 pmol µL⁻¹] and 1 µL of DNA template in a reaction volume of 25 µL per sample. They were performed on a thermocycler (Flex Cycler, Analytik Jena) with following settings: initial denaturation at 94 °C for 2 min, 10 touchdown cycles with initial annealing at 65 °C for 40 s and a reduction of 1 °C per cycle; elongation at 72 °C for 40 s; and denaturation at 94 °C for 30 s. This was followed by 25 cycles of annealing at 55 °C for 40 s, elongation at 72 °C for 40 s and denaturation at 94 °C for 30 s. Final annealing was at 42 °C for 60 s, and final elongation at 72 °C for 5 min. Correct sequence length (233 bp, including the 40 nucleotide GC clamp) of 2.5 µL of PCR amplicons per sample was controlled (100 bp standard ladder, Biozym) via electrophoresis on a 1.5% agarose gel (1× TBE). PCR amplicons were then separated according to GC content on a DGGE gel. A double-gradient gel was cast with a structural gradient of 6–8% acrylamid–bisacrylamid (37.5 : 1) and with a denaturing gradient of 40–80% [100% denaturant is defined as 7 M urea and 40% formamide (v/v)] (Petri & Imhoff, 2001). This gradient was shown to be suitable for the analysis of bacteria associated with *F. vesiculosus* (Lachnit *et al.*, 2009). Electrophoresis was run in 0.5× TAE buffer at 60 °C and 80 V for 14 h in a C.B.S. Scientific DGGE system. The gel was stained with SYBR Gold (Life Technologies GmbH, Darmstadt, Germany) for 45 min and rinsed with 1× TAE buffer for 30 min prior to gel documentation via UV transilluminator (Vilber).

Statistical analysis

The assumptions of normality and homogeneity of variances were tested with Shapiro–Wilks *W* test and Levene's test, respectively. Non-normally distributed data were square-root-transformed. The settlement assays were analysed by one-way ANOVA using the STATISTICA 8 software package (StatSoft). *Post hoc* comparisons were made with Tukey's HSD.

Bands of the DGGE fingerprint were called with the bare eye, and a Bray–Curtis similarity matrix was constructed based on the presence or absence of bands using the PRIMER 6 software package. The matrix served as basis for analysis of similarity (ANOSIM), and data were visualized by cluster analysis and nonmetric multi-dimensional scaling. *R* values generated by ANOSIM indicate the degree of separation between groups and usually lie between 0 and 1 (large values of *R*, close to 1, indicate complete separation of groups; small values close to 0 imply that they are not or barely separated. Mid-range values of *R* (0.5) indicate that groups are distinguishable but overlap to a certain degree) (Clarke & Warwick, 2001).

Results

Larval attachment assays with monospecies bacterial films

In general, a higher proportion of *A. improvisus* cyprids settled on the unfiled surfaces than on the surfaces with a monospecies bacterial film.

Cyprid attachment on films of the strain affiliated to *S. basaltis* significantly differed from the attachment on the unfiled surfaces (Fig. 2). Although the effects of *P. halotolerans* and *U. litoralis* films on cyprid attachment were not significantly different from the attachment on the unfiled surfaces, cyprids tended to attach less to the filmed surfaces than to nonfiled surfaces (Fig. 2). Under high settlement pressure, the initially observed inhibitive effects of bacterial films on cyprid attachment of all bacterial films disappeared (Fig. 3). When exposure time was reduced (at high settlement pressure), cyprid attachment on all filmed surfaces differed significantly from the unfiled surfaces (Fig. 4). The repellent effect of *S. basaltis* tended to be strongest (Fig. 4). No significant difference of cyprid attachment between filmed and unfiled surfaces was seen after bacterial films were killed (Fig. 5). During the experiments with *Pseudoalteromonas*, cyprid attachment in the field was low, and on average, only four cyprids attached after 24 h (Fig. 6). Cyprid attachment on *P. mariniglutinosa* and *P. tunicata* films did not differ in comparison with the unfiled surfaces. These two films even showed a slight but nonsignificant inductive effect on cyprid attachment (Fig. 6). In contrast, *P. arctica* repelled cyprids (Fig. 6).

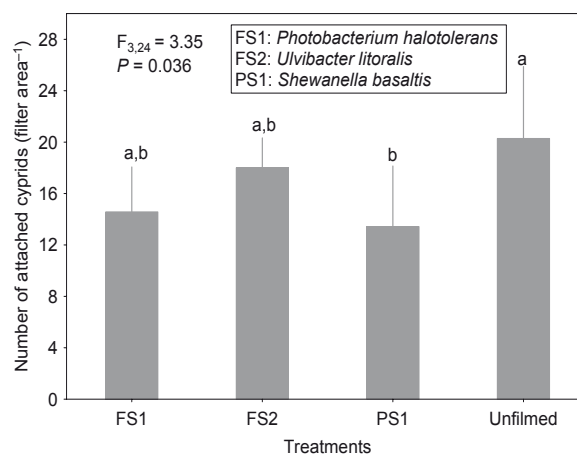


Fig. 2. Experiment I. Effect of monospecies bacterial films isolated from *Fucus serratus* (FS) and *Polysiphonia stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: mean numbers of cyprids attached after 24 h of exposure in the Kiel Fjord. The unfiled surface was a filter substrate without bacterial film. Each bar represents the mean of seven replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

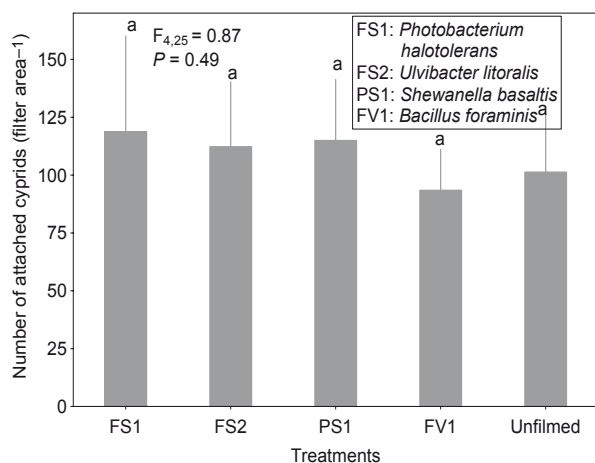


Fig. 3. Experiment II. Effect of monospecies bacterial films isolated from *Fucus vesiculosus* (FV), *Fucus serratus* (FS) and *Polysiphonia stricta* (PS) on cyprid attachment of *Amphibalanus improvisus* at the very peak settlement: mean numbers of cyprids attached after 24 h of exposure in the Kiel Fjord. The unfiled surface was a filter substrate without bacterial film. Each bar represents the mean of six replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

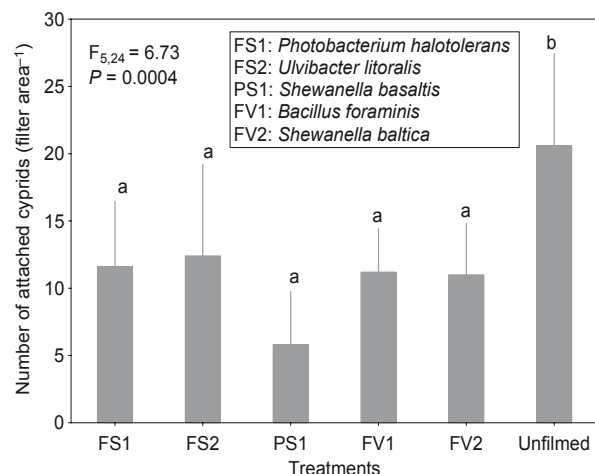


Fig. 4. Experiment III. Effect of monospecies bacterial films isolated from *Fucus vesiculosus* (FV), *Fucus serratus* (FS) and *Polysiphonia stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: mean numbers of cyprids attached after 7 h of exposure in the Kiel Fjord. The unfiled surface was a filter substrate without bacterial film. Each bar represents the mean of five replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

Epifluorescence microscopy revealed that the density of bacteria on filters ranged from 1.26×10^6 to 19.3×10^6 cells per filter area (Table 1). There was no significant correlation between the density of bacteria and the number of attached cyprids (Table 1).

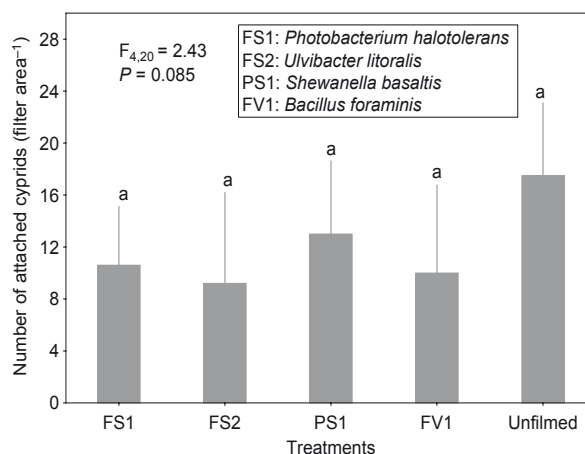


Fig. 5. Experiment IV. Effect of nonviable monospecies bacterial films isolated from *Fucus vesiculosus* (FV), *Fucus serratus* (FS) and *Polysiphonia stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: mean numbers of cyprids attached after 7 h of exposure in the Kiel Fjord. The unfiled surface was a filter substrate without bacterial film. Each bar represents the mean of six replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

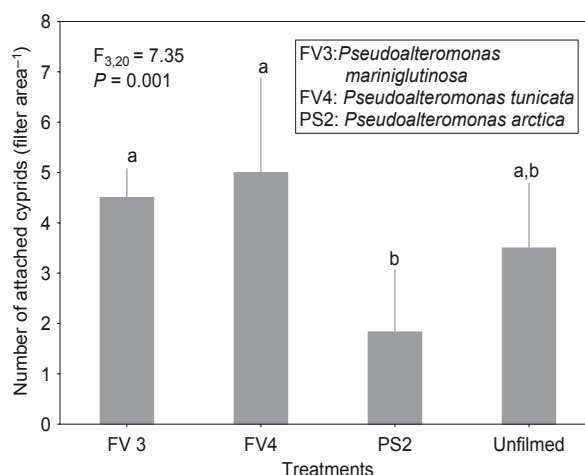


Fig. 6. Experiment V. Effect of *Pseudoalteromonas* species isolated from *Fucus vesiculosus* (FV) and *Polysiphonia stricta* (PS) on cyprid attachment of *Amphibalanus improvisus*: mean numbers of cyprids attached after 24 h of exposure in the Kiel Fjord. The unfiled surface was a filter substrate without bacterial film. Each bar represents the mean of five replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

Larval attachment assays with microbial assemblages

The cyprid attachment assays revealed that on average, 24–54% fewer cyprids attached to substrata with differently treated microbial assemblages than to the filters without

Table 1. Density of attached bacteria and the number of attached cyprids on the filter area of the apparatus

Bacteria	Mean density of bacteria × 10 ⁶ cells per filter area	Mean number of cyprid attachment
PS1	7.699154	13.42
FS1	3.508008	12.5
FS2	7.963794	18
PS1	6.499046	5.8
FS1	12.40112	11.6
FS2	16.30916	12.4
FV1	2.289437	11.2
FV2	13.2935	11
PS1	19.29404	10
FS1	1.261652	8.6
FS2	12.5919	8
FV1	8.413065	7.5

Data on attached bacterial density and number of attached cyprids from experiment I, III and IV in the monospecies bioassay. PS1, *Shewanella basaltis*; FS1, *Photobacterium halotolerans*; FS2, *Ulva litoralis*; FV1, *Bacillus foraminis*; FV2, *Shewanella baltica*. The mean density of bacteria was based on counts of five fields at ×1000 magnification under an epifluorescence microscope. There is no correlation between bacterial density and the amount of larval attachment ($r^2 = 0.007$, $P = 0.417$).

microorganisms underneath (controls). Lowest numbers of attached cyprids were found on substrata with microbial assemblages developed at 5 and 15 °C, an effect that was significant relative to control substrata but not relative to the substrata with microbial assemblages developed at 20 °C (Fig. 7). The 20 °C microbial assemblages showed a repellent trend, but this result was not significantly different from the control.

Epibacterial community composition

Analysis of the DGGE banding pattern (Fig. 8) of 16S rRNA gene sequences of bacteria sampled from their host, *F. vesiculosus*, showed that the different temperature treatments led to significant shifts in the epibacterial community composition (ANOSIM global $R = 0.524$, $P = 0.001$). The bacterial communities from the different temperature treatments formed clusters with different degrees of similarity (Fig. 9, Fig. S1), as shown by the results of the pairwise permutation tests (Table 2). The bacterial communities of 5 and 20 °C differed as indicated by a high value of R (0.756) (Table 2). The bacterial communities of 5 and 15 °C differed slightly less ($R = 0.501$), whereas communities from the 15 and 20 °C treatments largely overlapped ($R = 0.256$). The number of bacterial cells in the cavities of the filter apparatuses during the field attachment assays did not significantly differ between the temperature treatments and was on average 270–360 × 10⁶ cells per cavity. The number of other microor-

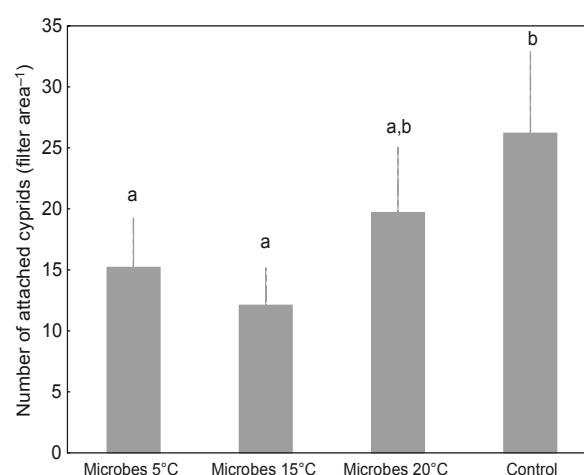


Fig. 7. Mean number of attached *Amphibalanus improvisus* cyprids on apparatus with polycarbonate filters during exposure in the Kiel Fjord for 16 h. Biofilms were detached from their macroalgal host, *Fucus vesiculosus*, which had been cultured at different temperatures (5, 15, and 25 °C). Filters without the harvested microbial assemblage served as a control substrate. Each bar represents the mean of 10 replicates. Error bars represent 95% CI. Significant differences at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars.

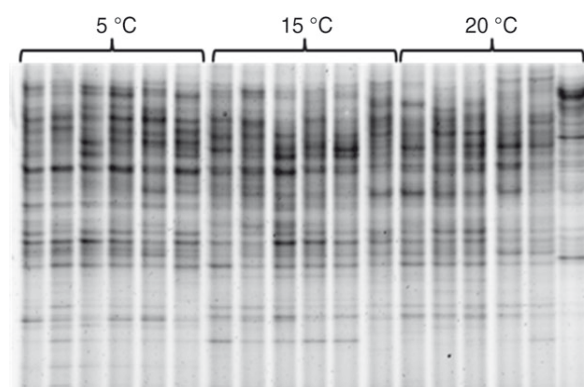


Fig. 8. DGGE fingerprint of 16S rRNA gene sequences of epibacteria from *Fucus vesiculosus* cultured at different temperatures (5, 15 and 20 °C; $n = 6$ per temperature level).

ganisms (mostly diatoms) was on average approximately 100-fold smaller and also did not significantly differ between treatments (Fig. 10a and b).

Discussion

Knowing that bacteria are key components of the biofilms, especially with regard to their inhibitory effects on macrofoulers (e.g. Khandeparker *et al.*, 2002, 2003; Dobretsov & Qian, 2006), we used bacterial strains isolated from macroalgal surfaces and tested their effects on larval attachment of *A. improvisus* in field experiments. As only

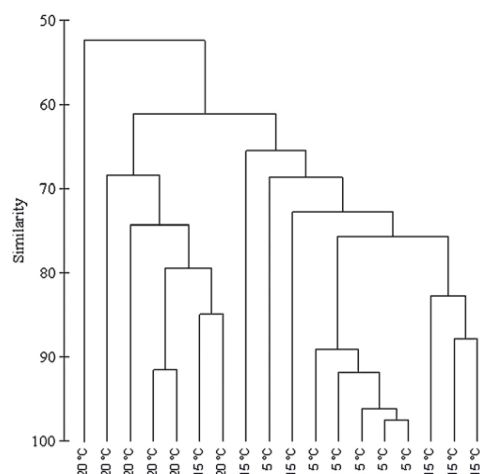


Fig. 9. Dendrogram based on DGGE epibacterial community fingerprint showing similarities in% (group-average linking of Bray–Curtis similarities).

about 5% of bacteria from the natural environment are culturable (Eilers *et al.*, 2000) and, therefore, the results of experiments with bacterial isolates may not reflect interactions in the field, we also used natural microbial assemblages that were harvested from their host *F. vesiculosus* grown under different temperature conditions.

Marine bacteria associated with basibionts have the potential to protect hosts from micro- and macrofouling (Holmström *et al.*, 1992; Holmström & Kjelleberg, 1999; Steinberg *et al.*, 2002; Dobretsov & Qian, 2004). Our results showed that there were inhibitory effects of monospecies bacterial films on cyprid attachment of *A. improvisus*. Six isolates from the surfaces of macroalgae tested in this study were inhibitive and two were noninhibitive. O'Connor & Richardson (1996) developed laboratory and field assays with the same barnacle species used in our study but with different monospecies bacterial films (*Cobetia marina* formerly known as *Deleya marina*, *Alteromonas macleodii* and *Pseudomonas fluorescens*). The authors also found repellent effects on barnacle attachment for all tested strains, although this was substratum-dependent. It seems that several different strains can repel *A. improvisus* as shown by the study mentioned previously and supported by our results.

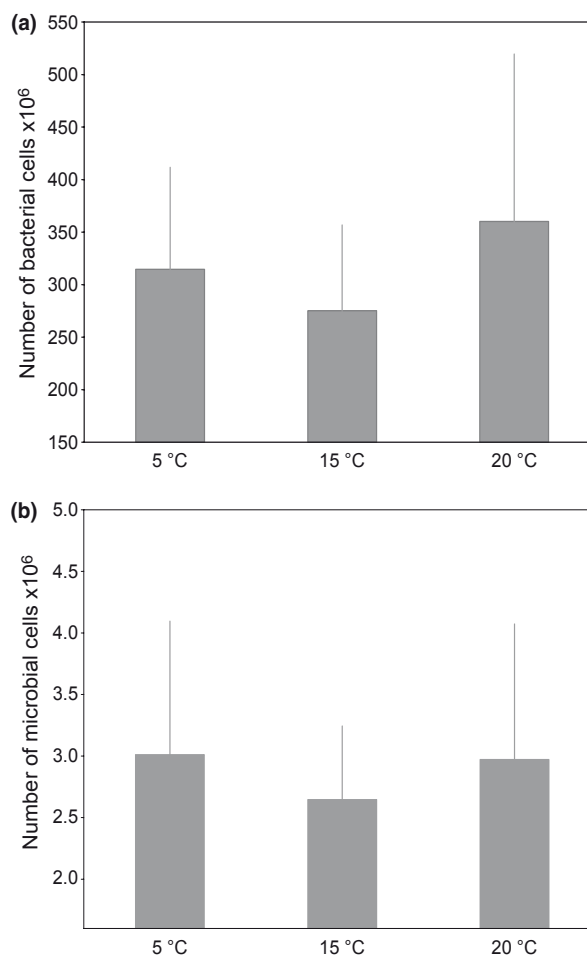


Fig. 10. (a) Mean number of bacterial cells and (b) mean number of other microbial cells (mostly diatoms) used in the field attachment assays per temperature level. Cells were enumerated by epifluorescence microscopy. Each bar represents the mean of five replicates per temperature level. Error bars represent 95% CI; N.S.

In the present study, *S. basaltis* and *P. arctica*, isolated from a biofilm associated with the red alga *P. stricta*, were the most potent inhibitors among all tested isolates. They reduced the number of attached cyprids by 35–67%. *Shewanella baltica* and *B. foraminis* isolated from *F. vesiculosus* had inhibitory effects (~48%) similar to the effects of bacterial strains isolated from *F. serratus* (*P. halotolerans* and *U. litoralis*). *Pseudoalteromonas*

Table 2. Analysis of similarity (ANOSIM) pairwise tests results of epibacterial community samples ($n = 6$ per temperature level)

Groups	<i>R</i> statistic	Significance level	Possible permutations	Actual permutations	# Observed permuted statistics $\geq R$
5 °C, 15 °C	0.501	0.002	462	462	1
5 °C, 20 °C	0.756	0.002	462	462	1
15 °C, 20 °C	0.256	0.024	462	462	11

tunicata and *P. mariniglutinosa* isolated from *F. vesiculosus* did not have any repellent effects on cyprid attachment. *Pseudoalteromonas tunicata*'s neutral effect on attachment of cyprids is surprising because many studies have reported inhibitive effects of this species against settlement of algal spores, invertebrate larvae, bacteria and fungi (e.g. Holmström *et al.*, 2002 and references therein). However, inductive effects of some other *Pseudoalteromonas* species on marine larval invertebrates have been shown. For instance, *Pseudoalteromonas luteoviolacea* was identified to be one of the strongest settlement inducers for an Australian echinoid, *Heliocidaris erythrogramma* (Hugett *et al.*, 2006). Some bacteria express larval-specific activity, that is, inhibit settlement of one species and induce settlement of another species (Maki *et al.*, 1989, 1992; Lau *et al.*, 2003). Different species of barnacle cyprids may also respond differently to similar or even identical cues (e.g. Johnson & Strathmann, 1989; Keough & Raimondi, 1996). In our study, *P. tunicata* did not have any repellent effect on *A. improvisus* larvae, whereas this isolate strongly repelled *A. amphitrite* larvae in the study conducted by Holmström *et al.* (2002). This target organism specificity can be one explanation for the discrepancy of results from different studies.

Our results show that reduction in barnacle attachment by bacterial films was not density-dependent. These observations coincide with findings of Kavouras & Maki (2004) on the inhibitive effect of monospecies biofilms on zebra mussel larvae. Results of our study are, however, in disagreement with those of Lau *et al.* (2003), who found a negative correlation between bacterial density and the percent settlement of cyprids. It seems that a certain cell density evokes a repellent effect, and above this density, there is no additional activity. There may have been slight changes in the microbial cell densities and also in the bacterial community composition during the course of the assays that we could not measure. We experienced difficulties in sampling the microbial assemblages at the end of the assays without cell loss, and microscopic enumeration of cells densities was not possible owing to particles and barnacles attached to the filters. However, the assays were of relatively short duration, so we assume that the changes are negligible.

We used the procedure of Lau *et al.* (2003) in our study and tested cellular viability as a parameter for the settlement inhibition activity of bacterial films. Lau *et al.* (2003) found that cyprid settlement of *A. amphitrite* showed no difference on live and nonviable bacterial films. Another study by Lau & Qian (2000) showed that inductive effects of *Rhodovulum* sp. (Alphaproteobacteria) on larval settlement of the marine polychaete, *Hydroides elegans*, were strictly dependent on bacterial viability. In our study, inhibitive effects of nonviable (i.e. no

metabolic activity) bacterial films on cyprid attachment were weaker than those of live bacterial films. However, all isolates still exhibited inhibitive trends on larval attachment compared to the unfilmed surfaces, suggesting that the inhibitive effect was mediated by both bacterial metabolic activity and direct larval contact with the bacterial film surface (physical existence of bacterial cells).

In our study, inhibitive effects of bacterial films were not observed when cyprid settlement pressure in the field was high. In contrast to our results, O'Connor & Richardson (1998) reported that settlement of *A. amphitrite* on bacterial films was independent of the number of cyprids they used in their assays in the laboratory. During surface exploration, barnacle cyprids use a proteinaceous secretion to attach temporarily and walk across surfaces, leaving behind a so-called footprint. This secretion also functions as settlement cue for subsequently exploring larvae (Dreanno *et al.*, 2006a, b). Barnacles often settle gregariously (Crisp, 1974), which could explain the high settlement rates of cyprids when large numbers of cyprids are present in the field. In laboratory experiments, however, cyprid density is usually lower than that in natural habitats during peak settlement. Consequently, the magnitude of settlement observed in laboratory experiments may not be ecologically representative (Gotelli, 1990; Clare *et al.*, 1994).

During peak settlement of barnacles in the Kiel Bight, we have observed that *F. vesiculosus* thalli can become heavily fouled by *A. improvisus* (Rohde *et al.*, 2008; Fig. S2). This observation indicates that results from the monospecies bacterial films, that is, no repelling effect on cyprids when settlement pressure was severe, reflect realistic scenarios in the field. The thallus surface is essential for light and nutrient uptake (Wahl, 2008). Severe overgrowth by macrofoulers can, therefore, be disadvantageous for the host alga because photosynthesis is reduced and thus growth (Rohde *et al.*, 2008). Obviously, *F. vesiculosus* is not covered by a monospecies biofilm in the field but by a complex bacterial community (Lachnit *et al.*, 2011). Therefore, in addition to the monospecies films, we tested the effect of natural epibiotic microbial assemblages on cyprid attachment, which closer resemble the natural biofilm state than monospecies films do. We could not conduct the attachment assays with biofilms that were identical with those on the alga, because we removed the biofilm from its host, possibly losing biofilm members in the cotton swabs. We followed this procedure to exclude or at least minimize the potential direct effects of the host alga on barnacle attachment. Despite this limitation, the microbial assemblages had repellent effects on barnacle larvae, most pronounced with the microorganisms from algae treated at 5 and 15 °C. The repellent effect of bacterial biofilms on invertebrate larval attach-

ment can be explained by bacterial abundance (e.g. Dobretsov & Qian, 2006) and by the bacterial community composition (e.g. Qian *et al.*, 2003), both of which were analysed in our study. Neither the number of bacterial cells nor the number of other microorganisms (mostly diatoms) differed between the biofilms from different temperatures. Apparently, the number of microbial cells was not responsible for the strongest repellent effects of the 5 and 15 °C microbial assemblages. These results coincide with previous studies on the attachment of two barnacle species that were not affected by the abundance of microorganisms (Qian *et al.*, 2003; Thiyagarajan *et al.*, 2006). Our results can rather be explained by the bacterial community composition, which differed most between 5 and 20 °C and least between 15 and the 20 °C, respectively. The 15 °C microbial assemblages repelled on average about 39% more and the 5 °C microorganisms on average 23% more barnacle larvae than the 20 °C microbial assemblages did. We would, therefore, have expected the microbial communities from the lower (5 and 15 °C) and high (20 °C) temperature treatments to form distinct communities responsible for the observed effects on cyprid attachment. However, the picture was not as clear as expected: the 15 and 20 °C microbial communities did not form distinct clusters but largely overlapped in their community composition. In a previous study, temperature had a direct effect on the bacterial community composition on artificial substrate, which in turn affected the attractiveness of the biofilm to barnacle cyprids (Lau *et al.*, 2005). In their study, the bacterial communities differed more among each other than the ones in our study did. Our results indicate that not large but small shifts in the community composition (which in our study resulted from a temperature shift of 5 °C) or in the relative abundance of single community members may have accounted for a stronger repellent effect of the microbial assemblage. It remains to be tested which members of the bacterial community are responsible for the repellent effects. Candidates could be the single strains that showed repellent effects in the study at hand. However, it remains unclear whether they would play the same role in a complex community. We cannot rule out that other members of the microbial assemblage (e.g. diatoms) or metabolites from the host algae may have caused or at least contributed to the repulsion of barnacles. *Fucus vesiculosus* has been shown to produce secondary metabolites (phlorotannins) with the potential to directly repel cyprids (Brock *et al.*, 2007). However, the microbial assemblages used in our study were thoroughly washed to remove residual host algal water-soluble phlorotannins and other water-soluble compounds. Microalgal cells (mostly diatoms) were present in the tested microbial community. They were not identified further, and therefore, we do

not know whether bioactive species were present. In any case, diatom cells were outnumbered by bacteria by far (100-fold). Considering the repellent effect of several monospecies biofilms in this study and the undisputed key role of bacteria in larval settlement (reviewed in Hadfield, 2011), it is likely that the bacteria in the natural microbial assemblages were at least in part responsible for the repellent effects against barnacle larvae. There is evidence that bacteria produce water-soluble compounds that play an inhibitive role in the attachment of the polychaete *H. elegans* (Dobretsov & Qian, 2002). To our knowledge, in most studies (e.g. Qian *et al.*, 2003; Lau *et al.*, 2005) on the inhibitive effects of complex biofilms on barnacle attachment, assays were conducted with larvae being in direct contact with the biofilm, thus testing surface bound and water-soluble compounds simultaneously. In our study, the attachment apparatus was constructed in a way that only allowed water-soluble compounds from the natural microbial assemblage to diffuse through the filters. The observed repellent effect must, therefore, have been caused by one or several water-soluble compounds emitted by the bacteria (and possibly diatoms) rather than by surface bound compounds of the extracellular polymeric substances. The nature of the compounds was not analysed and their identification will need to be addressed in future studies.

Many antibacterial compounds have been extracted from algae (reviewed in Goecke *et al.*, 2010), indicating that many algae species actively defend their surface from epibiosis (for examples see Harder, 2008). Some algal species have been shown to regulate their surface-associated bacteria to their own benefit (*Delisea pulchra*: Maximilien *et al.*, 1998; *Bonnemaisonia asparagoides*: Nylund *et al.*, 2010; *Dictyosphaeria ocellata*: Sneed & Pohnert, 2011). This regulatory capacity has also been suggested for *F. vesiculosus* (Lachnit *et al.*, 2010). In our study, the microbial assemblages from algae treated at the lower temperatures (5 and 15 °C) were the most repellent. Therefore, it seems possible that the alga is in a healthy state at lower temperatures, allowing it to maintain a beneficial epibacterial community, and that the alga's chemical control of the biofilm could be impaired at higher temperatures causing stress. Although this idea is speculative, we find it deserves attention in future studies investigating effects of climate change. In the course of climate change, water surface temperatures in the Baltic Sea could increase by up to 4 °C by 2100 (BACC, 2008). This rise in temperature could indirectly reduce the general performance of *F. vesiculosus* via weakening of the protective biofilm, leading to higher fouling of its surface and consequently reduced available solar energy. Besides this, higher water temperatures during the summer months coincide with high settlement pressure of barnacles as well as increased grazing pressure, and

therefore, can generate synergistic stressful interactions (Wahl *et al.*, 2011).

We conclude that the complex biofilm associated with macroalgae can protect its host from unwanted colonizers. This indirect defence mechanism may strengthen the direct antifouling defence of the macroalga against macrofoulers. As indicated by the results of the monospecies films, this protective effect may not withstand severe fouling pressure but may minimize biotic stress for the algae caused by fouling of its surface at times when fouling pressure is moderate. Temperature stress may also reduce the protective effect of the alga's biofilm. Thus, an increase in water temperature owing to global warming may result in multiple stressors reducing the performance of the macroalga.

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Authors' contribution

A.N. and S.B.S. contributed equally to this work.

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Supporting Information

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

Fig. S1. Non-metric multidimensional scaling (n-MDS) plot visualizing differences and overlaps between epibacterial community compositions.

Fig. S2. *Fucus vesiculosus* collected in the Kiel Fjord with severe fouling by barnacles.

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Discussion and Conclusion

6 Discussion

In this study, I have demonstrated that the abiotic factors temperature, salinity, and irradiance lead to shifts in epibacterial community composition and/or diversity levels in short term laboratory and outdoor experiments with the macroalgal host *Fucus vesiculosus*. Furthermore, I found that microbial assemblages harvested from the surface of *F. vesiculosus* reduced attachment rates of barnacle larvae in a bioassay conducted in the field and that this observation was strongest for microbial assemblages that were kept at lower vs. higher temperatures.

6.1 Which Factors Structure Epibacterial Communities?

Not surprisingly, the abiotic factors tested in my study only accounted for a part of the variation in epibacterial communities observed between treatment levels. There are numerous other (abiotic and biotic) factors that determine the distribution, diversity levels, and community composition (i.e., structure) of bacterial communities. Figure 4 depicts a conceptual model of these factors and their interactive links, some of which will be further discussed in the following. As stated in Paper III, the influence of abiotic factors in structuring communities can be direct affecting bacterial physiology or indirect via shifts in the biotic interactions within the macroalgal holobiont and its surrounding biotic environment.

6.1.1 Abiotic Factors

A meta-analysis of bacterial communities from different environments showed that salinity was the major determinant of bacterial phylogenetic diversity before extremes of temperature, pH, and other physico-chemical factors (Lozupone and Knight, 2007). In my study, salinity accounted for about 20% of the observed variation between samples, indicating that it is important in the configuration of epibacterial communities. Temperature also drove compositional shifts in the epiphytic community and also accounted for approx. 20% of the observed variation. These are clearly not the only abiotic factors that influence bacterial communities. Oxygen and organic compounds can also affect bacterial community structure in soil or sludge (Park and Noguera, 2004, reviewed in Kirchman, 2012). Considering that even on the surface of *F. vesiculosus* microhabitats with very low levels of oxygen were detected, this factor may influence the bacterial community structure on a microhabitat scale (see introduction). Bacteria enriched in the lab on different carbon sources from a kelp species were suggested to be opportunistic bacteria involved in the degradation of dead kelp tissue in natural communities of bacteria (Bengtsson *et al.*, 2011). Organic compounds from the algal cell wall are likely involved in structuring epibacterial communities on *F. vesiculosus* but their exact influence on bacterial populations

on living algae needs to be investigated. Furthermore, the composition of planktonic bacterial communities is restructured under different pH conditions (Krause *et al.*, 2012). The effect of pH on the bacteria associated with macroalgae and seagrass could be interesting issues because, as stated in the introduction, pH in the DBL is influenced by photosynthesis of the host alga. Future studies should incorporate several factors e.g., elevated temperatures, elevated pH, and lower salinity in order to account for more of the variability observed in natural bacterial communities.

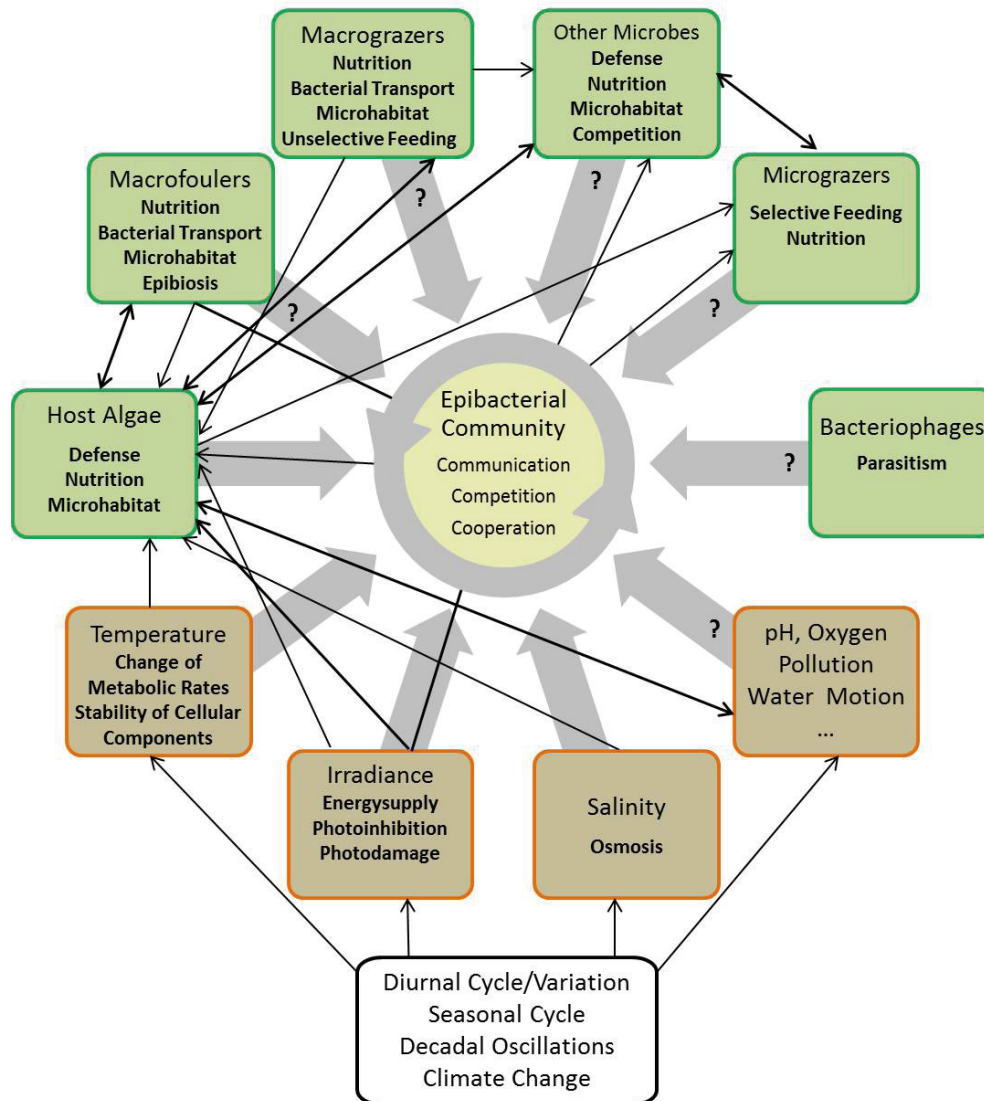


Figure 4 A conceptual model of biotic and abiotic factors and processes contributing to the structure of epibacterial communities. The structure of a bacterial community is made up of the sum of all bacterial phylotypes and their abundances. The model combines results from my doctoral project and from several studies on epibacterial communities on macroalgae cited in the introduction and discussion. Since there is only limited knowledge on the factors controlling the microbiota on macroalgae the model includes factors and processes that have been shown to structure other microbiomes. These are marked with a question mark indicating the necessity to include them in investigations on epiphytic bacterial communities. The black arrows indicate direct and indirect biotic and abiotic interactions, especially with the host macroalga and the variable environment, that may also affect epibacterial community structure.

6.1.2 Host Defense

Besides having defense mechanisms like sloughing of tissue (not all macroalgae can do this) or the release of reactive oxygen species (reviewed in Goecke *et al.*, 2010), macroalgae produce secondary metabolites that inhibit or promote the adhesion and/or growth of epibacteria (and other microbes e.g., diatoms and fungi) in single strain bioassays under laboratory conditions (Kubanek *et al.*, 2003, Lam *et al.*, 2008, Viano *et al.*, 2009, Goecke *et al.*, 2012). *F. vesiculosus* is chemically defended by producing metabolites such as Fucoxanthin, DMSP (Saha *et al.*, 2011, Saha *et al.*, 2012), and fucofluorethol (Sandsdalen *et al.*, 2003) with antibacterial activity and unidentified compounds with probiotic activity stimulating growth (Goecke *et al.*, 2012, Lachnit *et al.*, 2013). Mostly, the targeted bacteria were isolated strains, in part from macroalgal surfaces. This means that they first had to be cultured in the laboratory. Since only few bacteria are readily cultivable, as discussed in the introduction, the cultured ones possibly do not reflect their actual abundance and significance in complex epiphytic microbial communities. Some studies have complemented our knowledge about ecologically relevant host defenses by testing algal extracts against natural biofilm communities in the field. These studies have found that the alga indeed has the potential to structure their abundance (Nylund *et al.*, 2008) and composition (Persson *et al.*, 2011, Sneed and Pohnert, 2011, Lachnit *et al.*, 2013) by antibiotic substances or by metabolites interfering with the quorum sensing communication system of bacteria (reviewed for the red alga *Delisea pulchra* in Harder *et al.*, 2012). It remains unclear as to what extent host defense may have been impaired in my experiments, thus contributing to the observed variability in epibacterial diversity and community composition. The abiotic factors tested in my study can become stressful for *F. vesiculosus* (and congeneric species) under certain conditions (reviewed in Wahl *et al.*, 2011). A resulting reduction in the performance of the host alga has been observed with respect to antifeeding defenses (Weinberger *et al.*, 2011) and antifouling defenses (the latter targeting a small number of bacterial strains in the laboratory) (Wahl *et al.*, 2010). Thus, it seems likely that an altered host defense could have ecologically relevant effects on epibacterial community composition. Even though there is evidence from the above-mentioned studies that algae, including *Fucus* sp., regulate the community composition and abundance of epibacteria, the question as to what extent the interactions between bacteria within the biofilm itself influence composition remains unclear.

6.1.3 Interactions within the Epibiotic Biofilm

Bacteria communicate with each other by signalling molecules called autoinducers by which they regulate e.g., biofilm formation, virulence, bioluminescence, and the production of antibiotics (reviewed in Waters and Bassler, 2005). In multi-species biofilm, this interbacterial communication (termed quorum sensing) and other interactions, such as competition for nutrients

and cooperation (e.g., coaggregation between specific bacterial types) determines how the community develops and how it is structured (reviewed in Elias and Banin, 2012). Bacterial strains isolated from *F. vesiculosus* exhibit antibiotic activity against other bacterial strains isolated from macroalgae (Goecke *et al.*, 2013). This suggests that there is likely competition in the natural biofilm community that may affect composition. However, the processes in natural algal-associated biofilm communities remains unclear. Considering the high diversity of the bacterial communities found in my study (at least 200-500 phylotypes on one thallus section) these must be highly complex interactions about which we so far know nothing.

Despite the undoubtedly important role of bacteria in their building of complex biofilm, I suspect that microalgae in the biofilm in my study may also play a significant role for *F.vesiculosus*. Epiphytic diatoms were present in many samples in my study; in others, however, they were nearly absent. They never outnumbered bacteria, but in some samples they could be considered the dominant members of the biofilm due to their larger size. The potential role of diatoms in the microbial assemblages for repulsion of cyprids was briefly discussed in Paper III. For future investigations on the interactions between algal hosts and microbes, eukaryotic microbes (microalgae, fungi, and others) should be taken into consideration.

Protist grazers (such as amoeba, ciliates, nanoflagellates) can enforce top-down control on bacteria in biofilm communities (Huws *et al.*, 2005) and in various planktonic and soil microbial communities (reviewed in Kirchman, 2012). They may influence epibacterial community structure on macroalgae by selective feeding. Furthermore, top-down control of viruses that infect bacteria (bacteriophages) are important in the concept of the holobiont (Zilber-Rosenberg and Rosenberg, 2011). The viral community associated with corals has been investigated (Marhaver *et al.*, 2008). The authors identified bacteriophages thus suggesting that they may have an important role in structuring the bacterial members of the holobiont. Although research on bacteriophages associated with macroalgae is in its infancy, bacteriophages are likely to contribute to the structure of epibacterial communities on macroalgae. They may be very important drivers, further complicating the studies on algal associated bacterial communities and should be considered in future studies on macroalgal holobionts.

6.1.4 Macrofoulers and Grazers

As briefly mentioned in the discussion of Paper II, macroscopic organisms that either graze or settle on the host alga may also play a role in structuring the biofilm. Although macroscopic grazers do not graze selectively on the microscopic biofilm members, they may reduce cell densities while grazing on the macroalgal host. Although speculative, it is conceivable that macroscopic grazers, such as gastropods or isopods, may bring along their own specific bacterial community, thus acting as vectors for “invasive” bacteria that may establish and restructure the

algal community. The same may be true for macrofouler larvae (barnacle cyprids, mussel veligers) that attach to the macroalga or bryozoans that create special microhabitats on an algal surface.

6.1.5 Random Processes

While the above-mentioned influencing forces are deterministic, there is evidence for random processes contributing to the composition of epibacterial communities. This was briefly discussed in Paper I referring to the Burke studies on epibacteria associated with the green alga *Ulva australis*. The authors found that there was a large variability in epibacterial community composition based on taxonomy between algal individuals of the same species. This variability was confirmed for *F. vesiculosus* in my study. Considering this and the fact that the communities significantly differed between algae and seawater (similarly confirmed in my study by the comparison between epiphytic and epilithic bacterial communities), there seemed to be two processes at work. Niche-based processes explain the difference in epibacteria between algal surfaces and seawater (or stones) due to obvious differences in the type of substrate/medium. Random processes account for the large variability between individuals in observed phylotypes. The authors suggested that the “lottery hypothesis” - originally a theory in macroecology proposed to explain the high diversity of fishes on coral reefs and meanwhile confirmed with empirical evidence (Sale, 1976, Munday, 2004) - can be extrapolated to microbial studies. It states that an empty niche is colonized from within a functional group by any bacterial phylotype that, due to chance events, gets there first. Indeed, when analyzing functional genes of epibacteria deemed important in the host-associated lifestyle (e.g., genes involved in biofilm formation), the variability between algal host individuals was reduced and a core of functional bacterial genes was consistently present. Considering the vast amount of bacterial phylotypes that I detected in each experiment (>2000), the functional redundancy of epibacteria observed in the Burke study is likely to be confirmed in future metagenomic studies on epibacteria from *F. vesiculosus*.

6.2 The Role of Epibacteria in a Changing Environment

In my study, it became apparent that the epibiotic biofilm reorganizes driven by changes in temperature, salinity, and irradiance. Furthermore epibiotic assemblages reared at different temperatures had differing effects on macrofouler larvae. The environmental factors naturally fluctuate and are predicted to change in the future as stated in the introduction. Especially, elevated water temperatures cause damage to algal beds (Case *et al.*, 2011), to sponges (Fan *et al.*, 2013), and to coral reefs (e.g., Hoegh-Guldberg, 1999, McWilliams *et al.*, 2005) by interfering with the healthy state of the holobiont.

6.2.1 Pathogens

An increase of temperature can boost the risk of disease for host organisms (reviewed in Harvell *et al.*, 2002). Since sea surface temperatures are expected to increase globally (IPCC, 2007), this may affect the resilience of ecosystems (Case *et al.*, 2011). In a study on bleaching observed in the red alga *Delisea pulchra*, a tight link between increased temperature and bacterial virulence was proven. The bacterial pathogen that infected algal thalli at 24°C (but not at 19°C) was identified as a strain of the Roseobacter clade, (family Rhodobacteriaceae). Based on these results and previous results that showed that the Roseobacter were often equipped with QS (Quorum sensing) and secretion systems, the authors suggested that this group may pose a particular threat in marine ecosystems that will be affected by predicted higher temperatures (Case *et al.*, 2011). In my study, the Rhodobacteraceae were more abundant at the higher temperatures than at the lower ones, and as briefly discussed in paper I, all OTUs that correlated positively with temperature were classified as members of this family. During my experiments, I observed that after about four weeks of temperature incubation the algae showed signs of necrosis at 20°C and these signs were more severe at 25°C. I was only able to analyze the epibacterial community in depth by pyrosequencing after two weeks of temperature incubation, so I cannot state whether the Rhodobacteraceae were still correlated with the higher temperatures after three or four weeks of temperature incubation, when the alga shows signs of stress. Furthermore, not all members of the Roseobacter or Rhodobacteraceae, respectively, are pathogenic. In my experiment they were also among the most abundant groups present at cold and cool temperatures (5°C and 15°C), where the host alga showed no signs of necrotic tissue. Necrosis may have resulted from an infection by pathogenic bacteria in stressed algae similar to the infection described in the above-mentioned study. There, bacterial cells penetrated algal tissue, spreading the infection finally resulting in necrosis (Case *et al.*, 2011). Considering that the Rhodobacteraceae were also found to be present at the low temperatures in my experiment, it is conceivable that they are opportunistic pathogens for stressed algae at higher temperatures (possibly due to algae degrading enzymes). Based on my results from the temperature treatments, the bacterial family Rhodobacteraceae seems to be a good candidate for a closer look using specific primers and probes in order to prove their abundance on stressed algae and further investigate their role in algal disease.

In my study, a conspicuous increase of known pathogenic bacteria was not observed at the low salinity level or at low sunlight levels - those conditions that could be stressful for the alga. However, since the low salinity level clearly influenced community composition, coupled with another stressor (e.g., high temperature), it may further boost the risk of attack by pathogens.

6.2.2 Mutualistic Role of Microbial Assemblages

Based on the results from the bioassays testing the impact of epimicrobial assemblages on the attachment rates of barnacle larvae, it is likely that the biofilm plays an important role in the health of the host alga by functioning as an effective barrier against attachment of macrofoulers. Temperature had an impact on the magnitude of the effect with the most pronounced repulsive effect from biofilm cultured at 15°C and 5°C and a weakening of the effect at 20°C. Therefore, it seems possible that temperature, besides driving an increase of pathogens, may also have a more subtle effect by weakening the protective layer of biofilm.

One interesting aspect that should be investigated in the future is whether the observed trend for higher epibacterial diversity (richness and evenness) in the temperature experiment at 15°C can be linked to the slightly stronger repulsive effect observed in the field bioassays. For this purpose the experiment should be repeated, and the bacterial community should be analyzed by sequencing rather than by DNA fingerprinting methods (only the latter was applied in the field bioassay in order to investigate shifts in the bacterial community after temperature treatment). In order to assess the impact of my results for *Fucus* in natural settings in the future, the actual threat for healthy individuals of being overgrown by macrofoulers would have to be determined in field surveys. If the biofilm is important in reliably assisting the defense of the host, it makes sense that the microbes responsible are reliably transmitted from one generation to the next (one of the principles of the hologenome theory introduced in the first chapter). Or they are reliably acquired from the environment, which would imply very fine-tuned selective mechanisms by the host. Alternatively, many different phylotypes randomly acquired from the environment could be involved in protecting the host, indicating a high level of functional redundancy. The similarity or dissimilarity of epibacterial communities from parents and offspring would have to be investigated.

Conclusion

This study has shown that the epibacterial microbial assemblage found on the surface of *Fucus vesiculosus* has the potential to maintain the healthy status of the host throughout periods of intense fouling pressure by assisting the defense of the host against macrofouler larvae. The protective role may be influenced by temperature-driven shifts in the epibiotic community composition. When abiotic conditions (temperature, as well as irradiance and salinity) change, shifts in the epibacterial community composition and taxonomic diversity can occur - as demonstrated in my study. It seems that only a few dominant members of the diverse bacterial biofilm contribute to the observed shifts. They may play key roles in interactions with the macroalgal host. Considering these important interactions between macroalgal host, epiphytic microbes, and other larger organisms,

the beneficial role of bacteria can be negatively affected by e.g., weakened antifouling properties of the biofilm at elevated temperatures. However, whether this will be the case under environmental conditions affected by climate change is a question that at this time remains a matter of speculation for *Fucus vesiculosus* in the Baltic Sea. My conclusions are based on short term experiments neglecting the adaptability of the host and the bacteria and their combined adaptability within the holobiont concept. Whether the observed taxonomic shifts in my study will have consequences for the development and maintenance of healthy algal hosts in their environment will depend on the functional redundancy within the epibacterial community. For the time being, we have gained insight into bacterial community composition and diversity by inferring taxonomy which will be further broadened if we identify functional genes important in the interactions with the macroalgal host.

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Supporting Information

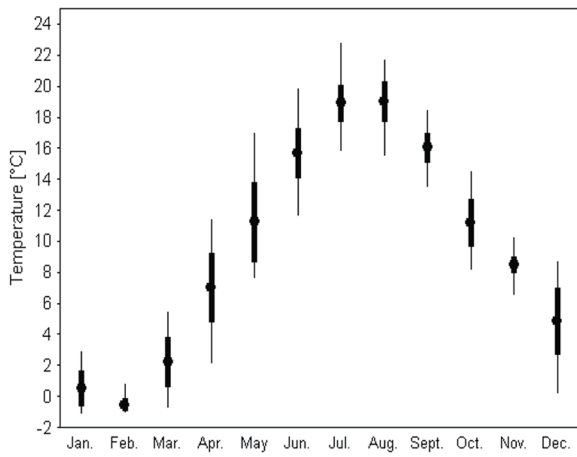


Figure S 1 Mean (circle in the center) water temperature at approx. 1.5 m water depth at the sampling site of *Fucus vesiculosus* during the course of one year (May 2009-May 2010) with standard deviation (thick lines) and minimum and maximum values (thin lines).

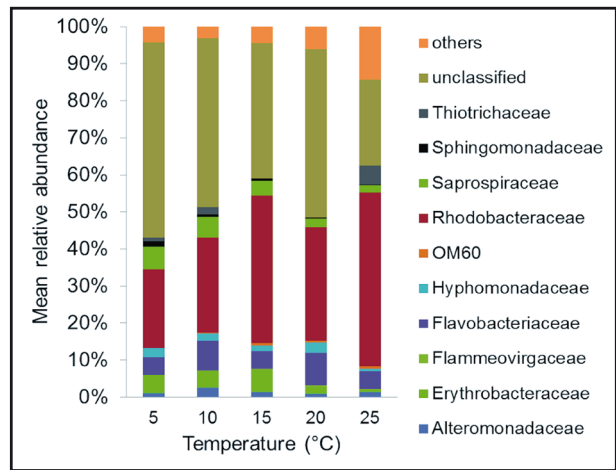


Figure S 3 Relative abundances of bacterial OTUs at the family level. For clarity, only the ten most abundant families are individually depicted in the bars; the rest of the classified families are compiled in “others”.

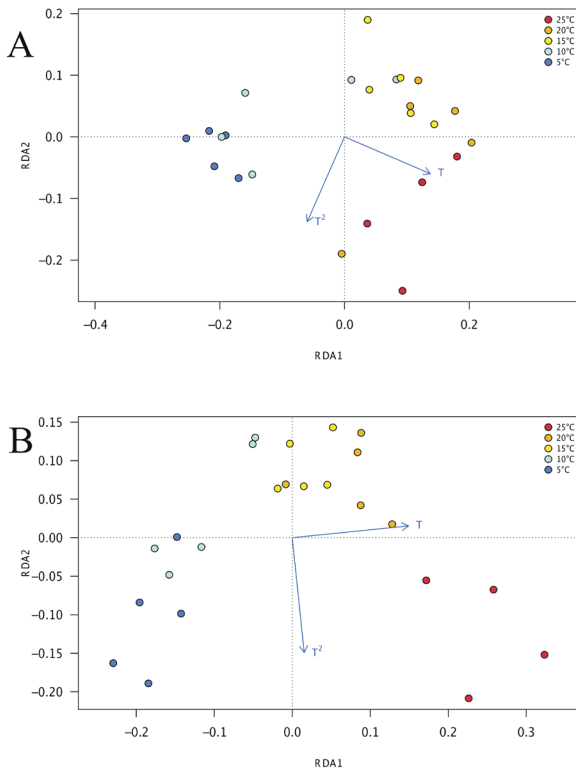


Figure S 2 Redundancy analysis of the Hellinger-transformed presence absence data matrix based on bands of the DGGE fingerprint of epibacteria from *Fucus vesiculosus* cultured at different temperatures for 7 days (A) and 21 days (B) (5, 10, 15, 20, and 25°C; n = 5 per temperature level, except for n = 4 at 25°C). Distance biplot showing relationships between samples (scaling 1) as depicted by the sample points and the temperature gradient vectors (T for linear relations and T² for unimodal relations).

Table S 1 Good's coverage in %. Mean of 5 replicates of each temperature level with standard deviation.

Temperature	5°C	10°C	15°C	20°C	25°C
Coverage %	0.85 ± 0.05	0.81 ± 0.04	0.78 ± 0.06	0.79 ± 0.03	0.81 ± 0.05

Table S 2 Amount of variation explained by temperature (T) and the squared temperature term (T²) in the redundancy analysis of the Hellinger-transformed presence absence data matrix based on bands of the DGGE fingerprint of epibacteria from *Fucus vesiculosus* cultured at different temperatures for 7 days and 21 days (see also Figure S 2A and B).

Data set	% variation explained	
	T	T ²
DGGE 7 days	12	4
DGGE 21 days	16	4

Table S 3 Statistical analysis of community diversity: Comparison of goodness of fit of regressions (AIC: Akaike Information Criterion) and statistical output.

Diversity	AIC		Adjusted R ²		p	
	linear	binomial	linear	binomial	linear	binomial
OTU richness	286	282.7	0.1	0.24	0.07	0.02
Inverse Simpson Index	218.3	218.8	0.12	0.13	0.05	0.08

Table S 4 The top 15 OTUs: Number of cumulative reads of the respective OTU across all replicates (n=5) of one temperature level (the number of total reads per temperature level was 6 740).

# OTU	Number of reads				
	5°C	10°C	15°C	20°C	25°C
77	1466	452	114	40	2
46	184	235	109	70	3
42	425	529	577	277	170
110	207	119	127	147	11
29	87	119	120	69	9
112	2	39	189	123	26
45	297	638	265	441	89
483	8	26	27	9	3
27	33	50	117	63	57
41	0	10	13	7	0
325	4	37	151	332	114
51	34	52	258	146	340
261	0	0	0	5	112
28	30	46	115	189	213
507	1	0	3	6	153

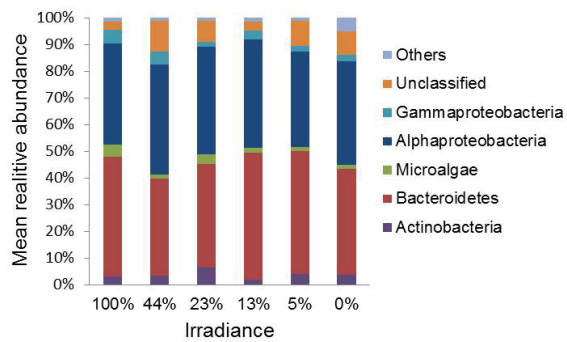


Fig. S1 OTUs (97% sequence similarity) with the highest mean relative abundances at the phylum and subphylum level, respectively, after ten days of irradiance treatment. OTUs with mean abundances < 1% are compiled in „others“. Microalgal chloroplasts are included. (n = 5 per irradiance level, except for n = 4 at 23% irradiance).

Table S 1 Statistical analysis of epibacterial diversity after ten days of irradiance treatment: Comparison of goodness of fit of regressions (AIC: Akaike Information Criterion)

Diversity	linear	binomial
OTU richness	198.96	194.66
Inverse Simpson Index	104.66	100.64

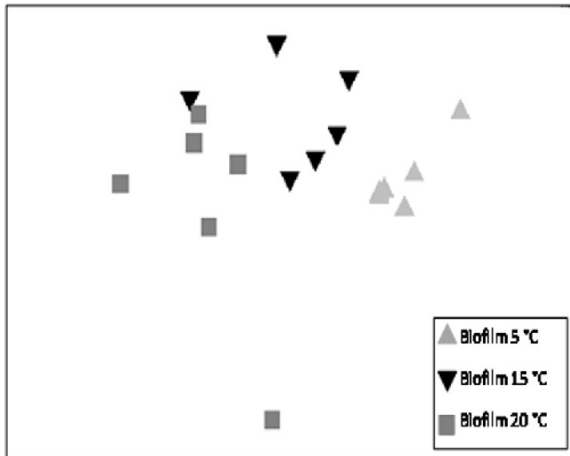


Fig. S1. Non-metric Multidimensional Scaling (n-MDS) plot visualizing differences and overlaps between epibacterial community compositions. Scaling is based on Bray-Curtis Similarities; 2D Stress: 0.11.



Fig. S2. *Fucus vesiculosus* collected in the Kiel Fjord with severe fouling by barnacles.

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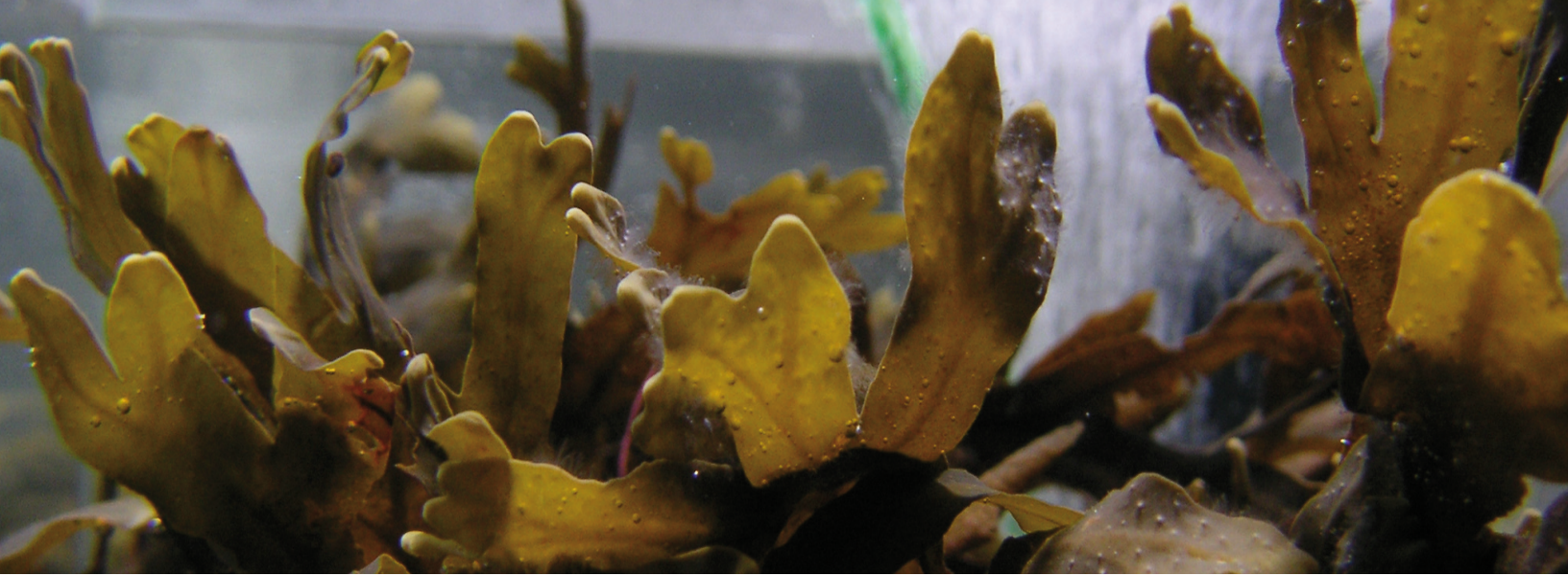
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Eidesstattliche Erklärung

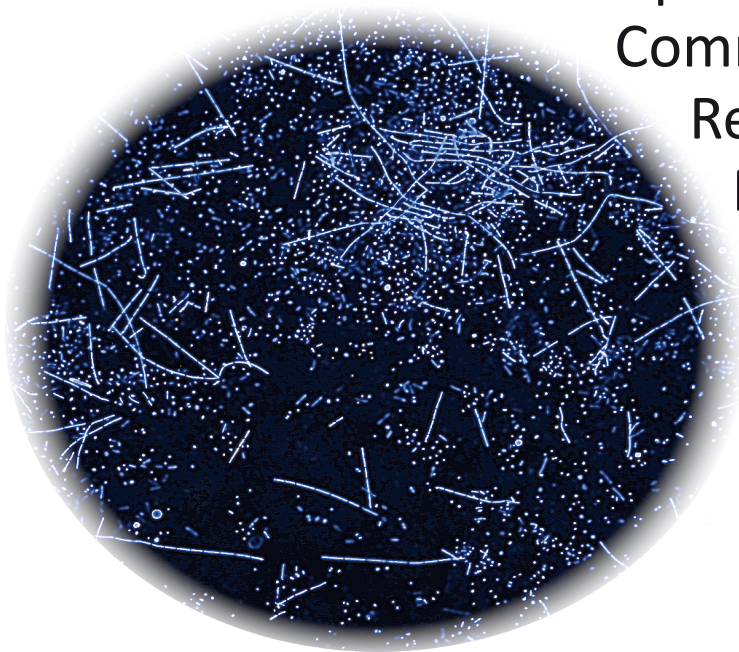
Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation mit dem Titel „*Ecology of Microbes Associated with a Macroalgal Host: Effects of Environmental Conditions on the Composition of Epibacterial Communities and Repellant Effects of Microbial Epibionts on Barnacle Larvae*” selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt und habe die Dissertation nach den Regeln guter wissenschaftlicher Praxis (Standard wissenschaftlichen Arbeitens nach den Empfehlungen der DFG) verfasst.

Kiel, den _____



Ecology of Microbes Associated with a Macroalgal Host:

Effects of Environmental Conditions on the Composition of Epibacterial Communities and Repellant Effects of Microbial Epibionts on Barnacle Larvae



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
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