Zur Homepage der Dissertation

Allelopathy of marine macroalgae against microbial epibiosis

Allelopathie mariner Makroalgen gegen mikrobiellen Aufwuchs

Von der Fakultät für Mathematik und Naturwissenschaften
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angenommene Dissertation
von

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To the people who I care and love

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DECLARATION

PUBLICATIONS

Results of my PhD thesis have been published in or submitted to international journals (Chapters 2 – 4). Some important findings have been referenced from the Master theses of Annika Grage and Dirk Schulz (Chapter 4.1); whilst the extension of my work has been addressed in the Master thesis of Rebecca Neumann. Details of publications and Master theses of A. Grage, D. Schulz and R. Neumann are listed as follows:

Lam C, Grage A, Schulz D, Harder T (submitted) Antifouling activity of North Sea macroalgae against benthic diatoms. Biofouling

Lam C, Stang A, Schulte A, Harder T (submitted) Planktonic epiphytic bacteria and fungi are selectively eliminated by marine macroalgae. FEMS Microbiol Ecol

Harder T, Lam C (2006) Waterborne allelochemicals of marine macroalgae affect bacterial diversity and abundance in close proximity. Proceedings of the GfÖ, Hoffmeister T, Diekmann M (eds), vol. 36, pp 306

Lam C, Harder T (in press) Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity. J Phycol

Grage A (2005) Allelochemischer Einfluss von Makroalgenmetaboliten auf benthische Diatomeen: Ausarbeitung eines Biotests. Master thesis, University of Oldenburg, Germany

Schulz D (2005) Quantitative and qualitative Untersuchungen von Makroalgen besiedeInden Bakteriengemeinschaften. Master thesis, Unviersity of Oldenburg, Germany

Neumann R (2007) The allelochemical effect of macroalgae on quantitative and qualitative composition of bacterial communities in plankton and biofilms. Master thesis, University of Oldenburg, Germany

CONFERENCES AND SEMINARS

Lam C, Harder T (2006) North Sea macroalgae affect bacterial community patterns in ambient water. Annual Conference of the Association for the General and Applied Microbiology (VAAM), Jena, Germany. **Oral presentation**.

Lam C, Harder T (2006) Waterborne metabolites of North Sea macroalgae affect bacterial community patterns in ambient seawater. 11th International Symposium on Microbial Ecology (ISME-11), Vienna, Austria. **Poster presentation**.

Harder T, Lam C (2006) Waterborne allelochemicals of marine macroalgae affect bacterial diversity and abundance in close proximity. 36th Annual Conference of the Ecological Society of Germany, Switzerland and Austria (GfÖ), Bremen, Germany. **Oral presentation**.

Lam, C., Harder, T. (2007) Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity. 4th Marine Biological Association, Annual Postgraduate Workshop, Liverpool, United Kingdom. **Poster presentation**.

OTHER PUBLICATIONS DURING THE COURSE OF DOCTORAL STUDY

Lam C, Harder T, Qian PY (2005a) Induction of larval settlement in the polychaete *Hydroides elegans* by benthic diatoms: the role of extracellular polysaccharides (EPS). Mar Ecol Prog Ser 286:145-154

Lam C, Harder T, Qian PY (2005b) Growth conditions of benthic diatoms affect quality and quantity of extracellular polymeric larval settlement cues. Mar Ecol Prog Ser 294:109-116

CONTENTS

KURZZUSAMMENFASSUNG

Die Oberflächen mariner Makrophyten sind oftmals frei von mikro- und makroskopischen Aufwuchsorganismen. Diese Beobachtung legt nahe, dass die Makrophyten über Verteidigungsstrategien gegen Aufwuchs (Fouling) verfügen. Bisherige Studien zu diesem Thema basierten im Wesentlichen auf der Untersuchung von Gesamtextrakten der Makrophyten auf eine kleine Auswahl von Bakterienisolaten. Dabei wurden nicht nur ökologisch relevante Bakterien unterschlagen, sondern die natürlichen Freisetzungsmechanismen der antibakteriellen Stoffe aus Makrophyten blieben weitgehend unberücksichtigt. Die vorliegende Arbeit hatte zum Ziel, sich diesen Fragestellungen in einer ökologisch relevanten und unvoreingenommenen Herangehensweise zu nähern, und unterteilt sich in folgende Teilgebiete: 1) Zunächst wurde der Effekt der von Makrophyten ins Wasser abgegebenen Substanzen in einem kulturunabhängigen Ansatz auf die Diversität von Freiwasserbakterien und Pilzen untersucht. Dabei wurde zwischen Makrophytenmetaboliten in Abwesenheit und Anwesenheit von Makrophten unterschieden; 2) Der Effekt von Makrophytenmetaboliten auf phylogenetisch unterschiedliche Gruppen von Freiwasserbakteriengemeinschaften wurde durch die Verwendung von phylogenetisch selektiven Oligonukleotidsonden untersucht; 3) Der Einfluss von Makrophytenextrakten wurde auf Ansiedlungs- und Wachstumsinhibition von benthischen Diatomeen untersucht. Die Ergebnisse dieser Arbeiten zeigten einen qualitativen und quantitativen Einfluss der Makrophytenmetabolite auf die Zusammensetzung von planktonischen Freiwasserbakteriengemeinschaften. Es wurden insbesondere Bakterien der *Bacteriodetes* und der *Roseobacter* Gruppe

von den Metaboliten beeinflusst, was den Schluß nahelegte, dass diese epiphytischen Bakterientypen besonders problematisch für den Makrophytenwirt sind. Die Pilzdiversität im Freiwasser wurde ebenfalls durch die Makrophytenmetabolite beeinflusst, allerdings zeigte sich hier ein anderes Aktivitätsspektrum im Vergleich zu den Freiwasserbakterien. Makrophytenextrakte zeigten ein ausgeprägtes Aktivitätspotential gegen benthische Diatomeen, so dass eine gezielte und selektive Verteidigung gegen diese Gruppe von Aufwuchsorgansimen geschlussfolgert werden kann.

ABSTRACT

Marine macroalgae are often devoid of micro- and macro-colonizers, suggesting some sort of host-derived antifouling defense. Previous studies mainly focused on tissue extracts of entire algae against few bacterial isolates, thereby not only neglecting ecologically relevant bacteria but also natural delivery mechanisms of algal antimicrobial agents. Several hypotheses were made in my study: 1) investigated potential antimicrobial effects of waterborne macroalgal metabolites utilizing a culture-independent approach to compare the bacterial and fungal community richness in seawater in the presence and absence of macroalgae; 2) tested antibacterial activity of macroalgal metabolites against phylogenetically specific bacterial colonizers in the presence of algae; 3) studied the inhibitory effects of macroalgae against epiphytic diatoms. Results revealed that macroalgae affected planktonic bacteria qualitatively and quantitatively by waterborne algal metabolites. Both the *Bacteriodetes* and the *Roseobacter* clade were greatly eliminated by macroalgae, implying these epiphytic bacteria may pose a particular threat to the host. Fungal community pattern was also affected in the presence of algae, but with a different specificity of algae. Furthermore, algal extracts exhibited a distinct pattern of activities against epiphytic diatoms, suggesting a targeted and selective effect of macroalgal metabolites on individual fouling diatoms. My study firstly demonstrates macroalgae exhibit antimicrobial activities against a wide range of epiphytic microorganisms based on natural delivery mechanisms of allelochemicals utilizing a culture-independent approach, thus minimizing the ecological bias based on few microbial isolates.

ABBREVIATIONS

ACW	Algae conditioned seawater
ANOSIM	Analysis of similarity
CFB	*Cytophaga/Flavobacteria Bacteroidetes*
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
DAPI	DNA-binding fluorochrome 4,6-diamidino-2-phenylindole
DCM	Dichloromethane
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethylsulfoxide
HPLC	High Perfromance Liquid Chromatography
ITS	Internal Transcribed Spacer
MeOH	Methanol
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NSW	Natural seawater
SEM	Scanning Electron Microscopy
TAE buffer	Tris-acetate-EDTA buffer
TE buffer	Tris-EDTA buffer

1. INTRODUCTION

1.1 North Sea macroalgae

Species diversity. Marine macroalgae used for my PhD work are collected from the rocky shore of Helgoland, southeastern of the North Sea (Fig. 1; N: 7^{o} 52' 60", W: 54^{o} 10' 0"). In the mid-19th century, 274 marine macroalgal species have been recorded by scientists at the Marine Station (Biologische Anstalt Helgoland) on the island: 77 species of Chlorophycota, 100 species of Phaeophycota and 97 species of Rhodophycota (Bartsch & Kuhlenkamp, 2000). The flora of Helgoland has relatively small number in comparison with the surrounding cold-temperate coastlines of Britain and Ireland, France and Scandinavia. For instance, the species directory for the British Isles and the surrounding seas comprise of 820 macroalgal species (Guiry, 1997), whilst 325 species are listed in the northern part of the Baltic Sea and 370 species are found in the Norwegian coastlines (Nielsen et al. 1995; Rueness et al. 1997).

Figure 1. General view of rocky shore with a variety of Rhodophyceae, Phaeophyceae and Chlorophyceae on Helgoland.

The species diversity of macroalgae on Helgoland is continuously changing over time, especially in the period between the beginning and the end of the 20th century (Bartsch & Kuhlenkamp, 2000). The sensitivity of macroalgal species detection is one of the reasons to cause for the difference. Generally, species detection depends on their life histories, abundance, time period and location of sampling, change of natural habitats, invasion of introduced species and other environmental factors such as water temperature, light intensity and nutrients

(Lobban & Harrison, 1994). According to Bartsch & Kuhlenkamp (2000), macroalgae with a life cycle comprising perennial or pseudoperennial macroscopic stages are reported to be dominant on Helgoland. Although the natural substrata on Helgoland are comprised of special red sandstone, chalk and flint stone, the building of protective moles, harbour constructions and breakwaters from the early 1920s onwards provided diverse new habitats for different kinds of macroalgae to colonize the island. Interestingly, an introduction of foreign species, such as *Bonnemaisonia hamifera*, *Codium fragile*, *Mastocarpus stellatus* and *Sargassum muticum*, is contributed to the change in species diversity and composition. The first two species have either a restricted habitat or occur infrequently, whilst the red alga *Mastocarpus stellatus* and the brown alga *Sargassum muticum* altered the appearance of some biotopes with their massive growth and continued its colonization around the island (Bartsch & Kuhlenkamp, 2000; Bartsch & Tittley, 2004). Additionally, physical parameters such as nutrients and seawater temperature might affect the species diversity on Helgoland when they are varied over time (Becker et al. 1997; Franke et al. 1999). As the overall world climate is increasing every year due to global warming, the species diversity on Helgoland could be influenced as expected in the coming future.

Laboratory cultivation. Macroalgae collected from the field are quickly rinsed with natural seawater (NSW) to get rid of isopods prior to experiment. As the algae are removed from the natural habitats, they could release stressful compounds which disturb the overall effectiveness or performance of antimicrobial activity in the conditioning experiment (Lobban & Harrison, 1994). Therefore, the

algae should be acclimatized at the laboratory culture room for 48 h in 2 L aerated Erlenmeyer flasks containing *ƒ*/2 nutrient medium made from the same batch of NSW (Guillard & Ryther, 1962) at 12 ± 1 ˚C with several daylight-type fluorescent lamps at an irradiance level of 20 µmol photons m^{-2} sec^{-1} under a 15:9 h light:dark photo cycle. Although all abiotic factors are adjusted similarly to the field according to the sampling time period, algal fitness would decrease when they are cultured at the laboratory for a certain period of time. Therefore, the same batch of algae is not recommended to perform the conditioning experiment more than once in the study.

1.2 Macroalgal defence

Marine epibiosis. By definition, marine epibiosis refers to the phenomenon that surface of living organisms are rapidly covered with a layer of adsorbed organic matters and subsequently colonized by micro- (i.e. bacteria, diatoms, fungi, viruses) and macro- (i.e. algal spores, larvae) organisms (Wahl, 1989). Marine macroalgae are easily susceptible to epibiosis as they are sessile and restricted to photic zone where optimal growth conditions for phyto- and zooplankton occur (de Nys et al. 1995). Epibiosis can pose both beneficial and detrimental effects to host macroalgae. Some green algae are benefited from hosting epiphytic bacteria for a proper development; otherwise atypical morphology is exhibited when it is grown in axenic culture (Tatewaki et al. 1983). Other advantages like symbiotic epibionts protect the host from UV radiation (Koch & Brandt, 2003) and desiccation (de O Figueiredo et al. 2000) or facilitate nitrogen-fixation (Thevanathan et al. 2000) are described in literature. Importantly, the adverse effects of microbial epibiosis may reach beyond pathogenicity and virulence. Since microbial films are important

sources of chemical cues for larval settlement in many benthic marine invertebrates (Lau et al. 2002; Harder et al. 2002), microbial epibiosis may promote subsequent colonization by rigid crustose epibiotic macroorganisms, which in turn significantly impair the basibionts' ability to exchange gases and nutrients (Jagels, 1973), damage the tissue by increased weight, rigidity and drag (Dixon et al. 1981), and decrease the growth rate of photosynthetic basibionts by cutting surface irradiance levels (Sand-Jensen, 1977; Silberstein et al. 1886).

Interestingly, marine macroalgae like other soft-bodied invertebrate species such as corals and sponges, are virtually free of epiphytes on its surfaces in comparison to co-occurring biofilms on inanimate substrata (Steinberg et al. 1997; Maximilien et al. 1998; Dobretsov & Qian, 2002). Physical defenses such as mucus secretion, periodic sloughing of outermost cell layers to remove attached epiphytes on algal blades are hypothesized to suppress epibiosis (Fig. 2; Moss, 1982; Keats et al. 1997; Corre & Prieur, 1990). Increasingly, macroalgae are chemically defended themselves by producing bioactive compounds into the seawater (de Nys et al 1995; Steinberg et al 1997; Steinberg et al 1998; Harder et al 2004; Engel et al. 2006; Paul et al 2006). Such observations have inspired large-scale algal screening programs to discover novel antimicrobial compounds. It will be described in details in the up-coming paragraphs.

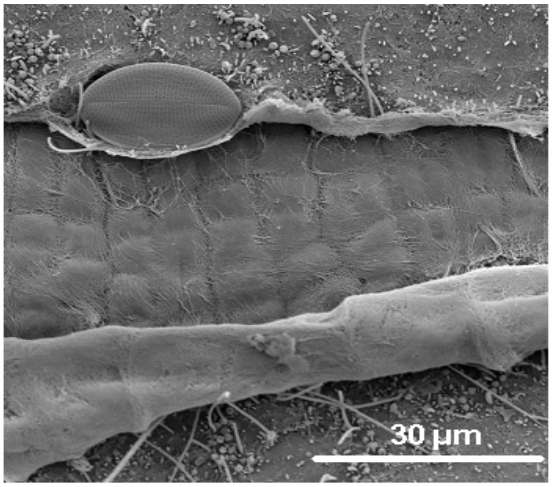

Figure 2. Scanning electron microscopy (SEM) picture showing periodic sloughing of epidermal cells of the brown alga *Laminaria digitata*.

Allelopathy of macroalgae. In order to compete for limited resources such as light, space and nutrients in marine environment, most photoautotrophs, macroalgae in particular, have to release bioactive secondary metabolites (or allelochemicals) interfering with settlement and/or growth of competitors in their vicinity (Gross, 1999; 2003). Different from terrestrial plants, macroalgae have special structures with no stomata, a reduced or no cuticle and less tight cell connections (Hutchinson, 1975), all of which should facilitate the direct release of water-soluble compounds into seawater through open stomata or from epidermal cells. Generally, there are two types of allelopathic interactions in marine systems. First, macroalgae produce sufficient amount of bioactive metabolites, excrete into the seawater and reach target species at an effective, active concentration (Gross, 2003). However, dilution and short half-life cycle of these compounds are the major problems of this type of allelopathic interactions (Lewis, 1986; Weinberger et al. 1999; Küpper et al. 2001; Küpper et al. 2002). Second, macroalgae release metabolites and target particular epiphytes by direct contact, which is, compounds located in secretory trichomes, epidermal glands, or otherwise associated with the surfaces of the algae. The chemistry of these compounds is mostly hydrophobic and non-polar in nature in order to maximize its concentrations at or close the host surfaces without rapid dissolution away from the surface of the affected organisms (Jennings & Steinberg, 2001).

Macroalgal allelochemicals. Provided that macroalgae are a rich source of bioactive metabolites, there are increasing investigations aiming at discovering novel antimicrobial compounds against bacteria (Hellio et al. 2000; 2001; Pelegrin & Morales, 2004; Paul et al. 2006), diatoms (Hellio et al. 2002; Amsler et al. 2005),

fungi (Ballesteros et al. 1992; Hellio et al. 2000; Engel et al. 2006), virus (Caccamese et al. 1981), algal spores (Bazes et al. 2006) and invertebrate larvae (Harder & Qian, 2000; Hellio et al. 2002; Walters et al. 2003; Harder et al. 2004; Maréchal et al. 2004). In the majority of these studies, crude-extracts of entire algal material were directly screened for antimicrobial activities with a small number of laboratory-cultivable bacterial, diatom and fungal isolates (Hellio et al. 2000; 2001; 2002; Engel et al. 2006; Paul et al. 2006). Although this procedure provided an insight of bioactive algal metabolites, the natural delivery mechanisms, as well as the susceptibility of ecologically relevant bacteria to naturally released algal compounds remain largely unknown (Kubanek et al. 2003; Dworjanyn et al. 2006; Engel et al. 2006; Nylund et al. 2007).

The isolation, identification and elucidation of macroalgal bioactive metabolites with potent antifouling activity have been intensively studied recently (Smit, 2004; Bhadury & Wright, 2004; Mayer & Hamann, 2004; Paul et al. 2006). So far, compounds with non-polar, halogenated properties such as fatty acids, furanones, lipopeptides, amides, alkaloids, terpenoids, lactones, pyrroles and steroids are described to be good antifoulants (Bhadury & Wright, 2004). For example, ultrastructural studies revealed vesicle cells near the thallus surface of the red alga *Delisea pulchra* containing lipophilic, halogenated furanones, which inhibit growth and attachment of ecologically relevant bacteria both in the field and the laboratory (Steinberg et al. 1997; Maximilien et al. 1998). In the red alga *Asparagopsis armata,* bromoform and dibromoacetic acid have been demonstrated to exhibit antibacterial effects (Paul et al. 2006). Lipid extracts from the brown algae *Cytoseira balearica* and *Zanardinia prototypes*, and the green alga *Codium coralloides* exhibited antibacterial and antifungal activities

(Caccamese et al. 1981). Furthermore, a broadband inhibition was observed in the red alga *Laurencia obtusa* by producing different kinds of bioactive metabolites (i.e. Palisol, Palisadin A, 5*β* – Hydroxyaplysistain, Laurencienyne) against colonizing bacteria, algal spores and invertebrate larvae (Caccamesse et al. 1981; Steinberg et al. 1998).

Although the former studies suggest the majority of bioactive compounds are to be of low molecular weight and non-polar in nature, other studies recommend large polar macromolecules with similar antimicrobial effects are observed in direct proximity on algal surfaces (Regan & Glombitza, 1986; Dobretsov & Qian, 2002; Harder et al. 2004; Bazes et al. 2006). In the past, phlorotannins from brown algae were often considered to function as chemical deterrents against marine herbivores (Sieburth & Conover, 1965; Jennings & Steinberg, 1997). Recent study has shown that the deterrence of the amphipods *Ampithoe valida* and *A. longimana* and the sea urchin *Arbacia punctulata* was lost following storage or fractionation of the active, water soluble extracts of the brown alga *Fucus vesiculosus* (Kubanek et al. 2004). Compounds other than phlorotannins appear to be chemical deterrents of *F. vesiculosus*. Unfortunately, these compounds still remain unknown due to the chemical instability in nature.

1.3 Objectives

In this study, I investigated the antimicrobial effects of waterborne macroalgal metabolites against potential epiphytic colonizers in seawater. Firstly, I tested the hypothesis that the observed antimicrobial effects of waterborne metabolites from macroalgae targeted the whole spectrum of potential epiphytic bacteria in seawater utilizing culture-independent approach. Secondly, I studied whether these compounds targeted specific bacterial phyla and planktonic fungi. Thirdly, I tested whether macroalgae exhibited potent inhibitory effects against epiphytic diatoms. Briefly, I collected a variety of macroalgae from Rhodophyceae, Phaeophyceae and Chlorophyceae from the North Sea (Helgoland), Germany. Culture-independent methods comprised the collection of planktonic bacteria and fungi in algal culture water (ACW) on membrane filters and followed by filter PCR and denaturant gradient gel electrophoresis (DGGE) of 16S rDNA gene sequences of harvested bacteria and fungi with different primers, respectively. These objectives were experimentally addressed with oligonucleotide probes, specifically targeting different bacterial phyla (*Cytophaga/Flavobacteria Bacteroidetes*, alpha-*Proteobacteria*, the *Roseobacter* clade) and fungi (ITS 1 region) in seawater that has been exposed to the macroalgae under investigation. As for epiphytic diatoms examination, macroalgae were firstly examined by scanning electron microscopy and then performed chemical extraction for diatom attachment assays against four ecologically relevant benthic diatoms. My experimental designs provided valuable insight to the natural delivery mechanisms of bioactive waterborne algal metabolites against a wide range of different potential groups of epiphytic micro-colonizers (i.e. bacteria, fungi and diatoms) in seawater.

1.4 References

Amsler CD, Okogbue LN, Landry DM, Amsler MO, McClintock JB, Baker BJ (2005) Potential chemical defenses against diatom fouling in Antarctic macroalgae. Bot Mar 48:318-322

Ballesteros E, Martin D, Uriz MJ (1992) Biological activity of extracts from some Mediterranean macrophytes. Bot Mar 35:481-485

Bartsch I, Kuhlenkamp R (2000) The marine macroalgae of Helgoland (North Sea): an annotated list of records between 1845 and 1999. Helgol Mar Res 54:160-189

Bartsch I, Tittley I (2004) The rocky intertidal biotopes of Helgoland: present and past. Helgol Mar Res 58:289-302

Bazes A, Silkina A, Defer D, Bernède-Bauduin C, Quéméner E, Braud JP, Bourgougnon N (2006) Active substances from *Ceramium botryocarpum* used as antifouling products in aquaculture. Aquaculture 258:664-674

Becker GA, Frohse A, Damm P (1997) The northwest European shelf temperature and salinity variability. Dtsch Hydrogr Z 49, H.2/3:145-161

Bhadury P, Wright PC (2004) Exploitation of marine algae: biogenic compounds for potential antifouling applications. Planta 219:561-578

Caccamese S, Azzolina R, Furnari G, Cormai M, Grasso S (1981) Antimicrobial and antiviral activities of some marine algae from Eastern Sicily. Bot Mar 14:365-367

Corre S, Prieur D (1990) Density and morphology of epiphytic bacteria on the kelp *Laminaria digitata*. Bot Mar 33:515-23

de Nys R, Steinberg PD, Willemsen P, Dworjanyn SA, Gabelish CL, King RJ (1995) Broad spectrum effects of secondary metabolites from the red algae *Delisea pulchra* in antifouling assays. Biofouling 8:259-71

de O Figueiredo MA, Kain JM, Norton TA (2000) Responses of crustose corallines to epiphytic and canopy layer. J Phycol 36:17-24

Dixon J, Schroeter SC, Kastendick J (1981) Effects of encrusting bryozoan, *Membranipora membranacea* on the loss of blades and fronds by the giant kelp, *Macrocystis pyrifera* (Laminariales). J Phycol 17:341-345

Dobretsov S, Qian PY (2002) Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. Biofouling 18:217-28

Dworjanyn SA, de Nys R, Steinberg PD (2006) Chemically mediated antifouling in the red alga *Delisea pulchra*. Mar Ecol Prog Ser 318:153-163

Engel S, Puglisi MP, Jensen PR, Fenical W (2006) Antimicrobial activities of extracts from tropical Atlantic marine plants against marine pathogens and saprophytes. Mar Biol 149:991-1002

Franke HD, Gutow L, Janke M (1999) The recent arrival of the oceanic isopod *Idotea metallica* Bosc off Helgoland (German Bight, North Sea): an indication of a warming trend in the North Sea? Helgol Meeresunters 52:347-357

Gross E (1999) Allelopathy in benthic and littoral areas: Case studies on allelochemicals from benthic cyanobacteria and submersed macrophytes, in *Principles and Practices in Plant Ecology*: Allelochemical Interactions, Inderjit, Dakshini, K. M. M., and Foy, C. L. Eds., CRC Press, Boca Raton, pp. 179-199

Gross E (2003) Allelopathy of aquatic autotrophs. Crit Rev Plant Sci 22:313-339

Guillard R, Ryther J (1962) Studies of marine planktonic diatoms. Can J Microbiol 8:229-239

Guiry MD (1997) Benthic red, brown and green algae. In: Howson CM, Picton BE (eds) The species directory of the marine fauna and flora of the British Isles and the surrounding seas. Ulster Museum and the Marine Conservation Society, Belfast, pp 341-367

Harder T, Qian PY (2000) Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitive cues for larval attachment and metamorphosis in the polychaete *Hydroides elegans*. Biofouling 16:205-214

Harder T, Lam C, Qian PY (2002) Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. Mar Ecol Prog Ser 229:105-12

Harder T, Dobretsov S, Qian PY (2004) Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. Mar Ecol Prog Ser 274:133-41

Hellio C, Bremer G, Pons AM, Le Gal Y, Bourgougnon N (2000) Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. Appl Microbiol Biotechnol 54:543-549

Hellio C, de La Broise D, Dufossé L, Le Gal Y, Bourgougnon N (2001) Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar Environ Res 52:231-247

Hellio C, Berge JP, Beaupoil C, Le Gal Y, Bourgougnon N (2002) Screening of marine algal extracts for anti-settlement activities microalgae and macroalgae. Biofouling 18:205-215

Hutchinson GE (1975) A Treatise on Limnology. Vol. III. Limnological Botany, John Wiley & Sons, New York

Jagels R (1973) Studies of a marine grass, *Thalassia testudinum* I. Ultrastructure of the osmoregulatory leaf cells. Am J Bot 60:1003-1009

Jennings JG, Steinberg PD (1997) Phlorotannins vs. others factors affecting epiphyte abundance on the kelp *Ecklonia radiate*. Ocealogia 109:461-473

Keats DW, Knight MA, Pueschel CM (1997) Antifouling effects of epithallial shedding in three crustose coralline algae (Rhodophyta, Coralinales) on a coral reef. J Exp Mar Biol Ecol 213:281-93

Koch EW, Brandt LA (2003) Epiphytic layers as UV-B Filters on Seagrass Leaves. Gulf Mex Sci 21:102-3

Kubanek J, Jensen PR, Keifer PA, Sullards MC, Collins DW, Fenical W (2003) Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. Proc Natl Acad Sci USA 100:6916-6921

Kubanek J, Lester SE, Fenical W, Hay ME (2004) Ambiguous role of phlorotannins as chemical defenses in the brown alga *Fucus vesiculosus*. Mar Ecol Prog Ser 277:79-93

Küpper FC, Kloareg B, Guern J, Potin P (2001) Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata*. Plant Physio 125:278-291

Küpper FC, Müller DG, Peters AF, Kloareg B, Potin P (2002) Oligoalginate recognition and oxidative burst play a key role in natural and induced resistence of sporophytes of Laminariales. J Chem Ecol 28:2057-2081

Lau SCK, Mak KKW, Chen F, Qian PY (2002) Bioactivity of bacterial strains from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*. Mar Ecol Prog Ser 226:301-310

Lewis WM, Jr. (1986) Evolutionary interpretation of allelochemical interactions in phytoplanktonic algae. Am Nat 127:184-194

Lobban CS, Harrison PJ (1994) Seaweed ecology and physiology. Cambridge University Press, United Kingdom, pp 69-72

Maréchal JP, Culioli G, Hellio C, Thomas-Guyon H, Callow ME, Clare A, Ortalo-Magné (2004) Seasonal variation in antifouling activity of crude extracts of the brown alga *Bifurcaria bifurcate* (Cystoseiraceae) against cyprids of *Balanus amphitrite* and the marine bacteria *Cobetia marina* and *Pseudoalteromonas haloplanktis*. J Exp Mar Biol Ecol 313:47-62

Mayer AMS, Hamann MT (2004) Marine pharmacology in 2000: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. Mar Biotechnol 6:37-52

Maximilien R, de Nys R, Holmström C, Gram L, Givskov M, Crass K, Kjelleberg S, Steinberg PD (1998) Chemical mediation of bacterial surface colonization by secondary metabolites fro the red alga *Delisea pulchra*. Aquat Microb Ecol 15:233-46

Moss BL (1982) The control of epiphytes by *Halidrys siliquosa* (L.) Lynbg. (Phaeophyta, Cystoseiraceae). Phycologia 21:185-91

Nielsen R, Kristiansen A, Mathiesen L, Mathiesen H (1995) Distributional index of the benthic macroalgae of the Baltic Sea area. Acta Bot Fenn 155:1-51

Nylund GM, Gribben PE, de Nys R, Steinberg PD, Pavia H (2007) Surface chemistry versus whole-cell extracts: antifouling tests with seaweed metabolites. Mar Ecol Prog Ser 329:73-84

Paul NA, de Nys R, Steinberg PD (2006) Chemical defense against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar Ecol Prog Ser 306:87-101

Pelegrin YF, Morales JL (2004) Antibacterial activity in marine algae from the coast of Yucatan, Mexico. Bot Mar 47:140-146

Regan M, Glombitza KW (1986) Phlorotannins, brown alga polyphenols. Progr Phyc Research (Round FE, Chapman DJ, eds) Biopress Ltd. Bristol 4:129-252

Rueness J, Brattegard T, Lein TE, Küfner R, Pedersen A, Sorlie AC (1997) In: Brattegard T, Holthe T (eds) Distribution of marine, benthic macro-organisms in Norway: a tabulated catalogue, preliminary eds. Research Report for DN Nr. 1997-1. Directorate for Nature Management, Trondheim, pp 22-64, 337-350

Sand-Jensen (1977) Effects of epiphytes on eelgrass photosynthesis. Aquat Bot 3:55-63

Sieburth JM, Conover JT (1965) *Sargassum* tannin, an antibiotic which retards fouling. Nature 208:52-53

Silberstein K, Chiffings AW, McComb AJ (1986) The loss of seagrass in Cockburn Sound, Western Australia. 3. The effect of epiphytes on productivity of *Posidonia australis* Hook. F Aquat Bot 24:355-371

Smit AJ (2004) Medical and pharmaceutical uses of seaweed natural products: A review. J Appl Phycol 16:245-262

Steinberg PD, Schneider R, Kjelleberg S (1997) Chemical defenses of seaweeds against microbial colonization. Biodegradation 8:211-20

Steinberg PD, de Nys R, Kjelleberg S (1998) Chemical inhibition of epibiota by Australian seaweeds. Biofouling 12:227-244

Tatewaki M, Provasoli L, Pinter IJ (1983) Morphogenesis of *Monostroma oxysperma* (Kutz.) Doty (Chlorophyceae) in axenic culture, especially in bialgal culture. J Phycol 19:404-416

Thevanathan R, Nirmala M, Manoharan A, Gangadharan A, Rajarajan R, Dhamothrarn R, Selvaraj S (2000) On the occurrence of nitrogen fixing bacteria as epibacterial flora of some marine green algae. Seaweed Res Utiln 22:189-97.

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. Mar Ecol Prog Ser 58:175-89

Walters LJ, Hadfield MG, Smith CM (1996) Waterborne chemical compounds in tropical macroalgae: positive and negative cues for larval settlement. Mar Biol 126:383-393

Weinberger F, Friedlander M, Hoppe HG (1999) Oligoagars elicit an oxidative burst in *Gracilaria conferta* (Rhodophyta). J Phycol 35:747-755

2. MARINE MACROALGAE AFFECT ABUNDANCE AND COMMUNITY RICHNESS OF BACTERIOPLANKTON IN CLOSE PROXIMITY

2.1 Introduction

Marine macroalgae have been frequently observed to lack epibionts, stimulating previous research to identify algal metabolites with antibacterial effects. The majority of these studies utilized solvent extracts of algal tissues against bacterial isolates. Both the ecological role of algal metabolites, especially their release, and the pathogenicity of bacterial isolates were largely neglected in these studies. Utilizing a culture-independent approach, I investigated how the diversity of epiphytic bacteria was affected by waterborne metabolites of 9 macroalgae. Bacteria in algal culture water (ACW) were collected on filters without any purification or prior treatment of the cells and subjected to PCR („filter PCR“) with universal primers for eubacteria. The bacterial community profile in ACW was analyzed by denaturant gradient gel electrophoreses of PCR products and compared to a seawater control. In a species-specific pattern, some of the macroalgae under investigation inhibited up to 50% of bacterial bands in comparison to the control. My results suggest for the first time that the macroalgal defense system targets a wide spectrum of epiphytic bacteria in close proximity by waterborne algal metabolites. My work has been written in the format of manuscript and already accepted in the **Journal of Phycology** for publication in October, 2007 under vol. 43, no. 5 (JPY-06-221).

2.2 Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity

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Running head: Antibacterial waterborne macroalgal metabolites

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ABSTRACT

Many macroalgae employ chemical means to suppress epibiosis by micro- and macroorganisms. Previous studies mainly focused on tissue extracts of entire algae against few bacterial isolates, thereby not only neglecting ecologically relevant bacteria but also natural delivery mechanisms of algal antimicrobial agents. In this study, we investigated potential antimicrobial effects of waterborne macroalgal metabolites utilizing a culture-independent approach to compare the bacterial community richness in seawater in the presence and absence of macroalgae. The methodology comprised the collection of planktonic bacteria in algal culture water on membrane filters followed by filter PCR and denaturant gradient gel electrophoresis of 16S rDNA gene sequences of harvested bacteria with universal primers. Similarity analysis distinguished two groups of macroalgae under investigation, one of which showed more than 55% difference and the other less than 50% difference in bacterial community composition in comparison to natural seawater. The bacterial abundance in algal culture water of different algae was reduced between 20 and 50 percent. Further experiments demonstrated the observed effects to be caused by waterborne algal compounds; however some bacterial types were exclusively eliminated in the presence of algae indicating other causative modes of action than direct exposure of bacteria to waterborne compounds, such as surface mediated antimicrobial effects.

Keywords: Macroalgae, bacteria, waterborne metabolites, filter PCR, DGGE

INTRODUCTION

Surfaces exposed to seawater are rapidly covered with an organic layer of adsorbed organic matter and subsequently colonized by micro- (e.g. bacteria, diatoms, and fungi) and macroorganisms (e.g. larvae, algal spores). When surfaces of living organisms are colonized correspondingly, this phenomenon is referred to as epibiosis (Wahl, 1989). Macroalgae are particularly susceptible to epibiosis owing to their restriction to the photic zone where growth conditions for phyto- and zooplankton are optimal (de Nys et al. 1995). Epibionts can pose both beneficial and harmful effects to host macroalgae. For example, symbiotic epibionts protect their host from UV radiation (Koch and Brandt 2003) and desiccation (de O Figueiredo et al. 2000) or facilitate nitrogen-fixation (Thevanathan et al. 2000). The detrimental effects of epibiosis usually outweigh the beneficial ones causing diseases and tissue necrosis, decreased growth and photosynthesis, and result in competition for nutrients and interferences with reproductive processes (Armstrong et al. 2000).

In terms of their abundance and space occupation, bacteria are the most prominent colonizers of exposed surfaces in the marine environment. However, the bacterial abundance on many macroalgae is relatively low compared to co-occurring biofilms on inanimate substrata (Steinberg et al. 1997; Maximilien et al. 1998; Dobretsov and Qian 2002). It has been hypothesized that macroalgae have evolved physical and chemical defenses to suppress bacterial colonization or frequently shed microbial biofilms (Moss 1982, Keats et al. 1997, Corre and Prieur 1990). Increasingly, macroalgae have been reported to produce bioactive compounds with a variety of antibacterial (Hellio et al. 2000, 2001, Pelegrin and Morales 2004, Paul et al. 2006), antidiatom (Hellio et al. 2002) and antifungal

(Ballesteros et al. 1994, Hellio et al. 2000) activities. Such observations have inspired large-scale algal screening programs to discover novel antimicrobial compounds. In the majority of these studies, crude-extracts of entire algal material were directly screened for antimicrobial activities with a relatively small number of bacterial isolates under investigation (Hellio et al. 2000, 2001, 2002, Paul et al. 2006). Although these studies provided new bioactive algal metabolites, the natural delivery mechanisms, as well as the susceptibility of ecologically relevant bacteria to naturally released algal metabolites have been largely neglected (Hellio et al. 2001).

Recently, anti-epibiotic molecules were identified and quantified on and near the surface of macroalgae. For example, ultrastructure studies revealed vesicle cells near the thallus surface of the red alga *Delisea pulchra* containing non-polar, halogenated furanones, which inhibited growth and attachment of ecologically relevant bacteria both in the field and the laboratory (Steinberg et al. 1997, Maximilien et al. 1998). In the red alga *Asparagopsis armata* bromoform and dibromoacetic acid exhibited antibacterial effects (Paul et al. 2006). Whilst these studies indicated anti-epibiotic compounds to be of low molecular weight, other studies suggested large polar macromolecules with similar antimicrobial effects among waterborne algal compounds in direct proximity to algal surfaces (Regan and Glombitza 1986, Dobretsov and Qian 2002, Harder et al. 2004).

In this study, we revisited the hypothesis of waterborne antimicrobial macroalgal metabolites, utilizing a culture-independent approach to compare the planktonic bacterial community richness in algae-conditioned seawater, both in the presence and absence of macroalgae. Planktonic bacteria were collected on membrane filters without purification or prior treatment of cells and directly

subjected to PCR ("filter PCR") with universal eubacterial primers. The bacterial abundance and the community profiles in treatments and controls were analyzed by direct counts and fingerprint analyses of denaturant gradient gel electrophoresis (DGGE), respectively. This experimental design allowed the quantitative (i.e. direct counts) and qualitative assessment (cluster analysis of DGGE banding patterns) of macroalgal defenses against potentially epiphytic bacteria. Since commensalism between macroalgae and surface associated microorganisms is a common phenomenon, in the course of this manuscript we refer to macroalgae as the natural assemblage between plant and associated microorganisms.

MATERIALS AND METHODS

Macroalgae

Macroalgae were collected twice from the rocky shore of Helgoland (N: 54°11'18'', E: 07°52'38''), North Sea, between April and June 2005. The first collection for Experiment 1 (see below) was in April 2005 and included six algal species of Rhodophyceae (*Ceramium rubrum*, *Corallina officinalis*, *Palmaria palmata*, *Porphyra umbilicalis*, *Mastocarpus stellatus* and an unclassified red alga), 2 Phaeophyceae (*Laminaria digitata*, *Fucus serratus*) and one Chlorophyceae (*Ulva lactuca*) The second batch of algae was collected in June 2005 and comprised *Ceramium rubrum*, *Laminaria digitata* and *Mastocarpus stellatus* for Experiments 2 to 4 (see below). Prior to conditioning experiments, macroalgae were rinsed with aged natural seawater to detach isopods. Algae were acclimatized for 48 h in 2 L aerated Erlenmeyer flasks containing *f*/2 nutrient medium made from the same batch of aged seawater (Guillard & Ryther 1962) at 12 ± 1 °C with several

daylight-type fluorescent lamps at an irradiance level of 9 µmol photons m^{-2} sec^{-1} under a 15:9 h light:dark photo cycle.

Experiment 1 A/B: Effect of macroalgae on planktonic bacterial community richness

Macroalgae (5 g of wet weight $litre^{-1}$ seawater) were incubated for 3 days in 2 L aerated Erlenmeyer flasks containing aged natural seawater (NSW) at 12 ± 1 ˚C under the temperature and light settings described above. These settings were averaged according to environmental conditions at the sampling site. Every day, the pH-value and nitrate and phosphate concentrations were monitored in culture flasks. A flask containing NSW only served as a control. (A) Aliquots (30 ml) of algae-conditioned seawater (ACW) and the control were collected at the beginning of the experiment (0 h) and after 72 h and subjected to membrane filtration and filter PCR with a eubacterial primer resulting in short amplicons of 193 basepairs (bps) (see filter PCR section for details). Each experimental setup was triplicated. (B) In a follow-up experiment a subset of algae (*Ceramium rubrum*, *Laminaria digitata*, *Mastocarpus stellatus*) was re-examined according to Experiment 1A but with a eubacterial primer resulting in long amplicons of 566 bps. Each experimental setup was triplicated.

Experiment 2: Detection of algae-specific bacteria in ACW

To verify a potential infection of the culture medium with algae specific bacteria *Ceramium rubrum*, *Laminaria digitata* and *Mastocarpus stellatus* (5 g L^{-1} wet weight) were individually incubated in sterile-filtered seawater as described above. A flask of NSW served as a control. Samples of ACW and the control were

subjected to filter PCR utilizing the primer for long amplicons. Each experimental setup was triplicated.

Experiment 3: Effect of waterborne algal compounds on planktonic bacterial community richness

To investigate the effect of waterborne algal compounds on bacterial community changes in the absence of algae, freshly collected *Ceramium rubrum*, *Laminaria digitata* and *Mastocarpus stellatus* were incubated in NSW for 72 h. The ACW was sterile-filtered through 0.22 µm membranes (Millipore, USA) and the bacteria-free ACW was mixed with natural (bacteria-containing) seawater in a sterile flask in a ratio of 1:1 and incubated for 24 h. A mixture of natural and sterile-filtered seawater in the same proportion served as a control. Samples of ACW and NSW were subjected to filter PCR utilizing long PCR products. Each experimental setup was triplicated.

Experiment 4: Distinction between different antibacterial modes of action

To distinguish between antibacterial effects of waterborne algal metabolites (in the absence of algae) and algal surface-mediated effects (in the presence of algae), samples obtained in Experiments 1 and 3 were compared on the same denaturant gradient gel utilizing long PCR products.

Filter PCR

The sampling procedure and the conditions for filter PCR were adopted from Kirchman et al. (2001) with modifications. Briefly, 30 ml of seawater were pre-filtered through 90 mm paper filters (12-15 µm, Sartorius, Göttingen, Germany,

FT-3-101-055) to remove phyto- and zooplankton and to minimize amplification of 16S rRNA genes in plastids of eukaryotes. Pre-filtered seawater was run through 25 mm polycarbonate filters (Osmonics Inc., GE Waters and Process Technologies, Hørsholm, Denmark, K02BP02500) with 0.22 µm pore size. After filtration the membrane was cut into 8 equal sections with sterile scissors. Three sections were fixed with 3 % formaldehyde in seawater on microscopy glass slides and kept at 4 ˚C for subsequent bacterial enumeration, the remaining 5 sections were individually kept in sterile 0.2 ml PCR caps. PCR caps containing one membrane section were filled with 50 µl master mix composed of 10 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Roth, Karlsruhe, Germany), 25 mM $MgCl_2$, 1 % bovine serum albumin (BSA; Sigma, Seelze, Germany, A-7030), 2.5 units taq DNA Polymerase (Molzym, Bremen, Germany) and 10 µM of each universal primer: (i) 341F (5'-ATTACCGCGGCTGCTGG-3') and GC-534R (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') (short PCR products of 193 bps) (ii) GC-341F (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTC(AC)TTTGAGTTT-3') (long PCR products of 566 bps). PCR amplification of 16S rRNA was performed at 95 ˚C for 3 min; 10 touchdown cycles of 95 ˚C for 30 s, 64 ˚C for 30 s and 72 ˚C for 1 min; the annealing temperature started at 64 ˚C and was lowered to 54 ˚C in increments of 1 ˚C per cycle; 16 cycles of 94 ˚C for 30 s, 54 ˚C for 30 s and 72 ˚C for 1 min; and final extension at 72 ˚C for 10 min. Amplified DNA was verified by electrophoresis of 4 µl of PCR products in 1 % agarose in 1 × TAE (2 M Tris, 1 M sodium acetate, 50 mM EDTA at pH 7.4) buffer.

DGGE analysis of bacteria in algal culture medium

Equal amounts of PCR products obtained by filter PCR were analyzed by standard DGGE methods (Muyzer et al. 1993) using 6 % (w/v) acrylamide/bisacrylamide (37.5:1) gels containing a linear gradient ranging from 50 to 70 % denaturant (100 % denaturant solution containing 40 % formamide and 7 M urea) for short PCR products, and 35 to 65 % denaturant for long PCR products. Electrophoresis was carried out at 100 V for 18 h, maintained at 60 ˚C using an Ingeny DGGE apparatus (PhorUII). The gel size was 25.4 cm x 21.0 cm x 0.75 mm. DNA standards were prepared with the same PCR protocol as above utilizing 7 bacterial isolates, i.e. 2 γ-proteobacteria (*Vibrio* sp. & *Pseudoalteromonas* sp.), 1 flavobacteriaceae/salegentibacter (*Salegentibacter nodsonii*) and 4 bacillaceae (*Bacillus mycoides*, *Bacillus* sp. YY, *Bacillus* sp. VAN04, *Bacillus* sp. TP1). Gels were stained with SYBRGold® (Invitrogen, Karlsruhe, Germany) and destained with 1 × TAE buffer. Gel images were taken with an AlphaImager® (AlphaInnotech, San Leandro, CA, USA).

Chemical parameters in natural and algae-conditioned seawater

The pH-value in seawater treatments and controls, and the concentrations of nitrate, nitrite and phosphate in natural and ASW were monitored with commercial testing kits (Sera, Heinsberg, Germany) at the beginning of the experiment and after 3 days of incubation.

Bacterial enumeration on filter membranes

Bacteria were visualized with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc., Burlingame, CA, USA). The

formalin-fixed filter sections were stained with DAPI at a concentration 0.5 µg ml^{-1} for 5 min at room temperature. Bacteria on filter sections were recorded at a magnification of 1250 × in 15 randomly chosen fields of view (Zeiss Axiophot, Göttingen, Germany; λ_{EX} = 359 nm, λ_{EM} = 441 nm).

Statistical analysis

The software packages Statistica v.6.1 (StatSoft, Tulsa, OK, USA) and SPSS v.11 (Chicago, IL, USA) were used for statistical analyses of bacterial abundance on filter membrane. The normality assumption of data was checked with Shapiro-Wilk's *W*-test (Shapiro & Wilk 1965). Data that failed to meet the homogeneity criterion were analyzed non-parametrically. This was achieved by normal ANOVA and Tukey's multiple comparison tests (Conover & Iman 1981). The data presented in the figures are not transformed. DGGE banding patterns of the bacterial communities in ACW and NSW were subjected to one-way analysis of similarity (ANOSIM) in order to test for significant differences between groups of different treatments. Computed values were graphically illustrated by Bray-Curtis cluster analysis. All calculations were performed with the PRIMER v.5 computer program (PRIMER-E Ltd., Plymouth, UK).

RESULTS

Experiment 1 A&B: Qualitative and quantitative effects of algae-conditioned seawater (ACW) on planktonic bacterial community richness

Compared to the control of NSW, the planktonic bacterial community in ACW differed in quality and quantity after 3 days of incubation together with algae. Among the macroalgae under investigation, DGGE fingerprint analysis based on

short PCR products (Exp. 1A) revealed the highest dissimilarity of 80 % in *Ceramium rubrum* in comparison to the seawater control, followed by *Fucus serratus* (55 %), *Laminaria digitata* (55 %), *Corallina officinalis* (50 %), *Palmaria palmata* (50 %), *Mastocarpus stellatus* (45 %), an unclassified red alga (45 %), *Ulva lactuca* (30 %) and *Porphyra umbilicalis* (25 %, Fig. 1A). Bacterial abundance in algal treatments and the seawater control ranged from 1.5 ± 0.23 × 10^5 to 13.9 ± 2.30 × 10^5 cells ml^{-1} (Fig. 1B). Generally, the planktonic bacterial abundance in ACW was significantly lower than in the NSW control (Tukey's test, $p < 0.05$), with the only exception of *Porphyra umbilicalis* (Tukey's test, $p > 0.05$). Although there was a slight increase in bacterial density in the seawater control from day 0 to 3 ($p < 0.05$), the similarity of these two controls scored more than 90 % in the cluster analysis, indicating that the bacterial richness in seawater was nearly unchanged after 3 days of incubation.

After omitting algae-specific bacteria, DGGE fingerprint analysis based on long PCR products (Exp. 1B) showed the highest dissimilarity of 42 % in the brown alga *Laminaria digitata* compared to the seawater control, followed by *Ceramium rubrum* (35 %) and *Mastocarpus stellatus* (35 %, Fig. 2A). The similarity of two NSW controls scored more than 95 % in the cluster analysis, indicating the bacterial richness in seawater was nearly unchanged during the experimental period. Two out of 3 replicates of each treatment were mostly identical in the community profiles (> 95 %; Table 2).

Experiment 2: Detection of algae-specific bacteria in ACW

The comparison of DGGE banding patterns in lanes representing algae-conditioned water samples and controls revealed specific bacterial types that

originated from macroalgae. Due to their unique retention positions, these bacterial types were phylogenetically different from the pool of bacterial types observed in the seawater control (Fig. 3A). The community profiles observed in replicated samples were mostly identical (> 90 %; Fig. 3B, Table 2).

Experiment 3: Effect of waterborne algal compounds on planktonic bacterial community richness

The bacterial community richness in the *Laminaria* and *Ceramium* treatments shared only 20 % similarity in comparison to the NSW control, whilst the *Mastocarpus* treatment showed 12 % similarity compared to the NSW control (Fig. 4A & B). The community profiles observed in replicated samples were mostly identical (> 95 %; Fig. 4B, Table 3). These results indicated that waterborne compounds of both *Ceramium* and *Laminaria* eliminated a certain fraction of planktonic bacteria in natural seawater.

Experiment 4: Distinction between different antibacterial modes of action

DGGE fingerprint analysis revealed different numbers of affected bacterial phylotypes in the treatments with waterborne algal metabolites in the absence and presence of algae (Expts. 1 and 3, Fig. 5). Certain phylotypes were eliminated both in presence and absence of algae in comparison to the NSW control indicating that waterborne algal metabolites were responsible for the deletion effect. Contrary, certain phylotypes were eliminated only in the presence of algae indicating another mode of action.

Chemical parameters in natural and algae-conditioned seawater

Throughout the incubation period of 72 hours the pH in NSW controls and algal treatments was 7.5. The nitrite and nitrate concentrations were unchanged at 0.1 and 5.0 mg L^{-1}, respectively. The phosphate concentration in algal treatments decreased from 0.2 to 0.1 mg L^{-1}.

DISCUSSION

Based on field observations marine macroalgae have long been hypothesized to employ chemical means to deter microbial epibionts (Sieburth and Conover 1965, Steinberg et al. 1997, Maximilien et al. 1998, Harder and Qian 2000, Steinberg 2001, Dobretsov and Qian 2002, Harder et al. 2004, Paul et al. 2006). Largely motivated by potential pharmaceutical applications, these studies primarily aimed to identify novel marine natural products (Alejandro et al. 2004) utilizing crude organic extracts from whole plant tissue against a variety of standard bacterial isolates, diatoms and fungi (Ballesteros et al. 1994, Hellio et al. 2000, 2001, 2002, Pelegrin and Morales 2004, Paul et al. 2006). Although these assays provided insight into the diverse chemistry of antimicrobial compounds of macroalgae, the ecological roles of these compounds remained largely unknown.

In this study, we randomly selected 9 marine macroalgae (red, brown and green) and investigated if the bacterial richness and abundance in algae-conditioned water was influenced by antimicrobial effects originating from these algae. In order to pick up ecologically meaningful changes, a bacterial culture-independent approach was taken to compare the potential influence of algae on planktonic bacteria, both in algae-conditioned water in the presence and absence of macroalgae. The methodology comprised the collection of suspended bacteria in algal treatments on membrane filters followed by filter PCR of 16S rDNA gene

sequences of harvested bacteria with 2 pairs of universal primers producing PCR amplicons of different length. The community patterns of bacterial phylotypes were analyzed by denaturant gradient gel electrophoresis (DGGE). Together with the enumeration of bacteria on membrane filters this experimental design allowed a qualitative and quantitative assessment of antimicrobial effects of macroalgae against ubiquitous bacteria in seawater. The incubation of naturally occurring planktonic bacteria in seawater together with macroalgae for a period of 3 days affected both, bacterial community richness and abundance in algal culture water. With the exception of the *Porphyra* treatment, bacterial densities in other algal treatments were 20 – 50 % reduced in comparison to natural seawater (Fig. 1B). Given that the planktonic bacterial community pattern in natural seawater was resolved as a number of discernible bands, representing different bacterial phylotypes, the bacterial community composition in algal treatments was qualitatively affected due to the lack of certain bands in comparison to the seawater control (Fig. 1A). Since nutrient concentrations and pH in algae conditioned seawater did not change drastically within the limits of detection over the course of study compared to the control, the observed effects were hypothesized to stem from interactions between algae and planktonic bacteria.

Although algae were thoroughly rinsed with natural seawater prior to their utilization in experiments, their incubation in the same quality of seawater changed the qualitative composition of the bacterioplankton due to the release of bacteria formerly associated with algae. The introduction of these bacterial types into experimental setups was observed as additional gel bands in comparison to the band patterns obtained from natural seawater (Fig. 3A). As long as the bacterial contaminants from algae were also present in the seawater community,

the qualitative interpretation of community changes by statistical similarity analysis was not affected. In the case of additional phylotypes in algal treatments due to the release of algae-specific bacteria, such as in the case of the red algae *Ceramium rubrum* and *Mastocarpus stellatus* and the brown alga *Laminaria digitata* (Fig. 3A), these gel bands were omitted in a revised similarity analysis.

The revised similarity analysis grouped the 9 macroalgae under investigation into a cluster of algae with a pronounced effect of more than 55% difference (i.e. *Ceramium rubrum*, *Fucus serratus, Laminaria digitata*) and a cluster of less than 50% difference (i.e. *Corallina officinalis*, *Palmaria palmata*, *Mastocarpus stellatus*, an unclassified red alga, *Ulva lactuca*, *Porphyra umbilicalis*) in bacterial community richness in comparison to the NSW control. The re-examination of antibacterial effects of 3 algae utilizing a longer PCR amplicon showed that *L. digitata* had a pronounced effect of more than 40 % difference, followed by *C. rubrum* and *M. stellatus* with a cluster of less than 35 % difference in bacterial community richness in comparison to the NSW control (Fig. 2).

To investigate whether the observed antimicrobial effects were due to waterborne algal compounds, conditioned water of 3 algal species was sterile-filtered, mixed in a 1:1 ratio with untreated seawater and incubated for another day. In this experimental setup similarity analysis of bacterial community richness revealed only 80% similarity of the *Ceramium* and *Laminaria* treatment in comparison to the control (Fig. 4B) indicating that bacteria were indeed affected by waterborne algal compounds, i.e. in the absence of algae. Waterborne compounds obtained from *Mastocarpus stellatus* also affected the bacterial community richness, but to a lower extent than in the case of *Ceramium* and *Laminaria* (Fig. 4B). Interestingly, the number of affected bacterial phylotypes by

waterborne algal compounds in the absence of algae was not the same as the conditioning experiment in the presence of algae. Certain bacterial phylotypes were only eliminated in the presence of algae indicating that other modes of action than direct exposure of bacteria to waterborne compounds were causative for the observed effects (Fig. 5). The nature of these effects remains elusive, but surface-mediated processes between the algal blade and pelagic bacteria may be relevant for the observed effects. This could be due to steep chemical gradients of antimicrobial compounds with high bioactivity thresholds within the boundary layer of the blade surface or due to antimicrobial effects mediated by bacteria in biofilms on the blade surface as reported earlier by others (Boyd et al. 1999, Dobretsov and Qian 2002). An alternative explanation may be the short half-life of known algal defense agents, which may degrade within short time and consequently would have vanished in our experiments with algae-conditioned seawater in the absence of algae. For example, potent reactive oxygen species with short half-lives caused by oxidative burst have been reported in the red alga *Gracilaria conferta* (Weinberger et al. 1999) and the brown alga *Laminaria digitata* (Küpper et al. 2001, Küpper et al. 2002).

In summary, our study supported the concept of waterborne biologically active compounds from macroalgae. Whilst these effects have previously been reported for eukaryotic organisms such as larvae (Walters et al. 1996, Harder and Qian 2000, Harder et al. 2004), this study emphasized the role of waterborne algal compounds against planktonic bacteria for the first time.

Due to the rather long incubation of algae in seawater for 3 days the observations made in this study did not allow to identify the site of action of antimicrobial effects, e.g. within the thallus boundary layer, the water column in

close proximity to algae or both. The exclusive elimination of certain phylotypes in the presence of algae (Fig. 4) highlighted the possibility of surface-mediated effects whilst the experiments in the absence of algae indicated that waterborne antibacterial compounds were causative for the observed effects. Whether these compounds were formerly associated with the thallus and reached bioactive threshold concentrations due to long incubation times cannot be answered in this study. Importantly, our study suggests that epibiotic bacteria can already be targeted in much greater distance to the algal thallus under still water conditions than previously assumed.

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REFERENCES

Alejandro, M., Mayer, S. & Hamann, M. T. 2004. Marine Pharmacology in 2000: Marine compounds with antibacterial, anticoagulant, antifungak, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; Affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. *Mar. Biotechnol.* 6:37-52.

Armstrong, E., Boyd, K. G. & Burgess, J. G. 2000. Prevention of marine biofouling using natural compounds from marine organisms. *Biotechnol. Ann. Rev.* 6:221-41.

Ballesteros, E., Martin, D. & Uriz, M. J. 1994. Biological activity of extracts from some Mediterranean macrophytes. *Bot. Mar.* 35:481-5.

Boyd, K. G., Adams, D. R. & Burgess, J. G. 1999. Antibacterial and repellent activity of marine bacteria associated with algal surfaces. *Biofouling* 14:227-36.

Conover, W. J. & Iman, R. L. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat.* 35:124-9.

Corre, S. & Prieur, D. 1990. Density and morphology of epiphytic bacteria on the kelp *Laminaria digitata*. *Bot. Mar.* 33:515-23.

de Nys, R., Steinberg, P. D., Willemsen, P., Dworjanyn, S. A., Gabelish, C. L. & King, R. J. 1995. Broad spectrum effects of secondary metabolites from the red algae *Delisea pulchra* in antifouling assays. *Biofouling* 8:259-71.

de O Figueiredo, M. A., Kain, J. M. & Norton, T. A. 2000. Responses of crustose corallines to epiphytic and canopy layer. *J. Phycol.* 36:17-24.

Dobretsov, S. & Qian, P. Y. 2002. Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. *Biofouling* 18:217-28.

Guillard, R. & Ryther, J. 1962. Studies of marine planktonic diatoms. *Can. J. Microbiol.* 8:229-39.

Harder, T. & Qian, P. Y. 2000. Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitive cues for larval attachment and metamorphosis in the polychaete *Hydroides elegans*. *Biofouling* 16:205-14.

Harder, T., Dobretsov, S. & Qian, P. Y. 2004. Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. *Mar. Ecol. Prog. Ser.* 274:133-41.

Hellio, C., Bremer, G., Pons, A. M., Le Gal, Y. & Bourgougnon, N. 2000. Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. *Appl. Microbiol. Biotechnol.* 54:543-9.

Hellio, C., de La Broise, D., Dufossé, L., Le Gal, Y. & Bourgougnon, N. 2001. Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. *Mar. Environ. Res.* 52:231-47.

Hellio, C., Berge, J. P., Beaupoil, C., Le Gal, Y. & Bourgougnon, N. 2002. Screening of marine algal extracts for anti-settlement activities microalgae and macroalgae. *Biofouling* 18:205-15.

Keats, D. W., Knight, M. A. & Pueschel, C. M. 1997. Antifouling effects of epithallial shedding in three crustose coralline algae (Rhodophyta, Coralinales) on a coral reef. *J. Exp. Mar. Biol. Ecol.* 213:281-93.

Kirchman, D. L., Yu, L., Fuchs, B. M. & Amann, R. 2001. Structure of bacterial communities in aquatic systems as revealed by filter PCR. *Aquat. Microb. Ecol.* 26:13-22.

Koch, E. W. & Brandt, L. A. 2003. Epiphytic layers as UV-B Filters on Seagrass Leaves. *Gulf. Mex. Sci.* 21:102-3.

Küpper, F. C., Kloareg, B., Guern, J. & Potin, P. 2001. Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata*. *Plant Physio.* 125:278-91.

Küpper, F. C., Müller, D. G., Peters, A. F., Kloareg, B. & Potin, P. 2002. Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of Laminariales. *J. Chem. Ecol.* 28:2057-81.

Maximilien, R., de Nys, R., Holmström, C., Gram, L., Givskov, M., Crass, K., Kjelleberg, S. & Steinberg, P. D. 1998. Chemical mediation of bacterial surface colonization by secondary metabolites fro the red alga *Delisea pulchra*. *Aquat. Microb. Ecol.* 15:233-46.

Moss, B. L. 1982. The control of epiphytes by *Halidrys siliquosa* (L.) Lynbg. (Phaeophyta, Cystoseiraceae). *Phycologia* 21:185-91.

Muyzer, G., De Waal, E. C. & Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction –amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695-700.

Paul, N. A., de Nys, R. & Steinberg, P. D. 2006. Chemical defense against bacteria in the red alga *Asparagopsis armata*: linking structure with function. *Mar. Ecol. Prog. Ser.* 306:87-101.

Pelegrin, Y. F. & Morales, J. L. 2004. Antibacterial activity in marine algae from the coast of Yucatan, Mexico. *Bot. Mar.* 47:140-6.

Regan, M. & Glombitza, K. W. 1986. Phlorotannins, brown alga polyphenols. Progr Phyc Research (Round FE, Chapman DJ, eds) Biopress Ltd. *Bristol* 4:129-252.

Shapiro, S. S. & Wilk, M. B. 1965. An analysis of variance test for normality (complete samples). *Biometrika* 52:591-611.

Sieburth, J. M. & Conover, J. T. 1965. *Sargassum* tannin, an antibiotic which retards fouling. *Nature* 208:52-3.

Steinberg, P. D. 2001. Chemical mediation of interactions at seaweed surfaces. *J. Phycol.* 37:46-7.

Steinberg, P. D., Schneider, R. & Kjelleberg, S. 1997. Chemical defenses of seaweeds against microbial colonization. *Biodegradation* 8:211-20.

Thevanathan, R., Nirmala, M., Manoharan, A., Gangadharan, A., Rajarajan, R., Dhamothrarn, R. & Selvaraj, S. 2000. On the occurrence of nitrogen fixing bacteria as epibacterial flora of some marine green algae. *Seaweed. Res. Utiln.* 22:189-97.

Wahl, M. 1989. Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Mar. Ecol. Prog. Ser.* 58:175-89.

Walters, L. J., Hadfield, M. G. & Smith, C. M. 1996. Waterborne chemical compounds in tropical macroalgae: positive and negative cues for larval settlement. *Mar. Biol.* 126:383-93.

Weinberger, F., Friedlander, M. & Hoppe, H. G. 1999. Oligoagars elicit an oxidative burst in *Gracilaria conferta* (Rhodophyta). J. Phycol. 35:747-55.

FIGURES & TABLES

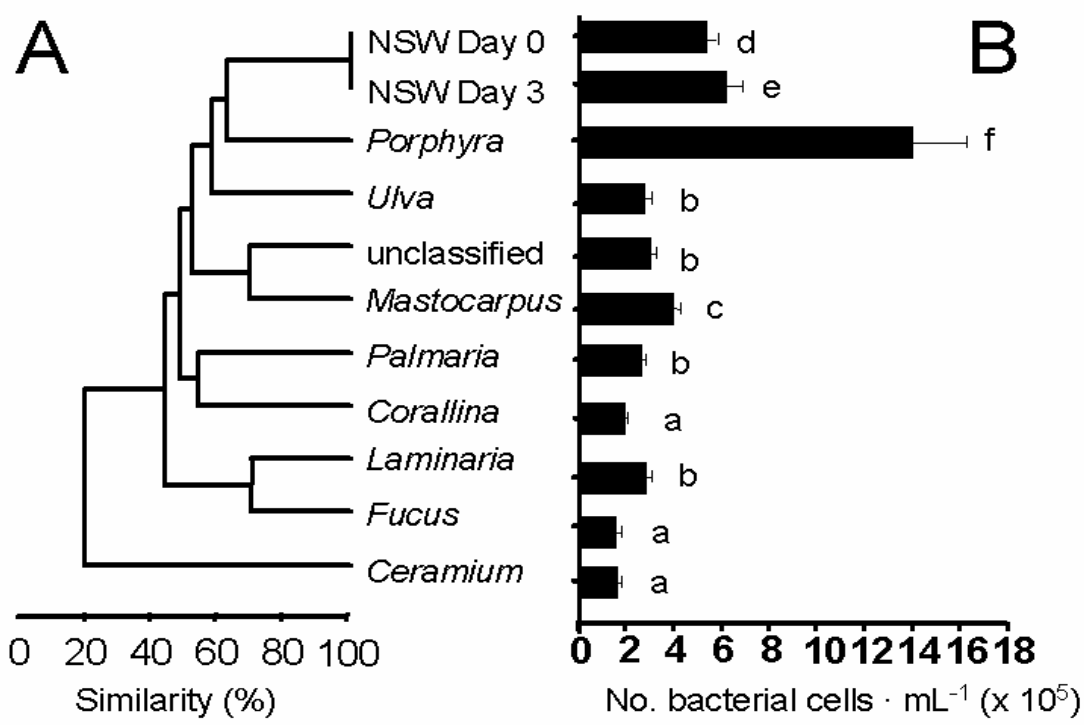


Figure 1: **A:** Cluster analysis of DGGE banding patterns of planktonic bacteria in algae-conditioned seawater after 3 days of incubation and controls of natural seawater (NSW) at day zero (the beginning of the experiment) and after 3 days . Bacterial richness was analyzed by DGGE of short PCR products obtained with primers 341F and GC-534R. The dendrogram was constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method. **B:** Mean bacterial abundance in the same treatment shown in panel 1A at the beginning of the experiment and after days of incubation. Data that differed significantly (Tukey's test, p=0.05) are indicated by different letters. Data are means ± SD of 3 replicates.

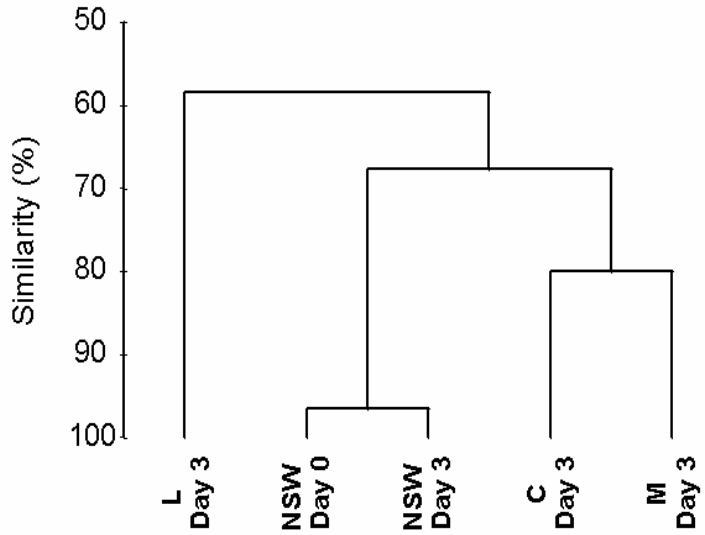


Figure 2. Cluster analysis of DGGE banding patterns of planktonic bacterial communities in algae-conditioned seawater of *Laminaria*, *Ceramium* and *Mastocarpus* after 3 days of incubation and natural seawater (NSW) at the beginning of the experiment (Day 0) and after 3 days. Bacterial diversity was analyzed by DGGE of long PCR products obtained with primers GC-341F and 907R. The dendrograms were constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method. Algae-specific bacteria introduced by macroalgae were omitted in the similarity matrix.

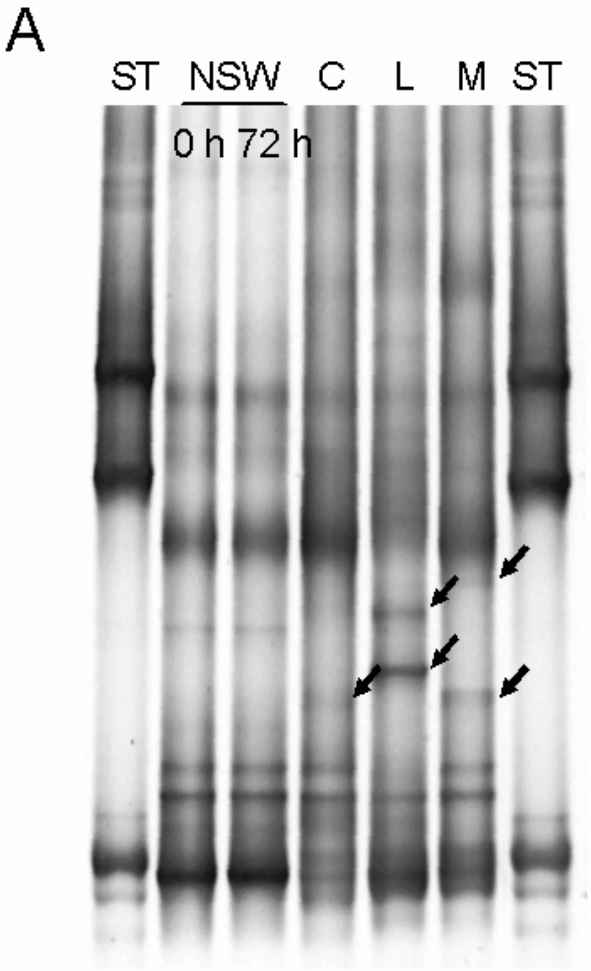
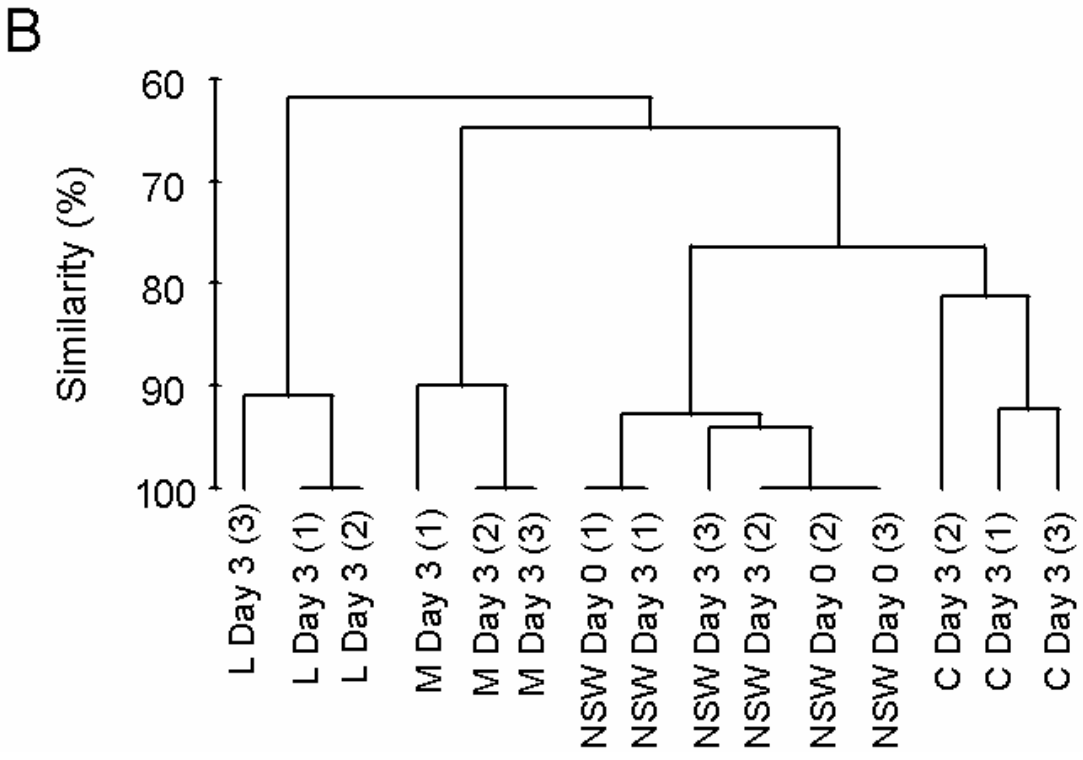


Figure 3: **A (left)**: DGGE fingerprints showing the presence of additional bacterial phylotypes in seawater incubated with the macroalgae *C. rubrum* (C), *L. digitata* (L) & *M. stellatus* (M) for 3 days compared to a control of natural seawater (NSW) after zero and 3 days. Algae-specific bacterial types are labeled with arrows. All treatments were compared on the same gel. DNA standards (ST) comprised 7 bacterial isolates. **B (down)**: Cluster analysis of all replicate treatments. The dendrogram was constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method. Algae-specific bacteria introduced by macroalgae were omitted in the similarity matrix.



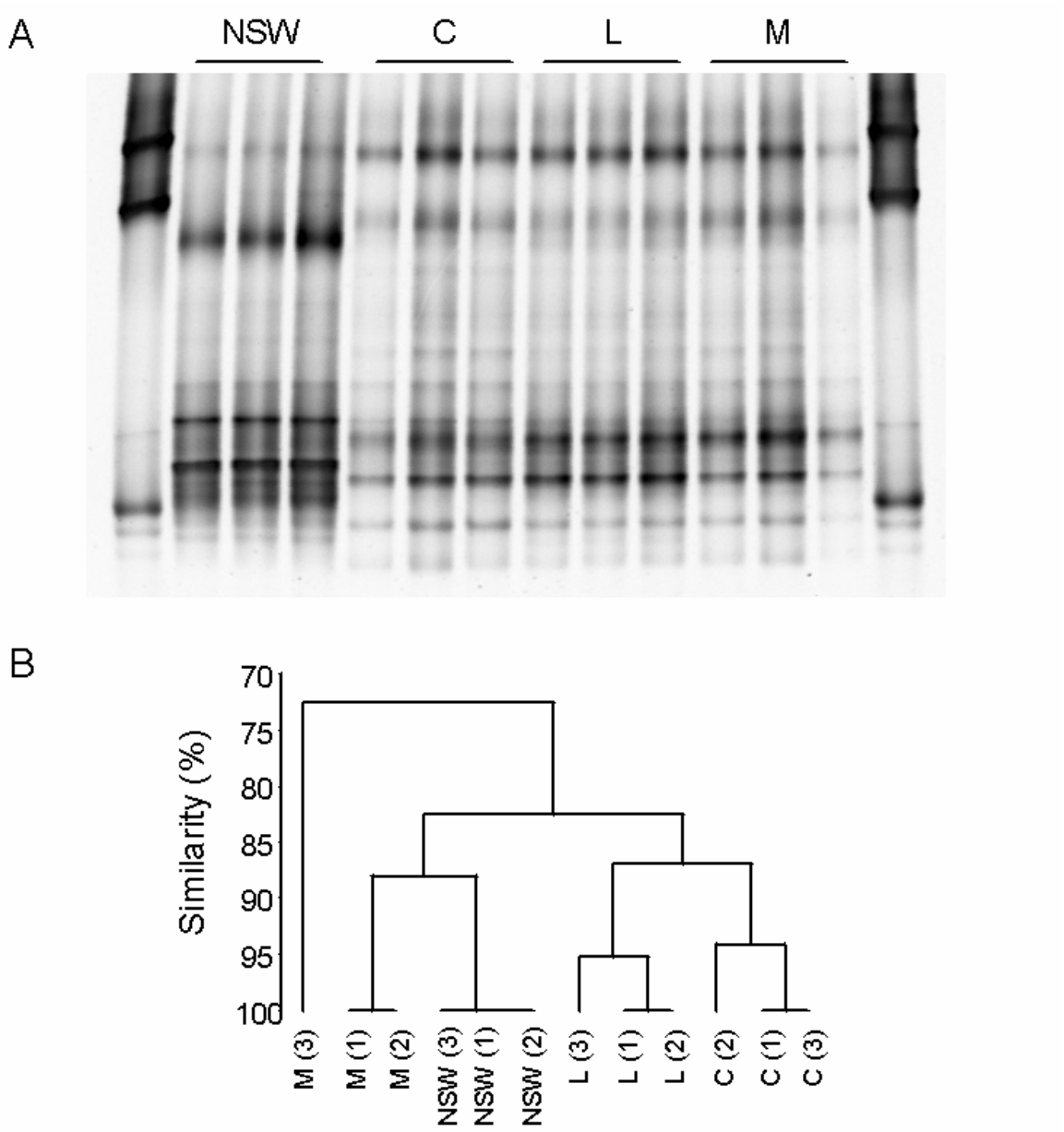


Figure 4 **A**: Denaturant gradient gel showing 3 replicates of planktonic bacterial communities in seawater after 24 h exposure to waterborne compounds of *C. rubrum* (C), *L. digitata* (L) & *M. stellatus* (M). Sterile-filtered algae-conditioned water was mixed in a 1:1 ratio with natural seawater and incubated for 24 h. A 1:1 mixture of natural and sterile-filtered seawater served as control (NSW). **B:** Cluster analysis of all replicate treatments. The dendrogram was constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method. Algae-specific bacteria introduced by macroalgae were omitted in the similarity matrix.

Figure 5: DGGE fingerprints distinguishing between antibacterial effects of waterborne algal metabolites and algal surface-mediated antibacterial effects of *C. rubrum* (C), *L. digitata* (L) & *M. stellatus* (M) in comparison to a control of natural seawater (NSW). The symbols '+' and '-' denote treatments were performed in the presence (Experiment 1) and absence (Experiment 3) of macroalgae, respectively. Arrows indicate band positions in the absence of algae which are deleted in the presence of algae, asterisks indicate algae-specific bacterial phylotypes introduced with algae. All treatments were run and compared on the same gel. DNA standards (ST) comprised 7 bacterial isolates.

Table 1. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic bacteria in algae-conditioned seawater of *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* after 72 h and natural seawater control (NSW) at day 0 and 72 h.

	NSW (0)	NSW (72)	*C. rubrum* (72)	*L. digitata* (72)	*M. stellatus* (72)
NSW (0)	-				
NSW (72)	0.185	-			
C. rubrum (72)	0.778 *	0.759 *	-		
L. digitata (72)	1.0 *	1.0 *	1.0 *	-	
M. stellatus (72)	1.0 *	1.0 *	1.0 *	1.0 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 341-907 (*Escherichia coli* numbering system; Brosius et al. 1978). The asterisk marks statistical significance (α = 0.05).

Table 2. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic bacteria in algae-conditioned seawater of *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* after 72 h and natural seawater control (NSW) at 0 h and 72 h. Algae-specific bacteria introduced by macroalgae were omitted in the similarity matrix.

	NSW (0)	NSW (72)	*C. rubrum* (72)	*L. digitata* (72)	*M. stellatus* (72)
NSW (0)	-				
NSW (72)	0.222	-			
C. rubrum (72)	1.0 *	1.0 *	-		
L. digitata (72)	0.63 *	0.63 *	1.0 *	-	
M. stellatus (72)	0.704 *	0.704 *	0.148	1.0 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 341-907 (*Escherichia coli* numbering system; Brosius et al. 1978). The asterisk marks statistical significance ($\alpha = 0.05$).

Table 3. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic bacterial communities in natural seawater (NSW) after 24 h exposure to waterborne compounds of *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus*.

	NSW	*C. rubrum*	*L. digitata*	*M. stellatus*
NSW	-			
C. rubrum	1.0 *	-		
L. digitata	1.0 *	1.0 *	-	
M. stellatus	0.556 *	0.407	0.556 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 341-907 (*Escherichia coli* numbering system; Brosius et al. 1978). The asterisk marks statistical significance (α = 0.05).

3. PLANKTONIC EPIPHYTIC BACTERIA AND FUNGI ARE SELECTIVELY ELIMINATED BY MARINE MACROALGAE

3.1 Introduction

In my previous study, community richness of epiphytic bacteria was both qualitatively and quantitatively affected by waterborne algal metabolites. Based on this laboratory observation, I further extended the hypothesis by testing whether the waterborne algal metabolites targeted specific bacterial phyla. Additionally, I tested whether other microepiphytes, such as fungi, were affected by these compounds. These objectives were experimentally addressed with selective oligonucleotide probes that (1) distinguished between different bacterial phyla (the *Bacteroidetes*, alpha-*Proteobacteria*, *Roseobacter*), and (2) specifically targeted planktonic fungal community in seawater. My results showed that the community composition of bacterial phyla was reduced to a greater extent in presence of *Ceramium rubrum* and *Mastocarpus stellatus*, whilst a more pronounced antifungal effect was observed in presence of *Laminaria digitata* and *M. stellatus*. The most affected bacterial phylotypes were belonged to the *Bacteroidetes* and the *Roseobacter* clade, implying these phylotypes may pose a particular threat to the algae. My results are in good agreement with other studies that macroalgae affect epiphytic colonizers by producing bioactive metabolites targeting different bacterial phyla slectively. Most importantly, my study firstly demonstrates macroalgal defenses against epiphytic microorganisms based on natural delivery mechanism of allelochemicals utilizing a culture-independent approach. My work has been written in the format of manuscript and already submitted to the **FEMS Microbiology Ecology** for revision.

3.2 Planktonic epiphytic bacteria and fungi are selectively eliminated by marine macroalgae

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Running head: Antibacterial and antifungal waterborne macroalgal metabolites

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ABSTRACT

Marine macroalgae employ chemical means to deter fouling of epiphytic bacteria. In order to test whether antibacterial macroalgal metabolites affect the pool of potential bacterial colonizers in a phylum-specific fashion, the community richness of different bacterial phyla and fungi was analyzed with oligonucleotide probes selectively targeting the *Bacteroidetes,* alpha-*Proteobacteria* and *Roseobacter group*, and the ITS1 region of fungi. The community composition of bacterial phyla was reduced to a greater extent in presence of the red algae *Ceramium rubrum* and *Mastocarpus stellatus* than in the brown alga *Laminaria digitata*, indicating that either the quality of algal metabolites was different among macroalgae, or that the susceptibility of planktonic bacteria towards alga-derived antimicrobial effects correlated with their phylogenetic affiliation. The majority of affected phylotypes belonged to the *Bacteroidetes* and the *Roseobacter* clade, implying that epiphytic bacterial colonizers belonging to these groups may pose a particular threat to the algae. The fungal community pattern was also affected in the presence of macroalgae, but with a different specificity of algae, i.e. *L. digitata* and *M. stellatus* exhibited a more pronounced antifungal effect than *C. rubrum*. These results support earlier findings that macroalgae utilize allelochemical means to target epiphytic colonizers and provide experimental evidence that these effects differ among algae and affect different bacterial phyla selectively. To our knowledge, this is the first study that demonstrates macroalgal defenses against epiphytic microorganisms based on natural delivery mechanisms of allelochemicals utilizing a culture-independent approach, thus minimizing the ecological bias inherent to culture-dependent studies based on few microbial isolates. Keywords: Macroalgae, epibiosis, bacteria, fungi, allelochemistry

INTRODUCTION

Surfaces submerged in seawater are rapidly covered with an organic layer of adsorbed organic matter which favors subsequent colonization by micro- (i.e. bacteria, fungi, diatoms) and macro-organisms (i.e. algal spores, larvae). This process is generally considered as fouling. If the fouled surface belongs to living organisms the phenomenon is referred to as epibiosis (Wahl, 1989). Marine macroalgae are particularly susceptible to epibiosis because of their predominance in the photic zone where growth conditions for phyto- and zooplankton are optimal (de Nys et al., 1995). Epibionts, i.e. colonizers of living surfaces, can pose detrimental effects to host macroalgae such as disease, tissue necrosis, reduced photosynthesis and growth (Armstrong et al., 2000). Thus, the prevention of settlement and/or growth of epibionts on algal surfaces and the competition for space, light and nutrients between epibionts and hosts are the driving force for macroalgal allelopathy (Gross, 1999; 2003).

In terms of abundance and space occupation, bacteria are the dominant colonizers of surfaces in the marine environment, followed by diatoms and fungi. Although most bacteria do not compete with macroalgae for light and nutrients, they may enhance attachment of other photosynthetic epibionts, e.g. microalgae (Gross, 2003) and macroalgae (Tait et al., 2005). However, the bacterial abundance on many macroalgae is relatively low compared to co-occurring biofilms on inanimate substrata (Steinberg et al., 1997; Maximilien et al., 1998; Dobretsov & Qian, 2002). It has long been demonstrated that macroalgae have evolved antifouling mechanisms of physical nature (Moss, 1982; Keats et al., 1997; Corre & Prieur, 1990), but more recently increasing evidence indicates that macroalgae deter fouling of epiphytic bacteria also through chemical means (de

Nys et al., 1995; Steinberg et al., 1997; Steinberg et al., 1998; Harder et al., 2004; Paul et al., 2006). A variety of studies have shown that macroalgae are a rich source of bioactive compounds against colonizing bacteria (Hellio et al., 2000; 2001a; Pelegrin & Morales, 2004; Paul et al., 2006), diatoms (Hellio et al., 2002; Amsler et al., 2005) and fungi (Ballesteros et al., 1992; Hellio et al., 2000; Engel et al., 2006). These observations have inspired large-scale screening programs to discover novel antimicrobial compounds of algal origin. In the majority of these studies, crude-extracts of entire algal material were directly screened for antimicrobial activities with a small number of bacterial (Hellio et al., 2000; 2001a; 2001b; Freile-Pelegrin & Morales, 2004) and fungal isolates (Hellio et al., 2000; 2001b; Engel et al., 2006). The experimental approach taken in these studies provided valuable insight to a wide spectrum of bioactive algal metabolites, however the natural delivery mechanisms, as well as the susceptibility of ecologically relevant bacteria and fungi to naturally released algal metabolites often remained unknown (Gross 1999; 2003; Engel et al., 2006; Paul, 2006).

To cover any potential antimicrobial effects of algal compounds against the whole spectrum of potential bacterial colonizers in seawater, we previously utilized molecular fingerprinting tools to analyze the bacterial community richness in algal culture water (Lam & Harder, in press). The overall bacterial abundance in algae-conditioned treatments was reduced by 20 – 50 % in comparison to seawater controls. The observed effects were caused by waterborne metabolites, however some bacterial colonizers were exclusively eliminated in the presence of algae indicating other causative modes of action than direct exposure of bacteria to waterborne metabolites (Lam and Harder, in press).

The present study followed our previous investigation of antimicrobial effects of waterborne macroalgal metabolites against potential epiphytic colonizers. Firstly, we tested the hypothesis that the observed antimicrobial effects of algal metabolites targeted specific bacterial phyla. Secondly, we tested whether other microcolonizers, such as fungi, were affected by waterborne algal metabolites. Allelochemical effects of macroalgae against marine fungi have been described in green, brown and red algae, but their delivery mechanisms and their specificity are largely unknown (Paul & Fenical, 1984; Bouaicha et al., 1991; Smit, 2004). These objectives were experimentally addressed with selective oligonucleotide probes that (1) distinguished between different bacterial phyla (the *Bacteroidetes*, alpha-*Proteobacteria*, *Roseobacter*), and (2) specifically targeted the planktonic fungal community in seawater. Experiments were performed with the macroalgae *Ceramium rubrum*, *Laminaria digitata* and *Mastocarpus stellatus*.

MATERIALS AND METHODS

Macroalgae

Alge (Rhodophyceae: *Ceramium rubrum* & *Mastocarpus stellatus*; Phaeophyceae: *Laminaria digitata*) were collected on the rocky shore of Helgoland Island (N: 54^{o} 10' 0", E: 7^{o} 52' 60"), North Sea, in November 2006. Prior to experiments, macroalgae were treated and acclimatized at the laboratory according to Lam & Harder (in press). Since commensalism between epiphytic microorganisms and macroalgae is widespread, in the following the terminology “alga” refers to the assembly of plant and naturally associated microorganisms.

Experiment 1: Effect of macroalgae on community richness of planktonic bacteria

Macroalgae (5 g wet weight litre^{-1} seawater) were incubated for 3 days in 2 L aerated Erlenmeyer flasks containing fresh natural seawater (NSW) collected at the sampling site according to Lam & Harder (in press). The temperature and light settings were averaged according to environmental conditions at the sampling site. A flask containing NSW only served as a control. Aliquots (30 ml) of algal culture water (ACW) and the control were sampled after 72 h and subject to membrane filtration and filter PCR with three different sets of bacterial phylum-specific primers (see filter PCR for details). Each experimental setup was triplicated.

Experiment 2: Effect of macroalgae on community richness of planktonic fungi

The experimental setup was similar to Experiment 1, except that a larger volume of 1 L ACW and NSW control were collected and subjected to membrane filtration at the beginning of the experiment (0 h) and after 72 h. The filter membrane was subjected to standard DNA extraction (Zhou et al., 1996) prior to PCR with fungal primers (see fungal DNA extraction and PCR for details). Each experimental setup was triplicated.

Experiment 3: Effect of waterborne algal metabolites on community richness of planktonic fungi

The experimental setup to test waterborne algal metabolites in the absence of algae was performed in accordance to Lam & Harder (in press). Newly collected macroalgae were incubated in NSW for 72 h as described above and ACW was sterile-filtered through 0.22 µm membranes (Millipore, Billerica, USA). The bacteria-free ACW was mixed with natural (bacteria-containing) seawater in a sterile flask in a ratio of 1:1 and incubated for 24 h. A mixture of natural and

sterile-filtered seawater in the same proportion served as a control. Each experimental setup was triplicated.

Filter PCR

The sampling procedure and the conditions for filter PCR were described according to our previous study (Lam & Harder, in press). PCR amplification of 16S rRNA gene fragments was performed with three different primer pairs specific for: (i) the *Bacteroidetes* phylum (GC-CF 319f and 907R); (ii) alpha-*Proteobacteria* (GC-341f and ALF968r) and (iii) the *Roseobacter* clade within alpha-*Proteobacteria* (GC-ROSEO536Rf and GRb735r). Primer and GC clamp sequences, as well as PCR protocols of each bacteria-specific primer were adopted from Rink et al. (in press). Amplified DNA was verified by electrophoresis of 4 µl of PCR products in 1 % agarose in 1 × TAE (2 M Tris, 1 M sodium acetate, 50 mM EDTA [pH 7.4]) buffer.

Extraction and amplification of fungal DNA

To investigate whether the community richness of marine fungi and spores was affected by macroalgae, a modified filter PCR protocol was used. In comparison to the filtration volume required to generate sufficient fungal cell volume, more seawater had to be processed since the average abundance of marine fungi and spores is significantly lower than in case of bacteria (Kohlmeyer & Kohlmeyer, 1979). Briefly, filter membranes containing fungal biomass were homogenized in a sterile mortar together with 0.1 g of sterile sea sand (JT Baker, Netherlands) and 1 ml of CTAB extraction buffer (100 mM Tris at pH 7.5, 1 % (w/v) cetyltrimethylammoniumbromide, 0.7 M NaCl, 10 mM EDTA, 1 % (v/v)

mercaptoethanol. Fungal community DNA was extracted according to Zhou et al. (1996). Briefly the filter membrane slurry was incubated at 65 ˚C for 2 h together with proteinase K (20 mg ml^{-1}) and 20 % sodium dodecyl sulfate (SDS). An equal volume of chloroform:isoamylalcohol (24:1 vol/vol) was added to the mixture prior to centrifugation at 10,000 x g for 5 min. One ml of the aqueous supernatant was transferred into a 2 ml centrifuge tube and 0.6 volume of isopropanol was added to precipitate the DNA overnight. The DNA pellet was washed twice with ice cold 70% ethanol, air-dried at room temperature and resuspended in 30 µl TE buffer (10 mM Tris/Cl, 1 mM EDTA at pH 8.0).

Fungal PCR

A PCR cap was filled with 50 µl reaction volumes using 10 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Gene Craft, Germany), 25 mM MgCl2, 10 µM of ITS 2 (5'- GCTGCGTTCTTCATCGATGC -3') and GC-ITS 5 (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGAAGTAAAAGTCGTAACAAGG-3') primers, 1 % bovine serum albumin (BSA; Sigma A-7030), 2.5 units of taq DNA Polymerase (GeneCraft, Lüdinghausen, Germany) and 1 µl of DNA template. Amplification of the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) was carried out at 94 ˚C for 3 min; 37 cycles of 94 ˚C for 30 s, 56 ˚C for 30 s and 72 ˚C for 1 min; and final extension at 72 ˚C for 7 min. Amplified DNA was verified by electrophoresis of 4 µl of PCR products in 2 % agarose in 1 × TAE (2 M Tris, 1 M sodium acetate, 50 mM EDTA at pH 7.4) buffer.

DGGE analysis of bacteria and fungi in algal culture medium

Equal volumes of PCR amplicons were analyzed by standard DGGE methods (Muyzer et al., 1993) using a 6 % (w/v) acrylamide/bisacrylamide (37.5:1) gel containing a linear gradient ranging from 35 to 75 % denaturant (100 % denaturant solution containing 40 % formamide and 7 M urea) for primer pairs of the *Bacteroidetes* and alpha-*Proteobacteria*; whilst a 9 % (w/v) acrylamide/bisacrylamide (37.5:1) gel with a linear gradient ranging from 30 to 70 % denaturant was used for fungal and *Roseobacter* primer pairs. Electrophoresis was carried out at 60 ˚C under constant Voltage of 100 V for 18 h using an Ingeny DGGE apparatus (PhorUII). The gel dimension was 25.4 cm x 21.0 cm x 0.75 mm. DNA standards of the *Bacteroidetes*, alpha-*Proteobacteria*, *Roseobacter* and fungi were prepared using the same PCR protocol described above and summarized in Table 1. Gels were stained with SYBRGold® (Invitrogen, Karlsruhe, Germany) and destained with 1 × TAE buffer. Gel images were taken with an AlphaImager® (AlphaInnotech, San Leandro, CA, USA). Selected gel bands were excised with sterile gel cutters (Biozym, Bremen, Germany) and transferred into sterile PCR caps together with 30 µl of sterile-filtered Milli-Q water. Samples were stored at -20 ºC.

Cloning

Three DGGE bands (Roseobacter 1, 3 & 5) obtained by DGGE with *Roseobacter*-specific primers (Fig. 3A) were cloned using the pGEM®-T Vector System II (Promega, Madison, USA) following the manufacturers' instruction. Clones with inserts were picked, resuspended in SOC medium (stock solution: 2 % Bacto®-tryptone (w/v), 0.5 % Bacto®-yeast extract (w/v), 1M NaCl, 1M KCl, 2M

$MgCl_2$/$MgSO_4$, 2M glucose) and screened by DGGE to check if the insert positions matched with positions of the corresponding DGGE bands. Adequate clones were amplified and subsequently sequenced using the primers pUC/M13f and pUC/M13r with an annealing temperature of 48 ºC (Messing, 1983).

Phylogenetic analysis and nucleotide sequence accession number

PCR products were sequenced by Seqlab Sequence Laboratory, Göttingen, Germany, using the primers described above. All sequences were at least 400 bps in length. Phylogenetic affiliation of the sequences was compared to those in GenBank using the BLAST function of the NCBI server (http://www.ncbi.nlm.gov/BLAST/).

Statistical analysis

DGGE band patterns of the bacterial communities of both ACW and NSW were subjected to one-way analysis of similarity (ANOSIM) in order to test for significant differences between groups of samples of different treatments. Computed values were graphically illustrated by Bray-Curtis cluster analysis. All calculations were performed with the PRIMER v 5 computer programs (PRIMER-E Ltd., Plymouth, UK). Specific bacterial and fungal types that originated from macroalgae (defined as “algal-specific” bacteria/fungi) were omitted in the DGGE fingerprint analysis (Lam & Harder, in press).

RESULTS

Experiment 1: Effect of macroalgae on community richness of different phylogenetic groups of bacteria

The bacterial communities in algal treatments were characterized by the absence of bands in comparison to the control of natural seawater, which supposedly reflected a community of chemically unaffected bacteria. The algal treatments showed a few additional bands in comparison to the control. These phylotypes supposedly stemmed from macroalgae and were considered as contaminants and omitted in the analysis of bacterial community patterns. The community profiles observed in replicated samples were mostly identical (> 90 % similarity; Figs. 1-3B, Table 2-4).

(i) the *Bacteroidetes*

Under the combination of the *Bacteroidetes*-specific PCR primers the number of discernible gel bands obtained from algal treatments ranged from 6 to 7. In total, 10 band positions were used to create a similarity matrix based on Boolean character sets (1 or 0) corresponding to the presence or absence of a given band in a gel lane. The analysis of similarity (ANOSIM) of given bands per treatment produced a global R-value of 0.735 at the significance level of 0.2 %. Four phylotypes were eliminated in the *Ceramium* treatment, whilst 3 phylotypes were eliminated both in the *Laminaria* and the *Mastocarpus* treatments (Fig. 1A). The community composition of the *Bacteroidetes* in the *Laminaria* treatment was statistically the same as in the control of seawater (ANOSIM, R = 0.648, Fig. 1B, Table 2). Contrary, the community compositions in the *Ceramium* and the *Mastocarpus* treatments were significantly reduced sharing less than 70 %

similarity in comparison to the control of seawater (ANOSIM R = 1.0, Fig. 1B, Table 2).

(ii) alpha-*Proteobacteria*

Under the combination of alpha-*Proteobacteria* -specific PCR primers the number of discernible gel bands obtained from algal treatments ranged from 4 to 6. In total, 8 bands were used to create a similarity matrix. The analysis of similarity of given bands per treatment produced a global R-value of 0.556 at the significance level of 0.1 %. Two phylotypes were eliminated in the *Ceramium* treatment, whilst 1 and 4 phylotypes were eliminated in the *Laminaria* and the *Mastocarpus* treatments, respectively (Fig. 2A). The community composition of alpha-*Proteobacteria* in the *Laminaria* treatment was the same as in the control sharing more than 90 % similarity (ANOSIM, R = 0.074, Fig. 2B, Table 3). The bacterial community compositions in the *Ceramium* and the *Mastocarpus* treatment were significantly different from the control (ANOSIM, R = 0.741 (*Ceramium)*, 0.796 (*Mastocarpus*), Fig. 2B, Table 3) sharing less than 60 % similarity.

(iii) *Roseobacter*

Under the combination of *Roseobacter*-specific PCR primers the number of discernible gel bands obtained from algal treatments ranged from 8 to 10. In total, 12 bands were used to create a similarity matrix. The analysis of similarity of given bands per treatment produced a global R-value of 0.759 at the significance level of 0.1 %. Two phylotypes were eliminated in the *Laminaria* treatment, whilst 4 phylotypes were both eliminated in the *Ceramium* and the *Mastocarpus* treatments (Fig. 3A). The bacterial community compositions of *Roseobacter* in all algal treatments were statistically different from the seawater control sharing only 75 % similarity (ANOSIM, R = 1.0 (*Ceramium*), 0.796 (*Laminaria*), 1.0

(*Mastocarpus*), Fig. 3B, Table 4). Three gel bands which were selectively eliminated in algal treatments were excised from the control at corresponding positions (labeled *Roseobacter* 1, 3 & 5) and sequenced (Fig. 3A).

Phylogenetic affiliation and nucleotide sequence accession number of excised bands

The sequences of the excised bands *Roseobacter* 1, 3 & 5 were affiliated to different uncultured *Roseobacter* species (*Roseobacter* 1: 96 % similarity to DQ778278.1; *Roseobacter* 3: 94 % similarity to DQ778134.1; *Roseobacter* 5: 96 % similarity to DQ778280.1, Alonso-Sáez et al. 2007).

Experiments 2&3: Effects of macroalgae (in the presence of algae) and its metabolites (in the absence of algae) on planktonic fungal community richness

The fungal communities in algal treatments were characterized by the absence of bands in comparison to the seawater control, which supposedly reflected a community of chemically unaffected fungi. The algal treatments showed a few additional bands compared to the control. These phylotypes supposedly stemmed from macroalgae and were considered as contaminants and omitted in the analysis of fungal community patterns. The community profiles observed in replicated samples were mostly identical (> 90 % similarity; Figs. 4-5B, Table 5-6).

(i) in the presence of algae

Under the combination of fungal-specific PCR primers the number of discernible gel bands obtained from algal treatments ranged from 8 to 9. In total, 11 bands were used to create a similarity matrix. The analysis of similarity of given bands per treatment produced a global R-value of 0.91 at the significance level of 0.1 %. Two phylotypes were eliminated in the *Ceramium* treatment, whilst 3 phylotypes

were both eliminated in the *Laminaria* and the *Mastocarpus* treatments (Fig. 4A). The community composition of fungi in all algal treatments were statistically different compared to the controls (ANOSIM, R = 0.963 (*Ceramium*), 1.0 (*Laminaria* and *Mastocarpus*), Table 5) sharing less than 75 % similarity in fungal community in comparison to the control (Fig. 4B). In the seawater controls analyzed at the beginning and the end of the experiment the fungal community composition was more than 90 % similarity, verifying that the fungal community was not changed during the course of the experiment in chemically unaffected treatments (ANOSIM, R = 0.722, Fig. 4B, Table 5).

(ii) in the absence of algae

Under the combination of fungal PCR-primers the number of discernible gel bands obtained from algal treatments ranged from 12 to 13. In total, 14 bands were used to create a similarity matrix. The analysis of similarity of given bands per treatment produced a global R-value of 0.215 at the significance level of 3.0 %. Two phylotypes were eliminated in the *Laminaria* treatment, whilst only 1 phylotype was eliminated in the *Ceramium* and the *Mastocarpus* treatments (Fig. 5A). The fungal community patterns in the *Laminaria* and the *Ceramium* treatment were significantly different from the control (ANOSIM, R = 0.315 (*Ceramium, Laminaria*), Table 6). The fungal community composition in the *Mastocarpus* treatment was more than 90 % similarity to the control (Fig. 5B).

DISCUSSION

During the last 5 decades, marine macroalgae have been intensively screened for biologically active secondary metabolites (Pratt et al., 1951; Sieburth, 1964; Sieburth & Conover, 1965; Caccanese et al., 1985; Ballesteros et al., 1992; Harder & Qian, 2000; Steinberg, 2001; Engel et al., 2006). Chemical analyses of macroalgal extract components have led to the discovery of novel marine natural products (Alejandro et al., 2004) with potent efficacies against a variety of micro- and macrofouling organisms (Walters et al., 1996; Dobretsov & Qian, 2002; Bansemir et al., 2004; Harder et al., 2004; Bazes et al., 2006). In the majority of these studies crude extracts of whole plant tissue have been screened against a variety of microbial fouling organisms in culture dependent bioassays, e.g. with bacterial isolates, diatoms and fungi (Ballesteros et al., 1992; Hellio et al., 2000; 2001; 2002; Pelegrin & Morales, 2004; Amsler et al., 2005; Engel et al., 2006; Paul et al., 2006). Although these studies supported the notion of a diverse spectrum of bioactive plant metabolites, the identity of compounds, their ecological role as allelochemicals and their natural delivery mechanisms have been rarely investigated so far (de Nys et al., 1995; Steinberg et al., 1997; Maximilien et al., 1998).

Previously, we demonstrated antimicrobial effects of macroalgal metabolites against planktonic bacteria utilizing a culture-independent approach to compare the bacterial community richness in seawater in the presence and absence of different macroalgae. The eubacterial community profile in algal culture water was qualitatively and quantitatively reduced in the presence of macroalgae. This effect was mainly caused by waterborne algal metabolites, whilst some bacterial types

were additionally affected by surface-mediated processes between algae and bacteria (Lam & Harder, in press).

In order to test whether antibacterial macroalgal metabolites affected the pool of potential bacterial colonizers in a phylum-specific fashion, our previous experiments were repeated with oligonucleotide probes selectively targeting three different bacterial phyla, i.e. the groups of the *Bacteroidetes,* alpha-*Proteobacteria* and *Roseobacter*. The community composition within these bacterial phyla was reduced to a greater extent in presence of the red algae *Ceramium rubrum* and *Mastocarpus stellatus* than in the brown alga *Laminaria digitata* (Figs. 1-3), indicating that either the quality of algal metabolites was different among the three macroalgae under investigation, or that the susceptibility of planktonic bacteria towards alga-derived antimicrobial effects correlated with their phylogenetic affiliation. The majority of affected phylotypes belonged to the *Bacteroidetes* and the *Roseobacter* clade (Figs. 1 & 3), implying that epiphytic bacterial colonizers belonging to these groups may pose a particular threat to the algae. In an attempt to further characterize such potentially detrimental bacterial epibionts, 3 bands obtained from the *Roseobacter* gel were sequenced. The sequences were affiliated to three different uncultured *Roseobacter* species, thus further conclusions about their potential pathogenicity were not warranted. Nonetheless, these results clearly demonstrated potent effects of waterborne macroalgal metabolites against this important group of bacteria, which account for ca. 25% of marine microbial populations, particularly in coastal and polar regions (Selje et al., 2004; Buchan et al., 2005; Wagner-Döbler & Biebl, 2006). Although selective associations of members of the *Roseobacter* clade with red and green macroalgae have been shown (Ashen et al., 2000; Patel et al., 2003), the

ecological role of this clade in general and its interaction with eukaryotes in particular is poorly understood so far. However, our study provides first experimental evidence of a chemical ecological context between macroalgae and bacteria belonging to the *Roseobacter* clade.

To investigate whether the fungal community was similarly affected by macroalgae as in the case of planktonic bacteria, a modified filter PCR protocol targeting the fungal ITS 1 region was developed. The ITS regions are highly variable sequences of great importance to distinguish fungal species by PCR and molecular fingerprinting (White et al., 1990; Gardes & Bruns, 1993; Martin & Rygiewicz, 2005). Under the combination of fungal primers PCR amplicons ranging from 250 to 300 bps were obtained in this study. In comparison to the fungal community in seawater additional fungal phylotypes were observed in all algal treatments, suggesting these contaminants were specific to algae and thus originated from the host plant (Fig. 4A). Similar to the effects observed against bacteria, the fungal community pattern was affected in macroalgal treatments. Contrary to the antibacterial effects observed in the two red algae, the brown alga *Lamniaria digitata* and the red alga *Mastocarpus stellatus* exhibited a more pronounced antifungal effect than the other red alga *Ceramium rubrum* eliminating at least 3 fungal phylotypes compared to the control (Fig. 4A). Waterborne algal metabolites (i.e. in the absence of algae) did not reduce the fungal community composition (Table 6, Fig. 5B). This finding may be due to the high recalcitrance of fungal hyphae and spores towards antimicrobial compounds compared to bacteria (Miao & Qian, 2005). Alternatively, these results are in accordance with earlier studies demonstrating that antifungal effects of macroalgae were mediated

by microbes in biofilms on algal blade surfaces (Armstrong et al., 2001; Boyd et al., 1999; Dobretsov & Qian, 2002).

In summary, our experimental results clearly suggest that macroalgae utilize allelochemical means to target different potential epiphytic colonizers, i.e. bacteria and fungi. Regarding the elimination of bacterial colonizers, the allelochemical effects differ among algae and selectively affect bacterial phyla. To our knowledge, this is the first study that demonstrates macroalgal defenses against epiphytic microorganisms based on natural delivery mechanisms of allelochemicals utilizing a culture-independent approach, thus minimizing the ecological bias inherent to culture-dependent studies based on few microbial isolates.

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REFERENCES

Alejandro M, Mayer S & Hamann MT (2004) Marine Pharmacology in 2000: Marine compounds with antibacterial, anticoagulant, antifungak, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; Affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. *Mar Biotechnol* **6**:37-52.

Alonso-Sáez L, Balagué V, Sà EL, Sánchez O, González JM, Pinhassi J, Massana R, Pernthaler J, Pedrós-Alió C & Gasol JM (2007) Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiol Ecol* **60**:98-112.

Amsler CD, Okogbue IN, Landry DM, Amsler MO, McClintock JB & Baker BJ (2005) Potential chemical defenses against diatom fouling in Antarctic macroalgae. *Bot Mar* **48**:318-322.

Armstrong E, Boyd KG & Burgess JG (2000) Prevention of marine biofouling using natural compounds from marine organisms. *Biotechnol Ann Rev* **6**:221-241.

Armstrong E, Yan L, Boyd KG, Wright PC & Burgess G (2001) The symbiotic role of marine microbes on living surfaces. *Hydrobiologia* **461**:37-40.

Ashen JB & Goff LJ (2000) Molecular and ecological evidence for species specificity and co-evolution in a group of marine algal-bacterial symbiosis. *Appl Environ Microbiol* **66**:3024-3030.

Ballesteros E, Martin D & Uriz MJ (1992) Biological activity of extracts from some Mediterranean macrophytes. *Bot Mar* **35**:481-485.

Bansemir A, Just N, Michalik M, Lindequist U & Lalk M (2004) Extracts and sesquiterpene derivatives from the red alga *Laurencia chondrioides* with antibacterial activity against fish and human pathogenic bacteria. *Chem Biodivers* **1**:463-467.

Bazes A, Silkina A, Defer D, Bernède-Bauduin C, Quéméner E, Braud JP & Bourgougnon N (2006) Active substances from *Ceramium botryocarpum* used as antifouling products in aquaculture. *Aquaculture* **258**:664-674.

Bouaicha N, Magne F, Puel D & Pesando D (1991) Production of antifungal metabolites by the brown alga Dilophus ligulatus in culture and in the field. *Oebalia Taranto* Suppl. 2.

Boyd KG, Adams DR & Burgess JG (1999) Antibacterial and repellent activity of marine bacteria associated with algal surfaces. *Biofouling* **14**:227-236.

Buchan A, Gonzalez JM & Moran AM (2005) Overview of the marine *Roseobacter* lineage. *Appl Environ Microbiol* **71**:5665-5677.

Caccanese S, Toscano MG, Furnari M & Cormaci M (1985) Antimicrobial activity of red and brown algae from Southern Italy Coast. *Bot Mar* **28**:505-507.

Corre S & Prieur D (1990) Density and morphology of epiphytic bacteria on the kelp *Laminaria digitata*. *Bot Mar* **33**:515-523.

de Nys R, Steinberg PD, Willemsen P, Dworjanyn SA, Gabelish CL & King RJ (1995) Broad spectrum effects of secondary metabolites from the red algae *Delisea pulchra* in antifouling assays. *Biofouling* **8**:259-271.

Dobretsov S & Qian PY (2002) Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. *Biofouling* **18**:217-228.

Engel S, Puglisi MP, Jensen PR & Fenical W (2006) Antimicrobial activities of extracts from tropical Atlantic marine plants against marine pathogens and saprophytes. *Mar Biol* **149**:991-1002.

Freile-Pelegrin Y & Morales JL (2004) Antibacterial activity in marine algae from the coast of Yucatan, Mexico. *Bot Mar* **47**:140-146.

Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the idnetification of mycorrhizae and rusts. *Mol Ecol* **2**:113-118.

Gross E (1999) Allelopathy in benthic and littoral areas: Case studies on allelochemicals from benthic cyanobacteria and submersed macrophytes, in *Principles and Practices in Plant Ecology*: Allelochemical Interactions, Inderjit, (Dakshini KMM & Foy CL, eds), CRC Press, Boca Raton, pp. 179-199.

Gross E (2003) Allelopathy of aquatic autotrophs. *Crit Rev Plant Sci* **22**:313-339.

Harder T & Qian PY (2000) Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitive cues for larval attachment and metamorphosis in the polychaete *Hydroides elegans*. *Biofouling* **16**:205-214.

Harder T, Dobretsov S & Qian PY (2004) Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. *Mar Ecol Prog Ser* **274**:133-141.

Hellio C, Bremer G, Pons AM, Le Gal Y & Bourgougnon N (2000) Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. *Appl Microbiol Biotechnol* **54**:543-549.

Hellio C, de La Broise D, Dufossé L, Le Gal Y & Bourgougnon N (2001a) Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. *Mar Environ Res* **52**:231-247.

Hellio C, Thomas-Guyon H, Culioli G, Piovetti L, Bourgougnon N & Le Gal Y (2001b) Marine antifoulants from Bifurcaria bifurcate (Phaeophyceae, Cystoseiraceae) and other brown macroalgae. *Biofouling* **17**:189-201.

Hellio C, Berge JP, Beaupoil C, Le Gal Y & Bourgougnon N (2002) Screening of marine algal extracts for anti-settlement activities microalgae and macroalgae. *Biofouling* **18**:205-215.

Keats DW, Knight MA & Pueschel CM (1997) Antifouling effects of epithallial shedding in three crustose coralline algae (Rhodophyta, Coralinales) on a coral reef. *J Exp Mar Biol Ecol* **213**:281-293.

Kirchman DL, Yu L, Fuchs BM & Amann R (2001) Structure of bacterial communities in aquatic systems as revealed by filter PCR. *Aquat Microb Ecol* **26**:13-22.

Kohlmeyer J & Kohlmeyer E (1979) Marine mycology: the higher fungi. Academic Press, London, pp. 1-5.

Lam C & Harder T (in press) Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity. *J Phycol*

Martin KJ & Rygiewicz PT (2005) Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology* **5**:28-38.

Maximilien R, de Nys R, Holmström C, Gram L, Givskov M, Crass K, Kjelleberg S & Steinberg PD (1998) Chemical mediation of bacterial surface colonization

by secondary metabolites fro the red alga *Delisea pulchra*. *Aquat Microb Ecol* **15**:233-246.

Messing J (1983) New M13 vectors for cloning. Meth Enzymol 101:20-78

Miao L & Qian PY (2005) Antagonistic antimicrobial activity of marine fungi and bacteria isolated from marine biofilm and seawaters of Hong Kong. *Aquat Microb Ecol* **38**:231-238.

Moss BL (1982) The control of epiphytes by *Halidrys siliquosa* (L.) Lynbg. (Phaeophyta, Cystoseiraceae). *Phycologia* **21**:185-191.

Muyzer G, De Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction –amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**:695-700.

Patel P, Callow ME, Joint I & Callow JA (2003) Specificity in the settlement-modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. *Environ Microbiol* **5**:338-49.

Paul NA, de Nys R & Steinberg PD (2006) Chemical defense against bacteria in the red alga *Asparagopsis armata*: linking structure with function. *Mar Ecol Prog Ser* **306**:87-101.

Paul VJ & Fenical W (1984) Bioactive terpenoids from Caribbean marine algae of the genera *Penicillus* and *Udotea* (Chlorophyta). *Tetrahedron* **40**:2913-1918.

Pelegrin YF & Morales JL (2004) Antibacterial activity in marine algae from the coast of Yucatan, Mexico. *Bot Mar* **47**:140-146.

Pratt R, Mautner H, Gardner GMG, Sha H & Dufenoy J (1951) Antibiotic activity of seaweed extracts. *J Am Pharm Assoc* **40**:575.

Rink B, Seeberger S, Martens T, Duerselen CD, Simon M & Brinkhoff T (in press) Effects of a phytoplankton bloom in a coastal ecosystem on the composition of bacterial communities. *Aquat Microb Ecol*

Selje N, Simon M & Brinkhoff T (2004) A newly discovered *Roseobacter* clusters in temperate and polar oceans. *Nature* **427**:445-448.

Sieburth JM (1964) Antibacterial substances produced by marine algae. *Develop Ind Microbiol* **5**:124-34.

Sieburth JM & Conover JT (1965) *Sargassum* tannin, an antibiotic which retards fouling. *Nature* **208**:52-53.

Smit JA (2004) Medical and pharmaceutical uses of seaweed natural products: a review. *J Appl Phycol* **16**:245-262.

Steinberg PD (2001) Chemical mediation of interactions at seaweed surfaces. J *Phycol* **37**:46-47.

Steinberg PD, Schneider R & Kjelleberg S (1997) Chemical defenses of seaweeds against microbial colonization. *Biodegradation* **8**:211-220.

Steinberg PD, de Nys R & Kjelleberg S (1998) Chemical inhibition of epibiota by Australian seaweeds. *Biofouling* **12**:227-244.

Tait K, Joint I, Daykin M, Milton DL, Williams P & Cámara M (2005) Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga Ulva to bacterial biofilms. *Environ Microbiol* **7**:229-240.

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Mar Ecol Prog Ser* **58**:175-189.

Wagner-Döbler I & Biebl H (2006) Environmental biology of the marine *Roseobacter* lineage. *Annu Rev Microbiol* **60**:255-280.

Walter LJ, Hadfield MG & Smith CM (1996) Waterborne chemical compounds in tropical macroalgae: positive and negative cues for larval settlement. *Mar Biol* **126**:383-393.

White TJ, Bruns T, Lee S & Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications. (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds). New York, Academic Press Inc. pp. 315-22.

Zhou J, Bruns MA & Tiedje JM (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**:316-322.

FIGURES & TABLES

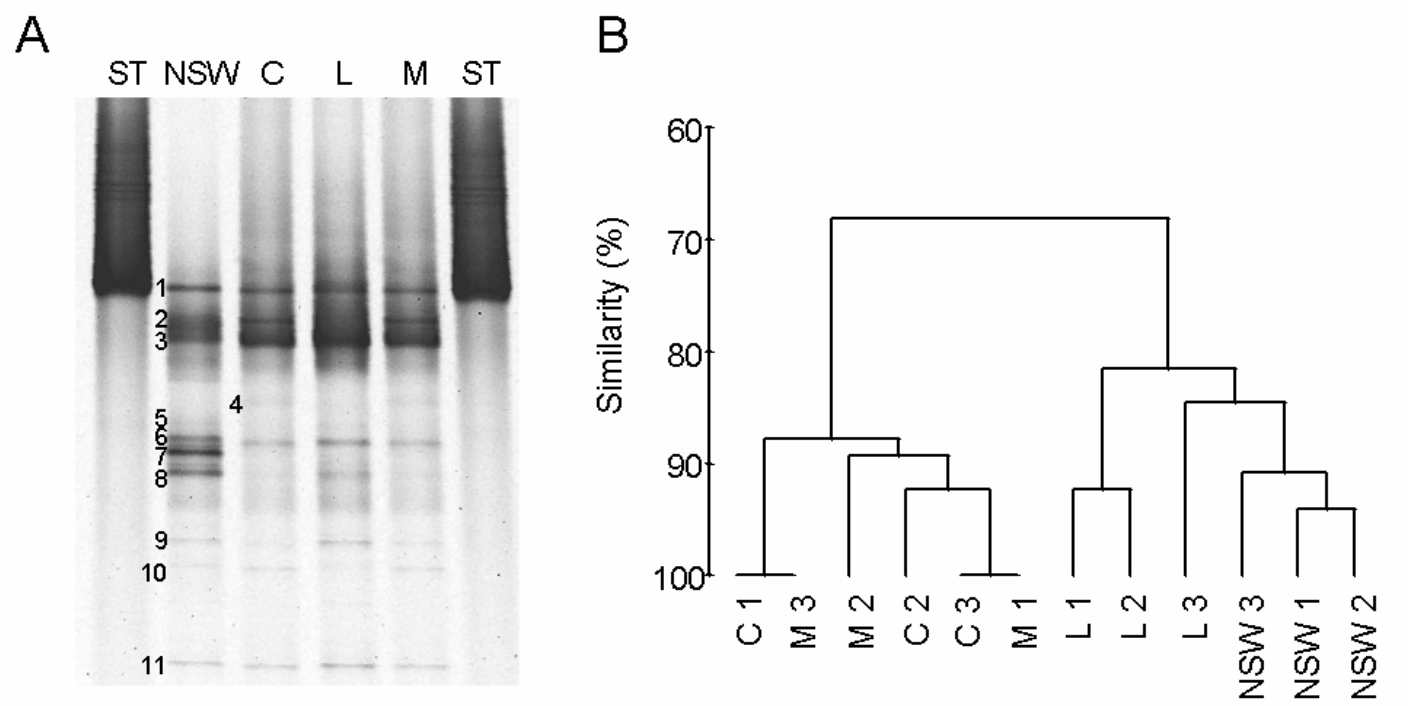

Figure 1: **A**: Denaturant gradient gel showing the bacterial community of the *Bacteroidetes* in macroalgal treatments of *Ceramium rubrum* (C), *Laminaria digitata* (L), *Mastocarpus stellatus* (M) and the control of natural seawater (NSW) after 72 h of incubation. DNA marker lanes are labeled as ST. All band positions are labeled with numbers. **B**: Cluster analysis of DGGE banding patterns of planktonic bacteria belonging to this group in replicated macroalgal treatments (1-3). The dendrograms were constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method.

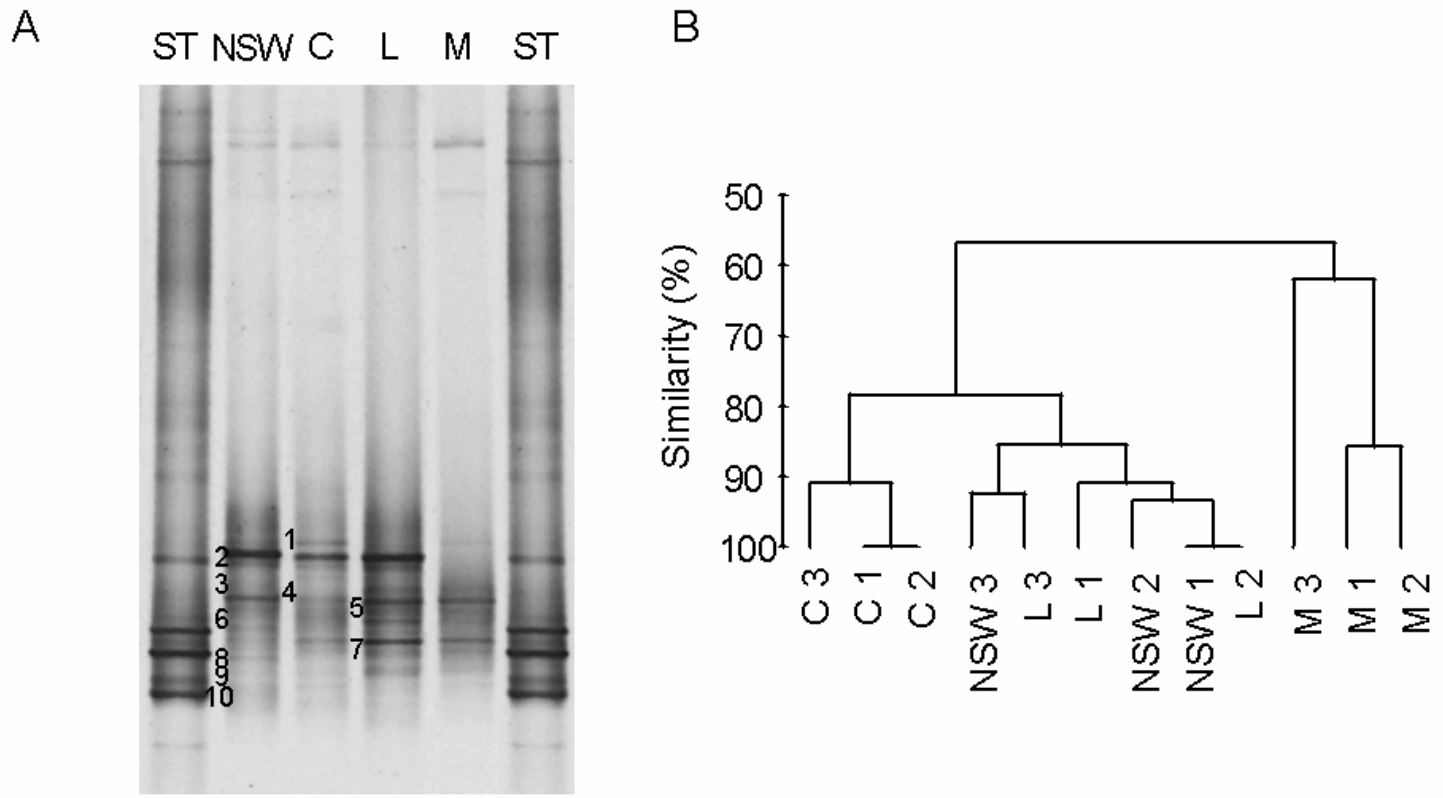

Figure 2: **A**: Denaturant gradient gel showing the bacterial community of the alpha-*Proteobacteria* in macroalgal treatments of *Ceramium rubrum* (C), *Laminaria digitata* (L), *Mastocarpus stellatus* (M) and the control of natural seawater (NSW) after 72 h of incubation. DNA marker lanes are labeled as ST. All band positions are labeled with numbers. **B**: Cluster analysis of DGGE banding patterns of planktonic bacteria belonging to this group in replicated macroalgal treatments (1-3). The dendrograms were constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method.

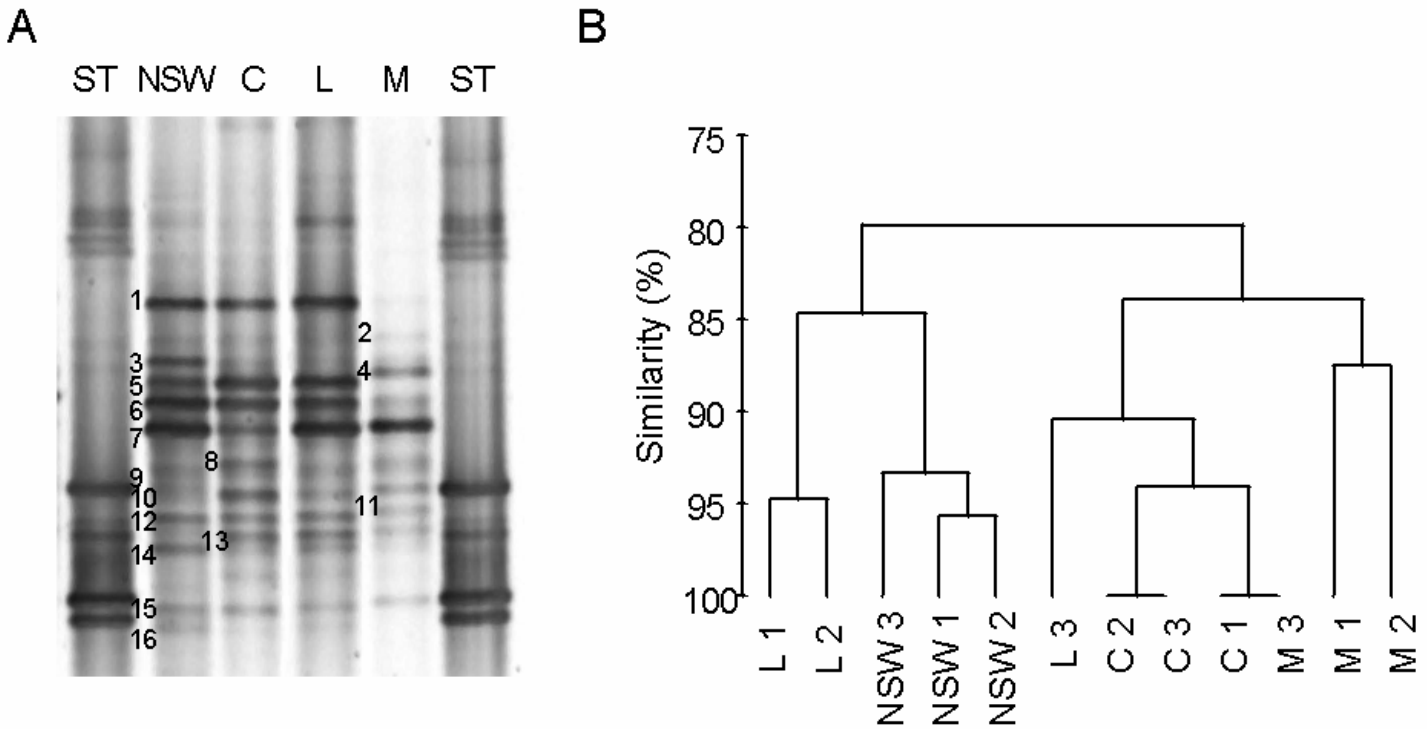

Figure 3 : **A**: Denaturing gradient gel showing the bacterial community of the Roseobacter in macroalgal treatments of *Ceramium rubrum* (C), *Laminaria digitata* (L), *Mastocarpus stellatus* (M) and the control of natural seawater (NSW) after 72 h of incubation. Bands 1,3 and 5 were excised and sequenced (referred to as Roseobacter 1, 3, & 5 in the Results). DNA marker lanes are labeled as ST. All band positions are labeled with numbers. **B**: Cluster analysis of DGGE banding patterns of planktonic bacteria belonging to this group in replicated macroalgal treatments (1-3). The dendrograms were constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method.

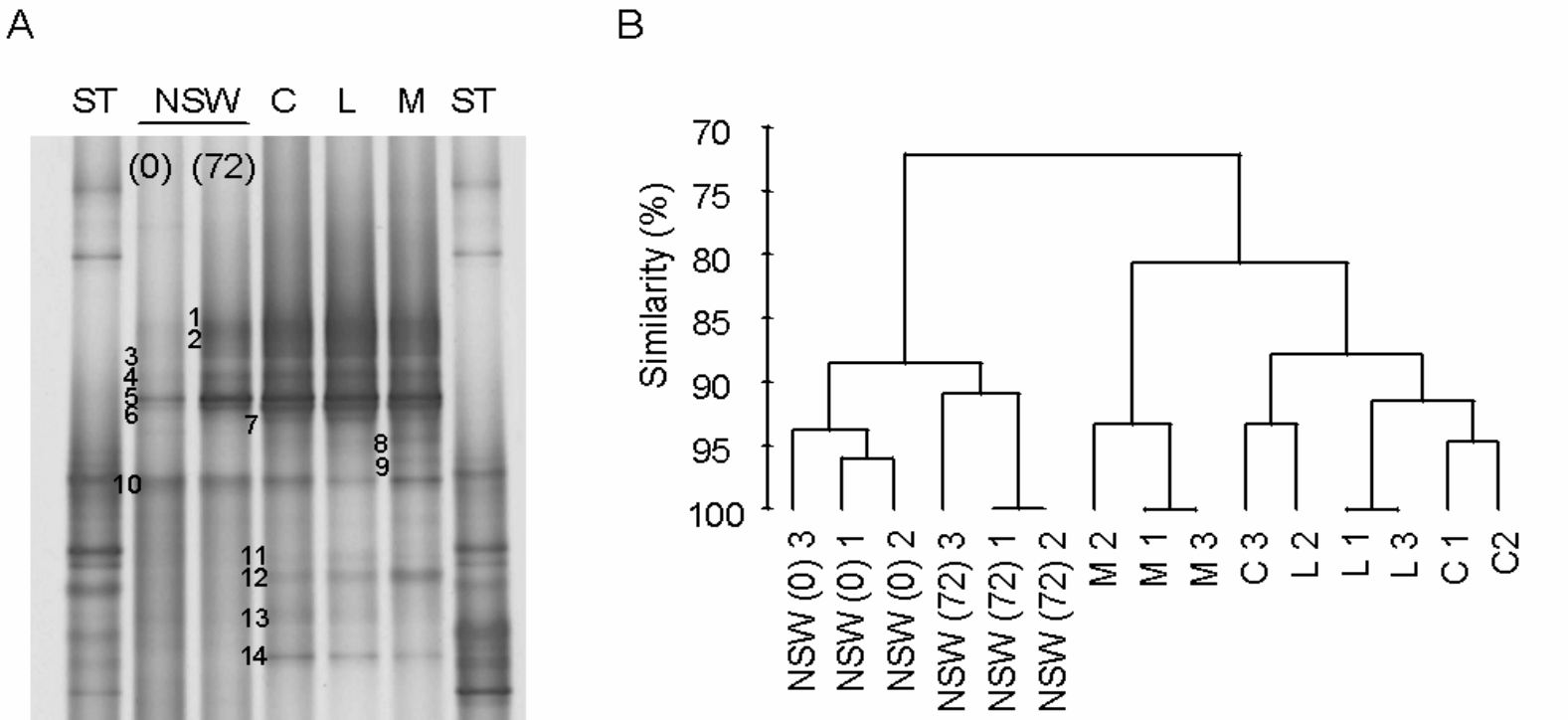

Figure 4: **A (left)**: Denaturant gradient gel showing the fungal community of ITS1 region in macroalgal treatments of *Ceramium rubrum* (C), *Laminaria digitata* (L), *Mastocarpus stellatus* (M) and the control of natural seawater (NSW) after 72 h of incubation. DNA marker lanes are labeled as ST. All band positions are labeled with numbers.
B (up): Cluster analysis of DGGE banding patterns of planktonic fungal in replicated macrolagal treatments (1-3). The dendrograms were constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method.

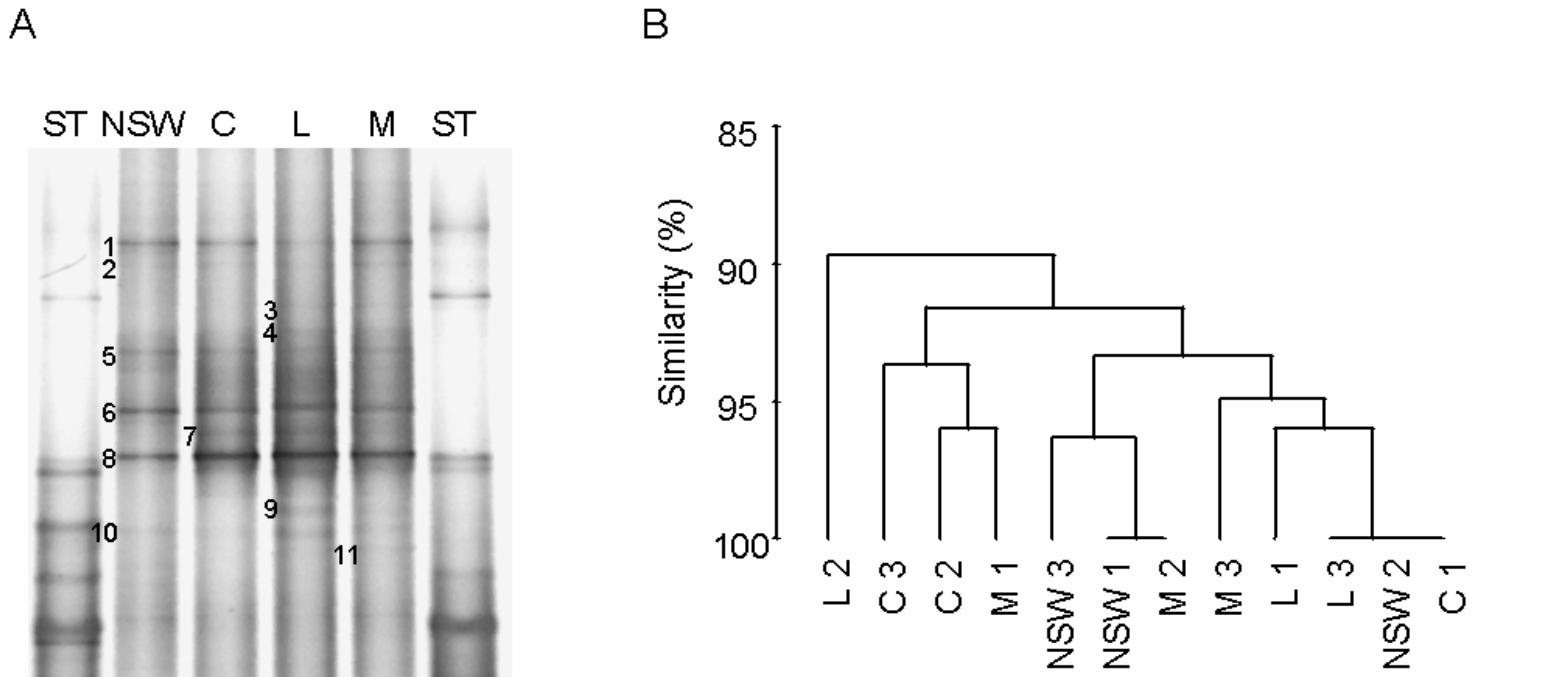

Figure 5: **A (left)**: Denaturant gradient gel showing the effects of waterborne metabolites in fungal community of ITS1 region in macroalgal treatments of *Ceramium rubrum* (C), *Laminaria digitata* (L), *Mastocarpus stellatus* (M) and the control of natural seawater (NSW) after 24 h of incubation. DNA marker lanes are labeled as ST. All band positions are labeled with numbers. **B (up)**: Cluster analysis of DGGE banding patterns of planktonic fungal in replicated macrolagal treatments (1-3). The dendrograms were constructed using a similarity matrix determined by Bray-Curtis coefficients and the unweighted pair-group average method.

Table 1. Bacterial/fungal species used as DNA standards.

the *Bacteriodetes*	*Roseobacter*
Cytophaga sp. I-377	*Phaeobacter inhibeus*
Cytophaga sp. KT0803	*Phaeobacter inhibeus; Mutant lacking production of antibiotics*
Cytophaga marinoflava	*Loktanella* sp.
Cytophaga sp.	*Sulfitobacter* sp.
	Roseobacter sp.

alpha-*Proteobacteria*	Fungi
Phaeobacter inhibeus	*Phoma glomerata*
Phaeobacter inhibeus; Mutant lacking production of antibiotics	*Penicillium* sp.
Loktanella sp.	*Trichoderma* sp.
Sulfitobacter sp.	*Mucor* sp.
Roseobacter sp.	*Aspergillus* sp.
	Cladosporium sp.

Table 2. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic bacteria of the *Bacteroidetes* in water conditioned with *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* and in a control of natural seawater (NSW) after 72 h of incubation.

	NSW	*C. rubrum*	*L. digitata*	*M. stellatus*
NSW	-			
C. rubrum	1.0 *	-		
L. digitata	0.648	0.87 *	-	
M. stellatus	1.0 *	0.148	0.87 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 319-907 (*Escherichia coli* numbering system; Brosius et al. 1978). The asterisk marks statistical significance (α = 0.05). Global R value is 0.735.

Table 3. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic bacteria of the alpha-*Proteobacteria* in seawater conditioned with *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* and in a control of natural seawater (NSW) after 72 h of incubation.

	NSW	*C. rubrum*	*L. digitata*	*M. stellatus*
NSW	-			
C. rubrum	0.741 *	-		
L. digitata	0.074	0.796 *	-	
M. stellatus	0.796 *	0.815 *	0.630 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 341-968 (*Escherichia coli* numbering system; Brosius et al. 1978). The asterisk marks statistical significance (α = 0.05). Global R-value = 0.556.

Table 4. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic bacteria of the *Roseobacter* in seawater conditioned with *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* and in a control of natural seawater (NSW) after 72 h of incubation.

	NSW	*C. rubrum*	*L. digitata*	*M. stellatus*
NSW	-			
C. rubrum	1.0 *	-		
L. digitata	0.796 *	0.722	-	
M. stellatus	1.0 *	0.389	0.759 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 536-735 (*Escherichia coli* numbering system; Brosius et al. 1978). The asterisk marks statistical significance (α = 0.05). Global R-value is 0.759.

Table 5. Analysis of similarity (ANOSIM) of DGGE banding patterns of planktonic fungi in seawater conditioned with *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* and in a control of natural seawater control (NSW) at the beginning of the experiment (0 h) and after 72 h of incubation.

	NSW (0)	NSW (72)	*C. rubrum* (72)	*L. digitata* (72)	*M. stellatus* (72)
NSW (0)	-				
NSW (72)	0.722	-			
C. rubrum	0.963 *	0.889	-		
L. digitata	1.0 *	1.0 *	0.352	-	
M. stellatus	1.0 *	1.0 *	0.963 *	1.0 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 2-5 within the Internal Transcribed Spacer regions (White et al. 1990). The asterisk marks statistical significance (α = 0.05). Global R-value is 0.91.

Table 6. Analysis of similarity (ANOSIM) of DGGE banding patterns of waterborne algal metabolites on community richness of planktonic fungi in seawater conditioned with *Ceramium rubrum*, *Laminaria digitata* & *Mastocarpus stellatus* and in a control of natural seawater control (NSW) after 24 h of incubation.

	NSW (24)	*C. rubrum* (24)	*L. digitata* (24)	*M. stellatus* (24)
NSW (24)	-			
C. rubrum	0.315 *	-		
L. digitata	0.315 *	0.296 *	-	
M. stellatus	0.074	0.148	0.352 *	-

Calculated *R*-values stem from analysis of amplified 16S rDNA sequence fragments derived from positions 2-5 within the Internal Transcribed Spacer regions (White et al. 1990). Global R-value = 0.215.

4 ANTIFOULING ACTIVITY OF NORTH SEA MACROALGAE AGAINST BENTHIC DIATOMS

4.1 Introduction

Based on my previous observations, marine macroalgae utilized allelochemical means to target different potential epiphytic colonizers, such as bacteria and fungi, in seawater. Provided that the competition for light and nutrients between epiphytic diatoms and macroalgae is particularly intense in the photic zone, few studies have addressed the allelochemistry of marine macroalgae against epiphytic diatoms so far. In this study, I tested the hypothesis that macroalgae from the North Sea exhibited potent inhibitory effects against epiphytic diatoms. Surfaces of a variety of macroalgae were visualized by scanning electron microscopy and crude organic extracts from the same batch of algae were immobilized in Phytagel® for diatom attachment and proliferation assays. My results revealed that macroalgae differed in terms of their epiphytic coverage with bacteria, fungi and diatoms and grouped them into 2 categories: 1) largely devoid of epiphytic diatoms on algal surfaces and, 2) heavily fouled with microepiphytes on its surfaces. Diatom attachment assays showed that algal extracts exhibited a distinct pattern of activities against four benthic diatoms. The susceptibility of diatoms to algal extracts was not species-specific. My experimental results support the concept that macroalgae not only target epiphytic bacteria and fungi by producing bioactive metabolites, but also individual epiphytic diatoms. This work has been written in the format of manuscript and already submitted to the **Biofouling** for revision.

Antifouling activity of North Sea macroalgae against benthic diatoms

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Running head: Chemical denfense of macroalgae against diatoms

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ABSTRACT

A variety of macroalgae was investigated under the scanning electron microscope to visualize epiphytic colonizers. The macroalgae differed in terms of their epiphytic coverage with bacteria, fungi and diatoms. Macroalgae largely devoid of epiphytic diatoms were hypothesized to employ effective antifouling means to reduce epiphytic coverage, whilst heavily fouled macroalgae were supposed to lack antifouling strategies. To test these hypotheses with regard to fouling diatoms from an allelochemical perspective, crude extracts of macroalgae were investigated in diatom attachment and proliferation assays with four benthic diatoms. Algal extracts exhibited a distinct pattern of activities against the diatoms under investigation suggesting a targeted and selective effect of macroalgal metabolites on individual fouling diatoms. Visual inspection of epiphytic colonizers on algal thalli is not a reliable predictor to group macroalgae into chemically defended and undefended categories.

Keywords: Macroalgae, diatoms, antifouling, epibiosis, allelopathy

INTRODUCTION

Complex communities of micro- (e.g. bacteria, diatoms, fungi) and macro- (e.g. algal spores, larvae) organisms often densely populate marine surfaces. If these surfaces belong to living organisms the phenomenon is referred to as epibiosis (Wahl, 1989). In the photic zone competition among phototrophic organisms for light, nutrients and space is particularly intense (de Nys et al. 1995; Harder et al. 2004a) causing a variety of detrimental effects to host organisms. For instance, algae are susceptible to disease and tissue necrosis induced by bacteria, fungi and microalgae (Mitchell & Chet, 1975; Bouarab et al. 2001; Cooney et al. 2002). The sometimes drastic changes of pH and redox conditions created by microepibionts may attack chemically sensitive algal thalli (Terry & Edyvean, 1981). Importantly, the adverse effects of microbial epibiosis may reach beyond pathogenicity and virulence. Since microbial films are important sources of chemical cues for larval settlement in many benthic marine invertebrates (Lau et al. 2002; Harder et al. 2002), microbial epibiosis may promote subsequent colonization by rigid crustose epibiotic macroorganisms, which in turn significantly impair the basibionts' ability to exchange gases and nutrients (Jagels, 1973), damage the tissue by increased weight, rigidity and drag (Dixon et al. 1981), and decrease the growth rate of photosynthetic basibionts by cutting surface irradiance levels (Sand-Jensen, 1977; Silberstein et al. 1886).

From a nutritional perspective it is evident that if the host and the epibiont share the same trophic requirements then planktonic nutrients reaching the basibiont may already be partially depleted after their passage through the epibiotic barrier. As epibionts may fall victim to predators of their hosts, so many

basibionts suffer from “shared doom”, i.e. damage due to grazers preying on epibionts (Dixon et al. 1981).

Many marine macroalgae are devoid of macroepiphytes (Steinberg et al. 1997; Maximilien et al. 1998; Dobretsov & Qian, 2002), suggesting some sort of host-derived antifouling defense. Epiphytes on algal thalli are physically removed by mucus secretion and periodic sloughing of outermost cell layers (Moss, 1982; Corre & Prieur, 1990; Keats et al. 1997; Nylund & Pavia, 2005), but increasing evidence suggests a chemical mode of antifouling defense. To create unfavorable or toxic conditions at or immediately above algal thalli is a wide-spread adaptation of macroalgae to cope with epibionts. The brown alga *Laminaria digitata* and the red alga *Gracilaria conferta* react with an oxidative burst to the presence of either alginate oligosaccharides or agar oligosaccharides, both of which are degradation products of their own cell walls (Küpper et al. 2001), resulting in the efficient elimination of bacterial epiflora (Weinberger et al. 2000). Moreover, many macroalgae feature a variety of chemical defense metabolites effective against different phyla of potential epibionts (Wium-Anderson, 1987; Steinberg et al. 1997; Harder & Qian, 2000; Harder et al. 2004a; Engel et al. 2006; Paul et al. 2006).

The chemical defense of marine organisms against epiphytic diatoms has received particular attention over the last decade. For example, the marine bacterium *Pseudoalteromonas tunicata*, isolated from the surface of the tunicate *Ciona intestinalis*, produced bioactive compounds against diatoms (Holmström et al. 1992; James et al. 1996). Waterborne metabolites of the sponge *Callyspongia* (*Euplacella*) *pulvinata* strongly inhibited the growth of the benthic diatom *Nitzschia paleaceae* (Dobretsov et al. 2004). Surprisingly few studies have addressed the allelochemistry of marine macroalgae against epiphytic diatoms although the

competition for light and nutrients between epiphytic diatoms and macroalgae is particularly intense in the photic zone (Gross, 2003). Several studies have reported specific epiphytic assemblages of diatoms on macroalgae in the field (Gough & Gough, 1981; Millie & Lowe, 1983; Blindow, 1987), implying that macroalgae may profoundly affect the composition of benthic diatoms in epiphytic biofilms. So far, crude extracts of macroalgae obtained in North East Atlantic waters (Hellio et al. 2002) and the Antarctic (Amsler et al. 2005) were reported to be toxic to typical epiphytic diatoms. As the antimicrobial activity of macroalgae is strongly influenced by environmental factors, such as water temperature, light, grazing pressure, season and geographical location (Hellio et al. 2004), we tested the hypothesis that macroalgae from the North Sea exhibited potent inhibitory effects against epiphytic diatoms.

A variety of red, brown and green macroalgae were investigated under the scanning electron microscope (SEM) to visualize epiphytic colonizers and to categorize algae regarding the presence or absence of epiphytic diatoms. Crude extracts of eight macroalgae were tested with regard to attachment and proliferation of diatoms abundant on inanimate substrata (i.e. *Nitzschia curvilineata*, *Navicula pelliculosa*, *Phaeodactylum tricornutum* & *Synedra* sp.). The macroalgae under investigation were assumed to be exposed to the same pool of diatoms, thus differences in the attachment assays were hypothesized to stem from algae-related attributes.

MATERIALS AND METHODS

Collection of macroalgae

Macroalgae were collected from the rocky shore of Helgoland Island (N: 54° 10' 0", E: 7° 52' 60") in June 2005. Different species of Rhodophyceae (*Ceramium rubrum*, *Corallina officinalis*, *Palmaria palmata*, *Mastocarpus stellatus*), Phaeophyceae (*Fucus serratus*, *Fucus vesiculosus*) and Chlorophyceae (*Cladophora rupestris*, *Ulva* sp.) were collected. Prior to experiments, algae were rinsed with sterile seawater to dissociate isopods and debris.

Preparation of algal extracts

Algae were briefly rinsed with sterile distilled water and blot-dried between paper towels. The samples were lyophilized overnight (Christ, Alpha 1-4, Germany). Dried algae were powdered and extracted twice in 1:1 dichloromethane:methanol at 4 ºC for 24 h. The combined extracts were filtered through paper filters (Sartoris, Göttingen, Germany) and reduced by rotary evaporation to a concentration that was volumetrically equivalent to one-tenth of the original algal tissue volume (i.e. 10 × tissue level concentration).

Diatom cultures

Four benthic diatoms abundant in North Sea water and biofilms were used in diatom attachment assays. The diatoms *Nitzschia curvilineata*, *Navicula pelliculosa* and *Phaeodactylum tricornutum* were obtained from the Culture Collection of Algae at the University of Göttingen, Germany. The diatom *Syndera* sp. was isolated from a biofilm harvested at Hooksiel (N: 53° 38' 31", E: 8° 04' 55"), North Sea. The purity of diatoms was checked under the light microscope. Diatom

culture conditions were adopted from Harder et al. (2002). To inhibit bacterial growth, antibiotics were added to diatom suspensions at a final concentration of 2.5 x 10^{-4} M streptomycin and 1.0 x 10^{-4} M penicillin. Prior to assays, diatom films were dislodged from the culture flask and separated by centrifugation.

Gel immobilization of algal extracts and antidiatom assays

The immobilization of macroalgal extracts was adopted from Harder et al. (2004b). Briefly, known volumes of algal extract at 10 × tissue level concentration were reduced by rotary evaporation, leaving a semi-solid crude extract residue. These extracts were dissolved in dimethylsulfoxide (DMSO) and transferred into 50 ml tubes. Gels were prepared by addition of Phytagel™ (Sigma, Seelze, Germany) to boiling distilled water at a final concentration of 2% (w/v). The hot gel solution was thoroughly mixed with the DMSO crude extract and diluted to 1 × tissue level concentration. Negative controls were prepared accordingly with DMSO only.

A PVC ring (70 mm in diameter, 20 mm wide, 10 mm thick) containing eight individual wells (10 mm in diameter, 8 mm deep each) was used for diatom attachment assays. Each well was filled with algal extract under replication (n = 3), the remaining two wells served as a solvent control (Fig. 1). The ring was placed into a 600 ml glass beaker containing sterile seawater enriched with f/2 medium (Guillard & Ryther, 1962) inoculated with concentrated monospecies diatom suspensions. The diatom suspension was stirred with a magnetic stirrer bar in the centre of the PVC ring. Diatom abundance on experimental gel surfaces were recorded after 24 h.

[enter Fig 1 about here]

Enumeration of diatom attachment

PVC rings were rinsed with sterile seawater to remove loose cells after 24 h of incubation. Gels were removed from wells and the top layer (ca. 1 mm) was sliced off and wet mounted on a microscope slide. Diatoms were enumerated at a magnification of 100 x in 15 randomly chosen fields of view by direct light microscopy (ZEISS Axiophot microscope, Göttingen, Germany).

Scanning electron microscopy of algal thalli

Macroalgae were rinsed with sterile seawater and fixed in 4 % formaldehyde in sterile filtered seawater. Fixed specimens were exposed to an ascending concentration series of ethanol (10 %, 30 %, 50 %, 70 %, 96 % and 100 %). In parallel, specimens were air-dried at room temperature overnight. All samples were critical-point dried with carbon dioxide (Bal-Tec, Liechtenstein, Germany) and sputter-coated with gold before visualization in a S-3200N scanning electron microscope (Hitachi, Japan) at 10 kV acceleration voltage.

Statistical analysis

Statistical differences of diatom abundances on gel surface were calculated with the SPSS computer package. The normality assumption of data was checked with Shapiro-Wilk's *W*-test (Shapiro & Wilk, 1965). Data sets that met the criteria of homogeneity of variance and normality were analyzed by ANOVA and multiple post hoc comparison (Conover & Iman, 1981); whilst data that failed to meet the criteria of parametric statistics were analyzed by Kruskal Wallis statistics followed by pairwise comparisons with Mann Whitney U tests.

RESULTS

Scanning electron microscopy of algal thalli

Visual analysis by SEM grouped the eight macroalgae under investigation into a category that was largely devoid of epiphytic diatoms, i.e. *Mastocarpus stellatus*, *Ulva* sp., *Fucus serratus*, *Fucus vesiculosus* (Fig. 2A), and a category that was densely populated with diatoms, i.e. *Corallina officinalis, Ceramium rubrum*, *Palmaria palmata* and *Cladophora rupestris* (Fig. 2B). The majority of diatoms on algal surface belonged to pennate diatoms. Bacteria were visible in critical-point dried samples, but not in air-dried samples. Fungal hyphae were particularly observed on the surfaces of *C. rubrum*, *F. vesiculosus* and *Ulva* sp.

[insert Figute 2 about here]

Diatom attachment assays

The experimental design allowed the simultaneous investigation of two different algal extracts on diatom attachment. The comparison of diatom abundance on experimental gel surfaces with the corresponding control grouped the algal extracts into three categories. Algal extracts that promoting higher diatom attachment than on the control ($\alpha = 0.05$) were classified as 'inductive', extracts evoking diatom attachment lower than in the control ($\alpha = 0.05$) were classified as 'inhibitive'. Algal extracts with no effect on diatom attachment ($\alpha = 0.05$) were classified as 'neutral'. The specificity of macroalgal extracts on diatom attachment and proliferation revealed a unique species-specific pattern (Table I; Fig. 3 A-D). There was no general trend of extract efficacies of macroalgae on diatoms under investigation. Among eight macroalgae under investigation, the green alga *C. rupestris* was the most potent alga to inhibit attachment of all four diatoms,

followed by the red algae *C. officinalis* and *C. rubrum*. With the exception of the red alga *P. palmata*, all the macroalgae under investigation inhibited attachment and proliferation of at least one diatom species (Table 1; Fig. 3A-D).

[insert Table I and Fig 3 A-D about here]

DISCUSSION

Macroalgae produce a wide range of secondary metabolites with inhibitory effects against bacteria (Hellio et al. 2000; 2001; Pelegrin & Morales, 2004; Paul et al. 2006), fungi (Ballesteros et al. 1994; Hellio et al. 2000; Engel et al. 2006), viruses (Damonte et al. 1994; Kolender et al. 1995), macroalgal spores (Hellio et al. 2004) and invertebrate larvae (Cho et al. 2001; Hellio et al. 2004). Surprisingly few studies have addressed the ability of macroalgae to deter diatom attachment and proliferation despite the fact that their abundance in marine biofilms is high (Harder et al. 2002) and that some macroalgae contain significantly less epiphytic diatoms than co-occurring biofilms on inanimate substrata (Dobretsov & Qian, 2002).

The macroalgae under investigation differed in terms of their epiphytic coverage with bacteria, fungi and diatoms (Fig. 2). Based on these snap-shot observations, macroalgae largely devoid of epiphytic diatoms on algal surfaces were hypothesized to employ effective antifouling means to reduce epiphytic coverage, whilst heavily fouled macroalgae were supposed to lack antifouling strategies. In order to test these hypotheses on fouling diatoms from an allelochemical perspective, crude extracts of macroalgae were investigated in diatom attachment and proliferation assays with four benthic diatoms. These diatoms were dominant in biofilms on inanimate surfaces and thus hypothesized

to be ecologically representative. Experiments were performed under hydrodynamically turbulent conditions since the settling velocity of diatoms is maximized this way (Ruiz et al. 2004).

Algal extracts exhibited a distinct pattern of activities against the diatoms under investigation. Only the extract of *Cladophora rupestris* inhibited all diatoms, whilst the other algal extracts affected less or none of the diatoms under investigation (Fig. 3). The susceptibility of diatoms to algal extracts was not species-specific (Table 1). Some algal extracts, such as the brown alga *Fucus serratus* inhibited attachment and proliferation of one diatom species (*Phaeodactylum tricornutum*) whilst promoting attachment of other diatoms (*Nitzschia curvilineata*, *Synedra* sp.), suggesting a targeted effect of macroalgal metabolites on individual fouling diatoms. Based on these data the hypothetical categorization of macroalgae into a group of supposedly defended and undefended algae was not warranted.

So far the modulatory role of macroalgal thalli on diatom attachment has been discussed controversially in the literature. Whilst some studies reported that algal surfaces accommodate a similar richness of benthic diatoms as artificial plants (Cattaneo & Kalff, 1978), other investigators have observed rather specific assemblages of diatoms on macroalgae in the field (Gough & Gough, 1981; Millie & Lowe, 1983; Blindow, 1987). Although the results of our study were limited to few diatoms the conclusions comply with the latter group of investigators and support the notion of rather targeted and specific allelochemical effects of macroalgae against fouling diatoms competing for the same resources, such as light, nutrients and space, in the same niche.

Other modes of antifouling defense of macroalgae may likely work in concert with algal metabolites. These modes include mucus secretion and sloughing of microcolonizers (Nylund & Pavia, 2005) and antimicrobial effects of host-associated microorganisms (Armstrong et al. 2001). For instance, epipyhtic bacteria and fungi associated with macroalgae were demonstrated to inhibit growth of fouling diatoms (Chen et al. 1996; Jenkins et al. 1998; Dobretsov & Qian, 2002). Since the microscopic assessment of algal thalli in this study revealed a significant colonization of some macroalgae by bacteria and fungi, it can be speculated that this epiphytic coverage may contribute to a target defense against certain epiphytes.

In summary, this study demonstrated that visual inspection of epiphytic colonizers on algal thalli is not a reliable predictor to group macroalgae into chemically defended and undefended categories. Instead, macroalgal allelochemistry seems to be rather targeted and selective on epiphytic microcolonizers.

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REFERENCES

Armstrong E, Yan L, Boyd KG, Wright PC, Burgess JG. 2001. The symbiotic role of marine microbes on living surfaces. Hydrobiologia 461:37-40.

Amsler CD, Okogbue LN, Landry DM, Amsler MO, McClintock JB, Baker BJ. 2005. Potential chemical defenses against diatom fouling in Antarctic macroalgae. Bot Mar 48:318-22.

Ballesteros E, Martin D, Uriz MJ. 1992. Biological activity of extracts from some Mediterranean macrophytes. Bot Mar 35:481-5.

Blindow I. 1987. The composition and density of epiphyton on several species of submerged macrophytes – the neutral substrate hypothesis tested. Aquat Bot 29:157-168.

Bouarab K, Potin P, Weinberger F, Correa J, Kloareg B. 2001. The *Chondrus crispus Acrochaete operculata* host-pathogen association, a novel model in glycobiology and applied phycopathology. J Appl Phycol 13:185-193

Cattaneo A, Kalff J. 1978. Seasonal changes in the epiphyte community of natural and artificial macrophytes in Lake Memphremagog (Que. and Vy.). Hydrobiologia. 60:135-144.

Chen C, Imamura N, Nishiijima M, Adachi K, Sakai M, Sano H. 1996. Halymecins, new antimicroalgal substances produced by fungi isolated from marine algae. J Antibiot 49:998-1005.

Cho JY, Kwon EH, Choi JS, Hong SY, Shin HW, Hong YK. 2001. Antifouling activity of seaweed extracts on the green alga *Enteromorpha prolifera* and the mussel *Mytilus edulis*. J Appl Phycol 13:117-25.

Conover WJ, Iman RL. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. Am Stat 35:124-9.

Cooney RP, Pantos O, Le Tissier MDA, Barer MR, O'Donnell AG, Bythell JC. 2002. Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. Environ Microbiol 4:401-413.

Corre S, Prieur D. 1990. Density and morphology of epiphytic bacteria on the kelp *Laminaria digitata*. Bot Mar 33:515-23.

Damonte EB, Neyts J, Pujol CA, Snoeck R, Andrei G, Ikeda S, Witvrouw M, Reymen D, Haines H, Matulewicz MC, Cerezo A, Coto CE, De Clercq E. 1994. Antiviral activity of a sulfated polysaccharide from the red seaweed *Nothogenia fastigiata*. Biochem Pharmacol 47:2187-92.

de Nys R, Steinberg PD, Willemsen P, Dworjanyn SA, Gabelish CL, King RJ. 1995. Broad spectrum effects of secondary metabolites from the red algae *Delisea pulchra* in antifouling assays. Biofouling 8:259-71.

Dixon J, Schroeter SC, Kastendick J. 1981. Effects of encrusting bryozoan, *Membranipora membranacea* on the loss of blades and fronds by the giant kelp, *Macrocystis pyrifera* (Laminariales). J Phycol 17:341-345.

Dobretsov S, Qian PY. 2002. Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. Biofouling 18:217-28.

Dobretsov S, Dahms HU, Qian PY. 2004. Antilarval and antimicrobial activity of waterborne metabolites of the sponge *Callyspongia* (*Euplacella*) *pulvinata*: evidence of allelopathy. Mar Ecol Prog Ser 271:133-46.

Engel S, Puglisi MP, Jensen PR, Fenical W. 2006. Antimicrobial activities of extracts from tropical Atlantic marine plants against marine pathogens and saprophytes. Mar Biol 149:991-1002.

Gough SB, Gough LP. 1981. Comment on “primary production of algae growing on natural and artificial aquatic plants: A study of interactions between epiphytes and their substrate” (Cattaneo and Kalff). Limnol Oceanogr 26:987-988.

Gross E. 2003. Allelopathy of aquatic autotrophs. Crit Rev Plant Sci 22:313-39.

Guillard R, Ryther J. 1962. Studies of marine planktonic diatoms. Can J Microbiol 8:229-39.

Harder T, Qian PY. 2000. Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitive cues for larval attachment and metamorphosis in the polychaete *Hydroides elegans*. Biofouling 16:205-14.

Harder T, Lam C, Qian PY. 2002. Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. Mar Ecol Prog Ser 229:105-12.

Harder T, Dobretsov S, Qian PY. 2004a. Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. Mar Ecol Prog Ser 274:133-41.

Harder T, Lau SCK, Tam WY, Qian PY. 2004b. A bacterial culture-independent method to investigate chemically mediated control of bacterial epibiosis in marine invertebrates by using TRFLP analysis and natural bacterial populations. FEMS Microbiol Ecol 47:93-99.

Hellio C, Bremer G, Pons AM, Le Gal Y, Bourgougnon N. 2000. Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. Appl Microbiol Biotechnol 54:543-9.

Hellio C, de La Broise D, Dufossé L, Le Gal Y, Bourgougnon N. 2001. Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar Environ Res 52:231-47.

Hellio C, Berge JP, Beaupoil C, Le Gal Y, Bourgougnon N. 2002. Screening of marine algal extracts for anti-settlement activities microalgae and macroalgae. Biofouling 18:205-15.

Hellio C, Marechal JP, Véron B, Bremer G, Clare AS, Le Gal Y. 2004. Seasonal variation of antifouling activities of marine algae from the Brittany Coast (France). Mar Biotechnol 6:67-82.

Holmström C, Rittschof D, Kjelleberg S. 1992. Inhibition of settlement by larvae of *Balanus amphitrite* and *Ciona intestinalis* by surface-colonising marine bacterium. Appl Environ Microbiol 58:2111-5.

Jagels R. 1973. Studies of a marine grass, *Thalassia testudinum* I. Ultrastructure of the osmoregulatory leaf cells. Am J Bot 60:1003-1009

James G, Holmström C, Kjelleberg S. 1996. Purification and characterization of a novel antibacterial protein from the marine bacterium D2. Appl Environ Microbiol 62:2783-88.

Jenkins KM, Toske SG, Jensen PR, Fenical W. 1998. Solanapyrones E-G, antialgal metabolites produced by a marine fungus. Phytochemistry 49:2299-304.

Keats DW, Knight MA, Pueschel CM. 1997. Antifouling effects of epithallial shedding in three crustose coralline algae (Rhodophyta, Coralinales) on a coral reef. J Exp Mar Biol Ecol 213:281-93.

Küpper FC, Kloareg B, Guern J, Potin P. 2001. Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata*. Plant Physio 125:278-91.

Kolender AA, Matulewicz MC, Cerezo AS. 1995. Structural analysis of antiviral sulfated α-D-(1 3)-linked mannans. Carbohyd Res 273:179-85.

Lau SCK, Mak KKW, Chen F, Qian PY. 2002. Bioactivity of bacterial strains from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*. Mar Ecol Prog Ser 226:301-310.

Maximilien R, de Nys R, Holmström C, Gram L, Givskov M, Crass K, Kjelleberg S, Steinberg PD. 1998. Chemical mediation of bacterial surface colonization by secondary metabolites fro the red alga *Delisea pulchra*. Aquat Microb Ecol 15:233-46.

Millie DF, Lowe RL. 1983. Studies on Lake Erie's littoral algae; Host specificity and temporal periodicity of epiphytic diatoms. Hydrobiologia 99:7-18.

Mitchell R, Chet I. 1975. Bacterial attack of corals in polluted seawater. Microb Ecol 2, 227-233.

Moss BL. 1982. The control of epiphytes by *Halidrys siliquosa* (L.) Lynbg. (Phaeophyta, Cystoseiraceae). Phycologia 21:185-91.

Nylund GM, Pavia H. 2005. Chemical versus mechanical inhibition of fouling in the red alga *Delisea carnosa*. Mar Ecol Prog Ser 299:111-121.

Paul NA, de Nys R, Steinberg PD. 2006. Chemical defense against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar Ecol Prog Ser 306:87-101.

Pelegrin YF, Morales JL. 2004. Antibacterial activity in marine algae from the coast of Yucatan, Mexico. Bot Mar 47:140-6.

Ruiz J, Macias D, Peters F. 2004. Turbulence increases the average settling velocity of phytoplankton cells. PNAS 101(51):17720-24.

Sand-Jensen. 1977. Effects of epiphytes on eelgrass photosynthesis. Aquat Bot 3:55-63.

Shapiro SS, Wilk MB. 1965. An analysis of variance test for normality (complete samples). Biometrika 52:591-611.

Silberstein K, Chiffings AW, McComb AJ. 1986. The loss of seagrass in Cockburn Sound, Western Australia. 3. The effect of epiphytes on productivity of *Posidonia australis* Hook. F Aquat Bot 24:355-371.

Steinberg PD, Schneider R, Kjelleberg S. 1997. Chemical defenses of seaweeds against microbial colonization. Biodegradation 8:211-20.

Terry LA, Edyvean RGJ. 1981. Microalgae and corrosion. Bot Mar 24:177-183.

Wahl M. 1989. Marine epibiosis. I. Fouling and antifouling: some basic aspects. Mar Ecol Prog Ser 58:175-89.

Weinberger F, Friedlander M, Hoppe HG. 1999. Oligoagars elicit an oxidative burst in *Gracilaria conferta* (Rhodophyta). J Phycol 35:747-55.

Wium-Andersen S, Anthoni U, Christophersen C, Houen G. 1982. Allelopathic effects on phytoplankton by substances isolated from aquatic macroaphytes (Charales). Oikos 39:187-190.

FIGURES & TABLES

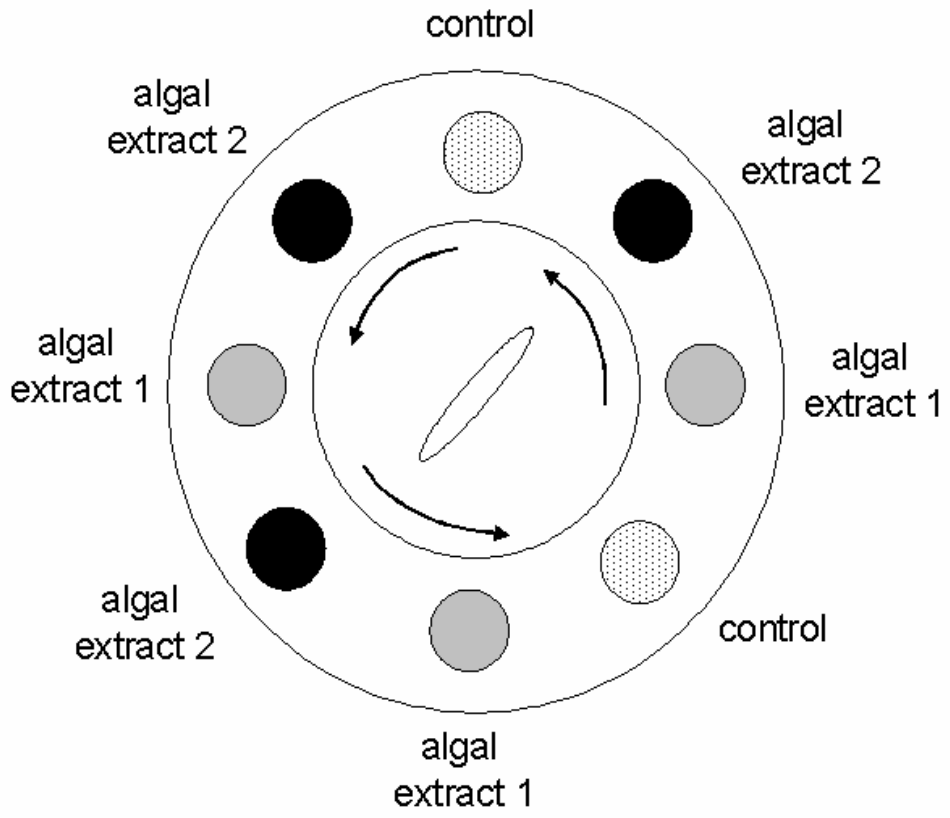


Figure 1: Schematic diagram showing the experimental design of diatom attachment assays. A PVC ring containing eight individual wells was filled with two different algal extracts (n = 3) and a solvent control (n = 2). A magnetic stirrer bar was placed in the middle of the ring.

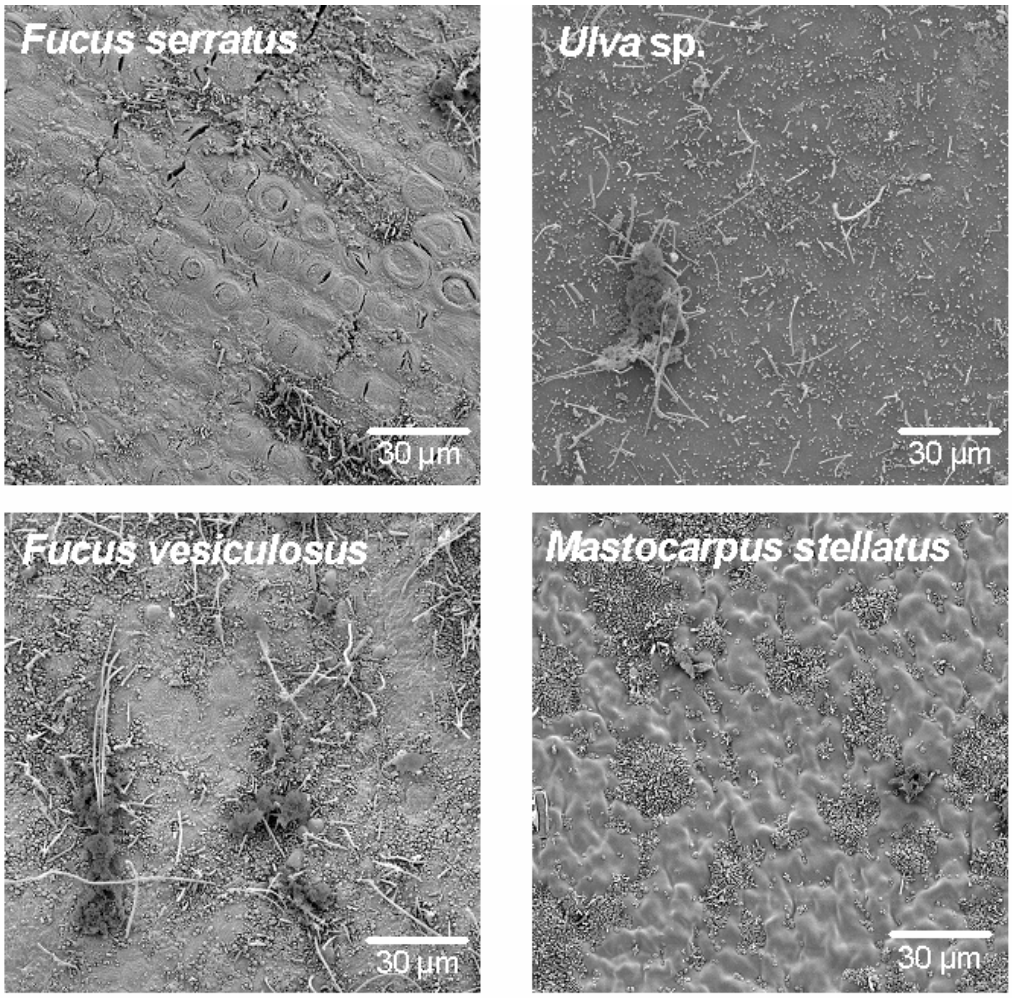


Figure 2A. Scanning electron micrographs (SEM) showing the category of macroalgae largely devoid epiphytic colonizers.

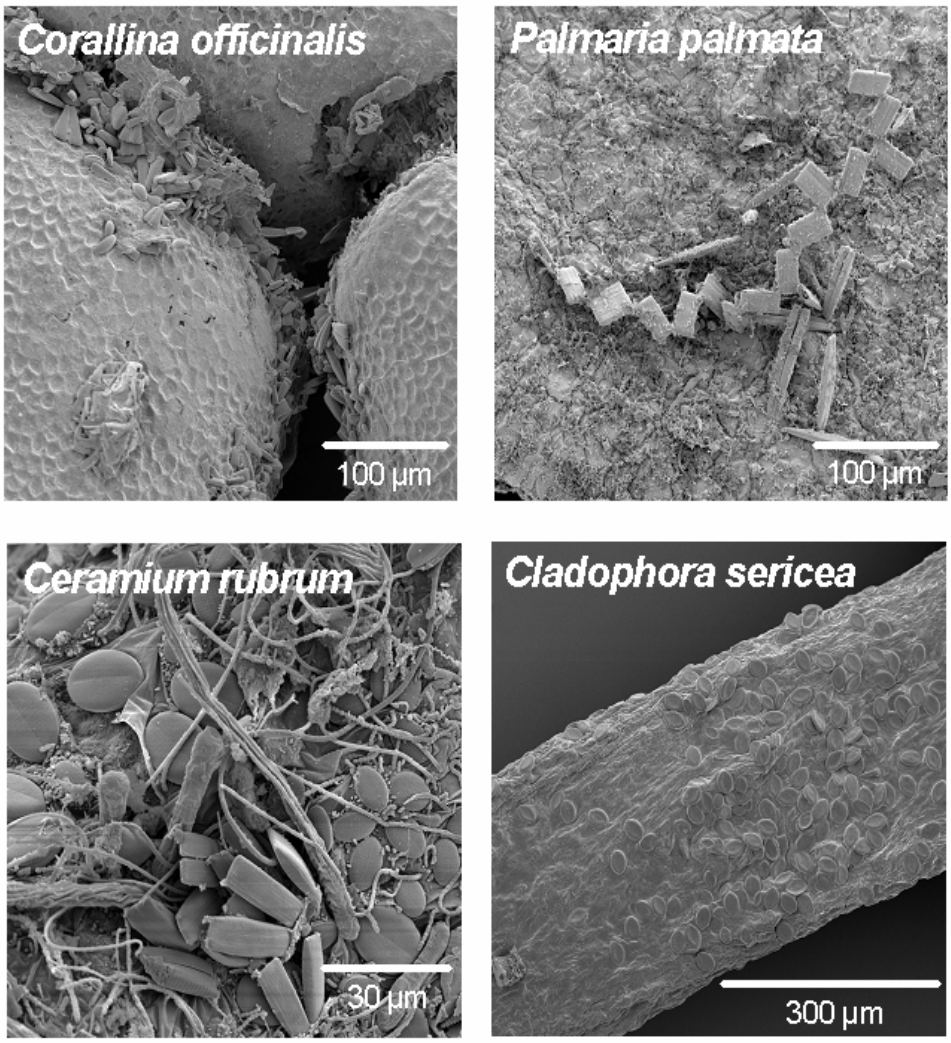


Figure 2B. Scanning electron micrographs (SEM) showing the category of macroalgae densely populated with epiphytic colonizers.

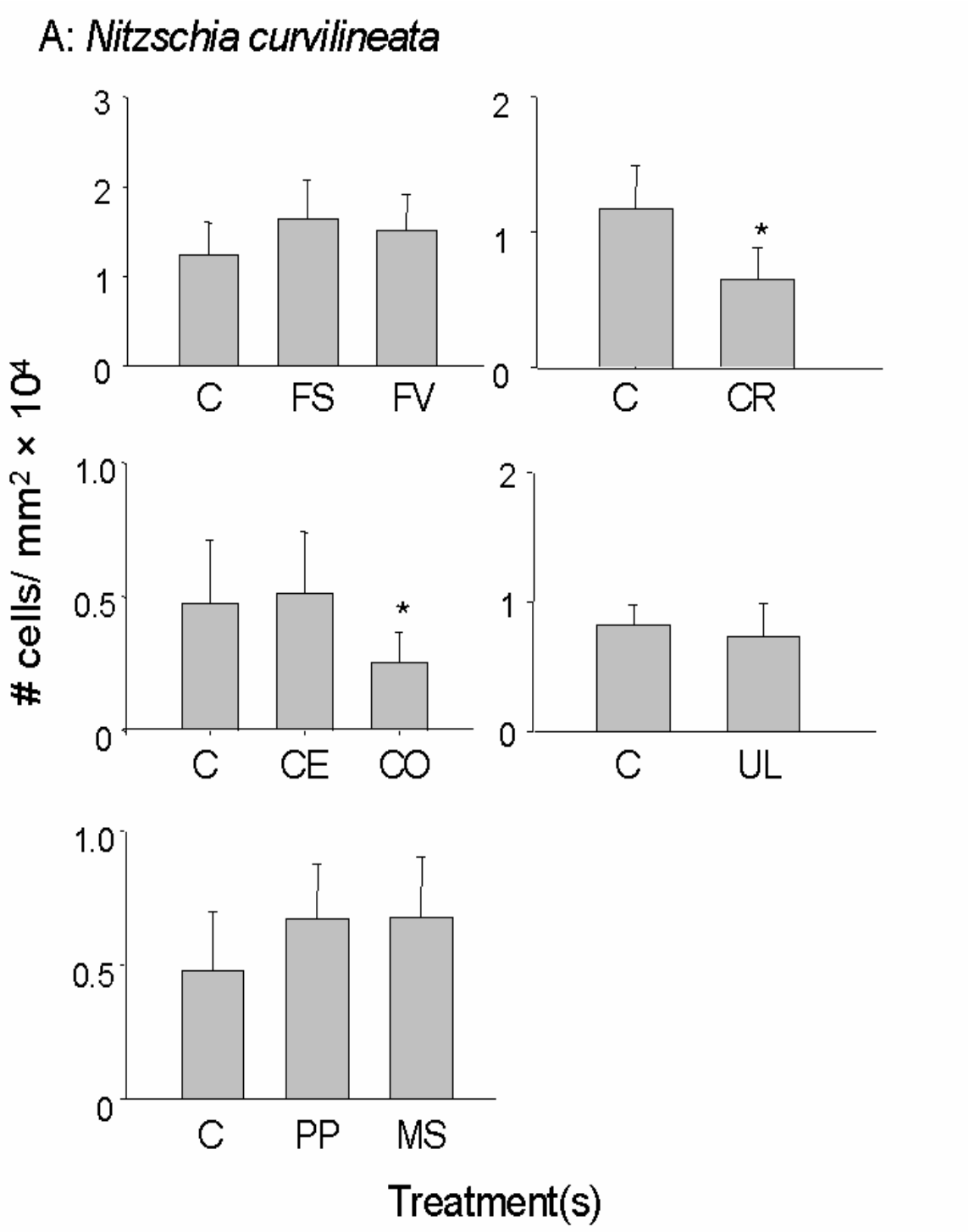
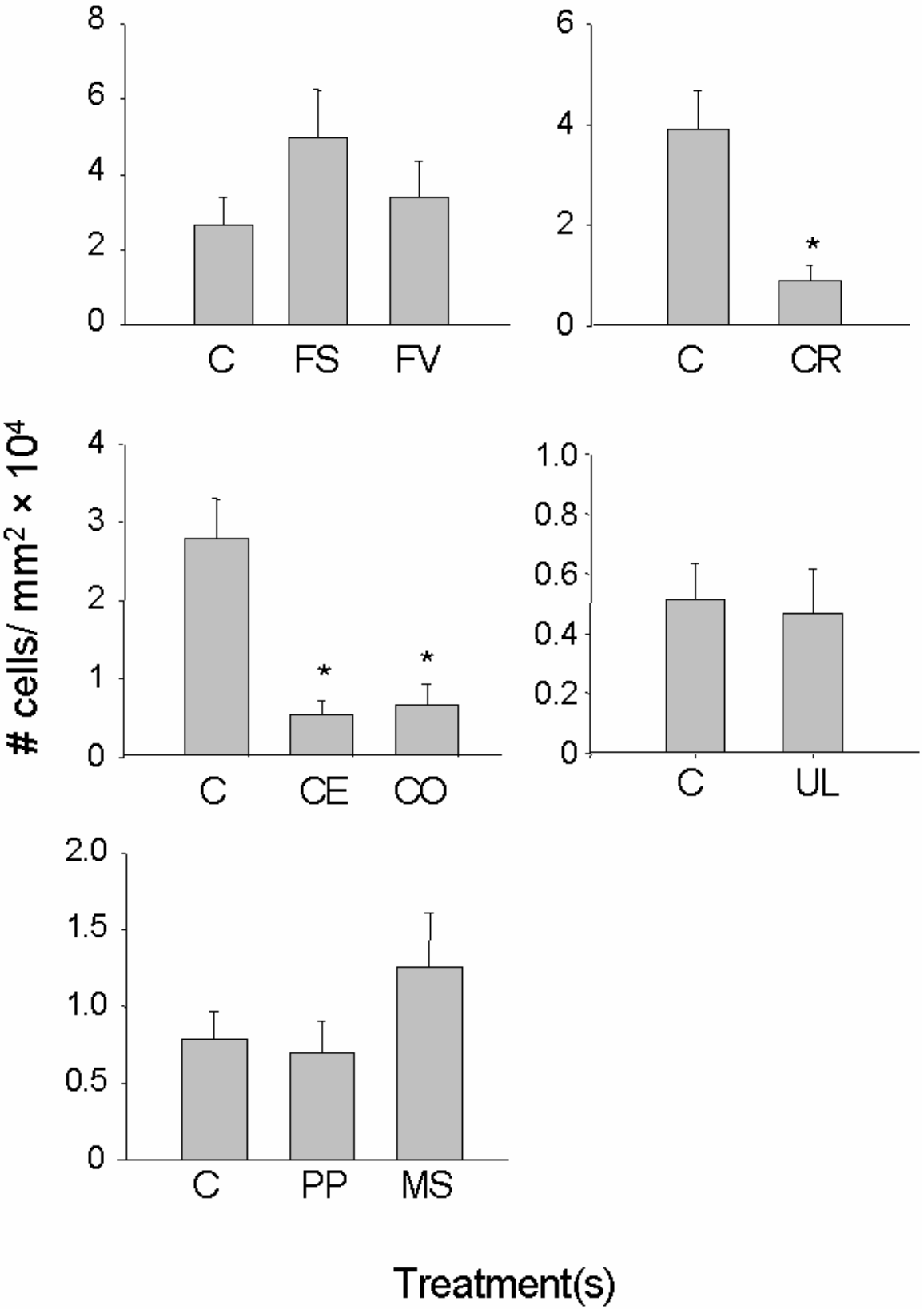
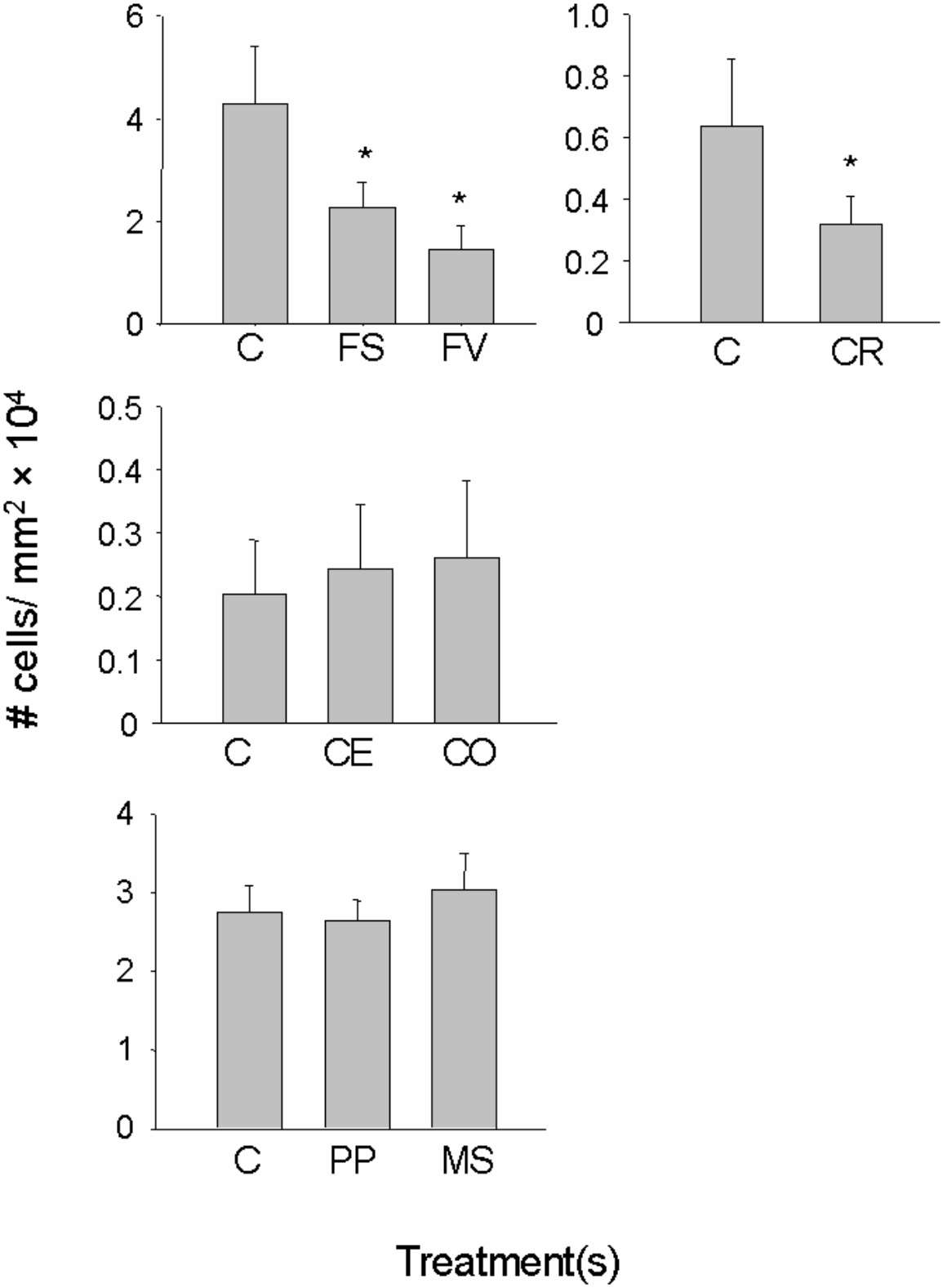


Figure 3. Mean density of diatoms (cells mm-2) on experimental gels containing macroalgal extracts (CE – *Ceramium rubrum*; CO – *Corallina officinalis*, CR - *Cladophora rupestris*, FS - *Fucus serratus*, FV - *Fucus vesiculosus*, MS - *Mastocarpus stellatus*, PP - *Palmaria palmata* & UL – *Ulva* sp.) and solvent controls after 24 h of incubation with diatoms (**A**. *Nitzschia curvilineata*, **B**. *Synedra* sp., **C**. *Phaeodactylum tricornutum* **D**. *Navicula pelliculosa*). Data plotted are means ± SD of replicates based on counts of 15 fields at 100 × magnification. Asterisks above bars denote significant differences in comparison to the solvent control.

B: *Synedra* sp.


8
6
4
2
0
C
FS
FV
6
4
2
0
*
C
CR
cells/ mm² × 10⁴
4
3
2
1
0
*
*
C
CE
CO
1.0
0.8
0.6
0.4
0.2
0
C
UL
2.0
1.5
1.0
0.5
0
C
PP
MS
Treatment(s)

C: *Phaeodactylum tricornutum*



D: *Navicula pelliculosa*

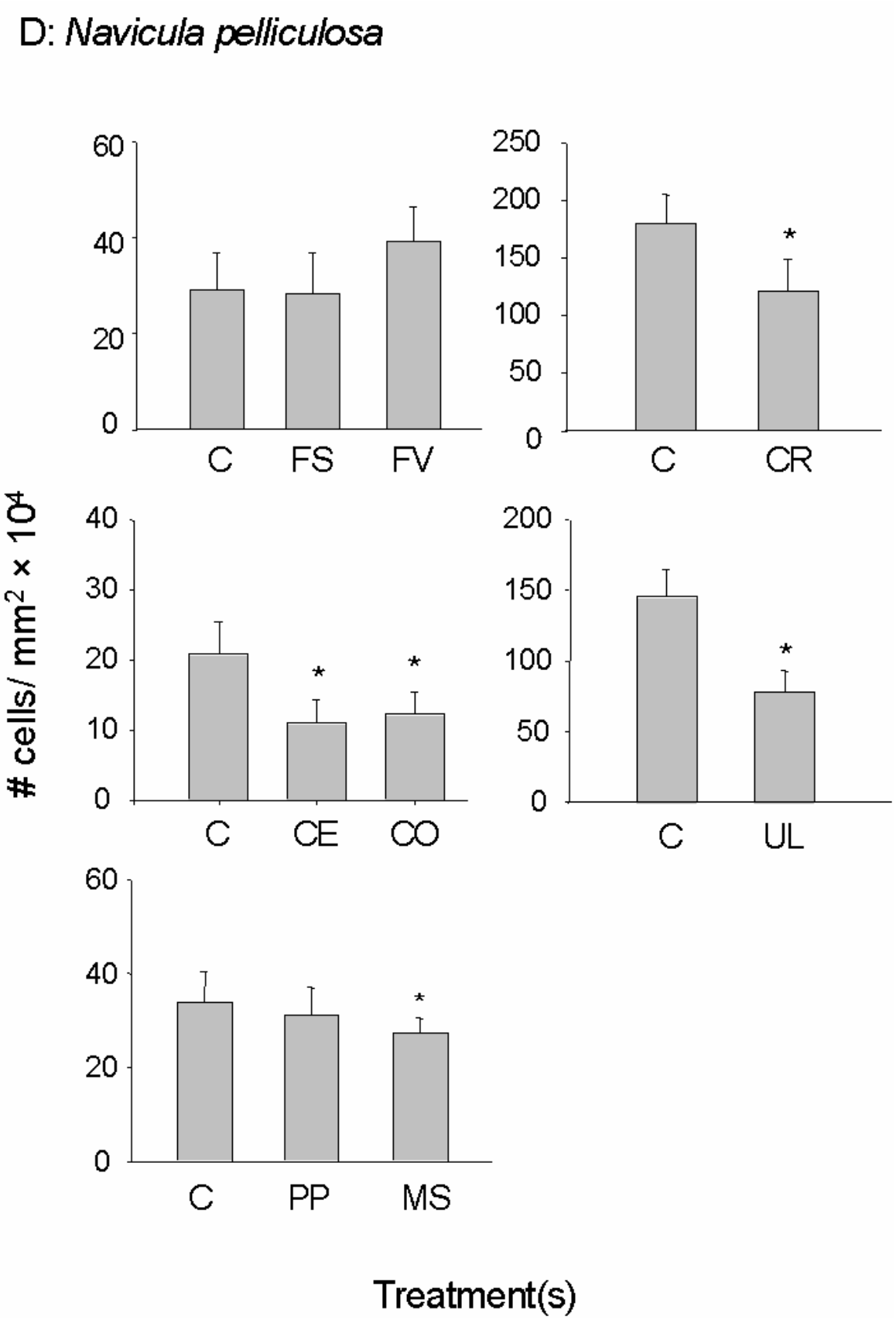


Table I. Effects of eight macroalgal extracts (CE – *Ceramium rubrum*; CO – *Corallina officinalis*, CR - *Cladophora rupestris*, FS - *Fucus serratus*, FV - *Fucus vesiculosus*, MS - *Mastocarpus stellatus*, PP - *Palmaria palmata* & UL – *Ulva* sp.) on attachment and proliferation of four diatoms (*Nitzschia curvilineata*, *Navicula pelliculosa*, *Phaeodactylum tricornutum* & *Synedra* sp.). Symbols denote the effect of algal extracts as significantly higher (□), lower (●), or equal (○) to a control containing the solvent DMSO only. 'nd' denotes not determined.

	N. curvilineata	*Synedra* sp.	*P. tricornutum*	*N. pelliculosa*
CE	○	●	○	●
CO	●	●	○	●
CR	●	●	●	●
FS	□	□	●	○
FV	○	○	●	□
MS	□	□	○	●
PP	□	○	○	○
UL	○	○	nd	●

5. SUMMARY

5.1 Antimicrobial activity of North Sea macroalgae against

(i) Bacteria. Macroalgae reduced community composition and abundance of planktonic bacteria in ambient seawater after 3 days of incubation (Fig. 1; Section 2.1). The magnitude of antibacterial effects was alga specific. Based on the results of similarity analysis of DGGE banding patterns, three out of nine macroalgae (*Ceramium rubrum*, *Laminaria digitata* and *Mastocarpus stellatus*) were chosen for further in-depth studies. The algal treatments showed a few additional bands in comparison to the control. These phylotypes supposedly stemmed from macroalgae and were considered as contaminants and omitted in the analysis of bacterial community patterns (Fig. 3; Section 2.1). Waterborne metabolites of three macroalgae reduced bacterial diversity, but the number of affected phylotypes in algal culture water (ACW) was dependent on the presence and absence of algae (Figs. 2 & 4; Section 2.1). Certain bacterial phylotypes were only eliminated in the presence of algae but not in the absence of algae, indicating surface-mediated effects were causative for the observed effects (Fig. 5; Section 2.1). This could be due to steep chemical gradients of antimicrobial compounds with high bioactivity thresholds within the boundary layer of the blade surface (Dworjanyn et al. 2006; Nylund et al. 2007) or due to antimicrobial effects mediated by bacteria in biofilms on the blade surface as reported earlier by others (Boyd et al. 1999; Armstrong et al. 2001; Dobretsov & Qian, 2002). An alternative explanation may be the short half-life of known algal defense agents, which may degrade within short period of time and consequently would have vanished in the experiments with ACW in the absence of algae (Weinberger et al. 1999; Küpper et al. 2001; Küpper et al. 2002).

Additionally, waterborne algal metabolites not only affect a wide spectrum of bacteria in seawater, but also target bacterial colonizers in a phylum-specific fashion. The community composition within the three different bacterial phyla (i.e. the *Bacteroidetes*, alpha-*Proteobacteria* & *Roseobacter*) was reduced to a greater extent in presence of the red algae *C. rubrum* and *M. stellatus* than in the brown alga *L. digitata* (Figs. 1-3; Section 2.2), indicating that either the quality of algal metabolites was different among the three macroalgae under investigation, or that the susceptibility of planktonic bacteria towards alga-derived antimicrobial effects correlated with their phylogenetic affiliation. The majority of affected phylotypes belonged to the *Bacteroidetes* and the *Roseobacter* clade (Figs. 1 & 3; Section 2.2), implying that these epiphytic bacteria may pose a particular threat to the algae. To further characterize such potentially detrimental bacterial epibionts, 3 bands obtained from the *Roseobacter* gel were sequenced. The sequences were affiliated to three different uncultured *Roseobacter* species (Fig. 3A; Section 2.2). Although the potential pathogenicity of the uncultured *Roseobacter* sp. on macroalgae remains unknown, our study provides first experimental evidence of a chemical ecological context between macroalgae and the *Roseobacter* clade.

(ii) Fungi. Under the combination of fungal-specific PCR primers targeting the fungal ITS 1 region, the fungal community was similarly affected by macroalgae as in the case of epiphytic bacteria. The fungal community pattern was affected in the presence of macroalgae after 3 days of incubation, but with a different specificity of algae, for example *L. digitata* and *M. stellatus* exhibited a more pronounced antifungal effect than *C. rubrum* (Fig. 4; Section 2.2). Waterborne algal metabolites (in the absence of algae) did not reduce the fungal community

composition (Table 6, Fig. 5B; Section 2.2). This may be due to the high recalcitrance of fungal hyphae and spores towards antimicrobial compounds compared to bacteria (Miao & Qian 2005). Alternatively, these results are in accordance with earlier studies demonstrating that antifungal effects of macroalgae were mediated by microbes in biofilms on algal blade surfaces (Bloor & England, 1989; Boyd et al. 1999; Armstrong et al. 2001). Additional fungal phylotypes were observed in all algal treatments but not in the controls, suggesting these contaminants were specific to algae and thus originated from the host (Fig. 4; Section 2.2). Nonetheless, this is the first time to investigate the effects of waterborne algal compounds in fungal community based on natural delivery system using cultural-independent approach.

(iii) Diatoms. Utilizing the scanning electron microscope to visualize epiphytic colonizers on algal surfaces, the eight macroalgae differed in terms of their epiphytic coverage with bacteria, fungi and diatoms (Fig. 2A-B; Section 4). By grouping them into 2 diferent categories, macroalgae largely devoid of epiphytic diatoms were hypothesized to employ effective antifouling means to reduce epiphytic coverage, whilst heavily fouled macroalgae were supposed to lack antifouling strategies. Crude extracts from the same batch of algae were used to study diatom attachment and proliferation assays with four benthic diatoms (i.e. *Nitzschia curvilineata*, *Navicula pelliculosa*, *Phaeodactylum tricornutum* & *Synedra* sp.). Results revealed algal extracts exhibited a distinct pattern of activities against four diatoms. Only the extract of *Cladophora rupestris* inhibited all diatoms, whilst the other algal extracts affected less or none of the diatoms under investigation (Fig. 3A-D; Section 4). The susceptibility of diatoms to algal

extracts was not species-specific (Table 1; Section 4). Some algal extracts, such as the brown alga *Fucus serratus* inhibited attachment and proliferation of one diatom species (*Phaeodactylum tricornutum*) whilst promoting attachment of other diatoms (*Nitzschia curvilineata*, *Synedra* sp.), suggesting a targeted effect of macroalgal metabolites on individual fouling diatoms. Visual inspection of epiphytic colonizers on algal thalli is not a reliable predictor to group macroalgae into chemically defended and undefended categories.

(iv) Summary. Apart from macroalgae, other marine soft-bodied organisms such as sponges and soft-corals control epibiosis by producing a diverse array of bioactive metabolites (Amade et al. 1987; Slattery et al. 1995; Harder et al. 2003; Lee & Qian, 2003; Dobretsov & Qian, 2004; Dobretsov et al. 2004; Lee & Qian, 2004; Yang et al. 2006). The majority of these researches employ similar culture-independent experimental tools to investigate potential chemical defenses of the soft-bodied organisms against epiphytic bacteria, which is comparable to my study. For instance, tissue extracts of the soft coral *Dendronephthya* sp. and waterborne products of coral-associated bacteria distinctly inhibited growth and attachment of indigenous bacterial isolates using terminal restriction fragment length polymorphism (TRFLP) analysis of bacterial community DNA (Harder et al. 2003). Apart from this observation, water-soluble and heat-stable polysaccharides with molecular weight larger than 100 kDa produced by coral-associated bacteria further inhibited larval settlement of the polychaete *Hydroides elegans* and the bryozoan *Bugula neritina* (Dobretsov & Qian, 2004).

Simiarly, marine sponges have evolved both physical and chemical defense strategies against a wide range of micro- (i.e. bacteria, diatoms) and macro-

colonizers (i.e. invertebrate larvae). TRFLP analysis revealed structural differences in bacterial communities colonizing the surface of the red sponge *Mycale adhaerens* or an inanimate reference surface (Lee & Qian, 2004), suggesting there were differences in chemical and/or physical characteristics between sponge and reference surfaces. Chemical extracts and conditioned seawater of this sponge further inhibited bacterial growth and larval settlement of *H. elegans* in a concentration-dependent fashion (Lee & Qian, 2003). Waterborne metabolites from another sponge *Callyspongia* (Euplacella) *pulvinata* inhibited growth of the benthic diatom *Nitzschia paleaceae* and settlement of the *H. elegans* larvae not only on its surface but also on inanimate reference surfaces nearby (Dobretsov et al. 2004).

In summary, marine soft-bodied organisms and macroalgae share some similarities and differences in controlling epibiosis. Firstly, they both defend themselves against epibiphytic colonizers by producing bioactive metabolites, or indirectly regulating the community of epibiotic microflora on its surface that affect subsequent fouling process. Secondly, the chemistry of these metabolites is highly hydrophilic and polar in nature. Thirdly, the metabolites released from these organisms facilitate broad spectrum inhibition against a wide range of potential epiphytic microorganisms, such as bacteria, diatoms and fungi for macroalgae; bacteria and diatoms in sponges and corals. However, my study firstly utilizes culture-independent method to study the allelochemical effects on phylogenetically different bacterial colonizers and planktonic fungi, which is rarely shown in coral and sponge researches. Although the antifouling effects of waterborne macroalgal metabolites against potential macroepiphytes have not been investigated in my study, the effects of macoalgal compounds against

different types of invertebrate larvae have already been verified in literature (Walters et al. 1996; Harder & Qian, 2000; Hellio et al. 2001a; 2001b). To my knowledge, this is the first study that demonstrates macroalgal defenses against epiphytic microorganisms based on natural delivery mechanisms of allelochemicals utilizing a culture-independent approach, thus minimizing the ecological bias inherent to culture-dependent studies based on few microbial isolates.

5.2 Methods discussion

(i) Cultivation of macroalgae. Macroalgae were maintained in 10 L aerated fish aquarium tank containing NSW enriched in *f*/2 nutrient medium (Guillard & Ryther, 1962) after experiments. Although all physical parameters (i.e. light intensity, water temperature) were assimilated to the field, algae are bleached or sporulated within one or two week(s) of incubation at the laboratory. This could be due to the lack of water current in the aquarium as wave motion keeps the algae in constant motion, providing maximum exposure to sunlight and contact with nutrients (Mann, 1973). Other possible explanation to the phenomenon could be due to the comparatively low light intensity (100 - 200 µmol m^2 s^1) in the culture room in comparison to the intertidal areas in Europe (500 – 2000 µmol m^2 s^1). Under the assumption that algal fitness decreases during the incubation period, freshly-collected macroalgae are directly consumed for the experiments without further subculture and use for other investigations.

(ii) Filter PCR. The method consists of filtering a small volume of water sample (i.e. 30 ml in this study) through a polycarbonate filter membrane and using a section of that filter directly in PCR without any purification or prior treatment of the cells (Kirchman et al. 2001). Given that filter PCR requires small volume of sample and short filtration time, it can restrict the application of molecular tools in extensive surveys of aquatic environments and in experiments calling for multiple treatments or repeated sampling. However, some concerns related to this method have been found during the course of the study.

Regarding to ecological significance of small sample volume in filter PCR, same batch of seawater at small (i.e. 30 ml) and large (i.e. 1 L) volumes were

filtered under same condition and subjected to direct PCR and standard PCR approach with DNA extraction, respectively. The PCR-products obtained by both approaches were analyzed by the standard DGGE methods (Muyzer et al. 1993). The bacterial community richness revealed by the filter PCR approach with small samples was comparable to that from the standard PCR approach (Fig. 1), which was in accordance with the results obtained by Kirchmann et al. (2001). The result indicates the small sample volume is ecologically representative to study the community richness of bacterioplankton in seawater.

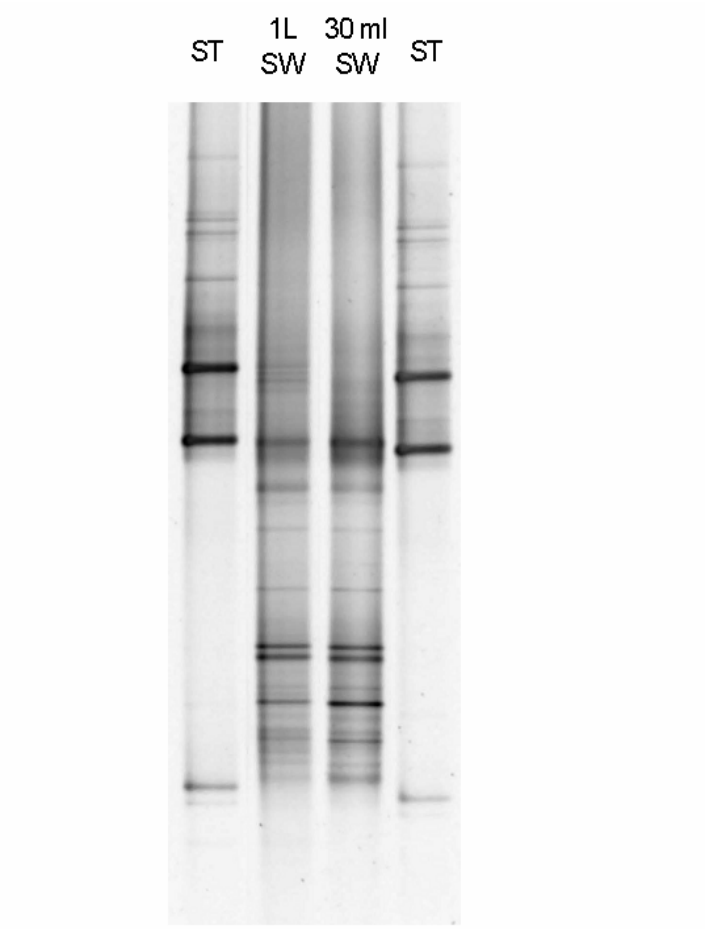


Figure 1. DGGE fingerprints compare the community richness of planktonic bacteria in seawater obtained by filter PCR from standard PCR protocols. Same batch of seawater with small (i.e. 30 ml) and large (i.e. 1 L) volumes were filtered under same condition and subjected to direct PCR and standard PCR with DNA extraction, respectively. All treatments were run and compared on the same gel. DNA standards (ST) comprised of 7 bacteria isolates.

Another concern was the application of mechanical suction pump during seawater filtration. As bacteria are lacking of protective cell wall, they could be easily damaged and filtered through the membrane under strong suction, resulting in decreasing the detection limits (or absence of bands) of certain bacterial phylotypes in DGGE gels. To test the hypothesis, the filtering method was compared to the centrifugation by spinning down same amount of seawater at a

certain centrifugation speed (i.e. 6,000 rpm, 15 min) prior to standard DNA extraction (Zhou et al. 1996). Under the assumption that all microbial cells have been span down by centrifugation without any damages, the community richness obtained by this protocol should represent the entire community in seawater. DGGE banding patterns revealed the bacterial community richness obtained by the filtration was comparable to that from the centrifugation (Fig. 2), indicating the majority of bacterial cells remain on membrane under suction.

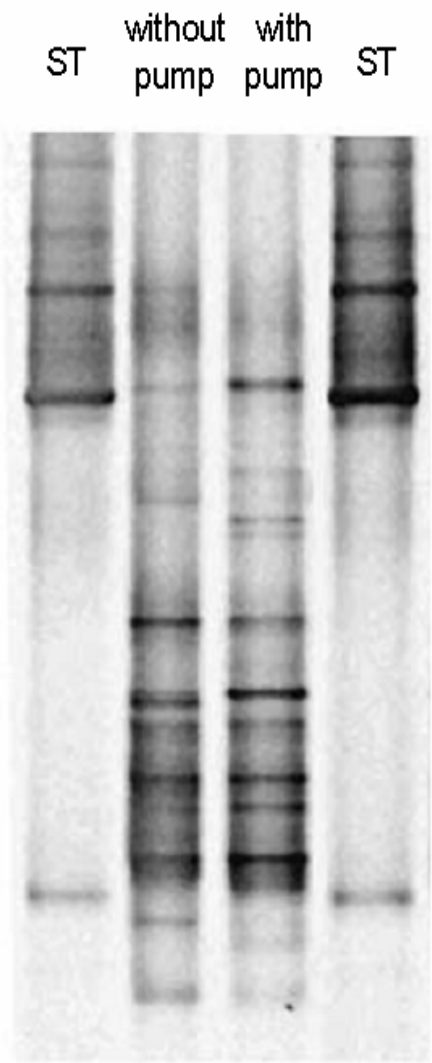


Figure 2 (left). DGGE fingerprints compare the community richness of planktonic bacteria in same amount of seawater between the filtering (with mechanical pump) and the centrifugation (without the pump) methods. The filtration method was adopted in the study for filter PCR; whilst the centrifugation was used to test if some bacterial phylotypes were eliminated under strong suction created by the pump. All treatments were run and compared on the same gel. DNA standards (ST) comprised of 7 bacteria isolates.

Pre-filtration is important in filter PCR although the filtered volume is small, especially when the seawater sample is turbid (Kirchman et al. 2001). Generally, 90 mm paper filters with 12 - 15 µm pore sizes are used to remove phyto- and zooplankton and to minimize amplification of 16S rRNA genes in plastids of eukaryotes (Lam & Harder, in press). If pre-filtration is ignored, DNA from environmental samples cannot be used in subsequent molecular analysis because of the presence of high humic acids contamination in DNA samples. However, an alternative approach ("boiled" filter PCR) could be adopted to improve the effectiveness of PCR amplification when pre-filtration was ignored. Filter section without pre-filtration was filled up with DNA-free water and boiled at

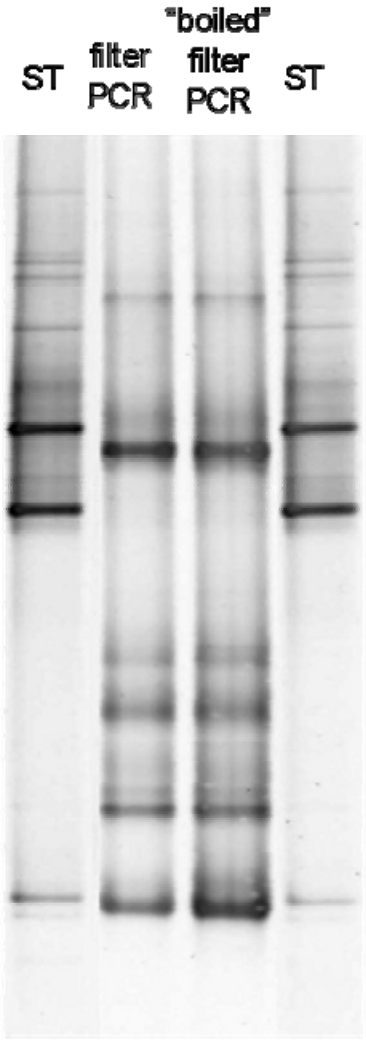


Figure 3. DGGE fingerprints compare the community richness of planktonic bacteria in seawater between filter PCR and "boiled" filter PCR. "Boiled" filter PCR was performed similarly as filter PCR except filter section was cooked and further diluted with DNA-free water prior to PCR. The DNA water with cooked filter section served as DNA template for subsequent PCR. All treatments were run and compared on the same gel. DNA standards (ST) comprised of 7 bacteria isolates.

94 ºC for 10 min as normal denaturation by PCR machine. The DNA water with the filter section was 10-fold diluted with DNA-free water before it served as DNA template for PCR. The bacterial community richness obtained by this approach was comparable to that from the standard filter PCR method (Fig. 3). However, filter PCR with pre-filtration is highly recommended in my study and this alternative method is used only when necessary.

Finally, in order to gain more knowledge on macroalgal defense mechanism against different epiphytic bacterial phyla between plankton and biofilms on algal surfaces, as well as some experimental discussions between seawater extraction and filter PCR methods, please refer to the Master thesis of Rebecca Neumann (2007).

(iii) Limitation of DGGE. Denaturing gradient gel electrophoresis (DGGE) has been used as a tool for profiling complex microbial populations without the biases of cultural analysis in environmental biology (Muyzer et al. 1993; Zwisler et al. 2003; Selje et al. 2005). This technique not only creats a genetic fringerprint or profile of total community diversity by separating mixed 16S rRNA PCR amplification products on the basis of their sequence melting behaviour, but also allows direct comparisons between samples simultaneously. However, there are some limitations of this technique. Firstly, the PCR products for DGGE analysis must be > 500 bp (Myers et al. 1985) to allow for efficient resolution and analysis. Although the sequences derived from the DGGE-PCR cloning of small product sizes are insufficient to allow great precision in the construction of phylogenetic trees, it does allow for a presumptive identification at least to genus level (Woese, 1987). For example, the *Roseo*-specific primers in my study yielded a very small

PCR product of about 200 bp, therefore the phylogenetic affilation of three excised gel bands belonged to three different uncultured *Roseobacter* species up to genus level. Possible solutions such as increase the length of primer products or target another variable region of the 16S rRNA are suggested for the problems. Furthermore, DGGE only separates the predominant species present in a community. Comigration can be another problem for retrieving clean sequences from individual DGGE cut bands. Another limitation of DGGE is that 16S rRNA gene sequences affiliated to specific bacterial species can be found in more than one position in DGGE gels, suggesting the presence of multiple *rrN* operons (Fig, 4; Nübel et al. 1996). Despite these shortcomings, DGGE is still a good, valuable technique providing substantial information about complex mixed bacterial populations.

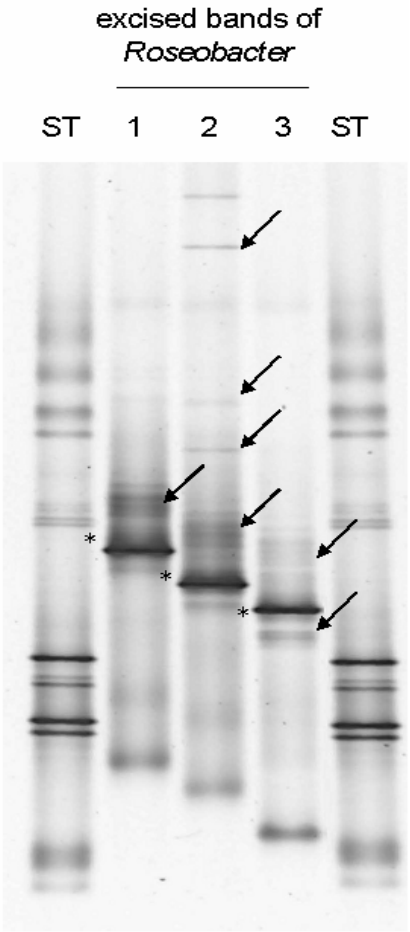


Figure 4 (right). DGGE fingerprints show the purity of PCR products of three excised bands from *Roseobacter*-DGGE gels prior to direct sequencing. The purity of PCR products of the excised band was achieved if the band was located at the right position without the presence of other bands in comparison to DNA standard (ST). Arrows indicated more than one band was shown on the gel other than the main band (*), suggesting the presence of *rrN* operons.

5.3 Outlook

Provided that macroalgae exhibited antimicrobial activities against colonizing planktonic bacteria and fungi by releasing waterborne compounds in direct proximity, the isolation and identification of a single biogenic compound from a single species of macroalgae could be one of the long-term directions in the coming future. This will be achieved by starting a series of bioassay guided fractionation with a few model species. Several preparative types of chromatography (i.e. flash chromatography, HPLC) will be set up for the bioassays at the laboratory. This is labor and time-consuming method, hence one or two model species with the highest antimicrobial efficacy will perform first. Purified compounds will be elucidated by MS or NMR. In order to reduce the continuously lost of bioactive metabolites from algal surfaces through dissolution, the majority of the identified bioactive compounds are described to be non-polar in nature, i.e. halogenated furanones of the Australian red alga *Delisea pulchra* (Dworjanyn et al. 2006; Paul et al. 2006). Although other researchers suggest large polar macromolecules with similar antimicrobial effects are observed in some macroalgae (Jennings & Steinberg, 1994; Harder & Qian, 2000; Dobretsov & Qian, 2002; Bazes et al. 2006), fewer waterborne algal metabolites have been successfully identified and elucidated so far. Surface extraction by dipping fresh algal material in non-polar solvent has been recently addressed to isolate non-polar extracts on algal surfaces (Nylund et al. 2007). Therefore, with careful choice of solvent and extraction time, this procedure of surface extraction could be useful to isolate and identify waterborne surface compounds from macroalgae.

Marine macroalage utilize allelochemical means to suppress epibiosis of micro- and macro-epiphytes. Although the antifouling activity of algal metabolites

against certain macroepiphytes has been described previously (Walters et al. 1996; Harder & Qian, 2000; Hellio et al. 2001a; 2001b), the effects of bioactive metabolites from my three model macroalgae (i.e. *Ceramium rubrum*, *Laminaria digitata*, *Mastocarpus stellatus*) against potential macro-epiphytes (i.e. macroalgal spores, invertebrate larvae) have not been investigated in my study. The extension of such work can be achieved by modifying the experimental protocols in Harder & Qian (2000). According to my previous study in Section 3, the most affected phylotypes by waterbone algal metabolites were belonging to the group of the *Bacteriodetes* and the *Roseobacter* clade. To further understand the pathogenicity of other groups of epiphytic bacteria towards macroalgae, further bands can be excised from other gels and sequenced. Similar suggestion is applied to the fungal work by utilizing a more specific primer pair targeting at different ITS regions of fungal DNA. Provided that the crude extracts from the stolon of Chlorophyceae exhibited the highest antibacterial activity compared to apical and basal areas (Freile-Pelegrin & Morales, 2004), further examination of the antimicrobial effects of waterborne metabolites released from different regions of Phaeophyceae and Rhodophyceae may be performed using the culture-independent techniques described in my thesis above.

5.4 References

Amade P, Charroin C, Baby C, Bacelet J (1987) Antimicrobial activities of marine sponges from the Mediterranean Sea. Mar Biol 94:271-275

Armstrong E, Yan L, Boyd KG, Wright PC, Burgess G (2001) The symbiotic role of marine microbes on living surfaces. Hydrobiologia 461:37-40

Bazes A, Silkina A, Defer D, Bernède-Bauduin C, Quéméner E, Braud JP, Bourgougnon N (2006) Active substances from *Ceramium botryocarpum* used as antifouling products in aquaculture. Aquaculture 258:664-674

Bloor S, England RR (1989) Antibiotic production by the cyanobacterium *Nostoc muscorum*. J Appl Phycol 1:367-372

Boyd KG, Adams DR, Burgess JG (1999) Antibacterial and repellent activity of marine bacteria associated with algal surfaces. Biofouling 14:227-236

Dobretsov S, Qian PY (2002) Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. Biofouling 18:217-228

Dobretsov S, Qian PY (2004) The role of epibiotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement. J Exp Mar Biol Ecol 299:35-50

Dobretsov S, Dahms HU, Qian PY (2004) Antilarval and antimicrobial activity of waterborne metabolites of the sponge *Callyspongia* (*Euplacella*) *pulvinata*: evidence of allelopathy. Mar Ecol Prog Ser 271:133-146

Dworjanyn SA, de Nys R, Steinberg PD (2006) Chemically mediated antifouling in the red alga *Delisea pulchra*. Mar Ecol Prog Ser 318:153-163

Freile-Pelegrin Y, Morales JL (2004) Antibacterial activity in marine algae from the coast of Yucatan, Mexico. Bot Mar 47:140-146

Guillard R, Ryther J (1962) Studies of marine planktonic diatoms. Can J Microbiol 8:229-239

Harder T, Qian PY (2000) Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitive cues for larval attachment and metamorphosis in the polychaete *Hydroides elegans*. Biofouling 16:205-214

Harder T, Lau SCK, Dobretsov S, Fang TK, Qian PY (2003) A distinctive epibiotic bacterial community on the soft coral *Dendronephthya* sp. and antibacterial activity of coral tissue extracts suggest a chemical mechanism against bacterial epibiosis. FEMS Microbiol Ecol 43:337-347

Hellio C, De La Broise D, Dufossé L, Le Gal Y, Bourgougnon N (2001a) Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar Environ Res 52:231-247

Hellio C, Thomas-Guyon H, Culioli G, Piovetti L, Bourgougnon N, Le Gal Y (2001b) Marine antifoulants from *Bifurcaria bifurcate* (Phaeophyceae, Cystoseiraceae) and other brown macroalgae. Biofouling 17:189-201

Jennings JG, Steinberg PD (1994) In situ exudation of phlorotannins by the sublittoral kelp *Ecklonia radiate*. Mar Biol 121:349-354

Kirchman DL, Yu L, Fuchs BM, Amann R (2001) Structure of bacterial communities in aquatic systems as revealed by filter PCR. Aquat Microb Ecol 26:13-22

Küpper FC, Kloareg B, Guern J, Potin P (2001) Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata*. Plant Physio 125:278-91

Küpper FC, Müller DG, Peters AF, Kloareg B, Potin P (2002) Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of Laminariales. J Chem Ecol 28:2057-81

Lam C, Harder T (in press) Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity. J Phycol

Lee OO, Qian PY (2003) Chemical control of bacterial epibiosis and larval settlement of Hydroides elegans in the red sponge *Mycale adhaerens*. Biofouling 19:171-180

Lee OO, Qian PY (2004) Potential control of bacterial epibiosis on the surface of the sponge *Mycale adhaerens*. Aquat Microb Ecol 34:11-21

Mann KH (1973) Seaweeds: their productvitiy and strategy for growth. Science 182:975-981

Miao L, Qian PY (2005) Antagonistic antimicrobial activity of marine fungi and bacteria isolated from marine biofilm and seawaters of Hong Kong. Aquat Micro Ecol 38:231-238

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction –amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695-700

Myers RM, Fischer SG, Lerman LS, Maniatis T (1985) Nearly all single base substitutions in DNA fragments joined to a GC-Clamp can be detected by denaturing gradient gel-electrophoresis. Nucleic Acids Res 13:3131-3145

Neumann R (2007) The allelochemical effect of macroalgae on quantitative and qualitative composition of bacterial communities in plankton and biolfilms. Master thesis, University of Oldenburg, Germany

Nübel U, Engelen B, Felske A, Sanidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAa in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J Bacteriol 178:5636-5643

Nylund GM, Gribben PE, de Nys R, Steinberg PD, Pavia H (2007) Surface chemistry versus whole-cell extracts: antifouling tests with seaweed metabolites. Mar Ecol Prog Ser 329:73-84

Paul NA, de Nys R, Steinberg PD (2006) Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar Ecol Prog Ser 306:87-101

Selje N, Brinkhoff T, Simon M (2005) Detection of abundant bacteria in the Weser estury using culture-dependent and culture-independent approaches. Aquat Microb Ecol 39:17-34

Slattery M, McClintock JB, Heine JN (1995) Chemical defenses in Antaractic soft corals: evidence for antifouling compounds. J Exp Mar Biol Ecol 190:61-77

Walters LJ, hadfield MG, Smith CM (1996) Waterborne chemical compounds in tropical macroalgae: positive and negative cues for larval settlement. Mar Biol 126:383-393

Weinberger F, Friedlander M, Hoppe HG (1999) Oligoagars elicit an oxidative burst in *Gracilaria conferta* (Rhodophyta). J Phycol 35:747-55

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221-271

Yang LH, Lee OO, Jin T, Li XC, Qian PY (2006) Antifouling properties of 10β-formamidokalihinol-A and kalihinol A isolated from the marine sponge *Acanthella cavernosa*. Biofouling 22:23-32

Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62:316-322

Zwisler W, Selje N, Simon M (2003) Seasonal patterns of the bacterioplankton community composition in a large mesotrophic lake. Aquat Microb Ecol 31:211-225

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1. Lam C, Grage A, Schulz D, Harder T (submitted) Antifouling activity of North Sea macroalgae against benthic diatoms. Biofouling
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3. Lam C, Harder T (2007) Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity. J Phycol
4. Harder T, Lam C (2006) Waterborne allelochemicals of marine macroalgae affect bacterial diversity and abundance in close proximity. Proceedings of the GfÖ, Hoffmeister T, Diekmann M (eds), vol. 36 306
5. Lam C, Harder T, Qian PY (2005b) Growth conditions of benthic diatoms affect quality and quantity of extracellular polymeric larval settlement cues. Mar Ecol Prog Ser 294: 109-116
6. Lam C, Harder T, Qian P Y (2005a) Induction of larval settlement in the polychaete *Hydroides elegans* by benthic diatoms: the role of extracellular polysaccharides (EPS). Mar Ecol Prog Ser 286: 145-154
7. Lam C, Harder T, Qian P Y (2003) Induction of larval settlement in the polychaete *Hydroides elegans* by surface-associated settlement cues of marine benthic diatoms. Mar Ecol Prog Ser 263: 83-92
8. Harder T, Lam C, Qian P Y (2002) Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. Mar Ecol Prog Ser 229: 105-112

ERKLÄRUNG

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