

**Antifouling Protection at Different Scales -
Multiple defence in *Mytilus edulis* and the
global performance of mytilid microtopographies**

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

Vorgelegt von
Anna Valeria Bers
Kiel 2006

Referent/in: Prof. Dr. M. Wahl

Korreferent/in: Dr. D. Piepenburg

Tag der mündlichen Prüfung: 12.12.2006

Zum Druck genehmigt: 20.12.2006

gez. Prof. Dr. J. Grotemeyer

TABLE OF CONTENTS

Summary	2
Zusammenfassung	4
General Introduction	6
Marine Biofouling	6
Molecular & Microfouling	7
Macrofouling & Epibiosis	8
Settlement Cues	8
Natural Defence Mechanisms	9
Mytilidae	11
Thesis Outline	12
References	13
Chapters	
I. A comparative study of the anti-settlement properties of mytilid shells	18
II. Relevance of mytilid microtopographies for antifouling defence and invasiveness - a global comparison	26
III. Chemical defence in mussels: antifouling effect of crude extracts of the periostracum of the blue mussel <i>Mytilus edulis</i>	41
IV. Bioassay guided investigation of one periostracal extract of <i>Mytilus edulis</i> with regard to its antifouling potential	54
Synthesis & Discussion	70
Danksagung	75
Curriculum Vitae	76
Erklärung	77

Marine organisms are constantly exposed to fouling by microorganisms, algal propagules and invertebrate larvae. Since being overgrown by epibionts influences to a great extent the interactions between substrate organism (basibiont) and its environment, basibionts have evolved defence mechanisms.

These natural antifouling systems of marine organisms have to target numerous taxa of potential settlers which exhibit a wide range of sensitivities. Consequently, these defence systems are highly complex, often consisting of a combination of mechanical, chemical, physical and associational mechanisms, which simultaneously or alternating affect attachment and growth of bacteria, fouling algae and invertebrates.

This study investigated the natural antifouling mechanisms of Mytilidae in general and *Mytilus edulis* specifically, by decoupling potential mechanical and chemical antifouling mechanisms, and examined whether one defence component, *i.e.* the microtopography, features regional optimisation. The latter was chosen with regard to the high invasive potential of Mytilidae, since a universal defence mechanisms that performs equally well in various biogeographic regions would favour the invasion of new habitats.

The distinct surface microtopography on the periostracum of several mytilid species was replicated and exposed to natural fouling in field experiments. Although repellent against barnacle cyprid larvae (Chapter I), all investigated microtopographies failed to prevent fouling in a persistent way (Chapter II) when exposed to a natural fouling community composed of bacteria, unicellular algae, macroalgal spores and invertebrate larvae. Decoupled from other shell properties, the microtopographies were rapidly covered by a thick biofilm, suggesting the existence of additional antifouling mechanisms that enables mussels to maintain a relatively clean surface for a longer time. In the following, six crude extracts of the periostracum of intact *M. edulis* shells were made using solvents of increasing polarity and tested against common fouling organisms (bacteria, diatoms and barnacle cyprid larvae) in laboratory based bioassays (Chapter III). Non-polar and moderately polar fractions showed the highest antifouling activities, providing first evidence of a chemical defence mechanism in *M. edulis*. Further fractionating and testing of the highly promising diethyl ether extract revealed that the antifouling compounds accumulate in the periostracum with increasing shell size and that they are ethanol-soluble (Chapter IV). Since the nature of the antifouling compound remains unclear, it may only be speculated about the accumulation process. They are either excreted by the mussel and transported into the periostracum via channels or pores, or they are not actively generated by the mussel, but are by-products of the sclerotisation and aging process of the periostracum.

The results of all experiments strongly support the idea of a multiple antifouling system in *Mytilus edulis*. I have been able to show that the mytilid surface topography repels macrofoulers, while surface-bound antifouling compounds affect microfoulers. Therefore, at least two antifouling mechanisms of *Mytilus edulis* are related to characteristics of the periostracum. These two mechanisms may be complemented by cumulative filtration in the mussel bed, and by the swiping movements of the mussel's foot to clean its shell on the outside. However, a regional optimisation of the surface microtopography has not been found (Chapter I and II). The invasive success of Mytilidae all over the world can therefore not be explained exclusively by a particularly efficient and broad-target antifouling performance of the surface structure.

Marine Organismen sind einem ständigen Besiedlungsdruck durch Mikroorganismen, Algensporen und Larvenstadien sessiler Invertebraten ausgesetzt. Weil die Gegenwart von Epibionten die Interaktionen zwischen Substratorganismus (Basibiont) und Umwelt stark beeinflusst, müssen Basibionten auf den Besiedlungsdruck reagieren.

Natürliche Verteidigungssysteme mariner Organismen gegen Aufwuchs müssen auf die verschiedensten Taxa potentieller Besiedler, die eine große Bandbreite an Empfindlichkeiten besitzen, abzielen. Aus diesem Grunde sind diese Abwehrsysteme hoch komplex, und bestehen meist aus einer Kombination mechanischer, chemischer, physikalischer und ökologischer Mechanismen, die gleichzeitig oder abwechselnd die Anheftung und das Wachstum von Bakterien, Algen und Wirbellosen beeinflussen.

Die vorliegende Arbeit untersucht die natürlichen Antifoulingmechanismen der Mytiliden generell und speziell von *Mytilus edulis* durch die Entkopplung potentieller mechanischer und chemischer Verteidigungsmechanismen. Des Weiteren wurde untersucht, ob eine dieser Verteidigungskomponenten, die Mikrotopographie, sich durch regionale Optimierung auszeichnet. Letztere wurde im Hinblick auf die hochinvasiven Mytiliden ausgewählt, da ein universeller, in den verschiedensten biogeographischen Regionen wirksamer Verteidigungsmechanismus die Invasion in neue Habitate erleichtern würde.

Die gleichmäßige Mikrotopographie auf dem Periostracum verschiedener Mytiliden-Arten wurde nachgebildet und in Feldversuchen dem natürlichen Besiedlungsdruck ausgesetzt. Obwohl diese Oberflächenstruktur wirksam gegen den Bewuchs durch Seepockenlarven ist (Kapitel I), konnte in der Gegenwart einer Besiedlergemeinschaft, bestehend aus Bakterien, einzelligen Algen, Makroalgen und Invertebraten-Larven der Bewuchs nicht dauerhaft verhindert werden (Kapitel II). Losgelöst von anderen Eigenschaften der Muschelschale, wurden die untersuchten Oberflächenstrukturen schnell von einem dichten Biofilm überzogen. Dies legt die Existenz zusätzlicher Antifoulingmechanismen nahe, die es den Miesmuscheln erlaubt, ihre Schalenoberfläche über längere Zeit sauber zu halten.

Im Folgenden wurden sechs Grobextrakte des Periostracums von intakten *M. edulis*-Schalen mit Lösungsmitteln steigender Polarität angefertigt. Diese wurden in Bioassays mit verbreiteten Foulingorganismen (Bakterien, benthischen Diatomeen und Balanidenlarven) auf ihre Antifoulingwirkung getestet (Kapitel III). Unpolare und mäßig polare Fraktionen zeigten die größten Antifouling-Aktivitäten, und erbrachten so den ersten Beleg für chemische Verteidigungsmechanismen in *M. edulis*. Weitere Fraktionierungsschritte und Untersuchungen des hochaktiven Diethylether-Extraktes zeigten, dass die Antifouling-Stoffe im Periostracum mit zunehmender Schalengröße angereichert werden, und dass die aktive Substanz in Ethanol löslich ist (Kapitel IV). Da

die Stoffgruppe der aktiven Substanzen noch nicht bekannt ist, kann man derzeit nur Vermutungen über die Anreicherung im Periostracum anstellen. Entweder werden diese Produkte aktiv von der Muschel produziert und über Schalenkanäle oder -poren ins Periostracum transportiert, oder aber die Substanzen wird nicht aktiv von der Muschel generiert, sondern ist ein Nebenprodukt der Sklerotisierung und des Alterungsprozesses des Periostracums

Die Ergebnisse aller Experimente unterstützen nachdrücklich das Konzept eines multiplen Verteidigungssystems bei der Miesmuschel *Mytilus edulis*. Ich konnte zeigen, dass die Oberflächenmikrostruktur Besiedlung durch Makrofouler abwehrt, während die Oberflächenchemie die Besiedlung durch Mikro fouler behindert. Daher stehen mindestens zwei Verteidigungsmechanismen mit dem Periostracum in Zusammenhang. Diese beiden Mechanismen könnten durch kumulative Filtration im Muschelbett und säubernde Bewegungen des Muschelfußes an der Schalenoberfläche noch unterstützt werden. Eine regionale Optimierung der Oberflächenmikrostruktur konnte jedoch nicht nachgewiesen werden (Kapitel I und II). Der weltweite Invasionserfolg der Mytiliden kann daher nicht ausschließlich mit einer für Makrofouler repulsiven Oberflächenstruktur erklärt werden.

Biofouling & Epibiosis - and why marine organisms need to defend themselves

Marine Biofouling

Any natural or man-made hard substratum submerged in the marine environment is rapidly colonized by sessile organisms. This phenomenon is termed marine biofouling, and comprises a complex series of chemical and biological events, resulting in the formation of single or multiple layers of attached molecules and organisms.

The establishment of biofouling may be best described as a temporal or competitive race for a substratum by various organisms, which is determined not only by the relative concentration of fouling organisms in the water column near the surface (Clare et al. 1992), but also by physical properties of the substratum and interactions between fouling organisms. There are principally two models to describe the fouling process. The classical view (Figure 1a) refers to fouling as a rather strict successional process with three stages (Davis et al. 1989, Maki & Mitchell 2002): *molecular fouling*, the instantaneous adsorption of organic molecules and formation of a conditioning film; *microfouling*, the attachment and growth of microorganisms, such as bacteria, fungi and unicellular

algae that result in the so-called biofilm formation; and *macrofouling*, the settlement and attachment of invertebrate larvae and macroalgal propagules. The classical model implies some causality between the different fouling levels, but may only be found under very particular circumstances. A more realistic and nowadays widely accepted model is the dynamic or probabilistic model (Figure 1 b) of the fouling process (Clare et al. 1992, Maki & Mitchell 2002). All fouling stages keep running continuously, and lead to a dynamic and complex netting of interactions: between water and substratum (or in absence of a substratum flocculation occurs), between water and specific fouling organisms, and interspecifically between fouling organisms, which again may interact with physical forces such as water flow or gravitation. Fouling therefore cannot be blocked by eliminating the initial stages of colonization, but the quality and/or composition of the fouling community may be influenced by changes in the initial biofilm. Successful antifouling solutions consequently need to target the different components of the colonization process simultaneously.

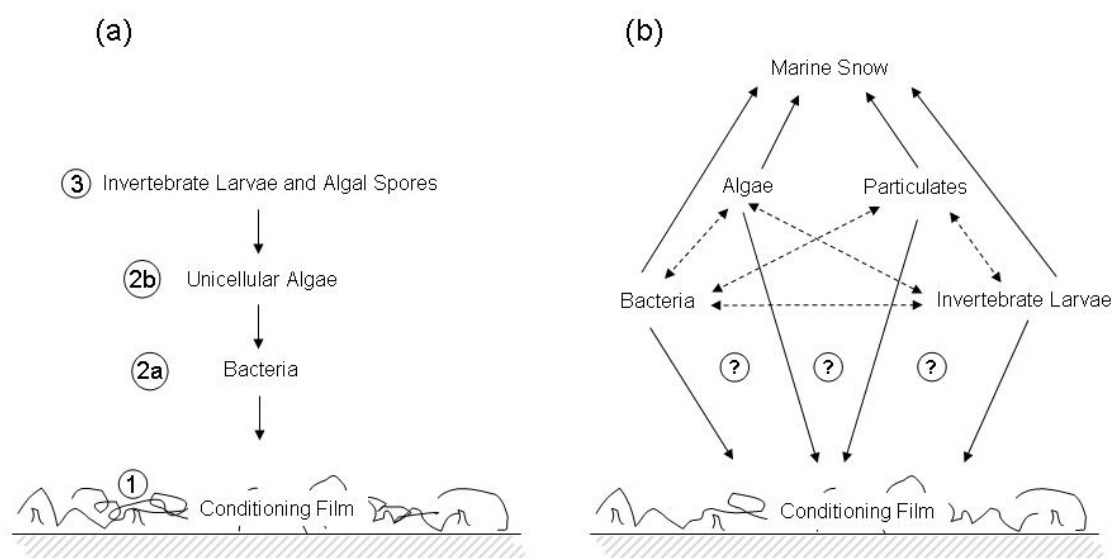


Figure 1. Diagrams of two models for biofouling succession. **(a)** The classical view of successional fouling on a substratum. Each fouling stage is a step that precedes the following stage and causality between levels is implied. **(b)** Surface fouling based on the probability that a particular fouling organism will encounter the substratum. After formation of the conditioning film, further colonisation will depend on which type of organism encounters the surface. In the absence of a substratum, molecules and organisms may attach to each other and participate in the formation of marine snow. Adapted from Clare *et al.* (1992).

Molecular & Microfouling

A surface immersed in seawater will be immediately covered by dissolved chemical compounds that adsorb to the surface and form a conditioning film within minutes (Wahl 1997). The presence of a conditioning film can change the characteristics of the substratum, and influences subsequent attachment of primary fouling organisms by facilitating settlement or providing carbon and other compounds as a food source (Maki & Mitchell 2002). Primary fouling organisms usually form

biofilms. These consist of attached bacteria, yeasts, unicellular algae, fungi and protozoa, all of which are enmeshed in a matrix of extracellular polymers (Lam et al. 2005). The extracellular matrix is generally composed of water and microbial macromolecules (Allison 2003) and provides a complex array of micro-environments, such as open spaces or channels (Davey & O'Toole 2000), thus marine biofilms possess dynamic structure and function (Maki & Mitchell 2002).

Macrofouling & Epibiosis

The unique characteristics of the medium water allow aquatic animals a sessile and filter-feeding life strategy. Settlement substratum can be – other than in terrestrial habitats – a limiting factor even for animals in an otherwise favourable habitat (Wahl 1989). The life cycle of a broad range of sessile marine invertebrates includes a pelagic larval stage. Motile larval stages of sessile organisms guarantee geographical dispersal of the species. The meroplanktonic larvae spend variable time (hours to months) within the water column and attach to a suitable substratum once they reach competency and subsequently metamorphose into sedentary juveniles (Pechenik 1990, Slattery 1997, Lam et al. 2005). For sessile organisms, settlement represents an important process, because it determines their future growth and survival (Hills & Thomason 1998).

Epibiosis is the association between sessile organisms living attached to the surface of living organisms and is, in most cases, facultative (Wahl & Mark 1999). The basibiont provides attachment surface for the epibiont. Besides having no effects on both organisms, this association can be advantageous for both epibiont (gain of attachment site, favourable hydrodynamics and exuded nutrients) and basibiont (camouflage, protection). In many cases, however, epibiosis has harmful effects for the basibionts, such as weight increase, drag and friction increase, mechanical or chemical damage to the body surface, impeded gas exchange, excretion and sensation, increased susceptibility to predation and competition for nutrients, leading to reduced fitness or even death (McKenzie & Grigolava 1996, Wahl 1997).

Settlement Cues

Larval stages are highly specialized in finding suitable settlement substratum, and their settlement is a crucial step in the colonization process. Both environmental cues and larval choice determine where larvae settle.

Environmental factors that influence larval settlement fall into physical, biological and chemical categories (Le Tourneux & Bourget 1988, Roberts et al. 1991, Rittschof et al. 1998). Physical factors include water flow, light, gravity, temperature, salinity and pressure, and also characteristics of the substratum, such as texture, colour, boundary flow or surface free energy. Biological cues originate from conspecifics (Knight-Jones & Crisp 1953), other sessile species and biofilm organisms (Keough

& Raimondi 1995, Wieczorek et al. 1995, Qian et al. 2003), or from exudates of potential basibionts (Wahl & Mark 1999) and can be coupled with chemical signals such as pheromones.

Most invertebrate larvae as well as algae propagules respond, for example, to surface cues such as roughness, topography and texture. Surface rugosities favour mussel settlement (Petraitis 1990), and blue mussel larvae are furthermore capable of selecting some particular scales of heterogeneity (Bourget et al. 1994). The influence of surface (micro-)topography on barnacle settlement has been studied intensively (Andersson et al. 1999, Berntsson et al. 2000a, Berntsson et al. 2000b, Petronis et al. 2000), demonstrating that certain topographies reduce barnacle settlement significantly. These topographies interfere with the cyprid's ability to adhere their antennular discs between or on the side of these structures. Even algal settlement is affected by microtopographies: both *Ulva* (syn. *Enteromorpha*) spores (Callow et al. 2002) and benthic diatoms (Scardino et al. 2006) select the most energetically favourable place for attachment, preferentially in valleys and against side walls.

Responses to natural chemical cues (derived from conspecifics, biofilms or basibionts) have been extensively studied in invertebrate larvae (reviewed by Hadfield & Paul 2001), and the responses vary from induced settlement and metamorphosis to settlement inhibition. Chemical cues from conspecifics generally seem to enhance settlement in invertebrate larvae (Knight-Jones & Crisp 1953, Clare & Matsumara 2000, Head et al. 2004, Dreanno et al. 2006). Bacteria and algae are also capable to sense chemical cues (reviewed by Amsler & Iken 2001). Algal spores may actively reject unsuitable surfaces (Callow et al. 2000), while the attachment and/or growth of bacteria can be mediated by surface chemistry (Knight-Jones & Crisp 1953, Bryan et al. 1996, Harder et al. 2003).

Natural Defence Mechanisms

Although their body surfaces provide potential settlement substratum, many marine organisms remain remarkably free of epibionts, despite facing the same fouling pressure as any other immersed solid substratum. This seems to be due to antifouling defence mechanisms, which are crucial to many marine organisms: the presence of fouling organisms (epibionts) changes most properties determining the basibiont's interactions with its abiotic and biotic environment (McKenzie & Grigolava 1996, Wahl 1997), as mentioned above.

To successfully prevent being overgrown, defence systems of marine organisms have to target numerous taxa of potential settlers which exhibit a wide range of sensitivities. Consequently, natural antifouling systems seem to be highly complex, simultaneously affecting attachment and growth of bacteria, fouling algae and invertebrates (Krug 2006). Some marine organisms are able to allow certain bacteria to colonise their body walls, and these bacteria have repulsive effects on other fouling organisms (Dobretsov et al. 2006). Previous research has revealed that defence mechanisms of marine organisms often occur as combinations of mechanical, chemical, physical or associational

mechanisms. They have often rather repulsive than toxic effects on fouling organisms (Wahl 1989, Wahl & Lafargue 1990, Wahl & Sönnichsen 1992, Wahl 1997, Wahl et al. 1998).

Complete and efficient protection is only assured, when different mechanisms act simultaneously or alternating (Schmitt et al. 1995, Clare 1996). Possible advantages of multiple defence systems may be constrictions for co-evolutionary adaptations (Wahl & Mark 1999). The production of single components at certain times may influence the energetic budget for growth, reproduction or the compensation of stress to a negligible extent. Defence systems are rarely constant in quality and quantity (Hay 1996). The variability with regard to concentration of active compounds comprises all possible scales: between cells or tissue of individuals, between conspecifics several meters or kilometres apart or distributed along a gradient (intertidal, salinity, eutrophication, predation pressure) and between sampling dates in different seasons (Ragan & Jensen 1978, Sieburth & Tootle 1981, Harvell 1986, Harvell et al. 1993, Pawlik et al. 1995, Swaeringen & Pawlik 1998, Van Alstyne et al. 2001). It is possible that on the population scale natural antifouling systems have been selected with regard to their efficiency under local fouling conditions, *i.e.* seasonality, fouling pressure and taxonomic composition of local fouling organisms. Thus, regionally optimised natural defence mechanisms should possess a high local efficiency with a rather narrow taxonomic mode of activity. Rittschof (2001) demonstrated that natural antifouling compounds indeed have a taxonomically relatively narrow activity, keeping in mind, that these compounds may only be one component of a multiple system.

Regional adaptations of defence systems have so far been barely investigated. The few existing studies deal with the anti-predation mechanism of sponges (Becerro et al. 2003, Jones et al. 2005). Geographical variability of secondary metabolites has been described for macrophytes of the Mediterranean, but without considering the aspect of regional optimisation (Valls 1993).

The putative regional optimisation of defence systems would also be of considerable relevance with regard to invasiveness. The rapid growth of international maritime trade has led to an exponential increase of invasions in the past decades (Carlton & Geller 1993, Ruiz et al. 1997); ballast water and ship hull fouling act as vectors of sessile organisms (Everett 2000, Gollasch 2002). If a translocated species survives in the new habitat due to its physiological tolerance or other favourable conditions, it will be able to reproduce and establish a new population (Vermeij 1996). The invasive success of introduced species, however, may not only depend on a wide tolerance to various environmental conditions, but may also depend on the efficiency of anti-predation or antifouling defence in the new biotic environment.

A recent study of the brown seaweed *Fucus evanescens* demonstrated an increased chemical resistance towards herbivory in invading populations when compared to populations from its native habitats (Wilkström et al. 2006): introduced *F. evanescens* had higher phlorotannin concentrations than the

native populations and are therefore avoided by generalist herbivores, leading to an increased invasive success. The regional optimisation of antifouling defences, however, has not been studied so far.

Mytilidae

Bivalves of the family Mytilidae are widely distributed in inter- and subtidal habitats all over the world (Figure 2). They are considered as important habitat engineers in benthic communities (Tsuchiya & Nishihira 1986, Commito et al. 2005, O'Connor et al. 2006). Mytilidae possess high productivity, high fecundity and a wide ecological tolerance with regard to salinity, temperature, desiccation, food concentration and quality and oxygen tension (Seed & Suchanek 1992). Their resulting competitiveness in various environments constitutes the basis for their remarkable invasive potential.

The northern hemisphere mytilid *Mytilus edulis* is widely distributed in European waters, extending from the White Sea, Russia, as far south as the Atlantic coast of Portugal (McDonald et al. 1991). Since the 19th century, however, *M. edulis* has spread far beyond its native ranges, and now also populates the South Chile and Argentina and the Kerguelen Islands (Carlton 1999), as well as the coast of the US states Maine and New York (McDonald et al. 1991).

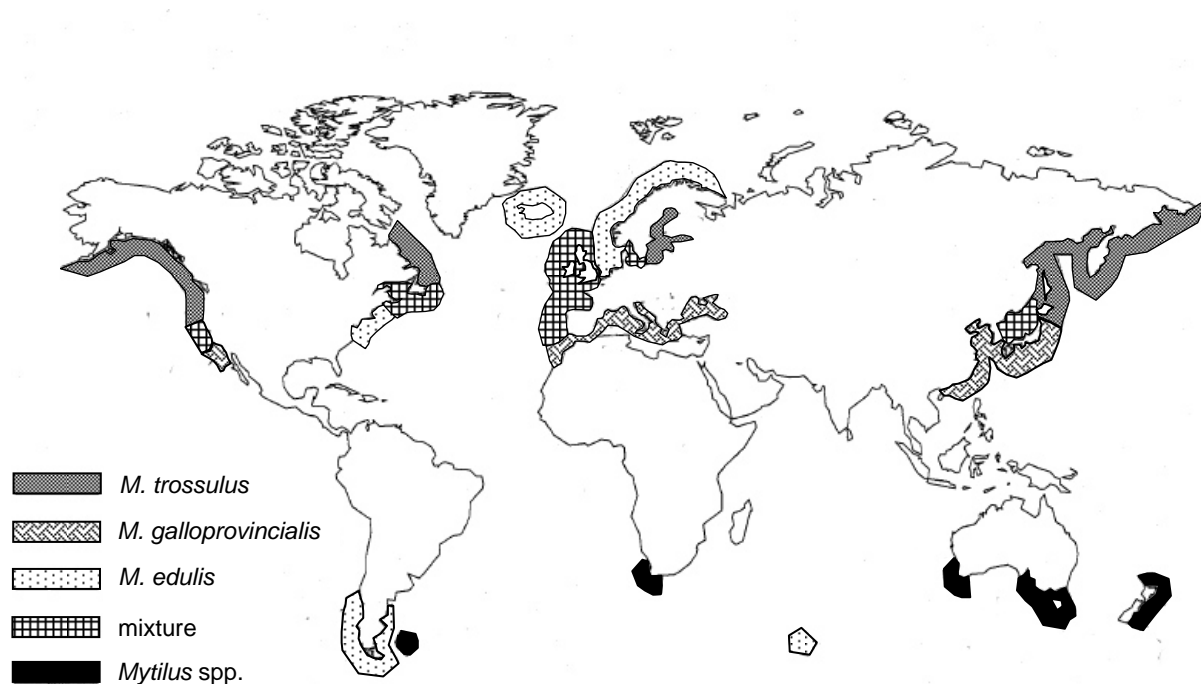


Figure 2. Global distribution of blue mussels, modified after Hilbisch et al. (2000). Approximate distribution of mussels were compiled from various sources, including Koehn (1991), McDonald et al. (1991), Gosling (1992) and Suchanek et al. (1997).

As epibenthic species, the body surface of mytilids that is potentially subject to fouling is the shell. The mytilid shell consists of three layers: the nacreous layer on the inside of the shell, followed by the

prismatic layer in the middle, and the waterproof periostracum on the outside (Bubel 1973, Saleuddin 1974). The outer layer is organic, while the inner two layers are calcareous. The periostracum is formed at the inner face of the outer fold of the mantle (Beedham 1958), secreted at the mantle edge and subsequently insolubilised, hardened and darkened (Saleuddin & Petit 1983). It is made up of three layers (Dunachie 1963) and consists of proteins, polysaccharides, lipids and hydrophobic amino acids (Bubel 1973, Saleuddin & Petit 1983, Waite 1983). The outermost layer of the periostracum, the so-called adhesive epithelium, appears to differ chemically from the rest of the periostracum (Kessel 1940); the second layer contains vacuoles and is thicker than the outermost layer and the layer beneath (Dunachie 1963), which does not contain vacuoles. The total thickness of the periostracum ranges from 20 – 50 μm , but may be as thick as 80 μm (Dunachie 1963, Harper 1997). Sclerotisation occurs during the aging of the periostracal protein (Waite 1983), when fibrous proteins are cross-linked by quinone tanning (Saleuddin & Petit 1983). The periostracum protects the calcareous layers of the shell from dissolution in seawater, serves as a matrix for the deposition of calcium carbonate crystals (Saleuddin & Petit 1983) and may also act as protection against boring organisms (Bottjer 1981, Harper & Skelton 1993, Wahl et al. 1998).

The natural antifouling defence of Mytilidae has been studied before and consist of mechanical, chemical and associational mechanisms: micro-ripples on the periostracum reduce macrofouling for a short period (Wahl et al. 1998, Scardino et al. 2003, Bers & Wahl 2004, Scardino & de Nys 2004) and secondary metabolites have previously been shown to affect ciliate fouling (Wahl et al. 1998). Young *Mytilus edulis* are able to clean their outer shell by swiping movement with their foot (Theisen 1972), and cumulative filtration reduces fouling in mussels beds, since larvae and spores are removed from the water column (Wahl et al. 1998). However promising this multiple system is, the details of its mode of functioning as well as its potential regional variability remain unclear.

The nature of *M. edulis*' defence system and the world-wide distribution of the family Mytilidae make them an excellent model organism to investigate on the one hand components of a natural, multiple defence system and potential synergisms among these, on the other hand a potential regional optimisation and differences in defence efficiency between resident and introduced mussels.

Thesis outline

The aim of this thesis was to (i) investigate the natural antifouling mechanisms of Mytilidae in general and *Mytilus edulis* specifically, by decoupling potential mechanical and chemical mechanisms, and (ii) to examine whether one defence component, *i.e.* the microtopography, features regional optimisation.

It is essential to firstly understand the single mechanisms (such as surface microtopography and chemistry) of a multiple system and their performance with regard to different fouling species before investigating a combination of two or more defence mechanisms.

An antifouling defence system may be specialised to the local settler pool. A universal defence mechanism that performs equally well in various biogeographic regions would possibly favour the invasion of new habitats. With regard to the highly invasive Mytilidae, the microtopography as one antifouling mechanism was chosen to investigate its efficiency on a global scale.

The following chapters represent independent studies, structured into abstract, introduction, methods, results and discussion. In Chapter I, the influence of mytilid microtopographies was investigated in a single-species field bioassay in Millport, Scotland. Settlement of barnacle cyprid larvae on natural microtopographies of 2 different mytilids (*Mytilus edulis* and *Perna perna*) was assessed *in situ*. Chapter II deals with the comparison of microtopographies of 4 different mytilid species of eight regional provenances. These microtopographies were exposed to fouling communities at eight experimental sites in the northern and southern hemisphere in order to investigate whether mytilid microtopographies are regionally adapted and perform differently when exposed to different settler pools. A further aim was to investigate the surface chemistry of *Mytilus edulis* (Chapter III). Extracts of the periostracum were made with solvents of varying polarity, and the resulting crude extracts were tested for activity in different bioassays at natural concentrations. Chapter IV comprises further work on one periostracal extract that proved to have antifouling activities, in relation to shell size and characteristics of the periostracum.

References

- Allison D (2003) The biofilm matrix. *Biofouling* 19:139-150
- Amsler CD, Iken KB (2001) Chemokinesis and Chemotaxis in Marine Bacteria and Algae. In: McClintock JB, Baker BJ (eds) *Marine Chemical Ecology*. CRC Press, Boca Raton, p 413-429
- Andersson M, Berntsson K, Jonsson P, Gatenholm P (1999) Microtextured surfaces: towards macrofouling resistant coatings. *Biofouling* 14:167-178
- Becerro MA, Thacker RW, Turon X, Uriz MJ, Paul VJ (2003) Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses. *Oecologia* 135:91-101
- Beedham GE (1958) Observations on the non-calcareous component of the shell of Lamellibranchia. *Quarterly Journal of Microscopical Science* 99:341-357
- Berntsson KM, Andreasson H, Jonsson PR, Larsson L, Ring K, Petronis S, Gatenholm P (2000a) Reduction of barnacle recruitment on micro-textured surfaces: analysis of effective topographic characteristics and evaluation of skin friction. *Biofouling* 16:245-261

- Berntsson KM, Jonsson PR, Lejhall M, Gatenholm P (2000b) Analysis of behavioural rejection of micro-textured surfaces and implications for recruitment by the barnacle *Balanus improvisus*. *Journal of Experimental Marine Biology and Ecology* 251:59-83
- Bers AV, Wahl M (2004) The influence of natural surface microtopographies on fouling. *Biofouling* 20:43-51
- Bottjer DJ (1981) Periostracum of the gastropod *Fusitriton oregonensis*: natural inhibitor of boring and encrusting organisms. *Bulletin of Marine Science* 31:916-921
- Bourget E, DeGuise J, Daigle G (1994) Scales of substratum heterogeneity, structural complexity, and the early establishment of a marine epibenthic community. *Journal of Experimental Marine Biology and Ecology* 181:31-51
- Bryan PJ, Rittschof D, McClintock JB (1996) Bioactivity of echinoderm ethanolic body-wall extracts: an assessment of marine bacterial attachment and macroinvertebrate larval settlement. *Journal of Experimental Marine Biology and Ecology* 196:79-96
- Bubel A (1973) An electron-microscope study of periostracum formation in some marine bivalves. I. The formation of the periostracum. *Marine Biology* 20:213 - 221
- Callow ME, Callow JA, Ista LK, Coleman SE, Nolasco AC, López GP (2000) Use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. *Applied and Environmental Microbiology* 66:3249-3254
- Callow ME, Jennings AR, Brennan AB, Seegert CE, Gibson A, Wilson L, Feinberg A, Baney R, Callow JA (2002) Microtopographic cues for settlement of zoospores of the green fouling alga *Enteromorpha*. *Biofouling* 18:237-245
- Carlton JT (1999) Molluscan invasions in marine and estuarine communities. *Malacologia* 41:439-454
- Carlton JT, Geller JB (1993) Ecological roulette: the global transport of nonindigenous marine organisms. *Science* 261:78-82
- Clare AS (1996) Marine natural product antifoulants: status and potential. *Biofouling* 9:211-229
- Clare AS, Matsumara K (2000) Nature and perception of barnacle settlement pheromones. *Biofouling* 15:57-71
- Clare AS, Rittschof D, Gerhart DJ, Maki JS (1992) Molecular approaches to non-toxic antifouling. *Invertebrate Reproduction and Development* 22:67-76
- Commito JA, Celano EA, Celico HJ, Como S, Johnson CP (2005) Mussels matter: postlarval dispersal dynamics altered by a spatially complex ecosystem engineer. *Journal of Experimental Marine Biology and Ecology* 316:133-147
- Davey ME, O'Toole G (2000) Microbial Biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* 64:847-867
- Davis AR, Targett NM, McConnell OJ, Young CM (1989) Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In: Scheuer PJ (ed) *Bioorganic Marine Chemistry, Vol 3*. Springer-Verlag, Berlin, p 85-114
- Dobretsov SV, Dahms HU, Qian PY (2006) Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling* 22:43-54
- Dreanno C, Kirby RR, Clare AS (2006) Smelly feet are not always a bad thing: the relationship between cyprid footprint protein and the barnacle settlement pheromone. *Biology Letters* doi:10.1098/rsbl.2006.0503
- Dunachie JF (1963) The periostracum of *Mytilus edulis*. *Transactions of the Royal Society of Edinburgh* 65:383 - 411
- Everett RA (2000) Patterns and pathways of biological invasions. *Trends in Ecology and Evolution* 15
- Gollasch S (2002) The importance of ship hull fouling as a vector of species introductions into the North Sea. *Biofouling* 18:105-121
- Gosling EG (ed) (1992) *The mussel Mytilus: ecology, physiology, genetics and culture, Vol 25*. Elsevier Science Publishing, Amsterdam
- Hadfield MG, Paul VJ (2001) Natural chemical cues for settlement and metamorphosis of marine invertebrate larvae. In: McClintock J, Baker B (eds) *Marine Chemical Ecology*. CRC Press, Boca Raton, p 431-461
- Harder T, Lau SCK, Dobretsov SV, 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 mechanisms against bacterial epibiosis. *FEMS Microbial Ecology* 43:337-347
- Harper EM (1997) The molluscan periostracum: an important constraint in bivalve evolution. *Palaeontology* 40:71-91
- Harper EM, Skelton PW (1993) A defensive value of the thickened periostracum in the Mytiloidea. *Veliger* 36:36-42
- Harvell C, Fenical W, Roussis V, Ruesink J, Griggs C, Green C (1993) Local and geographic variation in the defensive chemistry of a West Indian gorgonian coral (*Briareum asbestinum*). *Marine Ecology Progress Series* 93:165-173
- Harvell CD (1986) The ecology and evolution of inducible defenses in a marine bryozoan: cues, costs and consequences. *American Naturalist* 128:810-823
- Hay ME (1996) Marine chemical ecology: what's known and what's next? *Journal of Experimental Marine Biology and Ecology* 200:103-134
- Head RM, Berntsson KM, Dahlström M, Overbeke K, Thomason JC (2004) Gregarious settlement in cypris larvae: the effects of cyprid age and assay duration. *Biofouling* 20:123-128
- Hilbisch TJ, Mullinax A, Dolven SI, Meyer A, Koehn RK, Rawson PD (2000) Origin of the antitropical distribution pattern in marine mussels (*Mytilus* spp.): routes and timing of transequatorial migration. *Marine Biology* 136:69-77
- Hills JM, Thomason JC (1998) The effect of scales of surface roughness on the settlement of barnacle (*Semibalanus balanoides*) cyprids. *Biofouling* 12:57-69
- Jones AC, Blum JE, Pawlik JR (2005) Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? *Journal of Experimental Marine Biology and Ecology* 322:67-81
- Keough MJ, Raimondi PT (1995) Responses of settling invertebrate larvae to bioorganic films: effects of different types of films. *Journal of Experimental Marine Biology and Ecology* 185:235-253
- Kessel E (1940) Über den feineren Bau des Mytiliden-Periostracum, erschlossen aus der Optik. *Zeitschrift für Morphologie und Ökologie der Tiere* 36:581-594
- Knight-Jones EW, Crisp DJ (1953) Gregariousness in barnacles in relation to the fouling of ships and to the antifouling research. *Nature* 171:1109 - 1110
- Koehn RK (1991) The genetics and taxonomy of species in the genus *Mytilus*. *Aquaculture* 94:125-145
- Krug PJ (2006) Defense of benthic invertebrates against surface colonization by larvae: a chemical arms race. In: Fusetani N, Clare AS (eds) *Antifouling compounds*. Springer, Berlin, p 1-53
- Lam C, Harder T, Qian PY (2005) Growth conditions of benthic diatoms affect quality and quantity of extracellular polymeric larval settlement cues. *Marine Ecology Progress Series* 294:109-116
- Le Tourneux F, Bourget E (1988) Importance of physical and biological settlement cues used at different spatial scales by larvae of *Semibalanus balanoides*. *Marine Biology* 97:57-66
- Maki JS, Mitchell R (2002) Biofouling in the marine environment. In: Bitton G (ed) *Encyclopedia of Environmental Microbiology*. John Wiley & Sons, New York, p 610-619
- McDonald JH, Seed R, Koehn RK (1991) Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern hemisphere. *Marine Biology* 111:323-333
- McKenzie JD, Grigolava IV (1996) The echinoderm surface and its role in preventing microfouling. *Biofouling* 10:261-272
- O'Connor NE, Crowe TP, McGrath D (2006) Effects of epibiotic algae on the survival, biomass and recruitment of mussels, *Mytilus* L. (Bivalvia: Mollusca). *Journal of Experimental Marine Biology and Ecology* 328:265-276
- Pawlik JR, Chanas B, Toonen RJ, Fenical W (1995) Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrence. *Marine Ecology Progress Series* 127:183-194
- Pechenik JA (1990) Delayed metamorphosis by larvae of benthic marine invertebrates: Does it occur? Is there a price to pay? *Ophelia* 32:63-94
- Petratis PS (1990) Direct and indirect effects of predation, herbivory and surface rugosity on mussel recruitment. *Oecologia* 83:405-413
- Petronis S, Berntsson K, Gold J, Gatenholm P (2000) Design and microstructuring of PDMS surfaces for improved marine biofouling resistance. *Journal of Biomaterials Science - Polymer Edition* 11:1051-1072

- Qian PY, Thiyagarajan V, Lau SCK, Cheung SCK (2003) Relationship between bacterial community profile in biofilm and attachment of the acorn barnacle *Balanus amphitrite*. *Aquatic Microbial Ecology* 33:225-237
- Ragan MA, Jensen A (1978) Quantitative studies on brown algal phenols. II. Seasonal variations in polyphenol content of *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* (L.). *Journal of Experimental Marine Biology and Ecology* 34:245-258
- Rittschof D (2001) Natural product antifoulants and coatings development. In: McClintock J, Baker B (eds) *Marine Chemical Ecology*. CRC Press, Boca Raton, p 534-566
- Rittschof D, Forward Jr RB, Cannon G, Welch JM, McClary Jr M, Holm ER, Clare AS, Canova S, McKelvey LM, Bryan P, van Dover CL (1998) Cues and context: Larval responses to physical and chemical cues. *Biofouling* 12:31-44
- Roberts D, Rittschof D, Holm ER, Schmidt AR (1991) Factors influencing initial larval settlement: temporal, spatial and surface molecular components. *Journal of Experimental Marine Biology and Ecology* 150:203-219
- Ruiz GM, Carlton JT, Grosholz ED, Hines AH (1997) Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *American Zoologist* 37:621-632
- Saleuddin ASM (1974) An electron microscopic study of the formation and structure of the periostracum in *Astarte* (Bivalvia). *Canadian Journal of Zoology* 52:1463-1471
- Saleuddin ASM, Petit HP (1983) The mode of formation and the structure of the periostracum. In: Wilbur KM (ed) *The Mollusca*, Vol 4. Academic Press, New York, p 199-234
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctata imbricata*. *Biofouling* 19 (Supplement):221-230
- Scardino AJ, de Nys R (2004) Fouling deterrence on the bivalve shell *Mytilus galloprovincialis*: a physical phenomenon? *Biofouling* 20:249-257
- Scardino AJ, Harvey E, de Nys R (2006) Testing attachment point theory: diatom attachment on microtextured polyimide biomimics. *Biofouling* 22:55-60
- Schmitt TM, Hay ME, Lindquist N (1995) Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. *Ecology* 76:107-123
- Seed R, Suchanek TH (1992) Population and community ecology of *Mytilus*. In: Gosling E (ed) *The mussel Mytilus: ecology, physiology, genetics and culture*. Elsevier Science Publishers, Amsterdam, p 87-170
- Sieburth JM, Tootle JL (1981) Seasonality of Microbial Fouling on *Ascophyllum nodosum* (L.) Lejol, *Fucus vesiculosus* (L.), *Polysiphonia lanosa* (L.) Tandy and *Chondrus crispus* Stackh. *Journal of Phycology* 17:57-64
- Slattery M (1997) Chemical cues in marine invertebrate larval settlement. In: Nagabhushanam R, Thompson M (eds) *Fouling organisms of the Indian Ocean: biology and control technology*. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 135-158
- Suchanek TH, Geller JB, Kreiser BR, Mitton JB (1997) Zoogeographic distributions of the sibling species *Mytilus galloprovincialis* and *M. trossulus* (Bivalvia: Mytilidae) and their hybrids in the North Pacific. *Biological Bulletin* 193:187-194
- Swaeringen DC, Pawlik JR (1998) Variability in the chemical defense of the sponge *Chondrilla nucula* against predatory reef fish. *Marine Biology* 131:619-627
- Theisen BF (1972) Shell cleaning and deposit feeding in *Mytilus edulis* L. (Bivalvia). *Ophelia* 10:49-55
- Tsuchiya M, Nishihira M (1986) Islands of *Mytilus edulis* as a habitat for small intertidal animals - effect of *Mytilus* age structure on the species composition of the associated fauna and community. *Marine Ecology Progress Series* 31:171-178
- Valls R (1993) Séparation, identification, étude spectroscopique de métabolites secondaires d'algues brunes (Cystoseiracées). Dosage - Variations - Chimiotaxonomie. Saint-Jérôme, Marseille, France
- Van Alstyne KL, Dethier MN, O DD (eds) (2001) Spatial patterns in macroalgal chemical defenses, Vol. CRC Press LLC, Boca Raton, USA
- Vermeij GJ (1996) An agenda for invasion biology. *Biological Conservation* 78:3-9

- Wahl M (1989) Marine Epibiosis I. Fouling and antifouling. Some basic aspects. *Marine Ecology Progress Series* 58:175-189
- Wahl M (1997) Living attached: aufwuchs, fouling, epibiosis. In: Nagabushanam R, Thompson M (eds) *Fouling organisms of the Indian Ocean: biology and control technology*. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 31-83
- Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205-226
- Wahl M, Lafargue F (1990) Marine epibiosis II. Reduced fouling on *Polysyncraton lacazei* (Didemnidae, Tunicata) and proposal of an antifouling potential index. *Oecologia* 82:275-282
- Wahl M, Mark O (1999) The predominantly facultative nature of epibiosis: experimental and observational evidence. *Marine Ecology Progress Series* 187:59-66
- Wahl M, Sönnichsen H (1992) Marine Epibiosis IV. The periwinkle *Littorina littorea* lacks typical antifouling defences - why are some populations so little fouled? *Marine Ecology Progress Series* 88:225-235
- Waite JH (1983) Quinone-tanned scleroproteins. In: Hochachka PW (ed) *The Mollusca*, Vol 1. Academic Press, New York, p 467-504
- Wieczorek SK, Clare AS, Todd CD (1995) Inhibitory and facilitatory effects of microbial films on settlement of *Balanus amphitrite amphitrite* larvae. *Marine Ecology Progress Series* 119:221-228
- Wilkström SA, Steinarsdóttir MB, Kautsky L, Pavia H (2006) Increased chemical resistance explains low herbivore colonization of introduced seaweed. *Oecologia* 148:593-601

A comparative study of the anti-settlement properties of mytilid shells

Abstract

Marine organisms have evolved defence mechanisms to prevent epibiosis. This study investigated the anti-settlement properties of natural periostracal microtopographies of two mytilid species, *Mytilus edulis* (from North, Baltic and White Seas) and *Perna perna* (from the SW Atlantic). Resin replicas of shells were exposed to cyprids of the barnacle *Semibalanus balanoides*. Replicas with intact isotropic microtopographies and smooth controls were much less fouled than roughened anisotropic surfaces. This indicates that in both *M. edulis* and *P. perna* the periostracum possesses a generic anti-settlement property, at least against *S. balanoides* cyprids, which is not regionally adapted. Such a potential globally effective antisetlement mechanism possibly contributes to the invasive success of Mytilidae.

Introduction

Settlement of marine larvae is not a stochastic process and many species show some degree of choice (Keough & Downes 1982). Choices are made in response to diverse physical and chemical cues and may be highly specific (Rittschof & Costlow 1989), causing intense settlement pressure on any substratum in the sea, including other organisms. Epibiosis is a major problem for marine organisms, and yet the surfaces of most species are rarely completely overgrown. Since epibiosis may have harmful effects (Wahl 1997b), many marine species have evolved chemical, physical or mechanical defence mechanisms (Wahl 1989). The shells of epibenthic bivalves offer substantial space for larvae; however, the shells of mytilids often appear less fouled than adjacent non-biological substrata (Wahl et al. 1998, Bers & Wahl 2004).

Given that epibiosis reduces fitness (Wahl 1997a), then we might expect that surfaces of endemic species might be adapted for preventing settlement of the local epibiota. Conversely, more cosmopolitan species should exhibit a generalized antifouling activity. As the antifouling capacity of the shells of the mussels *Mytilus edulis* (Wahl et al. 1998, Bers & Wahl 2004) and *M. galloprovincialis* (Scardino et al. 2003) is related to the texture of the periostracum, then there is the possibility that periostracal textures may be adapted to locally abundant epibionts. Hence, the aim of this study was to investigate the anti-settlement properties of natural microtopographies of shells from different populations of mytilids and species with regard to one epibiont, the cyprid of the acorn barnacle *Semibalanus balanoides*. Shells from three different populations of the blue mussel *M. edulis*, and from one population of the brown mussel *Perna perna* were used in this study. Both of these species are cosmopolitan in northern and southern hemispheres, respectively. *P. perna* was chosen in order to include a closely related, but non-*Mytilus*, mytilid in the study as a reference point.

Material & Methods

M. edulis valves were obtained from the Baltic Sea (Kiel Fiord, Germany, 54°22'N, 10°09'E), White Sea (Matryonin Island, Russia, 66°18'N, 33°40'E), and North Sea (Hartlepool Marina, UK, 54°41'N, 1°11'W). *P. perna* valves were obtained from the southwest Atlantic (Itaipu beach, Brazil, 22°56'S, 43°03'W). All sites were sheltered bays.

To quantify the effect of periostracal microtopographies on cyprid settlement separately from other possible surface properties, resin replicas of valves were made. This method gives sub-micron replication and is non-toxic (Marrs et al. 1995). Microfouling on the shells was removed with a soft toothbrush and sterile filtered seawater (Sartorius 0.8 µm cellulose nitrate filter). Casts of shells were made using Kerr's Extrude[®] Wash Type 3 (KERR, USA) and high resolution resin replicas were made using Devcon[®] 2-TON[®] epoxy resin (DEVCON, UK). Replicas were coloured uniform grey using Coelan[®] Farbpaste (COELAN, Germany) and cured for 12 – 18 hours at room temperature. Smooth control surfaces were made by sealing a second resin replica of the same individual shell with resin to

give a glassy smooth surface. Rough control surfaces were made by standardised roughening of a third resin replica (Grade 70/Grit M2 abrasive paper, English Abrasives & Chemical Limited, UK). An experimental triplet comprised a smooth, natural and rough replica and three triplets were made for each provenance, each from a different individual's right or left valve.

Field experiments were performed from 5th – 18th April 2003 at Keppel Pier, Millport, Clyde Sea, UK (55°45'N, 4°54'W). No other species of barnacle settle at this time and the large numbers of cyprids exclude all other non-barnacle epibionts (Hills & Thomason 1998, Hansson et al. 2003).

Replicas in each triplet were fixed 2 cm apart on grey PVC sheet using non-toxic silicone adhesive. Triplets were randomized across the PVC sheet and the sheet attached intertidally (2.05 m above chart datum) in the middle of the *S. balanoides* settlement zone using 8 mm bolts. Digital photographs of each replica were taken every low tide for 25 tides (Canon D30 camera, 100 mm lens). Cyprids and metamorphs were counted using ImageTool 3.0 only in the central 1 cm² of each replica to reduce edge effects.

Microtopographies were examined by SEM. Three replicas from each provenance were sputter coated with a 20 nm thick gold—palladium alloy and viewed with a Zeiss DSM 940 SEM. Additionally, a 4 mm profile of each replica in one triplet was quantified with a Uniscan OSP100a laser profilometer. Data for the last tide (25) were tested for normality, transformed $((\text{count} + 1)^{0.07})$ and analysed using ANOVA (SPSS v.11): within-subjects factor was surface (natural, smooth, rough), between subjects factor was provenance, and the response was count. Sphericity of the data was assessed using Mauchly's test and where they were aspherical, Greenhouse–Geisser adjustments of F were used. All variances were homogeneous (Levene's test, $p > 0.05$).

Results

Valves from each provenance featured a rippled periostracal microtopography, with a wavelength of 1.5–2.0 mm running orthogonally to the growth rings of the shell (Figure 1). There was no significant difference in arithmetic mean roughness (R_a) between natural and roughened replicas (all provenances combined, ANOVA, $n = 8$, $F_{1,6} = 0.097$, $p = 0.77$, $\bar{x} R_{a, \text{natural}} = 14.01 \pm 4.26 \mu\text{m}$, $\bar{x} R_{a, \text{rough}} = 14.74 \pm 3.08 \mu\text{m}$). Smooth replicas could not be scanned in the laser profilometer due to their high reflectance.

Table 1. Contrast test with natural surface as reference level.

Source	Surface	df	F -value	P -value
Surface	natural vs smooth	1	1.802	0.216
	smooth vs rough	1	42.076	< 0.001

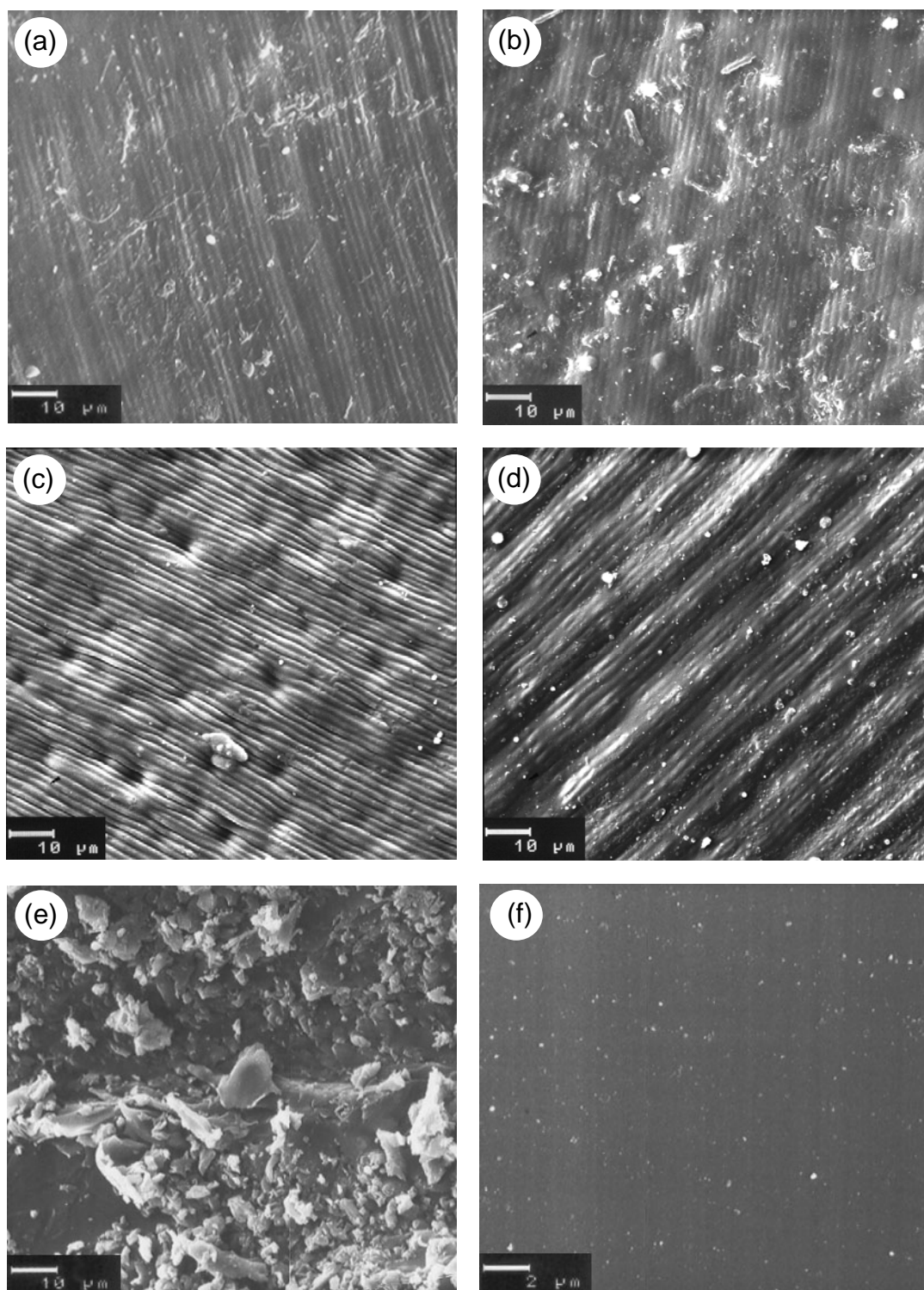


Figure 1. Scanning electron micrographs of: **(a)** *Perna perna* (Brazil); **(b)** *Mytilus edulis* (Russia); **(c)** *Mytilus edulis* (UK); **(d)** *Mytilus edulis* (Germany); **(e)** rough control; **(f)** smooth control.

The tide 25 data showed no significant interaction between country and surface ($F_{6, 16} = 1.38$, $p < 0.28$), and no significant main effects for country ($F_{3, 8} = 0.57$, $p = 0.65$). However, there were significant differences between surfaces ($F_{1, 2} = 33.79$, $p < 0.001$, Figure 2). An *a priori* contrast test (natural surface = reference) indicated that this difference was attributable to a significant difference between rough and both natural and smooth surfaces (Table 1). This suggests that the anti-settlement

property of the valves is the same irrespective of species or regional provenance. We did not differentiate between metamorphs and newly settled cyprids and the data include both. Reduction of space due to permanent attachment is seen as the plateaus after tide 17 (Figure 2).

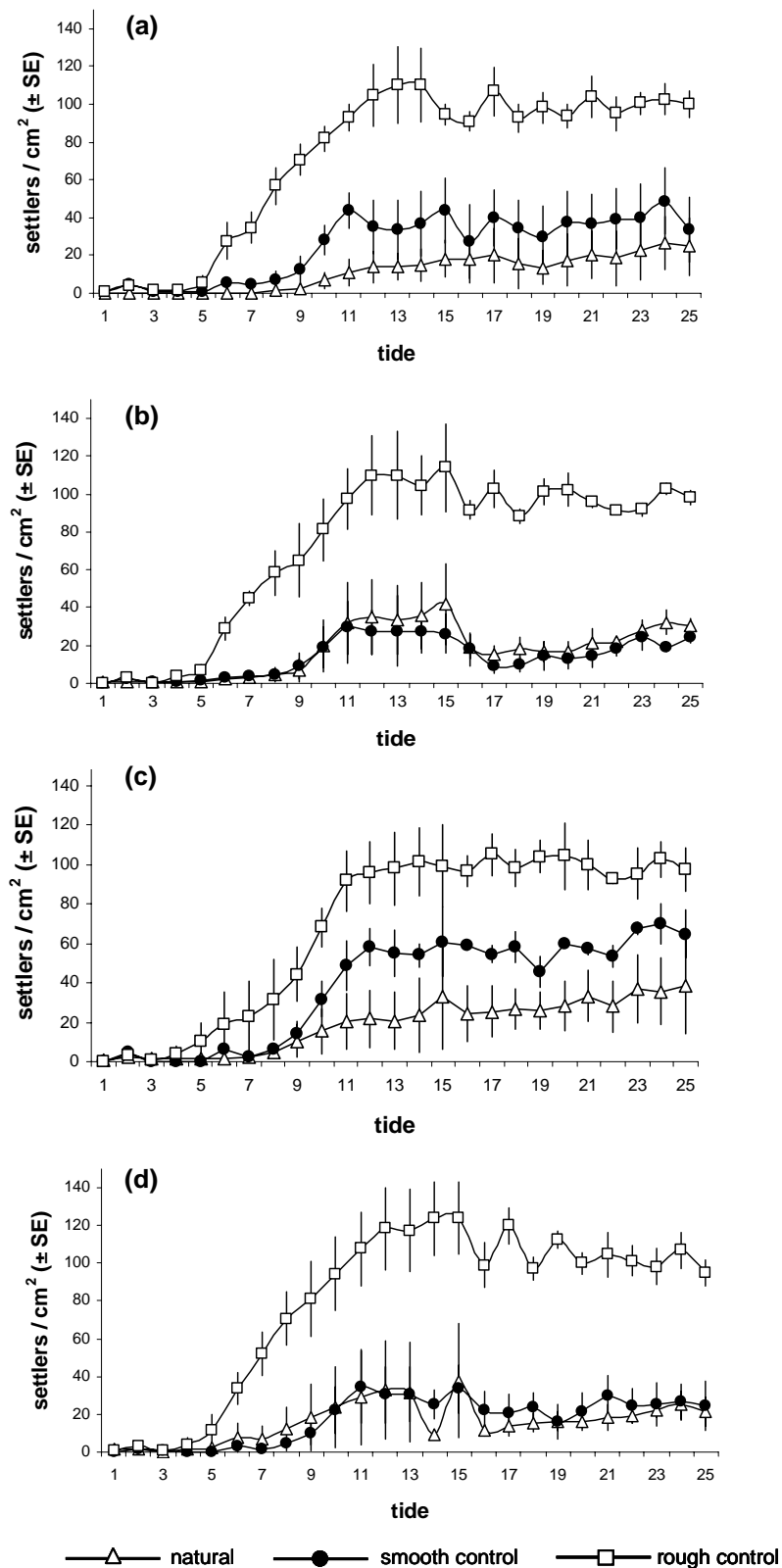


Figure 2. Barnacle settlement on natural, smooth and rough replica of (a) *Perna perna* (Brazil), (b) *Mytilus edulis* (Russia), (c) *Mytilus edulis* (UK), (d) *Mytilus edulis* (Germany). Error bars indicate SE.

Analysis of differences in the rate of settlement was undertaken using another ANOVA. Data for all tides were transformed and analysed with both tide number and surface as within subjects factors, and country as the between subjects factor. Initially, a fully factorial model was run and then main effects and interactions excluded from the model if $p > 0.1$. The final model was tide + surface + tide \times surface. There were significant interactions between surface and tide ($F_{6,04,66,4} = 9.3$, $p < 0.001$, Table 1). An *a priori* contrast test (tide 1 = reference) showed that significant differences ($p < 0.001$) only occurred after tide 4 (Figure 2). These results show that intense settlement leads to rapid differences between surfaces.

Discussion

This simple field assay-based study revealed remarkable similarities in the anti-settlement properties of mytilid shells against larvae of *Semibalanus balanoides*. There was no difference between smooth control and natural surfaces for both species, as well as no differences between *Mytilus edulis* shells from the Baltic, North and White Seas. In all cases smooth controls and natural surfaces were much less fouled than the rough control surfaces, and although none remained completely unfouled, this difference was seen after only four tides. As the magnitude of the surface topographies was the same for both natural and roughed valves, then this result is attributable to the change from a natural isotropic microtopography to the anisotropic roughened surfaces. It is known that the periostracum prevents boring organisms damaging the shell structure (Harper & Skelton 1993, Kaehler 1999) and it is now also known that intact periostracal textures maintain general fitness by reducing fouling (Scardino & de Nys 2004). External surfaces of most mollusc shells are generally rough. This is probably the ancestral condition and is governed by the difficulty of producing and maintaining a very smooth surface without frequent maintenance by the mantle. Mytilids appear to have adapted to reduce epibiosis by evolving an isotropic periostracum, not a smooth one. The consistency of these results indicates that all populations of *M. edulis* may possess an anti-settlement property, at least against *S. balanoides* cyprids, even when this species is absent, *i.e.* in the Baltic, where the dominant barnacle is *Balanus improvisus*. Hills & Thomason (1998) demonstrated that *S. balanoides* cyprids prefer surface roughness about the size of the larval body (0.5–1 mm), and do not like very smooth surfaces. They did not test rugosities at the scale presented here where the roughened control and natural shells both had roughness $\sim 14 \mu\text{m}$, somewhere between 35–70 times smaller than those tested by Hills & Thomason (1998). These results clearly show that cyprids of *S. balanoides* will settle on topographies at the micrometre scale as long as they are anisotropic. Thus, it can be cautiously inferred that the periostracal topography has anti-settlement activity that is not locally adapted. Additional support for this view comes from the similarity not only between *M. edulis* and *P. perna* as found by this study, but also from the similarity between these two species and *M. galloprovincialis*, which also

has antifouling properties (Scardino et al. 2003, Scardino & de Nys 2004). Artificial isotropic microtopographies have been shown to reduce barnacle settlement by reducing exploration time (Berntsson et al. 2000). It is therefore possible that the generalized anti-settlement property reported here is a reflection of this behaviour. It is possible that isotropic microtopographies will confer a general protection against the settlement of all barnacle larvae. Very little is known about biogeographical differences in defences of marine invertebrates (Becerro et al. 2003). Since the prevention of epibiosis, and hence maintenance of fitness, is essential, anti-settlement defences with global effectiveness would allow the invasion of new areas and may have contributed to the evolutionary and invasive capability of the mytilids. Understanding how these microtopographies function is attracting increasing attention given the urgent need to find alternatives to chemically active antifouling coatings (Andersson et al. 1999).

References

- Andersson M, Berntsson K, Jonsson P, Gatenholm P (1999) Microtextured surfaces: towards macrofouling resistant coatings. *Biofouling* 14:167-178
- Becerro MA, Thacker RW, Turon X, Uriz MJ, Paul VJ (2003) Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses. *Oecologia* 135:91-101
- Berntsson KM, Andreasson H, Jonsson PR, Larsson L, Ring K, Petronis S, Gatenholm P (2000) Reduction of barnacle recruitment on micro-textured surfaces: analysis of effective topographic characteristics and evaluation of skin friction. *Biofouling* 16:245-261
- Bers AV, Wahl M (2004) The influence of natural surface microtopographies on fouling. *Biofouling* 20:43-51
- Hansson LJ, Hudson IR, Seddon RJ, Shaw O, Thomason JC (2003) Massive recruitment of the barnacle *Semibalanus balanoides* in the Clyde Sea (Scotland, UK) in the spring of 2000. *Journal of the Marine Biological Association of the United Kingdom* 83:923-924
- Harper EM, Skelton PW (1993) A defensive value of the thickened periostracum in the Mytiloidea. *Veliger* 36:36-42
- Hills JM, Thomason JC (1998) The effect of scales of surface roughness on the settlement of barnacle (*Semibalanus balanoides*) cyprids. *Biofouling* 12:57-69
- Kaehler S (1999) Incidence and distribution of phototrophic shell-degrading endoliths of the brown mussel *Perna perna*. *Marine Biology* 135:505-514
- Keough MJ, Downes BJ (1982) Recruitment of marine invertebrates: the role of active larval choices and early mortality. *Oecologia* 54:346-352
- Marrs SJ, Thomason JC, Cowling MJ, Hodgkiess T (1995) A replica method for the study of marine biofilms. *Journal of the Marine Biological Association of the United Kingdom* 75:759-762
- Rittschof D, Costlow JD (1989) Surface determination of macroinvertebrate larval settlement. In: Klekowski R, Styczynska-Jurewicz E, Falkowsky L (eds) Twenty first European Marine Biology Symposium. Polish Academy of Sciences, Institut for Oceanology, Gdansk, Poland, p 155-163
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctata imbricata*. *Biofouling* 19 (Supplement):221-230
- Scardino AJ, de Nys R (2004) Fouling deterrence on the bivalve shell *Mytilus galloprovincialis*: a physical phenomenon? *Biofouling* 20:249-257
- Wahl M (1989) Marine Epibiosis I. Fouling and antifouling. Some basic aspects. *Marine Ecology Progress Series* 58:175-189

- Wahl M (1997a) Increased drag reduces growth of snails: comparison of flume and *in situ* experiments. *Marine Ecology Progress Series* 151:291-293
- Wahl M (1997b) Living attached: aufwuchs, fouling, epibiosis. In: Nagabushanam R, Thompson M (eds) *Fouling organisms of the Indian Ocean: biology and control technology*. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 31-83
- Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205-226

Relevance of mytilid microtopographies for antifouling defence and invasiveness - a global comparison

Abstract

Among the most important marine invasive species are the bivalves and, in particular, members of the family Mytilidae. We hypothesise that the success of certain mytilid species as invasives may not only be controlled by to their physiological adaptability, but also due anti-fouling characteristics of their shells. Field experiments were conducted at eight different sites in the northern and southern hemisphere to investigate the antifouling performance of the microtopographies of four mytilid species from different regional provenances when subjected to natural fouling. Repellent tendencies of the microtopography have been detected after 3 weeks. However, all investigated microtopographies failed to prevent fouling in a persistent manner when exposed to various fouling communities on a global scale and when decoupled from other shell properties. The invasive success of Mytilidae all over the world can therefore not be explained solely by a particularly efficient and broad-target antifouling performance of surface structure. We suspect that recently discovered chemical anti-microfouling properties of the periostracum complement the anti-macrofouling defence by shell microtopography.

Introduction

Invasive alien species can alter ecosystem functioning by suppressing or even displacing native species (Carlton 1999, Ruiz et al. 1999, Mack et al. 2000, Branch & Steffani 2004). Shipping is associated with the transport and release of ballast water and ship hull fouling, acting as a vector for larvae, spores and adult organisms (Carlton & Geller 1993, Gollasch 2002), and recent expansion of shipping routes has led to an increased dispersal of non-indigenous species. Similarly aquaculture, the aquarium trade and man-made canals and water ways all favour the introduction of species to new habitats (Carlton 1999, Branch & Steffani 2004, Streftaris et al. 2005).

Bivalves of the family Mytilidae are widely distributed in the inter- and subtidal all over the world. They are considered as key species and important habitat engineers in benthic communities. Mytilidae possess high productivity, high fecundity and wide ecological tolerance that allow them to adapt to various environments (Branch & Steffani 2004), highlighting their invasive potential. Since the 19th century, the northern hemisphere mytilids *Mytilus edulis* and *M. galloprovincialis* have spread far beyond their native ranges, and now populate the east coast of South America, the South Pacific Ocean (Carlton 1999), the northern Pacific coast (McDonald & Koehn 1988, Wonham 2004) and the South Atlantic. *M. galloprovincialis* invaded the South African shores in the mid-1970s (Griffiths et al. 1992), and now occupies the entire west coast of South Africa and the southern half of Namibia, with a spreading by as much as 115 km/yr (Branch & Steffani 2004). Other mytilids have been equally invasive: *Perna perna*, a southern hemisphere species, and *P. viridis*, an Indo-Pacific species have recently been found in the Gulf of Mexico (Hicks & Tunnell 1993, Hicks et al. 2001, Ingrao et al. 2001). In Brazil, *P. perna* has recently been classified as an old introduction, most likely dating from the 16th century (Souza et al. 2004). The Asian mytilid *Musculista senhousia* has been introduced to Australasia, the Pacific coast of North America and the Mediterranean, where it substantially changes the habitat complexity (Crooks & Khim 1999). The north-eastern Pacific mytilid *Mytilus trossulus* has not yet populated shores in the southern hemisphere, possibly being competitively inferior to the earlier invaders *M. edulis* and *M. galloprovincialis* (Carlton 1999).

Being liberated from parasites, predators and epibionts may facilitate invasive success (Colautti et al. 2004). We suspect that the success of certain mytilid species as invasives may not only be due to their physiological adaptability, but also due to (globally efficient) anti-fouling characteristics of their shells. As for all hard-shelled marine organisms, mytilids offer a potential settlement substratum for larval stages of invertebrates and for algal propagules. Being overgrown by epibionts may affect host organisms directly, by increasing drag, interfering with filter feeding, or indirectly by modulating ecological interactions (Lesser et

al. 1992, Wahl & Hay 1995, Wahl 1997a). Because epibiosis effects are often negative, many invertebrates and algae evolve antifouling defence mechanisms (Wahl 1997b). Yet, mytilid shells often remain remarkably free of epibionts, and defence mechanisms have been suggested in previous studies. One proposed defence is a distinct, ribbed microtopography on the periostracum that repels barnacle cyprid larvae (Wahl et al. 1998, Scardino et al. 2003, Bers et al. 2006b). We (Bers et al. (2006b)) have previously shown that there are regional differences in this microtopography and its effectiveness in preventing fouling. Yet, biogeographical differences in defence systems of marine invertebrates have been little studied (Becerro et al. 2003). Since the prevention of epibiosis is vital for the performance of most organisms, an antifouling defence system with an efficiency that is not regionally restricted would favour the invasion of new areas. Whether the mussel microstructure is specific for a certain fouling community, or, in contrast, universally efficient is of particular interest in the case of the Mytilidae which - as notorious hull foulers - are increasingly imported into new areas as a consequence of intensifying maritime traffic and the ban of highly toxic antifoulants (Champ 2000, Minchin & Gollasch 2003).

The aim of this study was to investigate the performance of the microtopographies of various mytilid species from different regional provenances when subjected to natural fouling in different biogeographic regions.

Material & Methods

Study Organisms

Four mytilid species from eight different regional provenances were used in this study:

1. *Mytilus edulis* from Kiel Fiord in the Western Baltic, Germany (54°22'N, 10°9'E), from Matryonin Island in the Kandalakshsky Gulf of the White Sea, Russia (66°18'N, 33°40'E), from Toothacker Cove on Swan's Island, Maine, USA (44°10'N, 68°25'W) and from Hartlepool Marina in the North East of England (54°41'N, 1°11'W).
2. *Perna perna* individuals from Itaipu Beach, Rio de Janeiro State, Brazil (22°56'S, 43°03'W) and from Bushman's, South Africa (33°42' S, 26°40'E).
3. *Perna viridis* from Long Harbour, South China Sea, Hong Kong (22°27'N, 114°21'E).
4. *Mytilus galloprovincialis planatus* from Townsville Breakwater Marina, Australia (19°15'S, 146°50'E).

Replication of Mussel Shells

To investigate the influence of mytilid surface topography on epibiosis separately from other possible anti-settlement properties such as chemical defences, high resolution resin replicates of the species were made (see Bers & Wahl 2004 and Bers et al. 2006b for details).

Smooth control surfaces were made by sealing a second resin replicate of the same individual shell with an additional layer of the epoxy resin. Rough control surfaces were made by sanding a third resin replicate of the same individual with a Dremel[®] rotary tool and sanding bands (no. 408/Grit 60) to provide standardised roughness. Thus, the surfaces of all replicates of each shell were identical in every respect (size, shape, colour, chemistry) except surface microtopography. All resin replicates were checked for the presence of micro-bubbles under a dissecting microscope, and poor replicates were discarded. The casts proved to be robust, non-toxic and highly accurate and therefore suitable for use in replicated experimental designs.

Field experiments

Eight individual mussels of each regional provenance were replicated as described above. The three casts of each individual mussel featuring the surface topographies 'natural', 'smooth' or 'rough' were combined as triplets and distributed to the experimental sites, so that at all sites the same set of eight mussel replicate triplets was exposed to fouling *in situ*. All mussel replicates were submerged at 1 m water depth, either hanging from piers or on floating buoys.

Identical experiments were conducted at eight sites (Germany, USA, Brazil, Chile, UK, Hong Kong, Australia and South Africa) in their respective summer season during 2004/2005 for six weeks (Table 1). *Mytilus edulis* from the USA were only tested at sites in the Northern Hemisphere, and at the US site they were only replicated 4 times due to space limitations. The field sites were all enclosed, sheltered bays or marinas, and therefore protected from major wave action.

Abundances of dominant (>1%) fouling organisms were checked weekly, by estimating percentage coverage under a dissecting microscope or by analysing digital photograph of the resin mussels using ImageTool 3.0. Only a central 1 cm² of each cast was used for the estimates to avoid possible edge effects. All replicates were re-deployed after counting. The abundances of fouling organisms on the microstructured surfaces were compared to those on the corresponding control surfaces.

Table 1. Details of the experimental sites

Country	Australia	Brazil	Chile	Germany	Hong Kong	South Africa	UK	USA
Longitude	146°50'E	43°08'W	71°22' W	10°9'E	114°21'E	26°40'E	1°11'W	68°25'W
Latitude	19°15'S	22°02' S	29°58' S	54°22'N	22°27'N	33°42' S	54°41'N	44°10'N
Start of experiment	29.09.2004	29.10.2004	12.10.2004	23.05.2005	14.05.2005	11.10.2004	06.05.2005	16.06.2005
End of experiment	10.11.2004	11.12.2004	26.11.2004	04.07.2005	25.06.2005	22.11.2004	19.06.2005	31.07.2005
Sea surface temperature (°C)	24 - 28	24 - 27	19 - 21	12-16	23 - 27	14 - 20	15-20	n/a
Tidal amplitude (m)	3.27	1.2	1.3-1.5	0.2	0.5 - 2.3	2	0	n/a
Species richness (with >1% cover)	6	8	5	8	4	4	4	4
Salinity [psu]	35.5-36	24 -34	34	12-17	20-35	35	35-38	n/a

Surface Characterisation

The surface topography of mussel shells from each species was visualized by Scanning Electron Microscopy (SEM). The shells were sputter coated with a 20 nm thick gold – palladium alloy using a Balzer Union SCD 004 instrument and viewed with a Zeiss DSM 940 Digital Scanning Microscope (Figure 1).

Statistical Analysis

Data for settlement after three and six weeks were analysed to determine the influence of time within the duration of the experiment. In order to compare settlement effects between experimental sites with very highly differing fouling communities (see Table 2) and between sampling dates, a meta-analysis based on the ln effect ratio was used to assess the relative and overall impact of surface microtopography on fouling (Gurevitch & Hedges 2001):

$$\ln \text{ effect ratio} = \ln \left(\frac{\text{total \% cover of recruits (natural surface structure)}}{\text{total \% cover of recruits (rough control)}} \right) \text{ and}$$

$$\ln \text{ effect ratio} = \ln \left(\frac{\text{total \% cover of recruits (natural surface structure)}}{\text{total \% cover of recruits (smooth control)}} \right), \text{ respectively.}$$

A non-overlap of confidence intervals of the ln effect ratios indicate significant differences or effects (level of significance $\alpha = 0.05$).

In addition, a Chi²-test was used to check whether the scatter of non-significant effects around zero was random, or whether either fouling-enhancing or fouling-reducing trends were more frequent than expected from an equal distribution.

Results

All Mytilidae used in this study possess a distinct microtopography of the periostracum. This surface structure consists of micro ripples with a width of 1-5 μm (Figure 1), running more or less at right angle to the growth rings. The microtopography of *M. edulis* exhibits a rather flat surface with micro ripples (1.5 – 2 μm), varying slightly at the 4 different sites. *M. galloprovincialis planatus*' microtopography is similar to its congeners. *P. perna* also features microripples, but on a less flat general surface, apparently forming larger sets of hills and valleys composed of smaller ripples; *P. viridis*, however, has a surface where sets of hills and valleys are accentuated, and ripples are attenuated. In the latter, the ripples are bigger ($\sim 5 \mu\text{m}$) than in the other species.

Settlement was highly variable with regard to macrofoulers (Table 2), however, in all instances the resin casts were rapidly covered by a thick biofilm. The total percentage cover only refers to macrofouling species, and both biofilm and benthic diatoms were excluded from the analysis. Due to technical problems, data from the German experimental site are missing for week 3, from the experimental sites in South Africa and the UK for week 6.

For all tested mytilid species at all experimental sites the general picture was similar: the microtopography did not have a persistent significant effect – either repellent or attractive – in comparison to the rough and smooth controls. In the few cases where a significant effect was found, natural microtopography almost exclusively reduced fouling:

(a) after 3 weeks exposure (Figure 2)

At the US site, the natural surface structure of the UK *M. edulis* significantly reduced fouling when compared to the rough control.

At the Hong Kong site, the natural microtopography of the autochthonous *P. viridis* significantly reduced fouling when compared to both rough and smooth control.

At the South African site, the natural surface structure of the German *M. edulis* reduced fouling in comparison to smooth and rough controls, while at the Hong Kong site the microtopography was less fouled than the smooth control.

The natural microtopography of *M. galloprovincialis planatus* of Australia performed best in Hong Kong, where it was significantly less fouled than both smooth and rough controls.

Table 2: Fouling species and their abundance at the different sites: +++ = very abundant, ++ = abundant, + = present, ○ = occasionally. Field site codes: AUS = Townsville, Australia, BRA = Mocangüê Island, Brazil, GER = Kiel, Germany, HK = Hong Kong, UK = Hartlepool, England, USA = Swan's Island, USA, ZA = Bushman's, South Africa; CHL = Bahía de Herradura, Chile

Fouling species	AUS	BR	CHL	GER	HK	UK	USA	SA
CHLOROPHYTA								
<i>Ulva linza</i>	○	++	+++	+	++			○
<i>Ulva fasciata</i>	○	+	○		+			
<i>Cladophora vagabunda</i>	○	+	○					
<i>Spongomorpha</i> sp.							+	
RHODOPHYTA								
<i>Polysiphonia</i> sp.			+++					
PHAEOPHYTA								
<i>Desmarestia viridis</i>							+++	
<i>Ectocarpus siliculosus</i>						++		
CILIATA								
<i>Folliculina</i> sp.			+					
ARTHROPODA								
<i>Amphibalanus amphitrite</i>	+				○			
<i>Balanus improvisus</i>				++				
<i>Austromegabalanus psittacus</i>			++					
ASCIDIA								
<i>Botrylloides nigrum</i>		+						
<i>Botrylloides leachi</i>	+					○		
<i>Bugula neritina</i>		+++						+
<i>Styela plicata</i>		++						
<i>Ascidia</i> sp.	○							
<i>Diplosoma listeranium</i>	++							
BRYOZOA								
<i>Watersipora subtorquata</i>	+							
<i>Schizoporella</i> sp.					+			
POLYCHAETA								
<i>Hydroides elegans</i>	++				++			
Serpulidae		+						
Spionidae		+++						
<i>Spirorbis spirorbis</i>					+			
HYDROZOA								
<i>Obelia dichotoma</i>		+++						
<i>Obelia</i> sp.			++		○			
BIVALVIA								
<i>Mytilus edulis</i>				++				
ANTHOZOA								
<i>Metridium senile</i>							○	

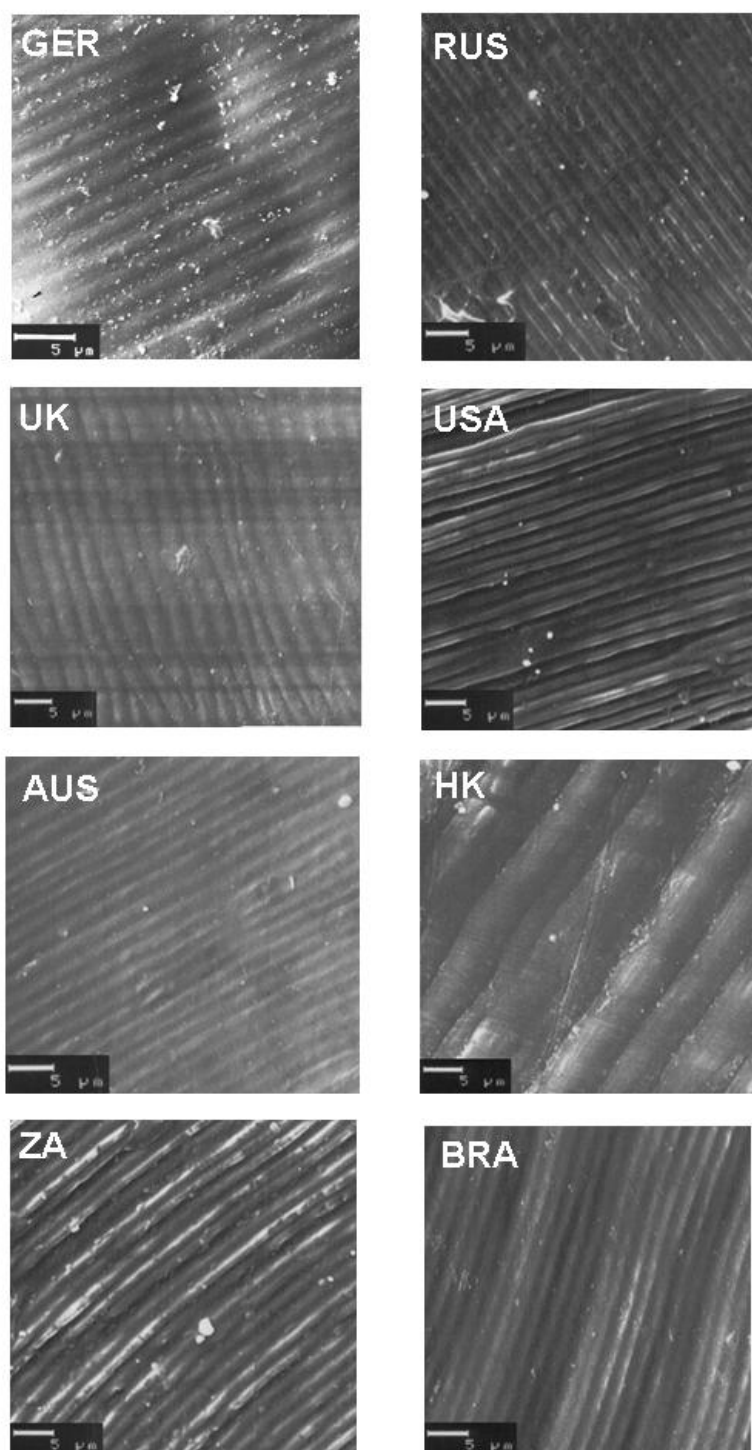


Figure 1. Scanning electron micrographs of the microtopographies of all investigated Mytilidae: GER = *M. edulis* from Germany, RUS = *M. edulis* from Russia, UK = *M. edulis* from the UK, USA = *M. edulis* from the US, AUS = *M. galloprovincialis planatus* from Australia, HK = *P. viridis* from Hong Kong, ZA = *P. perna* from South Africa and BRA = *P. perna* from Brazil.

(b) after 6 weeks exposure (Figure 3)

At the US site, the better performance of the UK *M. edulis* natural microtopography in comparison to the rough control persisted after six weeks, while all other effects detected after 3 weeks vanished. However, at the German field site, fouling on the natural surface structure of the South African *P. perna* was significantly lower than on the smooth and rough control surfaces. At the Brazilian field site, the natural microtopography of *M. galloprovincialis planatus* from Australia was marginally significantly less fouled than the rough control.

Minor attractive effect of natural microtopographies are found after 3 weeks at the Brazilian experimental site for both autochthonous and allochthonous *Perna perna*. The natural surface topography of the German *M. edulis* were rather attractive in comparison to the smooth control at the Chilean site, and the same is true for *M. edulis* from the UK at the Australian site.

After 6 weeks, the microtopography of the Russian *M. edulis* is more attractive than the smooth control at the Australian site, whereas the natural surface structure of *M. edulis* is significantly more fouled than the rough control at the Brazilian field site.

The Chi²-test showed that overall there were significantly more repellent tendencies and/or effects after three weeks (Chi² = 4.313, df = 1, p < 0.038) than attributable to chance, a trend not longer detected after 6 weeks (Chi² = 0.036, df = 1, p > 0.1).

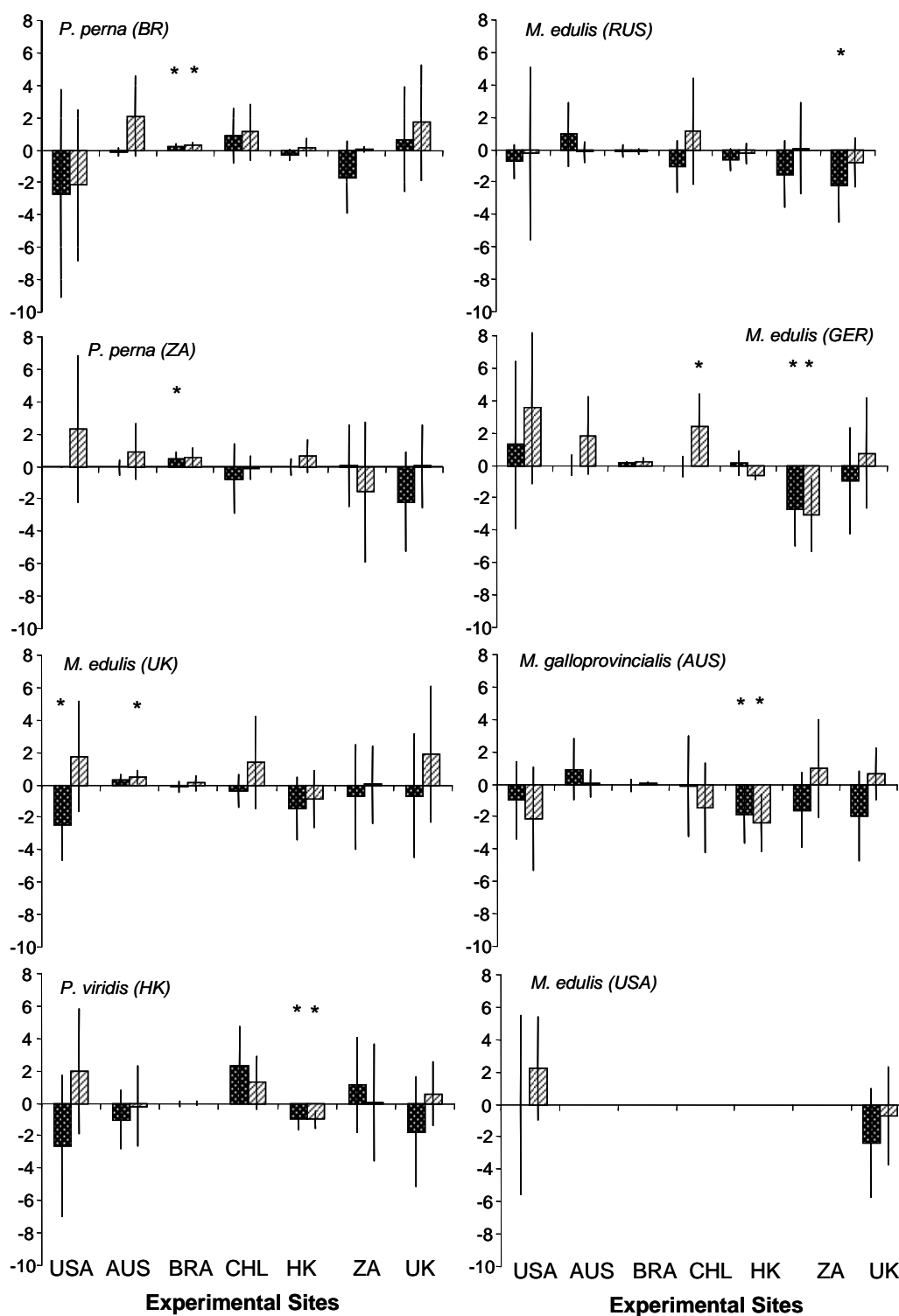


Figure 2. Fouling on natural microtopographies versus rough and smooth controls, using a ln effect ratio, after 3 weeks exposure to natural fouling communities. Error bars indicate the 95% confidence interval. \blacksquare = $\ln(\text{total \% cover on natural microtopography} / \text{total \% cover on rough control})$; \square = $\ln(\text{total \% cover on natural microtopography} / \text{total \% cover on smooth control})$. Experimental sites: USA = Swan's Island, USA; AUS = Townsville, Australia; BRA = Mocanguê Island, Brazil; CHL = Bahía de Herradura, Chile; HK = Hong Kong, ZA = Bushman's, South Africa, UK = Hartlepool, UK; stars indicate significant values.

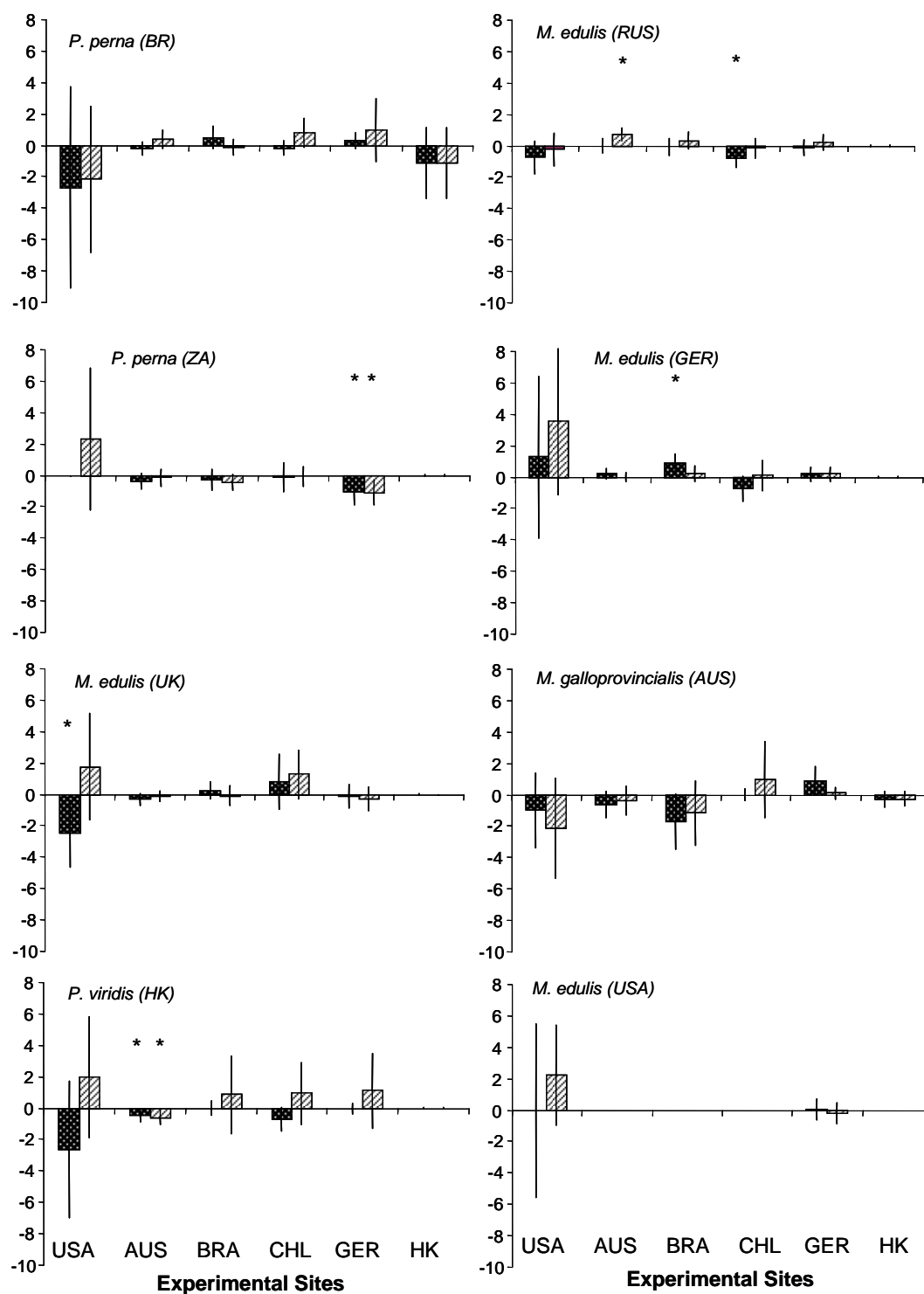


Figure 3. Fouling on natural microtopographies versus rough and smooth control, using a ln effect ratio, after 6 weeks exposure to natural fouling communities. Error bars indicate the 95% confidence interval. \blacksquare = \ln (total % cover on natural microtopography / total % cover on rough control); \square = \ln (total % cover on natural microtopography / total % cover on smooth control). Experimental sites: USA = Swan's Island, USA; AUS = Townsville, Australia; BRA = Mocanguê Island, Brazil; CHL = Bahía de Herradura, Chile; GER = Kiel fiord, Germany, HK = Hong Kong; stars indicate significant values.

Discussion

This study explored for the first time the performance of the shell microtopography of different mytilid species, facing fouling pressure in different bio-geographic regions. It was hypothesised that globally efficient antifouling properties of the shell would contribute to the invasive success of mytilid bivalves, as not being overgrown would be an advantage in populating new habitats.

All resin casts at all field sites, regardless of surface structure (roughened, smooth or naturally textured) were rapidly covered with a thick biofilm. Fouling micro-organisms fall into the size range of the micro ripples, which might offer multiple attachment points and therefore favour bacterial and diatom settlement (Scardino et al. 2006). The performance of a given surface texture is therefore dependent on the relation between surface rugosity and size of potential fouling organisms: microtopographies within the scale of microfoulers will be attractive, offering multiple attachment points and micro-refuges (Verran & Boyd 2001, Callow et al. 2002, Granhag et al. 2004), while the same structure may be unfavourable for macrofoulers (Bers et al. 2006b) due to fewer attachment points. It is furthermore possible that the bacteria and diatoms gradually fill up the mytilid microtopographies and masking them for macrofouling organisms: the Chi² test showed that during the first 3 weeks there were predominantly repulsive effects of the surface microtopography, but that these gradually disappeared during the following 3 weeks in the field. If there are repulsive effects, they are restricted to a relatively early period of colonisation and diminish afterwards. This supports the idea of the microtopographic ripples being filled up with biofilm, and is coherent with the findings of Scardino et al. (2003), Bers & Wahl (2004) and Scardino & de Nys (2004), who also described the temporary nature of antifouling effects of natural microtopographies. Though, we only studied the performance of the topographies at the initial stages (six weeks) of fouling. It may be possible that the microtopography interferes with the attachment strength of certain foulers. Once they exceed a certain size, they may be easily shoved off by grazers at a later stage or current shear stress. The invasive success of Mytilidae all over the world, however, cannot be explained by the antifouling performance of surface structure alone, since all investigated microtopographies failed to prevent fouling in a persistent manner when exposed to various fouling communities on a global scale and when decoupled from other shell properties. Consequently, shell microtopography alone does not seem to contribute substantially to the successful establishment of invasive Mytilidae in a new habitat.

Living mussels with intact periostracum, however, are well known to maintain a fouling-free surface over months to years. Hence, one or more additional mechanisms must be responsible for maintaining a clean shell over a long time periods. The surface chemistry of *Mytilus edulis* has been recently investigated (Bers et al. 2006a), revealing that the periostracum contains

several compounds that not only inhibit attachment of bacteria and diatoms, but also slow down diatom growth and prevent bacterial growth. It can therefore be argued that *Mytilus* is able to maintain a relatively biofilm-free surface with chemical defence mechanisms, while the surface microtopography repels macrofouling organisms, and that both mechanisms may act complementarily. The observation that on the resin casts featuring mechanical but not chemical defence components, defence weakens in the course of a few weeks is indicative of the masking effect of a developing biofilm.

The ecologically complex interactions of basi- and epibionts and/or predators may require a multi-level defence system with chemical, physical and mechanical mechanisms (Krug 2006). These natural defence systems with two or more synergistic mechanisms have been studied before. In the gorgonians *Leptogorgia* and *Pterogorgia* toxic secondary compounds slow down surface fouling and are combined with a periodical ablation of the outer surface (Targett et al. 1983). The ascidian *Polysyncraton lacazei* possesses one mechanical, one associational and four chemical mechanisms, targeting various types of fouling organism (Wahl & Banaigs 1991), and in four Caribbean sponges (*Agelas clathrodes*, *Cinachyrella alloclada*, *Clathria virgultosa* and *Xestospongia muta*) a chemical defence acts synergistically with glass spicules against generalist fish predators (Jones et al. 2005).

Another focus of this study was the regional optimisation of antifouling defence. If mussels adapt the microtopography of their periostracum to repel local coloniser species efficiently, then we would expect reduced defence performance when these mussels are faced with a different colonizer pool. Thus, allochthonous mussels should foul more intensively than autochthonous mussels. The inverse prediction applies when one expects that coloniser species have adapted to the microtopographies of naturally co-occurring mussels. Regional adaptations of mussels to local fouling organisms have not been detected in the present study; there were no obvious differences in how autochthonous and allochthonous species performed. It would have also been reasonable to expect that there was a higher pressure of epibiosis at lower latitudes, according to the latitudinal hypothesis (Gray 2001) in which tropical sites would have more fouling pressure (as a consequence of higher species diversity and warmer temperature) than temperate or cold sites; if this was true, we would expect mussels from warmer waters to perform better, which does not seem to be the case in our study. The question of regional optimisation, however, should be re-examined by taking into account more complete natural antifouling systems that combine both chemistry and microtopography in this kind of global comparison.

References

- Becerro MA, Thacker RW, Turon X, Uriz MJ, Paul VJ (2003) Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses. *Oecologia* 135:91-101
- Bers AV, D'Souza F, Klijnsma JW, Willemsen PR, Wahl M (2006a) Chemical defence in mussels: antifouling effect of crude extracts of the periostracum of the blue mussel *Mytilus edulis*. *Biofouling* 22:251-259
- Bers AV, Prendergast GS, Zürn CM, Head RM, Hansson L, Thomason JC (2006b) A comparative study of the anti-settlement potential of mytilid shells. *Biology Letters* 2:88-91
- Bers AV, Wahl M (2004) The influence of natural surface microtopographies on fouling. *Biofouling* 20:43-51
- Branch GM, Steffani CN (2004) Can we predict the effects of alien species? A case-history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck). *Journal of Experimental Marine Biology and Ecology* 300:189-215
- Callow ME, Jennings AR, Brennan AB, Seegert CE, Gibson A, Wilson L, Feinberg A, Baney R, Callow JA (2002) Microtopographic cues for settlement of zoospores of the green fouling alga *Enteromorpha*. *Biofouling* 18:237-245
- Carlton JT (1999) Molluscan invasions in marine and estuarine communities. *Malacologia* 41:439-454
- Carlton JT, Geller JB (1993) Ecological roulette: the global transport of nonindigenous marine organisms. *Science* 261:78-82
- Champ MA (2000) A review of organotin regulatory strategies, pending actions, related costs and benefits. *The Science of the Total Environment* 258:21-71
- Colautti RI, Ricciardi A, Grigorovich IA, Maclsaac HJ (2004) Is invasion success explained by the enemy release hypothesis? *Ecology Letters* 7:721-733
- Crooks JA, Khim HS (1999) Architectural vs. biological effects of a habitat-altering, exotic mussel, *Musculista senhousia*. *Journal of Experimental Marine Biology and Ecology* 240:53-75
- Gollasch S (2002) The importance of ship hull fouling as a vector of species introductions into the North Sea. *Biofouling* 18:105-121
- Granhag LM, Finlay JA, Jonsson PR, Callow JA, Callow ME (2004) Roughness-dependent removal of settled spores of the green alga *Ulva* (syn. *Enteromorpha*) exposed to hydrodynamic forces from a water jet. *Biofouling* 20:117-122
- Gray JS (2001) Marine diversity: the paradigms in patterns of species richness examined. *Scientia Marina* 65:41-56
- Griffiths CL, Hockey PAR, van Erkom Schurink C, Le Roux PJ (1992) Marine invasive aliens on South African shores: implications for community structure and trophic functioning. *South African Journal of Marine Sciences* 12:713-722
- Gurevitch J, Hedges LV (2001) Meta-Analysis: combining the results of independent experiments. In: Scheiner SM, Gurevitch J (eds) *Design and analysis of ecological experiments*. Oxford University Press, New York, p 347-370
- Hicks DW, Tunnell Jr JW, McMahon RF (2001) Population dynamics of the nonindigenous brown mussel *Perna perna* in the Gulf of Mexico compared to other world-wide populations. *Marine Ecology Progress Series* 211:181-192
- Hicks DW, Tunnell JW (1993) Invasion of the south Texas coast by the edible brown mussel *Perna perna* (Linnaeus 1758). *Veliger* 36:92-94
- Ingrao D, Mikkelsen PM, Hicks DW (2001) Another introduced marine mollusk in the Gulf of Mexico: the Indo-Pacific green mussel, *Perna viridis*, in Tampa Bay, Florida. *Journal of Shellfish Research* 20:13-19
- Jones AC, Blum JE, Pawlik JR (2005) Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? *Journal of Experimental Marine Biology and Ecology* 322:67-81

- Krug PJ (2006) Defense of benthic invertebrates against surface colonization by larvae: a chemical arms race. In: Fusetani N, Clare AS (eds) Antifouling compounds. Springer, Berlin, p 1-53
- Lesser MP, Shumway SE, Cucci T, Smith J (1992) Impact of fouling organisms on mussel rope culture: interspecific competition for food among suspension-feeding invertebrates. *Journal of Experimental Marine Biology and Ecology* 165:91-102
- Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, Bazzaz FA (2000) Biotic invasions: causes, epidemiology, global consequences, and control. *Ecological Applications* 10:689-710
- McDonald JH, Koehn RK (1988) The mussel *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. *Marine Biology* 99:111-118
- Minchin D, Gollasch S (2003) Fouling on ships' hulls: how changing circumstances and spawning events may result in the spread of exotic species. *Biofouling* 19:111-122
- Ruiz GM, Fofonoff P, Hines AH (1999) Non-indigenous species as stressors in estuarine and marine communities: assessing invasion impacts and interactions. *Limnology and Oceanography* 44:950-972
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctata imbricata*. *Biofouling* 19 (Supplement):221-230
- Scardino AJ, de Nys R (2004) Fouling deterrence on the bivalve shell *Mytilus galloprovincialis*: a physical phenomenon? *Biofouling* 20:249-257
- Scardino AJ, Harvey E, de Nys R (2006) Testing attachment point theory: diatom attachment on microtextured polyimide biomimics. *Biofouling* 22:55-60
- Souza RCCL, Fernandes FC, Silva EP (2004) Distribuição atual do mexilhão *Perna perna* no mundo: um caso recente de bioinvasão. In: Silva JSV, Souza RCCL (eds) Água de lastro e bioinvasão. Editora Interciência, Rio de Janeiro, RJ, p 157-172
- Streftaris N, Zenetos A, Papathanassiou E (2005) Globalisation in marine ecosystems: the story of non-indigenous marine species across European seas. *Oceanography and Marine Biology: an annual review* 43:419-453
- Targett NM, Bishop SS, McConnell OJ, Yoder JA (1983) Antifouling agents against the benthic marine diatom, *Navicula salinicola*: homarine from the gorgonians *Leptogorgia virgulata* and *L. setacea* and analogs. *Journal of Chemical Ecology* 9:817-829
- Verran J, Boyd RD (2001) The relationship between substratum surface roughness and microbiological and organic soiling: a review. *Biofouling* 17:59-71
- Wahl M (1997a) Increased drag reduces growth of snails: comparison of flume and *in situ* experiments. *Marine Ecology Progress Series* 151:291-293
- Wahl M (1997b) Living attached: aufwuchs, fouling, epibiosis. In: Nagabushanam R, Thompson M (eds) Fouling organisms of the Indian Ocean: biology and control technology. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 31-83
- Wahl M, Banaigs B (1991) Marine epibiosis III: Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). *Journal of Experimental Marine Biology and Ecology* 145:49-63
- Wahl M, Hay ME (1995) Associational resistance and shared doom: effects of epibiosis on herbivory. *Oecologia* 102:329-340
- Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205-226
- Wonham MJ (2004) Mini-Review: Distribution of the Mediterranean mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae) and hybrids in the Northeast Pacific. *Journal of Shellfish Research* 23:535-543

Chemical defence in mussels: antifouling effect of crude extracts of the periostracum of the blue mussel *Mytilus edulis*

Abstract

Shells of the blue mussel *Mytilus edulis* remain free of fouling organisms as long as they possess an intact periostracum, and a multiple antifouling defence that comprises a ripple-like microtopography and the production of chemical antifouling compounds has been suggested previously. This study investigates the chemical defence strategy of blue mussels for the first time. Six crude extracts of the periostracum of intact shells were made using solvents of increasing polarity. These extracts were tested against common fouling organisms in laboratory based bioassays. Non-polar and moderately polar fractions showed the highest activities: the diethyl ether fraction strongly inhibited attachment of *Balanus amphitrite* cyprids and the marine bacteria *Cobetia marina* and *Marinobacter hydrocarbonoclasticus*. Attachment of the benthic diatom *Amphora coffeaeformis* was significantly reduced by the dichloromethane extract, whereas both ethyl acetate and diethyl ether fractions slowed diatom growth. These results provide the first evidence of surface bound compounds that may moderate surface colonisation.

Introduction

Marine organisms face the same intense fouling pressure as any other submerged surface. Bacteria, diatoms and unicellular organisms initially settle on surfaces, forming a complex biofilm community (Lappin-Scott & Costerton 1989, Davey & O'Toole 2000), which may subsequently affect the settlement and attachment of micro- and macrofoulers (Kirchmann & Mitchell 1983, Wahl 1997, Wieczorek & Todd 1998, Maki & Mitchell 2002, Fusetani 2004). For algae and invertebrates, being overgrown is generally disadvantageous, leading to increased weight and surface friction, reduced elasticity and lower sensory performance, hindered gas exchange and enhanced susceptibility to predation (Wahl 1989, McKenzie & Grigolava 1996) and therefore reduced survival. Hence, defence mechanisms of body surfaces are common amongst marine organisms. Defence strategies consist of physical, chemical or ecological mechanisms, singly or in combination, in order to target a wide range of fouling organisms (Wahl 1997). Chemical defence mechanisms of marine organisms have been investigated more closely in the past decade in order to find possible alternatives to current commercial antifouling paints. A wide range of marine natural products has been screened for their activity with regard to antimicrobial, antifungal, antialgal and antilarval activities (reviewed by Clare, 1996; Fusetani, 2004; Dobretsov et al. 2006). Compounds with antifouling potential have been studied intensely in various marine sponges (Tsoukatou et al. 2002, Hellio et al. 2005) and algae (de Nys et al. 1995, Maximilien et al. 1998). Potential chemical defence mechanisms of molluscs in general, and of bivalves specifically, have been rarely studied and appear to function as general protection mechanisms against predators or to increase recruitment and fertilisation success (reviewed by Paul et al. 2006). For example, whole body extracts and extracts of opercula, egg masses and digestive glands of various gastropod species have been screened for antimicrobial activity (Benkendorff et al. 2001, Ramasamy & Murugan 2005) and antimicrobial peptides of bivalves have been characterised and purified (Mitta et al. 2000a, Mitta et al. 2000b). However, this type of defence is related more to innate immunity against molluscan pathogens than to the prevention of epibiosis. Moreover, the chemically active substances investigated are generally found within the body tissue, whereas antifouling defences need to be located at or released from the surface of the organisms (Steinberg et al. 2001). Shell surface bound metabolites of molluscs, however, have not been investigated so far. The blue mussel *Mytilus edulis* (L.) is a non-burrowing, epibenthic species with a world-wide distribution that often occurs in high abundance, thereby offering a valuable potential settlement substrate for both micro- and macrofoulers. However, a considerable proportion of *M. edulis* shells remains free of fouling organisms. Active cleaning of the shell by the swiping movements of the mussel's foot across the shell do not explain the reduction of surface fouling in this species because this cleaning mechanism is only possible for individuals < 3 cm in length (Theisen 1972). Thus, a multi-level antifouling defence of the mussel, consisting of cumulative filtration, a distinct microtopography and chemical repellents in the periostracum have been suggested. While the first two mechanisms have

been identified (Wahl et al. 1998, Bers et al. 2006), it remains unknown whether *M. edulis* possesses chemical mechanisms that might protect their shells from being overgrown.

The aim of this study was to investigate the antifouling potential of crude extracts of *M. edulis* periostracum against representatives of major fouling groups such as bacteria, diatoms and barnacles.

Material & Methods

Unfouled specimens of *M. edulis* (~5 cm shell length, n = 103) were collected from a mussel bed in Kiel Fiord, Western Baltic (54°19'N, 10°09'E). The soft body was completely removed from the shells. The outside of the valves was cleaned gently with a soft toothbrush and filtered seawater to remove debris and microfouling, then frozen at -20°C. Later, shells were freeze dried and consecutively extracted at room temperature for 1 h with petrol ether (extract 1), followed by hexane (extract 2), dichloromethane (extract 3), diethyl ether (extract 4), ethyl acetate (extract 5) and methanol (extract 6) at room temperature. The six resulting crude extracts (abbreviated with E 1-6) were reduced *in vacuo*. Stock solutions in dimethylsulphoxide (DMSO) of 10 000 ppm ($\mu\text{g ml}^{-1}$) were diluted subsequently in sterile seawater to test concentrations. The average concentration of the extracts (CE) in the periostracum (see Table 1) was re-calculated in relation to the volume of the periostracum, based on a mean periostracum thickness of 35 μm for *M. edulis* according to literature values (Harper 1997). Calculations were based on the following formulae:

- (i) $V_P = V_M \times (T_P / T_S)$, where V_P = periostracum volume, V_M = volume of mussel shells extracted, T_P = thickness of periostracum, and T_S = mean shell thickness, and
- (ii) $C_E = DW_E / V_P$, where DW_E = dry weight of extract and V_P = periostracum volume extracted.

The volume of the shells was calculated by displacement of water, shell thickness was measured with a manual thickness gauge. Mean shell thickness of the shells used in this study was 0.46 mm \pm 0.089 mm (SD).

Table 1. Amounts of periostracal extracts in six different solvents. Estimations of average concentrations are based on calculations of the periostracum volume. Periostracum thickness was assumed to be 35 μm (Harper 1997).

Solvent	Extract dry weight [mg]	Estimated Concentration [cm^3]
Petrol ether	22,54	1,481
Hexane	7,70	0,506
Dichloromethane	13,45	0,884
Diethyl ether	16,05	1,055
Ethyl acetate	7,83	0,515
Methanol*	73,48	4,829

* Presumably containing substantial amounts of NaCl

In laboratory based bioassays, the antifouling activity of periostracal components was tested against representatives of the major groups of fouling organisms, i.e. sessile invertebrates, algae and bacteria. All tests were carried out at the TNO laboratory in Den Helder, The Netherlands. STATISTICA (v. 6) was used for statistical analysis: for each target organism, bioactivities were assessed by 2-factorial ANOVA (factor 1 = extract, factor 2 = concentration) when assumptions were fulfilled. When homogeneity of variance was not given and could not be achieved by transformation, ANOVA was used nonetheless but with the alpha-level reduced to 0.01 in order to avoid type 1 errors (Underwood 1981, 1997, Glasby 1998).

Larval attachment assay

Mass reared cyprid larvae of the barnacle *Balanus amphitrite* were used for the assays. Adults were maintained in containers with vigorous aeration and controlled temperature ($27^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and light:dark conditions (15:9 h). They were fed on a diet of the diatom *Skeletonema costatum* and larvae of the brine shrimp *Artemia salina*. Mass spawned nauplii were collected, transferred to 8 l carboys and fed on *S. costatum*. The vessels were maintained in the same culture conditions as the adults. Larvae reached the cyprid stage after 4 d, and cyprids were matured for 3.5 d at $4\text{-}6^{\circ}\text{C}$ in the dark prior to use in the experiments. Attachment assays were carried out (6 replicates) in untreated polystyrene Greiner® multi-well plates (96 wells, flat bottom). Ten (± 1) cyprids were added to each well containing 130 μl of extract in DMSO or DMSO only control. According to Head et al. (2003), the critical threshold concentration of cyprid density beyond which overcrowding and therefore unreliable results are obtained, is 1 cyprid per 0.005 ml. Concentrations tested in this trial were 100, 30, 10, 3 and 1 ppm. The multi-well plates were incubated for 24 h at a temperature of 28°C under a 15:9 light:dark cycle. The assay was terminated after 24 h, when the plates were gently rinsed with sterile seawater and numbers of attached larvae counted under a dissecting microscope.

Diatom assay

The diatom bioassay determines biocidal activity of the test extracts by assessing the inhibition of microalgal attachment and growth. The benthic diatom *Amphora coffeaeformis*, a common species in benthic microalgal communities, was used for this test. Diatom cultures were maintained in a culture room at 17°C in filter sterilized seawater and silicate enriched F2 medium. Diatoms were cultured for 5 d prior the experiments.

Diatom attachment. Aliquots of 130 μl of diatom cell suspension were placed in untreated polystyrene Greiner® multi-well plates (96 wells, flat bottom); diatom cell suspensions were treated with the 6 extracts to give final concentrations of 100, 30 and 10 ppm and then incubated for 30 min at 25°C on a shaking table to allow the cells to attach to the surface. The plates were then gently rinsed with sterile

seawater to remove unattached diatoms and cell pigment fluorescence was quantified using a TECAN[®] GENios plate reader (software Magellan v 3.0). There were four replicates per treatment. DMSO controls of all concentrations were included, and fluorescence readings were re-calculated with a correction factor (for each extract and each concentration) which compensated for possible effects of auto-fluorescence of the extracts.

Diatom growth. 130 µl of diatom cell suspensions were placed in untreated polystyrene Greiner[®] multiwell plates (96 wells, flat bottom), incubated for 30 min at 25°C on a shaking table to allow cells to attach to the surface. The plates were then gently rinsed with sterile seawater to remove unattached diatoms and cell pigment fluorescence was quantified as before. Test solutions of all 6 extracts were prepared in concentrations of 100, 30, 10, 3 and 1 ppm in F2 medium and 130 ml were added into the well above the settled diatoms. Each concentration was replicated four times. DMSO in sterile filtered natural SW was used as a control. The plates were placed in a growth room at 17°C and growth was monitored over 96 hours.

Bacterial assays

Gram-negative marine bacteria, namely *Marinobacter hydrocarbonoclasticus* (ATCC 49840) and *Cobetia marina* (ATCC 25374) were grown in SPP medium for 18 – 20 h. Cells were washed, and diluted to an optical density (OD) of 0.2 at 595 nm. Bacteria were transferred to a Greiner[®] multi-well plate (96 wells, flat bottom), and crude extracts were added to obtain final concentrations of 100, 50 and 10 ppm. The filled multi-well plates were placed on a shaking table at 28°C and bacteria were allowed to attach for 30 min. Subsequently, the bacterial suspension was removed from the wells. Non-attached cells were removed by gently rinsing with sterile filtered seawater. Cells attached to the well walls were stained with fluorescent DNA-binding Syto-13, and fluorescence was subsequently measured (excitation 485 nm, emission 535 nm) in a TECAN[®] GENios plate reader (software Magellan v 3.0) to determine bacterial biomass as a proxy for bacterial attachment. Each concentration was replicated four times. DMSO controls of corresponding concentrations as well as seawater controls were included in the experiments.

Results

Larval attachment assay

Data were normally distributed (KS test $p > 0.05$) and variances homogenous (Levene's test $p > 0.05$). The two-factorial ANOVA detected significant differences with regard to the extract ($p < 0.001$) and the concentrations used ($p < 0.001$). The post hoc Tukey HSD test (MS = 3.4286, df = 175) revealed that these differences were due to the petrol ether fraction (E 1) and the diethyl ether fraction (E 4). The strength of this effect ('>' = significantly larger with $p < 0.002$, '~' = no significant

difference) depended on concentration: attachment was inhibited by both petrol ether fraction (E 1) and diethyl ether fraction (E 4) with concentrations of 100 ppm > 10 ppm ($p = 0.011$) ~ 3 ppm ($p = 0.006$) ~ 1 ppm ($p = 0.002$) (see Figure 1). There was no detectable influence of DMSO on larval attachment at any concentration.

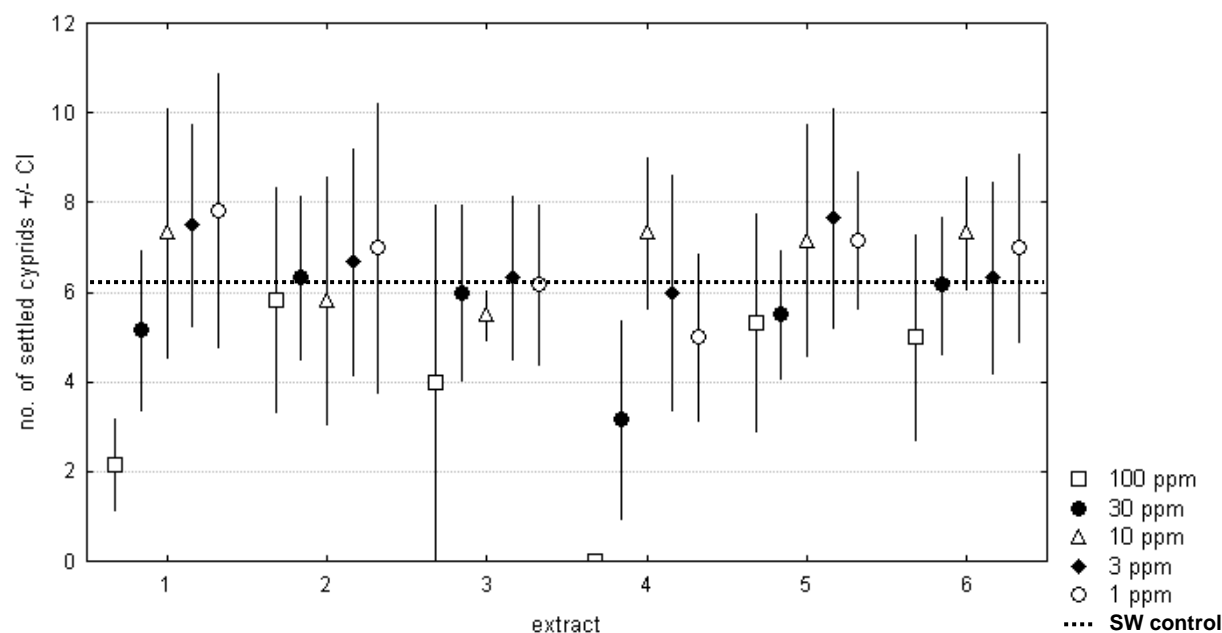


Figure 1. Effect of periostracal extracts on *Balanus amphitrite* cyprid attachment. Error bars indicate the 95% confidence interval. Extract (1) = Petrol ether, (2) = Hexane, (3) = Dichloromethane, (4) = Diethyl ether, (5) = Ethyl acetate, (6) = Methanol; the dotted line represents the mean settlement in the seawater control.

Diatom assays

Diatom attachment. Data were normally distributed (KS test $p > 0.2$), but non-homogenously distributed, so the α -level was reduced to 0.01. The two-factorial ANOVA detected significant differences ($p < 0.001$), and the Tukey HSD post hoc test ($MS = 46.608$, $df = 63$) revealed significant effects of both extract and concentration. No effect of DMSO on diatom attachment was detected. Both the dichloromethane fraction (E 3) and the methanol fraction (E 6) showed an attachment inhibiting activity at 100 ppm when compared to 10 ppm (both $p < 0.001$) (see Figure 2).

Diatom growth. A repeated measures ANOVA was performed to detect the effects of the extracts on diatom growth. The module in STATISTICA (v 6) automatically compensates the non-sphericity (Mauchly's test $p < 0.01$) of data. Data were generally normally distributed, but non-homogenously distributed, so the α -level was reduced to 0.01. The analysis detected a highly significant effect of time ($p < 0.001$), and this effect also interacts with concentration and the type of extract ($p < 0.001$). A Tukey HSD post hoc test was run to identify sources of variances ($MS = 1305.2$, $df = 257.08$). Differences were detected after 48 h (Figure 3): diatom growth was significantly inhibited by the diethyl ether fraction (E 4) at 100 ppm in comparison to 10 ppm ($p = 0.01$), but was only marginal

significant in comparison to 30 ppm ($p = 0.03$). The ethyl acetate fraction (E 5) significantly slows down growth at a concentration of 100 ppm when compared to all other concentration (all $p < 0.005$). For both extracts, the effect first appeared after 24 h and became significant after 48 h but disappeared thereafter (Figure 3).

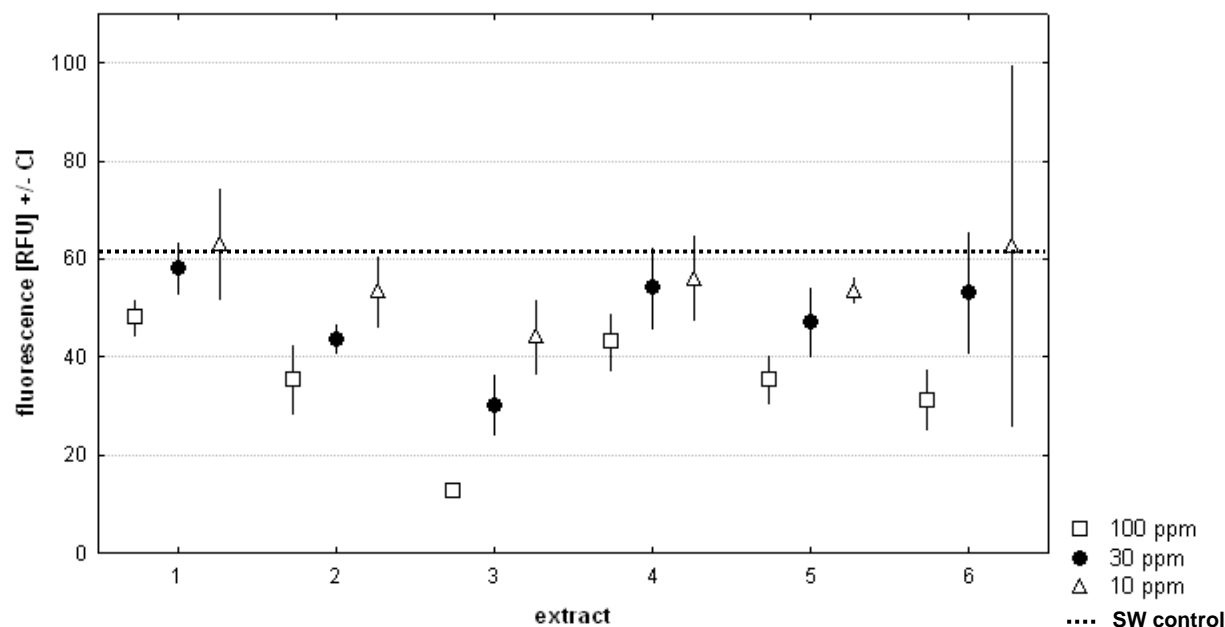


Figure 2. Effect of periostreal extracts on *Amphora coffeaeformis* attachment. Biomass is determined using diatom autofluorescence in relative fluorescence unit (RFU). Error bars indicate the 95% confidence interval. Extract (1) = Petrol ether, (2) = Hexane, (3) = Dichloromethane, (4) = Diethyl ether, (5) = Ethyl acetate, (6) = Methanol; the dotted line represents the mean settlement in the seawater control.

Bacterial assays

In both bacterial trials, no effect of DMSO was detected. Data sets of both experiments were normally distributed (KS test $p > 0.2$).

In the case of *Marinobacter hydrocarbonoclasticus* homogeneity of variances was achieved by square root transformation (Levene's test $p = 0.062$). Data were analysed with a two-factorial ANOVA, which detected a significant effects of extract and concentration ($p < 0.001$) as well as a significant interaction of both ($p < 0.001$). Tukeys HSD post hoc test ($MS = 7.0826$, $df = 72$) revealed significantly reduced bacterial adhesion in three extracts (Figure 4a). The strength of this effect depended on concentration with 100 ppm $>$ 50 ppm \sim 10 ppm in the dichloromethane fraction (E 3) and 100 ppm \sim 50 ppm $>$ 10 ppm for diethyl ether (E 4) and methanol fraction (E 6). All concentrations of the ethyl acetate fraction (E 5) significantly enhanced bacterial adhesion when compared to the DMSO control ($p < 0.001$).

For *Cobetia marina* no transformation achieved homogenous variances. Consequently, the α -level of ANOVA was reduced to 0.01. Two-factorial ANOVA detected significant effects of extract and

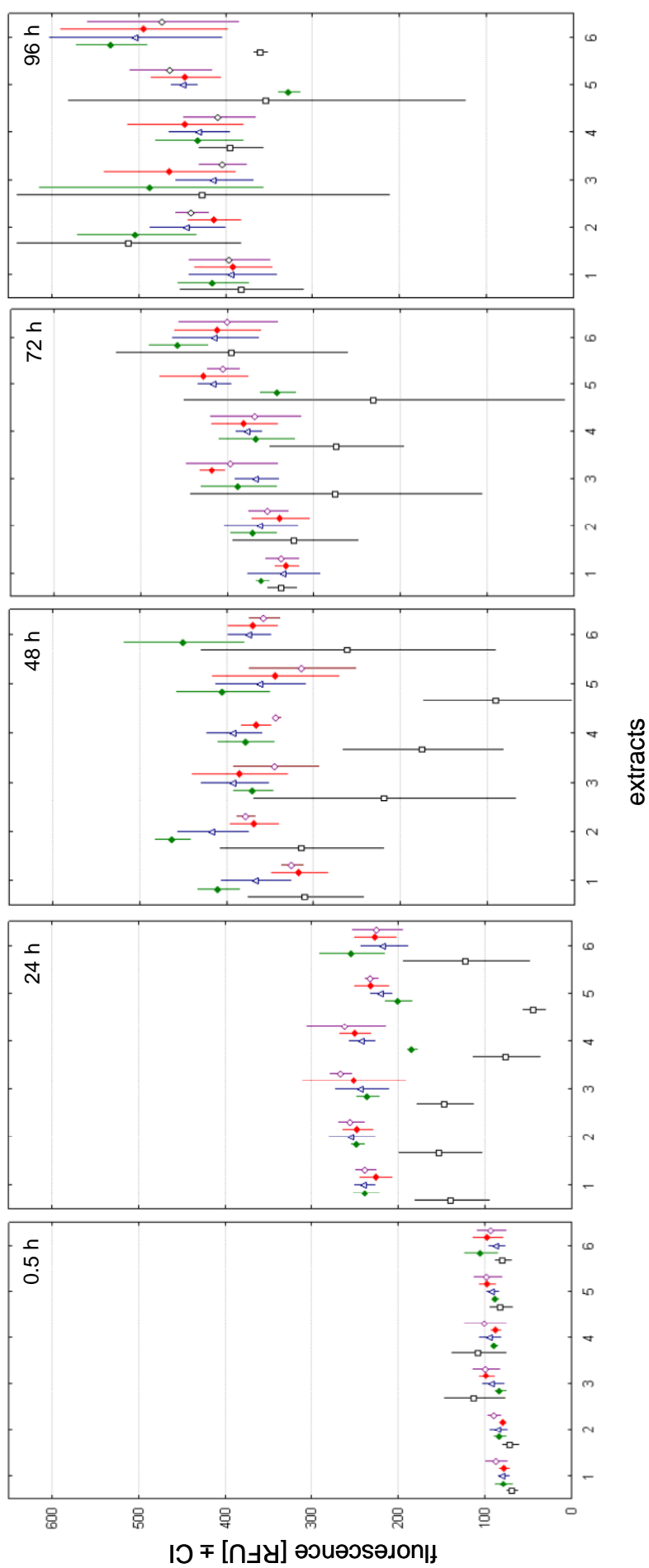


Figure 3. Growth of *Amphora coffeaeformis* in presence of periostracal extracts. Error bars indicate the 95 % confidence interval. Extract (1) = Petrol ether, (2) = Hexane, (3) = Dichloromethane, (4) = Diethyl ether, (5) = Ethyl acetate, (6) = Methanol; concentrations: \square = 100 ppm; \bullet = 30 ppm; \triangle = 10 ppm; \blacklozenge = 3 ppm; \circ = 1 ppm

concentration and significant interactions of both ($p < 0.001$). Tukey HSD post hoc test ($MS = 0.001141$, $df = 72$) showed that in the petrol ether fraction (E 1) and the hexane fraction (E 2) adhesion was significantly reduced in 100 ppm \sim 50 ppm $>$ 10 ppm; E 4 with a concentration of 100 ppm reduced adhesion significantly more than in 50 ppm and 10 ppm. For both the dichloromethane fraction (E 3) and the ethyl acetate fraction (E 5) a concentration of 100 ppm reduced adhesion significantly compared to 10 ppm (Figure 4b).

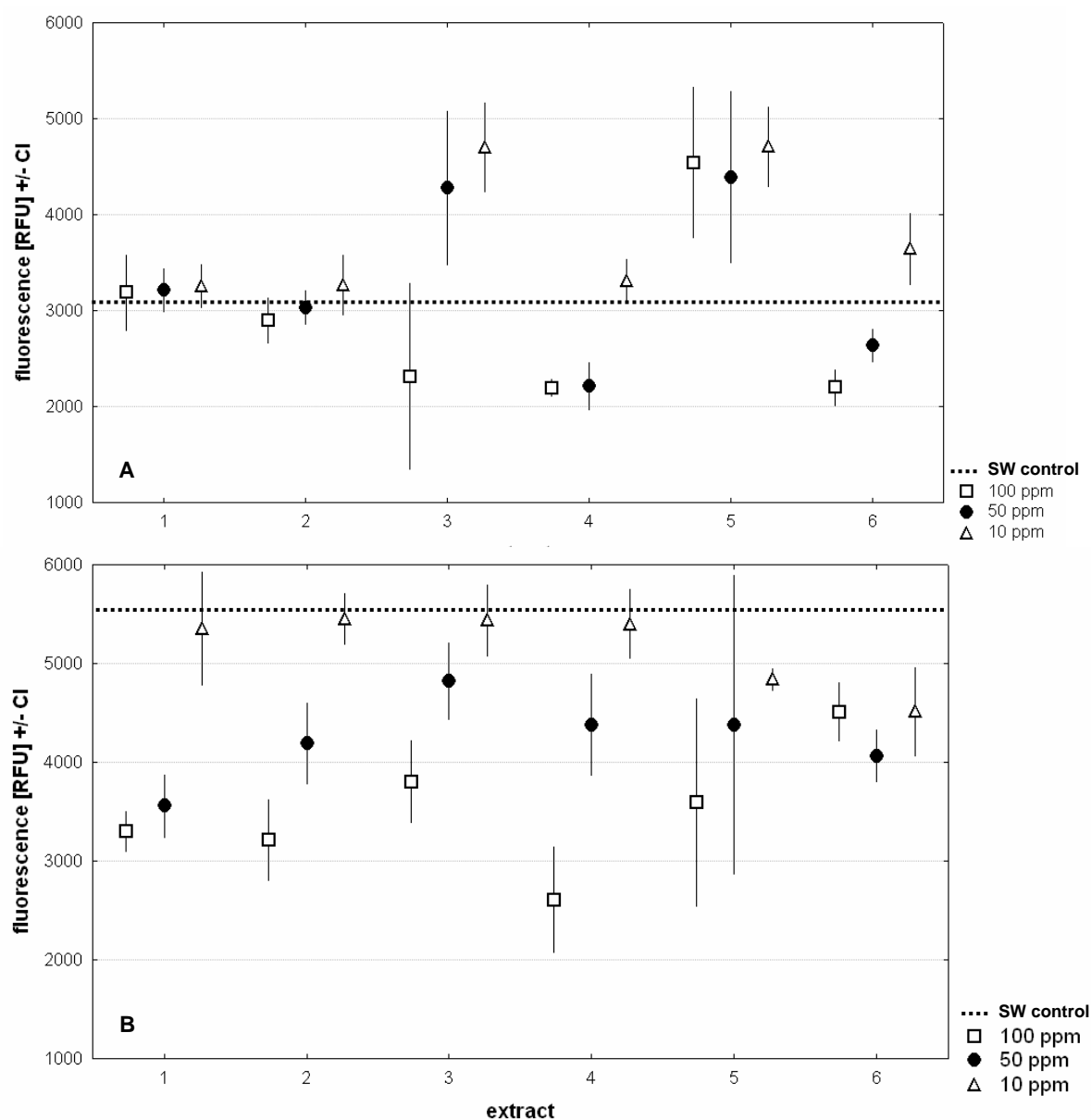


Figure 4. Effect of periostacial extracts on marine bacteria attachment. **(A)** *Marinobacter hydrocarbonoclasticus*. **(B)** *Cobetia marina*. Biomass is indicated as relative units of Syto 13-fluorescence (RFU). Error bars indicate the 95 % confidence interval. Extract (1) = Petrol ether, (2) = Hexane, (3) = Dichloromethane, (4) = Diethyl ether, (5) = Ethyl acetate, (6) = Methanol; the dotted line represents the mean settlement in the seawater control.

Discussion

This study represents the first evaluation of periostracal extracts of *M. edulis*. Anti-attachment activities of several fractions against common fouling species were revealed. At the given concentrations, the diethyl ether fraction showed strong inhibition of cyprid attachment and attachment of the marine bacteria *C. marina* and *M. hydrocarbonoclasticus*. Diatom attachment of *A. coffeaeformis* was significantly reduced by the dichloromethane fraction (E 3) whereas both ethyl acetate (E 5) and diethyl ether (E 4) fractions slowed down the growth of the benthic diatom *A. coffeaeformis* at 100 ppm concentration.

Bacterial attachment is one of the primary stages in the chronology of the fouling process (Lappin-Scott & Costerton 1989, Wahl 1989, Maki & Mitchell 2002). Bacterial cells, along with their extracellular material and other organic substances, form a biofilm that influences further surface colonisation (Maki et al. 1992, Wieczorek et al. 1995). Interactions between biofilm constituents and macrofoulers are complex and can be both positive and negative (Maki 1999, Unabia & Hadfield 1999). Previous studies have shown that metabolites of the bacterium *Cobetia (Halomonas) marina* inhibit settlement and development of *B. amphitrite* (Perry et al. 2001), while exopolysaccharides (EPS) of *Pseudomonas aeruginosa* promoted metamorphosis of *B. amphitrite* cyprids (Khandeparker et al. 2002), as does the EPS of benthic diatoms such as *A. coffeaeformis* (Patil & Anil 2005). Recent research, in contrast to the pharmaceutical screening for active compounds, also focuses on the ecological relevance of compounds that mediate surface colonisation. Body wall extracts of various echinoderms, for example, significantly inhibit settlement of barnacle and bryozoan larvae at or below natural tissue concentration (Bryan et al. 1996): the same compounds both stimulate and inhibit attachment and growth of different bacterial species, suggesting that the echinoderms investigated tolerate surface colonisation of certain bacteria that might promote their immune defences. Body extracts of the soft coral *Dendronephthya* sp. have shown antimicrobial activity (Harder et al. 2003). At the same time, this soft coral possess a distinct epibiotic bacterial community that again inhibits settlement of other bacteria and even macrofoulers (Harder et al. 2003, Dobretsov & Qian 2004), suggesting a coupled chemical and ecological defence mechanism. Similar observations have been made in sponges of the genera *Haliclona*, *Callyspongia* and *Mycale* (Dobretsov et al. 2005a, Dobretsov et al. 2005b), where metabolites can inhibit diatom and macrofouling, while bacterial densities on the surface of the sponge were similar to those on control surfaces. By regulating the composition of their surface biofilms, basibionts therefore may have a possibility to indirectly control macrofouling of their living surfaces.

In contrast to certain other invertebrates, very little is known about the chemical antifouling defences of Mytilidae. So far, studies of antifouling mechanisms in Mytilidae have focussed on the distinct microstructure of the periostracum, which has been shown to inhibit settlement of cypris larvae of various barnacle species (Scardino et al. 2003, Bers & Wahl 2004, Bers et al. 2006) and other fouling organisms (Scardino & de Nys 2004). This microtopography, however, appears to inhibit settlement of

macrofoulers rather than microfoulers, when tested without the chemical components of the periostracum (Bers, personal observations). In addition, the formation of a biofilm on resin replicas of periostracum microripples appears to reduce antifouling efficiency within a few weeks (Bers & Wahl 2004).

In the present study it is shown that several, often different, fractions affected microfouling organisms (bacteria and diatoms) by inhibiting attachment and/ or reducing growth. Additionally, the attachment of cypris larvae was inhibited. The concentrations tested were well below the estimated natural surface concentrations (Table I). Since concentrations of active compounds in the periostracum may be much higher than the concentration of substances diffusing to the boundary layer (where potential fouling organisms first come into contact with repellents or attractants), a concentration range of 100 to 1 ppm (*i.e.* 10x to 1000x below natural concentration) was tested. Furthermore, the natural concentration of active compounds might have been overestimated since whole shells were extracted although the possibility of the inclusion of substances from the prismatic and/or nacre layer cannot be excluded. However, the activities detected were sufficiently strong to deter foulers even when substantially diluted. Since the spatial distribution of compounds within the periostracum is not known, natural concentrations are based on the assumption that compounds are distributed homogeneously within the periostracum, which might not be the case in *M. edulis*. Therefore, the results may underestimate the true activity of periostracal chemistry, but nevertheless, they give valuable information on potential surface bound compounds or compounds that are released through slow leaching that mediate surface colonisation.

This study underlines the multiple character of the antifouling defence system of *M. edulis*, where mechanical (Bers et al. 2006), ecological (Wahl et al. 1998) and chemical mechanisms overlap and/or act in a complementary manner to inhibit a broad spectrum of fouling organisms, similar to the multiple antifouling defence of the tunicate *Polysyncrator lacazei* (Wahl & Banaigs 1991). The defence system of *Mytilus* appears to be highly complex: not only comprising complementary mechanisms (microtopography, surface chemistry and cumulative filtration), but also with the production of several bioactive components that potentially target the settlement or growth of more than one fouling organism. Identifying the active substances and quantifying their actual concentrations at the shell surface or in the surface boundary layer will be the next challenge in the process of unravelling the entire antifouling defence system of the blue mussel. It will also be of interest to evaluate the broad spectrum antifouling efficacy by re-combining the microtopographical and chemical components of the mussel's defence system.

References

- Benkendorff K, Davis AR, Bremner JB (2001) Chemical defense in the egg masses of benthic invertebrates: an assessment of antibacterial activity in 39 mollusks and 4 polychaetes. *Journal of Invertebrate Pathology* 78:109-118
- Bers AV, Prendergast GS, Zürn CM, Head RM, Hansson L, Thomason JC (2006) A comparative study of the anti-settlement potential of mytilid shells. *Biology Letters* 2:88-91
- Bers AV, Wahl M (2004) The influence of natural surface microtopographies on fouling. *Biofouling* 20:43-51
- Bryan PJ, Rittschof D, McClintock JB (1996) Bioactivity of echinoderm ethanolic body-wall extracts: an assessment of marine bacterial attachment and macroinvertebrate larval settlement. *Journal of Experimental Marine Biology and Ecology* 196:79-96
- Clare AS (1996) Marine natural product antifoulants: status and potential. *Biofouling* 9:211-229
- Davey ME, O'Toole G (2000) Microbial Biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* 64:847-867
- de Nys R, Steinberg PD, Willemsen PR, Dworjanyn SA, Gabelfish CL, King RJ (1995) Broad spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. *Biofouling* 8:259-279
- Dobretsov S, Dahms H, Tsoi M, Qian P (2005a) Chemical control of epibiosis by Hong Kong sponges: the effect of sponge extracts on micro- and macrofouling. *Marine Ecology Progress Series* 297:119-129
- Dobretsov S, Qian P (2004) The role of epibiotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement. *Journal of Experimental Marine Biology and Ecology* 299:35-50
- Dobretsov SV, Dahms HU, Qian PY (2005b) Antibacterial and anti-diatom activity of Hong Kong sponges. *Aquatic Microbial Ecology* 38:191-201
- Dobretsov SV, Dahms HU, Qian PY (2006) Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling* 22:43-54
- Fusetani N (2004) Biofouling and antifouling. *Natural Product Reports* 2:94-104
- Glasby TM (1998) Estimating spatial variability in developing assemblages of epibiota on subtidal hard substrata. *Marine and Freshwater Research* 49:429-437
- Harder T, Lau SCK, Dobretsov SV, 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 mechanisms against bacterial epibiosis. *FEMS Microbial Ecology* 43:337-347
- Harper EM (1997) The molluscan periostracum: an important constraint in bivalve evolution. *Palaeontology* 40:71-91
- Head RM, Overbeke K, Klijnstra J, Biersteker R, Thomason JC (2003) The effect of gregariousness in cyprid settlement assays. *Biofouling* 19:269-278
- Hellio C, Tsoukatou M, Maréchal JP, Aldred N, Beaupoil C, Clare AS, Vagias C, Roussis V (2005) Inhibitory effects of Mediterranean sponge extracts and metabolites on larval settlement of the barnacle *Balanus amphitrite*. *Marine Biotechnology* 7:279-305
- Khandeparker L, Anil AC, Raghukumar S (2002) Factors regulating the production of different inducers in *Pseudomonas aeruginosa* with reference to larval metamorphosis in *Balanus amphitrite*. *Aquatic Microbial Ecology* 28:37-54
- Kirchmann D, Mitchell R (1983) Biochemical interactions between microorganisms and marine fouling invertebrates. *Biodeterioration* 5:281-290
- Lappin-Scott HM, Costerton JW (1989) Bacterial biofilms and surface fouling. *Biofouling* 1:323-342
- Maki JS (1999) The influence of marine microbes on biofouling. In: Fingerman M, Nagabhushanam R, Thompson M (eds) *Recent Advances in Marine Biotechnology*, Vol 3. Science Publishers, Inc., Enfield, USA, p 147-171
- Maki JS, Mitchell R (2002) Biofouling in the marine environment. In: Bitton G (ed) *Encyclopedia of Environmental Microbiology*. John Wiley & Sons, New York, p 610-619
- Maki JS, Rittschof D, Mitchell R (1992) Inhibition of larval barnacle attachment to bacterial films: an investigation of physical properties. *Microbial Ecology* 23:97-106

- Maximilien R, de Nys R, Holmström C, Gram L, Giskov M, Crass K, Kjelleberg S, Steinberg PD (1998) Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga *Delisea pulchra*. *Aquatic Microbial Ecology* 15:233-246
- McKenzie JD, Grigolava IV (1996) The echinoderm surface and its role in preventing microfouling. *Biofouling* 10:261-272
- Mitta G, Vandenbulcke F, Hubert F, Salzet M, Roch P (2000a) Involvement of mytilins in mussel antimicrobial defense. *The Journal of Biological Chemistry* 275:12954-12962
- Mitta G, Vandenbulcke F, Noël T, Romestand B, Beauvillain JC, Salzet M, Roch P (2000b) Differential distribution and defence involvement of antimicrobial peptides in mussel. *Journal of Cell Science* 113:2759-2769
- Patil JS, Anil AC (2005) Biofilm diatom community structure: Influence of temporal and substratum variability. *Biofouling* 21:189-206
- Perry TD, Zinn M, Mitchell R (2001) Settlement inhibition of fouling invertebrate larvae by metabolites of the marine bacterium *Halomonas marina* within a polyurethane coating. *Biofouling* 17:147-153
- Ramasamy SM, Murugan A (2005) Potential antimicrobial activity of marine molluscs from Tuticorin, southeast coast of India against 40 biofilm bacteria. *Journal of Shellfish Research* 24:243-251
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctata imbricata*. *Biofouling* 19 (Supplement):221-230
- Scardino AJ, de Nys R (2004) Fouling deterrence on the bivalve shell *Mytilus galloprovincialis*: a physical phenomenon? *Biofouling* 20:249-257
- Steinberg PD, de Nys R, Kjelleberg S (2001) Chemical mediation of surface colonization. In: McClintock J, Baker B (eds) *Marine Chemical Ecology*. CRC Press, Boca Raton, p 355-387
- Theisen BF (1972) Shell cleaning and deposit feeding in *Mytilus edulis* L. (Bivalvia). *Ophelia* 10:49-55
- Tsoukatou M, Hellio C, Vagias C, Harvala C, Roussis V (2002) Chemical defense and antifouling activity of three Mediterranean sponges of the genus *Ircinia*. *Zeitschrift für Naturforschung* 57 c:161-171
- Unabia CRC, Hadfield MG (1999) Role of bacteria in larval settlement and metamorphosis of the polychaete *Hydroides elegans*. *Marine Biology* 133:55-64
- Underwood AJ (1981) Techniques of analysis of variance in experimental marine biology and ecology. *Annual Review of Oceanography and Marine Biology* 19:513-605
- Underwood AJ (1997) *Experiments in ecology: their logical design and interpretation using analysis of variance*, Vol. Cambridge University Press, Cambridge
- Wahl M (1989) Marine Epibiosis I. Fouling and antifouling. Some basic aspects. *Marine Ecology Progress Series* 58:175-189
- Wahl M (1997) Living attached: aufwuchs, fouling, epibiosis. In: Nagabushanam R, Thompson M (eds) *Fouling organisms of the Indian Ocean: biology and control technology*. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 31-83
- Wahl M, Banaigs B (1991) Marine epibiosis III: Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). *Journal of Experimental Marine Biology and Ecology* 145:49-63
- Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205-226
- Wieczorek SK, Clare AS, Todd CD (1995) Inhibitory and facilitatory effects of microbial films on settlement of *Balanus amphitrite amphitrite* larvae. *Marine Ecology Progress Series* 119:221-228
- Wieczorek SK, Todd CD (1998) Inhibition and facilitation of settlement of epifaunal marine invertebrate larvae by microbial biofilm cues. *Biofouling* 12:81-118

Bioassay guided investigation of one periostracal extract of *Mytilus edulis* with regard to its antifouling potential

Abstract

Chemical defence mechanisms are common in sessile marine invertebrates, since they evolved in a competitive environment where space is often a limiting resource. Only recently, crude extracts of the periostracum of *Mytilus edulis* have been studied and revealed antifouling activities in various extracts. Here, the diethyl ether extract has been investigated more closely by bioassay-guided fractionation. Especially fraction 4 and 5 showed high antifouling potential, inhibiting settlement and slowing down growth of the bacterium *Bacillus aquimaris* and the diatom *Amphora coffeaeformis*. Further fractionation and bioassay testing confirmed that the active compounds are soluble in ethanol - and therefore of rather polar nature -, but insoluble in water. The active compounds of these fractions furthermore accumulate in the periostracum of *M. edulis* with increasing shell size. Possible reasons and mechanisms for the accumulation of these compounds in the periostracum are discussed here. The identification of a chemical defence mechanism in *M. edulis* complements our previous findings with regard to the existence of a multiple natural defence system, consisting not only of a chemical, but also of a mechanical mechanism and an associational mechanism.

Introduction

Sessile marine invertebrates evolved in a competitive environment where space is often a limiting resource (Wahl 1997, Krug 2006). Adult organisms try to maintain a clean surface, while larvae must select and attach to any suitable substratum. Epibiosis, the association between sessile organisms living attached to the surface of living organisms, is therefore a common phenomenon in the marine environment (Wahl 1989). It is in most cases facultative (Wahl & Mark 1999). Besides having no effects on both organisms, this association can be advantageous for both epibiont (*e.g.* gain of attachment site, favourable hydrodynamics, and exuded nutrients) and basibiont (*e.g.* camouflage). In many cases, however, epibiosis has harmful effects for the basibionts, such as weight increase, drag and friction increase, mechanical or chemical damage to the body surface, impeded gas exchange, excretion and sensation, increased susceptibility to predation and competition for nutrients, leading to reduced fitness or even death (McKenzie & Grigolava 1996, Wahl 1997). Therefore, marine organisms have evolved a wide range of defence mechanisms to prevent being overgrown.

Chemical defence mechanisms are common in sessile marine invertebrates (Davis et al. 1989, Berenbaum 1995, Hadfield & Paul 2001, Dobretsov et al. 2005, Krug 2006, Paul et al. 2006). Especially sponges and ascidians are often chemically defended (Wahl et al. 1994), and numerous natural antifouling compounds have been isolated from these taxonomic groups (Sears et al. 1990, Willemsen 1994, Blunt et al. 2003, Hellio et al. 2005, Clavico et al. 2006). Molluscs, however, have been rarely studied with regard to their potential chemical defence mechanisms (reviewed by Paul et al. 2006). Mainly chemical protection mechanisms against predators have been identified so far, especially in sea slugs (Marín & Ros 2004), where deterrent substances from algae are incorporated in the snail's body. Hence, the investigated chemically active substances in molluscs are generally found within the body tissue, whereas antifouling defences need to be located at or released from the surface of the organisms (Steinberg et al. 2001).

With regard to the antifouling defence of the blue mussel *Mytilus edulis*, the microtopography of the periostracum has been studied intensively. Several studies could show that a micro-ribbed structure repels macrofoulers such as barnacle cyprid larvae (Scardino et al. 2003, Bers & Wahl 2004, Scardino & de Nys 2004, Bers et al. 2006b). However, the deterrence of the microtopography can be masked by microfoulers that fill up the surface structure (Bers, unpublished data). A complementary chemical antifouling component has therefore been suggested.

Despite an earlier study that indicated the possibility of a chemical defence mechanism in the periostracum of *Mytilus edulis* (Wahl et al. 1998), its surface chemistry has only recently been studied more detailed (Bers et al. 2006a): extracts of the periostracum of *M. edulis* were made using solvents of increasing polarity, and tested in bioassays for settlement and growth inhibition. The results suggested that the blue mussel possess surface bound antifouling compounds of non-polar and moderately polar nature. Especially the extract made with diethyl ether showed promising settlement

and growth inhibition of microfouling organisms such as bacteria and diatoms as well as anti-settlement properties with regard to barnacle cyprid larvae.

The periostracum - the outermost, waterproof organic layer of the mytilid shell - is formed at the inner face of the outer fold of the mantle (Beedham 1958), secreted at the mantle edge and subsequently insolubilised, hardened and darkened (Saleuddin & Petit 1983). It is made up of three layers (Dunachie 1963) and consists of proteins, polysaccharides, lipids and hydrophobic amino acids (Bubel 1973b, Saleuddin & Petit 1983, Waite 1983). Sclerotisation occurs during the aging of the periostracal protein (Waite 1983), when fibrous proteins are cross-linked by quinone tanning (Saleuddin & Petit 1983). The periostracum protects the calcareous layers of the shell from dissolution in seawater, serves as a matrix for the deposition of calcium carbonate crystals (Saleuddin & Petit 1983) and may also act as protection against boring organisms (Bottjer 1981, Harper & Skelton 1993, Wahl et al. 1998).

The aim of this study was to investigate more detailed the diethyl ether extract of the periostracum of *Mytilus edulis* that has previously proven to contain antifouling compounds. The results are related to its antifouling performance and the shell size in order to gain information about the compound's nature and accumulation in the periostracum.

Material & Methods

I. Fractionation of diethyl ether extract

Un-fouled specimens of *Mytilus edulis* (n = 2000) were collected in Kiel Fiord, Western Baltic (54°19'N, 10°09'E). The soft body was completely removed from the shells, and valves were cleaned with sterile filtered seawater to remove debris and microfouling. Shells were freeze dried and consecutively extracted at room temperature for two hours each with petrol ether, followed by hexane, dichloromethane, diethyl ether, ethyl acetate and methanol. 2.5 l of solvents were used in each extraction step. The six resulting crude extracts were reduced *in vacuo* at room temperature, but only the diethyl ether extract was used for further experiments.

The average concentration of the extract (C_E) in the periostracum was re-calculated in relation to the volume of the periostracum, based on a mean periostracum thickness of 35 μm for *Mytilus edulis* according to literature values (Harper 1997). Calculations were based on the following formulae:

(i) $V_P = V_M \times (T_P / T_S)$, where V_P = periostracum volume, V_M = volume of mussel shells extracted, T_P = thickness of periostracum, and T_S = mean shell thickness including periostracum, and

(ii) $C_E = DW_E / V_P$, where DW_E = dry weight of extract and V_P = periostracum volume extracted.

The volume of the shells was calculated by displacement of water, shell thickness was measured with a manual thickness gauge. The mean shell length of the shells used in this experiment was 41.69 mm \pm 7.53 mm (SD), mean shell thickness was 0.43 mm \pm 0.1mm (SD); extract dry weight was 14 mg, the average surface concentration (C_E) was therefore 0.202 mg ml⁻¹.

The dried diethyl ether extract was resuspended in 1700 μ l diethyl ether and separated by HPLC (KONTRON Instruments) on a C18 column (LiChroCART[®] 250-4, Merck) at a flow rate of 1 ml min⁻¹. A crude extract volume of 980 μ l of the crude extract was fractionated in an ethanol/methanol to hexane gradient (0-11 min. 100 % ethanol/methanol (5:1), 11-21 min isocratic to 100 % hexane, 21-31 min. 100 % hexane, 31-36 min. isocratic to 100 % ethanol/methanol (5:1)) in 32 fractions. The collection of the fractions started 1 min. after injection, and fractions were collected every minute (with exception of fraction 32 which includes the flow through of minute 33-36). The resulting fractions were again reduced in a rotary evaporator. Stock solutions of the fractions were made in DMSO and tested in various concentrations. The 32 fractions of the diethyl ether were tested for settlement and growth inhibition in laboratory based bacterial and microalgal bioassays at 300 ppm in a preliminary test to detect activities. Subsequently, the 18 most promising fractions were identified and tested with 5 replicates in the following bioassays:

Bacterial assays

The gram-positive bacterium *Bacillus aquimaris* (DSM 16205) and the gram-negative bacteria *Shewanella baltica* (DSM 9439) and *Pseudoalteromonas* sp. were grown in SPP medium for 18 – 20 hours. For settlement assays, cells were washed with sterile filtered sea water (FSW) and diluted to an optical density of 0.2 at 595 nm. A volume of 100 μ l of bacterial suspension was transferred to a Greiner[®] multi-well plate (96 wells, flat bottom), and fractions were added to obtain final concentrations of 200, 100 and 30 ppm. The filled multi-well plates were placed on a shaking table at 28°C and bacteria were allowed to attach for 1 hour. Subsequently, the bacterial suspension was removed from the wells. Non-attached cells were removed by gently rinsing the wells with sterile FSW. Cells attached to the well walls were stained with fluorescent DNA-binding Syto-13, and fluorescence (excitation wavelength: 485 nm; emission wavelength: 535 nm) was subsequently measured in a TECAN[™] GENios plate reader (software Magellan[™] v 3.0) to determine bacterial biomass as a proxy for bacterial attachment.

For bacterial growth assays, 100 μ l of bacterial suspensions (OD = 0.2) were filled in the wells and left to settle for 1 hour. After that, wells were rinsed two times with sterile FSW to remove non-attached cells. Wells were then filled with 100 μ l bacterial growth medium each, and fractions were added to obtain concentrations of 200, 100 and 30 ppm. The plates were then left on a shaking table at 28 °C and bacteria were allowed to grow for 4 h. After that, wells were rinsed again twice with sterile FSW and cells were stained with Syto-13, and fluorescence was measured as above.

Number of replicates was 5 in all cases. DMSO controls of corresponding concentrations were included in the experiments.

Diatom assays

The benthic pennate diatom *Amphora coffeaeformis*, a common fouling species in benthic communities, was used in these experiments. The culture was maintained in a culture room at 17 °C in sterile FSW and silicate enriched F2 media. Diatoms were cultured for 5 days prior the experiments.

For the settlement assay, diatom cell suspension (200 µl/ well) was placed in untreated polystyrene Greiner® multi-well plates (96 wells, flat bottom), and was treated with the fractions in final concentrations of 200, 100 and 30 ppm and then incubated for 30 minutes at 17°C to allow cells to attach to the surface. The plates were then gently rinsed with sterile FSW to remove unattached diatoms and cell pigment fluorescence (excitation wavelength: 430 nm; emission wavelength: 670 nm) was quantified using a TECAN™ GENios plate reader (software Magellan™ v 3.0). There were five replicates per treatment, and DMSO controls of all concentrations were included.

For the growth assay, 200 µl diatom suspension was placed in untreated polystyrene Greiner® multi-well plates (96 wells, flat bottom), incubated for 1 hour at 17°C to allow cells to attach to the surface. The plates were then gently rinsed with sterile FSW to remove unattached diatoms and cell pigment fluorescence was quantified as before. Test solutions of all fractions were prepared in concentrations of 200, 100 and 30 ppm in F2 medium and 200 µl were filled in the well on top of the settled diatoms. Each concentration was tested in fivefold replication. DMSO in sterile FSW was used as a control. The plates were placed in a culture room at 17°C and growth was monitored over 69 hours.

Statistical Analysis

STATISTICA (v. 6) was used for statistical analysis: for each target organism, bioactivities were assessed by 2-factorial ANOVA (factor 1 = extract, factor 2 = concentration) when assumptions were fulfilled. When normal distribution and homogeneity of variances were not given and could not be achieved by transformation, ANOVA was used nonetheless but with the alpha-level reduced to 0.01 in order to avoid type 1 errors (Underwood 1981, 1997, Glasby 1998).

A ln effect ratio was calculated for better comparison of bacterial and diatom settlement in the presence of the fractions (Figure 1). The ln effect ratio was calculated as described below:

$$\text{Effect Size} = \ln (\text{fluorescence}_{\text{treatment}} / \text{fluorescence}_{\text{DMSO control}})$$

In the case of *Amphora* growth, the ln effect ratios were used for a 1-factorial ANOVA to detect differences in the effect of the fractions between sampling times (T1/T0, T2/T1 and T3/T2, with T0 = after 1 h settlement without treatment, T1 = 21 h, T2 = 45 h and T3 = 61 h after adding the extract).

II. Correlation of shell size and anti-fouling activity

Specimens of *M. edulis* were collected and assorted into six samples, according to size and presence or absence of barnacle foulers (Table 1). Foulers and soft bodies were completely removed and the shells of all samples were extracted as described in section I. Aliquots of the dried diethyl ether extracts were analyzed by HPLC (identical conditions as before) and tested for inhibitory effects upon settlement of *A. coffeaeformis* as described in section I. STATISTICA (v. 6) and GraphPad Prism were used in order to calculate Spearman rang correlation coefficients and to plot best-fitting linear functions.

Table 1. Characterisation of six samples of *Mytilus edulis* shells used in experiment II.

Sample	Individuals	Shell length [mm]	Barnacles individuuum ⁻¹
1	11	71.5 +/- 5.5	16.9 +/- 7.1
2	16	50.4 +/- 6.3	9.1 +/- 7.6
3	28	32.3 +/- 4.4	3.8 +/- 4.6
4	20	38.7 +/- 3.5	0.4 +/- 1.0
5	35	34.5 +/- 2.0	0 +/- 0
6	71	20.9 +/- 1.5	0 +/- 0

III. Further fractionation of 2 active fractions

For further investigations, another 620 mussel shells (mean shell length: 56.57 mm \pm 1.15 mm (SD), mean shell thickness: 0.46 mm \pm 0.01 mm (SD)), were prepared in the same way as described in section I and extracted with 2.5 l dichloromethane and diethyl ether for 1 h at room temperature. The resulting crude extracts were reduced in a rotary evaporator ($DW_E = 17.13$ mg, $C_E = 0.323$ mg ml⁻¹). The dried diethyl ether extract was resolved in 2.5 ml diethyl ether and fractionated via HPLC (Waters™ 600 Pump + Controller, 486 Tunable Absorbance Detector) on a C18 column (LiChroCART® 250-4, MERCK) and a 200 μ l sampling loop with a methanol-ethanol to hexane gradient as described in section I in 32 fractions with a flow of 1 ml min⁻¹. The fractions 4 and 5 (that were collected 4-5 and 5-6 min after sample injection, respectively) were pooled and tested for activity in bacterial and diatom bioassays at natural surface concentrations (0.323 mg ml⁻¹). The bacteria *Bacillus aquimaris* (DSM 16205) and *Pseudoalteromonas* sp. and the diatom *Amphora coffeaeformis* were used for the assays and tests were carried out as above with regard to settlement and growth inhibition.

The pooled fractions were then fractionated by HPLC with a water-ethanol gradient (21 min 100 % H₂O, 20 min isocratic increase to 100 % ethanol, 40 min 100 % ethanol) at a flow of 0.5 ml min⁻¹. The resultant flow through was collected for further investigations, and divided into the water fraction, water-ethanol gradient fraction and the ethanol fraction. They were subsequently tested in bioassays at twice the natural concentration to ensure to detect activities. The number of replicates was always six.

Statistical analysis

STATISTICA (v. 6) was used for statistical analysis: for each target organism, bioactivities were assessed by 1-factorial ANOVA (factor = extract).

Results

I. Fractionation of diethylether extract

The three bacteria responded differently to the periostracal extract (Figure 1):

Settlement of *Shewanella baltica* was enhanced by fraction 4 ($p < 0.001$) at a concentration of 200 ppm, by fractions 2 ($p < 0.001$), and 24 ($p < 0.001$) at 100 ppm and by fractions 4-13 (all $p < 0.001$) at 30 ppm.

Settlement of *Bacillus aquimaris* was significantly inhibited by fractions 4 ($p < 0.001$) and 32 ($p < 0.001$), at a test concentration of 200 ppm, while at 100 ppm fraction 20 ($p < 0.001$) significantly increased settlement. At 30 ppm, no effects were detected at all.

The ANOVA did not reveal any significant effect of the fractions on settlement of *Pseudoalteromonas* sp. at an extract concentration of 200 ppm, although the ln effect ratio (Figure 1) suggests a slight settlement inhibition of fraction 4. However, no significant effects were detected for *Pseudoalteromonas* sp. at a concentration of 100 ppm, whereas settlement was significantly enhanced at 30 ppm by fraction 23 ($p = 0.004$) and 27 ($p = 0.001$).

Settlement of *Amphora coffeaeformis* was inhibited at 200 ppm by fractions 4 ($p = 0.001$) and 5 ($p < 0.001$), at 100 ppm by fraction 4 only ($p < 0.001$).

Growth of *S. baltica* was inhibited at 200 ppm by fraction 6 ($p = 0.003$) only, at 100 ppm by fractions 6 ($p < 0.001$), 7 ($p = 0.001$), 8 ($p = 0.003$), 9 ($p < 0.001$), 10 ($p < 0.001$) and 12 ($p < 0.001$). At 30 ppm, settlement was only inhibited by fraction 8 ($p < 0.001$).

Growth of *B. aquimaris* was significantly reduced by fractions 4 and 31 (both $p < 0.001$) at a concentration of 200 ppm, whereas all tested fractions did not affect growth of *Pseudoalteromonas* sp. at all 3 concentrations.

The growth of *Amphora coffeaeformis* was strongly affected by fractions 4 and 5. Figure 2a shows the diatom growth monitored over 69 h in the presence of the fractions. The values for 1 h (T0) hereby represent the values after 1h settlement without fractions, and measurements started after addition of F2-growth medium and the fractions. It becomes clear that fractions 4 and five significantly reduce the amount of diatoms per well, but that the surviving diatoms are able to recover and start growing after 21 h. Fractions are active at 200 ppm, which was the estimated natural surface concentration, and at 100 ppm. No growth inhibiting effect was detectable at a concentration of 30 ppm.

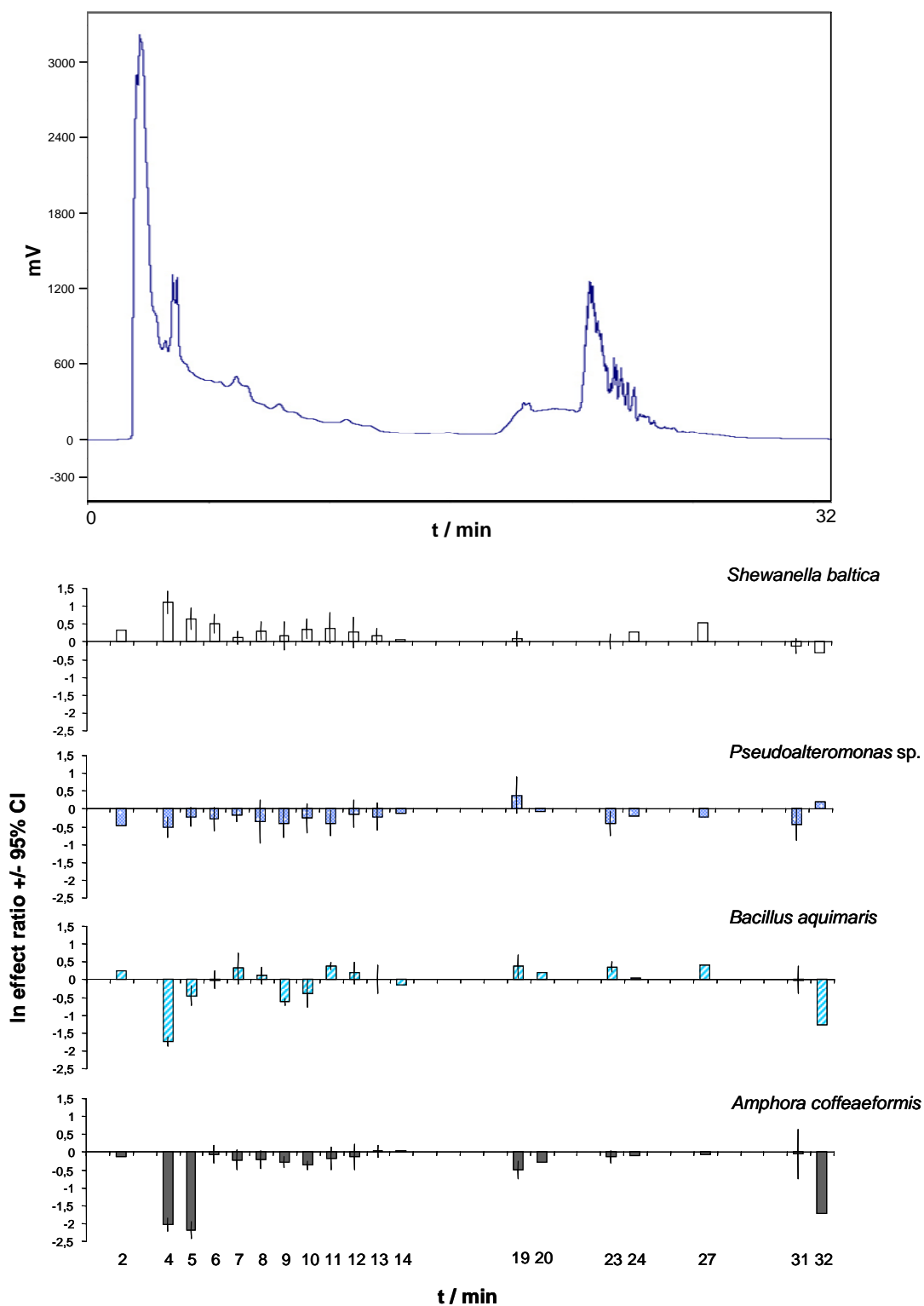


Figure 1. Top: HPLC chromatogram (absorbance at 205 nm) of the periostacial diethyl ether extracts. Fractions were collected every minute at a flow rate of 1 ml min^{-1} . Bottom: 18 out of 32 fractions were tested against settlement of the bacteria *Shewanella baltica*, *Pseudoalteromonas* sp. and *Bacillus aquimaris* and the benthic diatom *Amphora coffeaeformis*. For better comparison, an In effect ratio was calculated. Negative values therefore represent repellent effects. Error bars indicate the 95% confidence interval.

The ANOVA using \ln effect ratios revealed a highly significant reduction in fluorescence (Figure 2b) in fraction 4 ($p = 0.0002$) and 5 ($p = 0.0007$) in the comparison T0/T1 at a concentration of 200 ppm, whereas at 100 ppm the effect was smaller but still significant (fraction 4: $p = 0.0001$; fraction 5: $p = 0.001$). After 21 h (T1), the effect reversed since the remaining diatoms started growing (*i.e.* positive effect ratios).

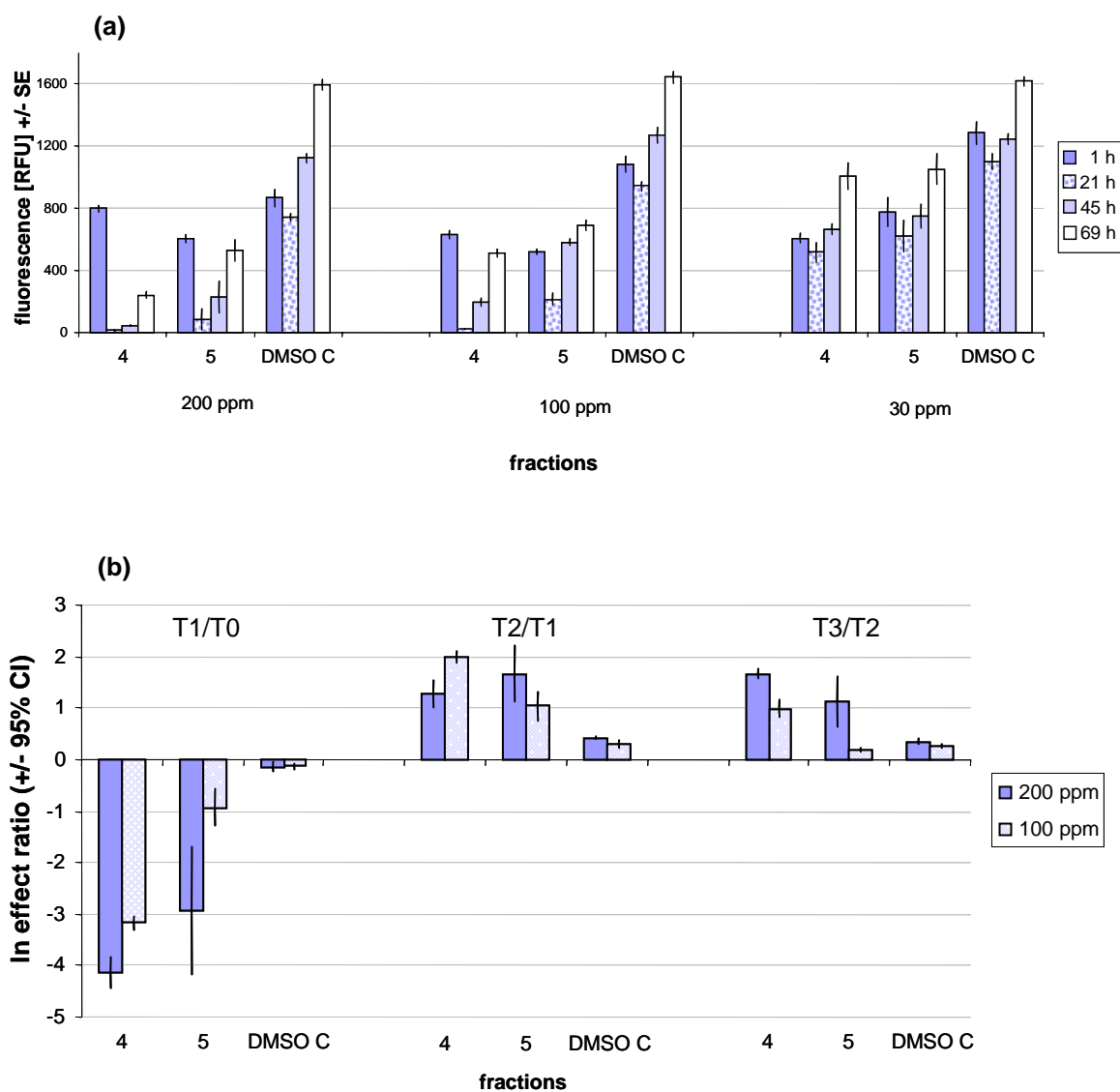


Figure 2. Growth of *Amphora coffeaeformis*, treated with fractions of the diethyl ether extract. **(a)** Growth in the presence of the two active fractions at 3 concentrations, compared to the DMSO control, monitored over 69 h; fluorescence of chlorophyll a is taken as a measurement of biomass. **(b)** ln effect sizes, calculated for the ratio of T1/T0, T2/T1 and T3/T2. T0 = after 1 h settlement without treatment, T1 = 21 h, T2 = 45 h and T3 = 61 h after adding the extract).

II. Correlation of shell size and anti-fouling activity

A particularly strong inhibition of *A. coffaeiformis* settlement was observed when it was exposed to diethyl ether extracts of *M. edulis* shells with a length of approximately 40 mm or more, while extract from 20 mm long shells had no visible inhibitory effect (Figure 3a). Overall, diatom settlement was significantly correlated with *M. edulis* shell size (Spearman $r = -0.906$, $p < 0.001$). A significant correlation existed also between diatom settlement during exposure to diethyl ether extracts and the amount of compounds in fraction 4 of these extracts (Spearman $r = -0.76$, $p < 0.001$, Figure 3b). Correspondingly, fraction 4 of extracts from large shells contained larger amounts of compounds than in fraction 4 of extracts from small shells (Figure 3c). However, the correlation between shell size and presence compounds in fraction 4 (Spearman $r = 0.657$) was not significant ($p = 0.156$), possibly due to lower replication. No significant rank correlation was detected between amount of compounds in fraction 4 of diethyl ether shell extracts and presence of barnacles upon the shells prior to extraction ($p = 0.42$). Similarly, no significant correlation existed between diatom settlement during exposure to diethyl ether extracts and presence of barnacles ($p = 0.41$).

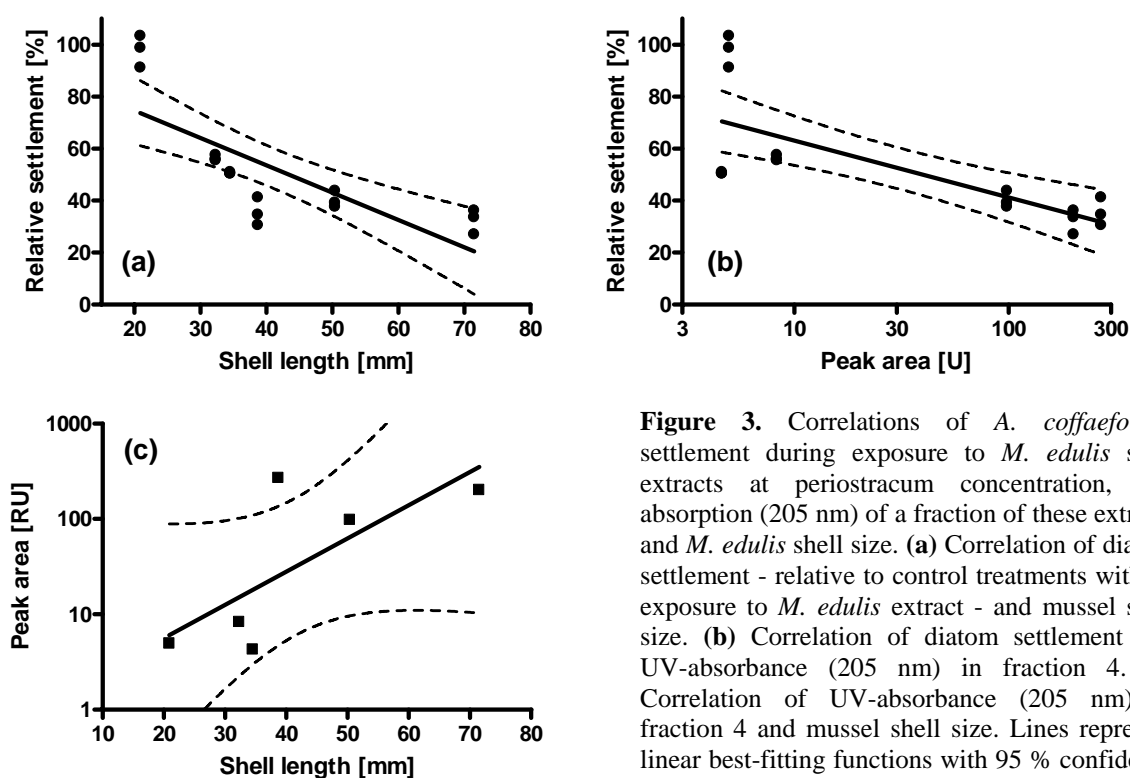


Figure 3. Correlations of *A. coffaeiformis* settlement during exposure to *M. edulis* shell extracts at periostracum concentration, UV absorption (205 nm) of a fraction of these extracts and *M. edulis* shell size. (a) Correlation of diatom settlement - relative to control treatments without exposure to *M. edulis* extract - and mussel shell size. (b) Correlation of diatom settlement and UV-absorbance (205 nm) in fraction 4. (c) Correlation of UV-absorbance (205 nm) in fraction 4 and mussel shell size. Lines represent linear best-fitting functions with 95 % confidence intervals.

III. Further fractionation of 2 active fractions

Settlement of *Pseudoalteromonas* sp. was significantly inhibited by the ethanol fraction ($p = 0.02$), while *Bacillus aquimaris* appeared to be more sensitive to the water fraction, although effects are not significant. Settlement of *B. aquimaris* was not affected by the gradient fraction, the settlement test of the latter with *Pseudoalteromonas* sp. failed due to contamination of the culture.

There was no effect on growth of both bacteria in the ethanol fraction. The water fraction, however, appeared to slightly inhibit growth of *B. aquimaris*, although the ANOVA did not detect significant differences between treatment and control, whereas the gradient fraction again did not affect growth of *B. aquimaris*.

Amphora settlement was significantly inhibited by the ethanol fraction ($p < 0.001$). The growth experiments with water, gradient and ethanol fraction failed due to technical problems.

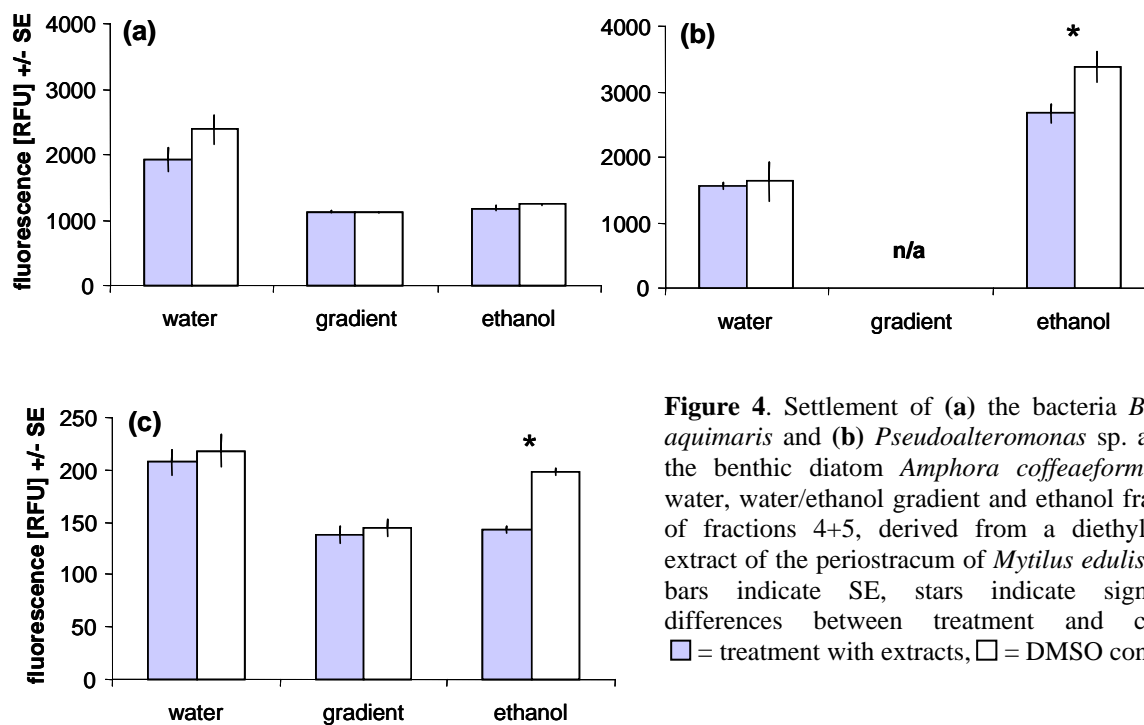


Figure 4. Settlement of (a) the bacteria *Bacillus aquimaris* and (b) *Pseudoalteromonas* sp. and (c) the benthic diatom *Amphora coffeaeformis* in water, water/ethanol gradient and ethanol fractions of fractions 4+5, derived from a diethyl ether extract of the periostracum of *Mytilus edulis*. Error bars indicate SE, stars indicate significant differences between treatment and control. \square = treatment with extracts, \square = DMSO control.

Discussion

The fractionation of a periostracal diethyl ether extract and subsequent bioassays revealed antifouling properties in several different fractions, demonstrating that this extract contained more than one active compound. Particularly promising antifouling properties were detected in two fractions, which were collected 4-6 min after sample injection in the HPLC. These fractions significantly inhibited settlement and slowed down growth of the bacterium *Bacillus aquimaris* and the diatom *Amphora coffeaeformis*. A marked peak in UV absorbance was observed during elution of fraction 4, which was

correlated in size with this activity. The solvent used for elution during minutes 4-6 was a mixture of methanol and ethanol and further fractionation and bioassay testing confirmed that the active compounds are soluble in ethanol - and therefore of rather polar nature - , but insoluble in water.

Additionally, we demonstrated that the active compounds of these fractions accumulate in the periostracum of *Mytilus edulis* with increasing shell size: the peak area of the HPLC chromatogram is higher with increasing mussel shell size and the greater the peak area, the lower the settlement of *Amphora coffeaeformis*.

Mussel growth is highly variable, especially in dense mussel clumps, so that small individuals are not necessarily the young ones (Kautsky 1982, and referenced therein). In the present study, however, the smallest mussels examined were about 3-4 month old, the biggest possibly 2 or more years. The described difference in mussels of different size classes indicates the chronological accumulation of active compounds in the periostracum, maybe as a consequence of the aging of the periostracum, the so called quinine-tanning (Waite et al. 1979, Waite 1983).

The periostracum is secreted at the mantle edge, and as an extracellular structure, it is then subject to aging. This process is not only characterised by deterioration, but also by considerable changes in the course of the so-called tanning. In the marine environment, quinone-tanned structures are found wherever a durable glue or protective coating is required in the life history of marine organisms (Waite 1990): typical quinone-tanned materials are egg capsules, byssal threads or carapace (Waite 1990), but also adhesive mucilages in algal zygotes (Vreeland et al. 1998).

The periostracum of *Mytilus edulis* is formed by tanning from a water soluble precursor protein, periostracin, which is rich in tyrosine (Waite et al. 1979). During the tanning process, which is particularly fast during the first months, but continues over 2 to 3 years (Waite 1983), tyrosine residues in periostracin are first hydroxylated to Dopa (Waite et al. 1979) and subsequently oxidized to o-quinones, which eventually form covalent cross-links and thereby the tanned scleroprotein (Bubel 1973a, Waite & Wilbur 1976). Due to the tanning process, periostracin becomes more and more hydrophobic (Waite 1983), which may potentially result in an increasing accumulation with time of hydrophobic compounds from the surrounding water. It is therefore not impossible that the polar, but water-insoluble antifouling-compounds present in the periostracum originate from the very close (biofilm) or more distant environment rather than from *M. edulis* itself. Further, hydroxylation and oxidation of phenolic amino acids during tanning are due to the activity of periostracum-located hydroxylases and phenol oxidases (Waite et al. 1979, Waite 1983). This demonstrates that functional enzymes are present in the periostracum months and even years after its excretion and a role of these or other enzymes in the biosynthesis of antifouling compounds is therefore not utterly impossible.

Another explanation for the accumulation of an active substance might be a continuous excretion of active substances by the mussel itself into the periostracum after its formation, e.g. via putative canals through the shell. Shell pores have been found in many mollusc groups like gastropods,

polyplacophorans and some bivalves (Araujo et al. 1994, Reindl & Haszprunar 1994, 1995). In Mytilidae, however, there are no channels penetrating the entire shell, but tubules that reach into the endostracum (Reindl & Haszprunar 1996), the innermost layer of the periostracum.

The nature of the compounds in question has not been determined in the present study, but is currently under investigation. Yet, this study clearly shows that the active compounds of the periostracum of *Mytilus edulis* contribute to the chemical defence in this species. They were repeatedly extracted from different samples of mussel shells, and the activity found particularly in fraction 4 consistently appeared in various experiments. The tested species, however, respond very differently to the periostracal compounds: while settlement of the bacterium *Shewanella baltica* was significantly enhanced by fraction 4, the same fraction strongly inhibited settlement of bacterium *Bacillus aquimaris* and the benthic diatom *Amphora coffeaeformis*.

Motility, settlement, biofilm formation and other forms of behaviour of many procaryotes and zoospores (e.g. of the marine alga *Ulva linza*) are known to be regulated by quorum sensing. Quorum sensing is the molecular communication between individual cells that results in synchronised changes in behaviour (Daniels et al. 2004). From the red alga *Delisea pulchra*, other plants and microorganisms secondary metabolites have been isolated which inhibit quorum sensing (Givskov et al. 1996, Bauer & Robinson 2002), suggesting that these organisms are able to interfere with the regulatory system of its surface associated bacteria. It may be speculated – since the nature of the active compound remains unclear – that *M. edulis* possesses a similar mechanism to affect its surface associated bacteria. Another potential mechanism would be the modulation the composition of surface fouling by “gardening” only bacteria which again repel other bacteria or even microalgae, as demonstrated for the natural defence system in the soft coral *Dendronephthya* sp. (Harder et al. 2003, Dobretsov & Qian 2004).

Furthermore, a (temporary) toxicity of the active compounds for at least microalgae can not be entirely excluded, due to the great impact on diatom growth revealed by our experiments.

The identification of a chemical defence mechanism in *Mytilus edulis* complements our previous findings (Wahl et al. 1998, Bers et al. 2006a, Bers et al. 2006b) with regard to the existence of a multiple natural defence system, consisting not only of a chemical, but also of a mechanical mechanism (i.e. the microtopography on the periostracum) and an associational mechanism (i.e. the cumulative filtration in the mussel bed). It is worth mentioning that the different mechanisms target different fouling organisms. The surface chemistry affects settlement and growth of microfoulers, and as shown before, also repels cyprid larvae, while the microtopography repels attachment of macrofoulers, but it is ineffective against microfoulers. The repelling of microfoulers, however, is essential to avoid the masking of the microtopography by a biofilm (Bers, unpublished data). The results of this study therefore suggest a cumulation of complementary or additive mechanisms in a multiple defence system.

References

- Araujo R, Ramos MA, Bedoya J (1994) Microtubules in the shell of the invasive bivalve *Corbicula fluminea* (Müller, 1774) (Bivalvia, Heterodonta). *Journal of Molluscan Studies* 60:405-413
- Bauer WD, Robinson JB (2002) Disruption of bacterial quorum sensing by other organisms. *Current Opinion in Biotechnology* 13:234-237
- Beedham GE (1958) Observations on the non-calcareous component of the shell of Lamellibranchia. *Quarterly Journal of Microscopical Science* 99:341-357
- Berenbaum MR (1995) The chemistry of defense: theory and practice. *Proceedings of the National Academy of Science* 92:2-8
- Bers AV, D'Souza F, Klijnstra JW, Willemsen PR, Wahl M (2006a) Chemical defence in mussels: antifouling effect of crude extracts of the periostracum of the blue mussel *Mytilus edulis*. *Biofouling* 22:251-259
- Bers AV, Prendergast GS, Zürn CM, Head RM, Hansson L, Thomason JC (2006b) A comparative study of the anti-settlement potential of mytilid shells. *Biology Letters* 2:88-91
- Bers AV, Wahl M (2004) The influence of natural surface microtopographies on fouling. *Biofouling* 20:43-51
- Blunt JW, Copp BR, Munro MHG, T NP, Prinsep MR (2003) Marine natural products. *Natural Product Reports* 20:1-48
- Bottjer DJ (1981) Periostracum of the gastropod *Fusitriton oregonensis*: natural inhibitor of boring and encrusting organisms. *Bulletin of Marine Science* 31:916-921
- Bubel A (1973a) An electron-microscope investigation into the distribution of polyphenols in the periostracum and cells of the inner face of the outer fold of *Mytilus edulis*. *Marine Biology* 23:3-10
- Bubel A (1973b) An electron-microscope study of periostracum formation in some marine bivalves. I. The formation of the periostracum. *Marine Biology* 20:213 - 221
- Clavico EEG, Muricy G, da Gama BAP, Batista D, Ventura CRR, Pereira RC (2006) Ecological roles of natural products from the marine sponge *Geodia corticostylifera*. *Marine Biology* 148:479-488
- Daniels R, Vanderleyen J, Michiels J (2004) Quorum sensing and swarming migration in bacteria. *FEMS Microbiology Reviews* 28:261-289
- Davis AR, Targett NM, McConnell OJ, Young CM (1989) Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In: Scheuer PJ (ed) *Bioorganic Marine Chemistry*, Vol 3. Springer-Verlag, Berlin, p 85-114
- Dobretsov S, Dahms H, Tsoi M, Qian P (2005) Chemical control of epibiosis by Hong Kong sponges: the effect of sponge extracts on micro- and macrofouling. *Marine Ecology Progress Series* 297:119-129
- Dobretsov S, Qian P (2004) The role of epibiotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement. *Journal of Experimental Marine Biology and Ecology* 299:35-50
- Dunachie JF (1963) The periostracum of *Mytilus edulis*. *Transactions of the Royal Society of Edinburgh* 65:383 - 411
- Givskov M, de Nys R, Manefield M, Gram L, Maximilien R, Eberl L, Molin S, Steinberg PD, Kjelleberg S (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *Journal of Bacteriology* 178:6618-6622
- Glasby TM (1998) Estimating spatial variability in developing assemblages of epibiota on subtidal hard substrata. *Marine and Freshwater Research* 49:429-437
- Hadfield MG, Paul VJ (2001) Natural chemical cues for settlement and metamorphosis of marine invertebrate larvae. In: McClintock J, Baker B (eds) *Marine Chemical Ecology*. CRC Press, Boca Raton, p 431-461
- Harder T, Lau SCK, Dobretsov SV, 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 mechanisms against bacterial epibiosis. *FEMS Microbial Ecology* 43:337-347

- Harper EM (1997) The molluscan periostracum: an important constraint in bivalve evolution. *Palaeontology* 40:71-91
- Harper EM, Skelton PW (1993) A defensive value of the thickened periostracum in the Mytiloidea. *Veliger* 36:36-42
- Hellio C, Tsoukatou M, Maréchal JP, Aldred N, Beaupoil C, Clare AS, Vagias C, Roussis V (2005) Inhibitory effects of Mediterranean sponge extracts and metabolites on larval settlement of the barnacle *Balanus amphitrite*. *Marine Biotechnology* 7:279-305
- Kautsky N (1982) Growth and size structure in a Baltic *Mytilus edulis* population. *Marine Biology* 68:117-133
- Krug PJ (2006) Defense of benthic invertebrates against surface colonization by larvae: a chemical arms race. In: Fusetani N, Clare AS (eds) *Antifouling compounds*. Springer, Berlin, p 1-53
- Marín A, Ros J (2004) Natural defenses in sacoglossan opisthobranchs: taxonomic trends and evolutive implications. *Scientia Marina* 68:224-241
- McKenzie JD, Grigolava IV (1996) The echinoderm surface and its role in preventing microfouling. *Biofouling* 10:261-272
- Paul VJ, Puglisi MP, Ritson-Williams R (2006) Marine chemical ecology. *Natural Product Reports* 23:153-180
- Reindl S, Haszprunar G (1994) Light and electron-microscopic investigations on shell pores (ceca) of fissurellid limpets (Mollusca, Archaeogastropoda). *Journal of Zoology* 233:385-404
- Reindl S, Haszprunar G (1995) Shell pores (caeca, aesthetes) of Mollusca: a case of polyphyly. In: Taylor J (ed) *Origin and evolutionary radiation of the Mollusca*. Oxford University Press, Oxford, p 115-118
- Reindl S, Haszprunar G (1996) Fine structure of caeca and mantle of arcoid and limpoid bivalves (Mollusca: Pteriomorpha). *Veliger* 39:101-116
- Saleuddin ASM, Petit HP (1983) The mode of formation and the structure of the periostracum. In: Wilbur KM (ed) *The Mollusca*, Vol 4. Academic Press, New York, p 199-234
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctata imbricata*. *Biofouling* 19 (Supplement):221-230
- Scardino AJ, de Nys R (2004) Fouling deterrence on the bivalve shell *Mytilus galloprovincialis*: a physical phenomenon? *Biofouling* 20:249-257
- Sears MA, Gerhart DJ, Rittschof D (1990) Antifouling agents from marine sponge *Lissodendoryx isodictyalis* Carter. *Journal of Chemical Ecology* 16:791-799
- Steinberg PD, de Nys R, Kjelleberg S (2001) Chemical mediation of surface colonization. In: McClintock J, Baker B (eds) *Marine Chemical Ecology*. CRC Press, Boca Raton, p 355-387
- Underwood AJ (1981) Techniques of analysis of variance in experimental marine biology and ecology. *Annual Review of Oceanography and Marine Biology* 19:513-605
- Underwood AJ (1997) *Experiments in ecology: their logical design and interpretation using analysis of variance*, Vol. Cambridge University Press, Cambridge
- Vreeland V, Waite JH, Epstein L (1998) Polyphenols and oxidases in substratum adhesion by marine algae and mussels. *Journal of Phycology* 34:1-8
- Wahl M (1989) Marine Epibiosis I. Fouling and antifouling. Some basic aspects. *Marine Ecology Progress Series* 58:175-189
- Wahl M (1997) Living attached: aufwuchs, fouling, epibiosis. In: Nagabushanam R, Thompson M (eds) *Fouling organisms of the Indian Ocean: biology and control technology*. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 31-83
- Wahl M, Jensen PR, Fenical W (1994) Chemical control of bacterial epibiosis on ascidians. *Marine Ecology Progress Series* 110:45-47
- Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205-226
- Wahl M, Mark O (1999) The predominantly facultative nature of epibiosis: experimental and observational evidence. *Marine Ecology Progress Series* 187:59-66
- Waite JH (1983) Quinone-tanned scleroproteins. In: Hochachka PW (ed) *The Mollusca*, Vol 1. Academic Press, New York, p 467-504
- Waite JH (1990) The phylogeny and chemical diversity of quinone-tanned glues and varnishes. *Comparative Biochemistry and Physiology B* 97:19-29

- Waite JH, Saleuddin ASM, Andersen SO (1979) Periostracin - a soluble precursor of sclerotized periostracum in *Mytilus edulis* L. *Journal of Comparative Physiology B* 130:301-307
- Waite JH, Wilbur KM (1976) Phenoloxidase in the periostracum of the marine bivalve *Modiolus demissus* Dillwyn. *Journal of Experimental Zoology* 195:359-368
- Willemsen PR (1994) The screening of sponge extracts for antifouling activity using a bioassay with laboratory-reared cyprid larvae of the barnacle *Balanus amphitrite*. *International Biodeterioration & Biodegradation*:361-373

Synthesis & Discussion

The periostracum is the key?

In the past two decades, marine organisms have been investigated with increasing intensity regarding their antifouling potential as a consequence of the proposed ban of the highly toxic, Tributyltin (TBT)-containing antifouling paints. This ban becomes worldwide effective in 2008, and has given new impulses in the understanding and development of non-toxic alternatives, especially in marine sciences. Various antifouling compounds have been isolated from marine organisms, often with a taxonomically broad activity. While in the late 1980s – 90s the focus was mainly on the pure identification of these compounds (Clare 1996), more recently the ecological role of antifouling defences has been investigated. Natural antifouling systems of marine organisms are highly complex, often with several mechanisms working alternating, complementarily or synergistically (Schmitt et al. 1995, Wahl 1997).

The ecologically complex interactions of basi- and epibionts and/or predators (Krug 2006) and the taxonomic and functional diversity of potential epibionts requires a multi-level defence system with chemical, physical and mechanical mechanisms (Wahl 1997). A multiple defence may prevent adaptations of the fouling organisms and takes into account the various types of epibionts and their varying requirements, tolerances and susceptibilities.

Natural defence systems with two or more synergistic mechanisms have been studied before. In the gorgonians *Leptogorgia* and *Pterogorgia* toxic secondary compounds slow down surface fouling and are combined with a periodical ablation of the outer surface (Targett et al. 1983). The ascidian

Polysyncraton lacazei possesses one mechanical, one associational and four chemical mechanisms, targeting various types of fouling organism (Wahl & Banaigs 1991), and in four Caribbean sponges (*Agelas clathrodes*, *Cinachyrella alloclada*, *Clathria virgultosa* and *Xestospongia muta*) a chemical defence acts synergistically with glass spicules against generalist fish predators (Jones et al. 2005).

The results produced within this project have contributed to a better understanding of the antifouling defence system of the blue mussel *Mytilus edulis* and of the possible role of the defensive surface microtopography of Mytilidae with regard to their invasion potential. Field and laboratory assays have been conducted to investigate the functioning of single mechanisms:

- i. The microtopographies of the periostracum of *Mytilus edulis* and *Perna perna*, when replicated using high resolution resin, have shown to prevent settlement of barnacles for some time (Chapter I).
- ii. When exposed to a natural fouling community, the effect of the microtopography is not sufficient to prevent fouling, though transient activity is also apparent (Chapter II).
- iii. The periostracum of *M. edulis* possesses antifouling compounds. These compounds are polar to moderately non-polar (Chapter III, IV) and prevent settlement of microfoulers, such as bacteria and diatoms, and slow down or inhibit their growth.

All mytilid species investigated in this study (*Mytilus edulis*, *M. galloprovincialis planatus*, *Perna perna*, *P. viridis*) possess similar ribbed, isotropic microtopographies on their periostracum. The antifouling properties of this microtopography target mainly macrofoulers. Artificial isotropic microtopographies have also been shown to reduce barnacle settlement by reducing exploration time (Berntsson et al. 2000a). It is therefore possible that the generalised anti-settlement property reported in Chapter I is a reflection of this behaviour, because cyprids of *Semibalanus balanoides* settled on topographies at the micrometre scale as long as they were anisotropic. It is furthermore possible that isotropic microtopographies will confer a general protection against the settlement of all barnacle larvae, since the microtopography of *Mytilus galloprovincialis planatus* already showed the same anti-settlement properties with regard to *Balanus amphitrite* (Scardino et al. 2003).

Although effective against cyprid larvae, all investigated microtopographies failed to prevent fouling in a persistent way (Chapter II) when exposed to a natural fouling community, being composed of bacteria, unicellular algae, macroalgal spores and invertebrate larvae. Decoupled from other shell properties, the microtopographies were rapidly covered by a thick biofilm. We suspect that the microfouling filled up the ripples and therefore masked the microtopographies, making them subsequently not detectable for invertebrate larvae and algal spores that generally exhibit active surface selection (Rittschof & Costlow 1989, Johnson 1994, Hills & Thomason 1998, Berntsson et al. 2000a, Berntsson et al. 2000b, Callow et al. 2000, Callow et al. 2002). These result underlined the

need of additional antifouling mechanism, which enables mussels to maintain a relatively clean surface for a longer time.

Consequently, the surface chemistry of the periostracum of the Baltic *Mytilus edulis* was investigated: some crude extracts, soluble in non-polar and moderately polar solvents, not only inhibit settlement of cyprid larvae, but also settlement and growth of two bacteria and a benthic diatom (Chapter III). The most promising compounds have been studied more detailed (Chapter IV), revealing that they are ethanol-soluble, and that the amount of this compound extracted from the periostracum increases with shell size. The accumulation of ethanol-soluble antifouling compounds in the periostracum may be a consequence of the aging of the periostracum, the so-called quinone tanning (Waite et al. 1979, Waite 1983). In the course of the tanning process, the periostracum becomes more and more hydrophobic (Waite 1983), which may potentially result in an increasing accumulation of hydrophobic compounds from the surrounding water or the surface-associated biofilm. The functional enzymes in the tanning process, phenol oxidases and hydroxylases, remain furthermore active month and even years after excretion of the periostracum, so a role of these or other enzymes in the biosynthesis of antifouling compounds cannot be excluded. Active substances may also be excreted by the mussel and transported via pores through the shell into the periostracum. In Mytilidae, however, there are no channels penetrating the entire shell, but tubules that only reach into the endostracum (Reindl & Haszprunar 1996), the innermost layer of the periostracum.

The species tested so far respond very differently to the periostracal compounds: while settlement of bacterium *Shewanella baltica* was significantly enhanced by “fraction 4”, the same compound strongly inhibited settlement of bacterium *Bacillus aquimaris* and the benthic diatom *Amphora coffeaeformis* (Chapter IV). It remains therefore arguable whether periostracal compounds affect the composition of surface fouling by modulating bacterial and macroalgal settlement, as found in other natural defence systems, e.g. in the soft coral *Dendronephthya* sp. (Harder et al. 2003, Dobretsov & Qian 2004).

Conclusions

The findings of all experiments strongly support the idea of a multiple antifouling system in *Mytilus edulis*, as previously suggested by Wahl et al. (1998). I have been able to show that the mytilid surface topography repels macrofoulers, while surface-bound antifouling compounds affect microfoulers. Without the chemical anti-microfouling activity, the efficiency of the microtopography is jeopardised by microbial masking. Therefore, at least two antifouling mechanisms of *Mytilus edulis* are related to characteristics of the periostracum. These two mechanisms may be complemented by cumulative filtration in the mussel bed, as well as with swiping movements of the mussel’s foot to clean its shell on the outside. The latter mechanism has only been reported for small, up to 3 cm long *Mytilus edulis* (Theisen 1972), and may therefore complete the picture with regard to the increasing accumulation of

antifouling compounds in the periostracum of older (>3 cm long) mussels. Cumulative filtration has previously been identified as an associational antifouling mechanism in aggregated mussels (Wahl et al. 1998), which are able to filter more than $100 \text{ m}^3 \text{ m}^{-2} \text{ d}^{-1}$ (Lassen et al. 2006) and therefore reduce the amount of algal propagules and invertebrate larvae in the surrounding water column that may potentially settle on their shells.

A regional optimisation of one antifouling defence mechanism, *i.e.* surface microtopography, has not been found (Chapter I and II). The invasive success of Mytilidae all over the world can therefore not be explained exclusively by a particularly efficient and broad-target antifouling performance of surface structure. However, it should be stressed that in the global comparison only an incomplete part of the mytilid defence system – microtopography without surface chemistry – was tested. Although the surface chemistry of other Mytilids, such as *Mytilus galloprovincialis*, *Perna perna* and *P. viridis*, has so far not been examined, it may also play a considerable part in local antifouling defence.

In conclusion, the present work revealed that the periostracum is the key in *Mytilus edulis*' antifouling defence, and gives new impulses for further investigation of the surface chemistry in Mytilidae and the microbial community on the shell of the living mussel.

References

- Berntsson KM, Andreasson H, Jonsson PR, Larsson L, Ring K, Petronis S, Gatenholm P (2000a) Reduction of barnacle recruitment on micro-textured surfaces: analysis of effective topographic characteristics and evaluation of skin friction. *Biofouling* 16:245-261
- Berntsson KM, Jonsson PR, Lejhall M, Gatenholm P (2000b) Analysis of behavioural rejection of micro-textured surfaces and implications for recruitment by the barnacle *Balanus improvisus*. *Journal of Experimental Marine Biology and Ecology* 251:59-83
- Callow ME, Callow JA, Ista LK, Coleman SE, Nolasco AC, López GP (2000) Use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. *Applied and Environmental Microbiology* 66:3249-3254
- Callow ME, Jennings AR, Brennan AB, Seegert CE, Gibson A, Wilson L, Feinberg A, Baney R, Callow JA (2002) Microtopographic cues for settlement of zoospores of the green fouling alga *Enteromorpha*. *Biofouling* 18:237-245
- Clare AS (1996) Marine natural product antifoulants: status and potential. *Biofouling* 9:211-229
- Dobretsov S, Qian P (2004) The role of epibiotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement. *Journal of Experimental Marine Biology and Ecology* 299:35-50
- Harder T, Lau SCK, Dobretsov SV, 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 mechanisms against bacterial epibiosis. *FEMS Microbial Ecology* 43:337-347
- Hills JM, Thomason JC (1998) The effect of scales of surface roughness on the settlement of barnacle (*Semibalanus balanoides*) cyprids. *Biofouling* 12:57-69

- Johnson LE (1994) Enhanced settlement on microtopographical high points by the intertidal red alga *Halosaccion glandiforme*. *Limnology and Oceanography* 39:1893-1902
- Jones AC, Blum JE, Pawlik JR (2005) Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? *Journal of Experimental Marine Biology and Ecology* 322:67-81
- Krug PJ (2006) Defense of benthic invertebrates against surface colonization by larvae: a chemical arms race. In: Fusetani N, Clare AS (eds) *Antifouling compounds*. Springer, Berlin, p 1-53
- Lassen J, Kortegård M, Riisgård HU, Friedrichs M, Graf G, Larsen PS (2006) Down-mixing of phytoplankton above filterfeeding mussels - interplay between water flow and biomixing. *Marine Ecology Progress Series* 314:77-88
- Reindl S, Haszprunar G (1996) Fine structure of caeca and mantle of arcoid and limpoid bivalves (Mollusca: Pteriomorpha). *Veliger* 39:101-116
- Rittschof D, Costlow JD (1989) Surface determination of macroinvertebrate larval settlement. In: Klekowski R, Styczynska-Jurewicz E, Falkowsky L (eds) *Twenty first European Marine Biology Symposium*. Polish Academy of Sciences, Institut for Oceanology, Gdansk, Poland, p 155-163
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctata imbricata*. *Biofouling* 19 (Supplement):221-230
- Schmitt TM, Hay ME, Lindquist N (1995) Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. *Ecology* 76:107-123
- Targett NM, Bishop SS, McConnell OJ, Yoder JA (1983) Antifouling agents against the benthic marine diatom, *Navicula salinicola*: homarine from the gorgonians *Leptogorgia virgulata* and *L. setacea* and analogs. *Journal of Chemical Ecology* 9:817-829
- Theisen BF (1972) Shell cleaning and deposit feeding in *Mytilus edulis* L. (Bivalvia). *Ophelia* 10:49-55
- Wahl M (1997) Living attached: aufwuchs, fouling, epibiosis. In: Nagabushanam R, Thompson M (eds) *Fouling organisms of the Indian Ocean: biology and control technology*. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, p 31-83
- Wahl M, Banaigs B (1991) Marine epibiosis III: Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). *Journal of Experimental Marine Biology and Ecology* 145:49-63
- Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205-226
- Waite JH (1983) Quinone-tanned scleroproteins. In: Hochachka PW (ed) *The Mollusca*, Vol 1. Academic Press, New York, p 467-504
- Waite JH, Saleuddin ASM, Andersen SO (1979) Periostracin - a soluble precursor of sclerotized periostracum in *Mytilus edulis* L. *Journal of Comparative Physiology B* 130:301-307

Viele Menschen haben mich mit praktischen Ratschlägen, unermüdlichem Einsatz und konstruktiven Diskussionen unterstützt, und damit direkt oder indirekt zum Gelingen dieser Arbeit beigetragen.

Zunächst danke ich Herrn Prof. Martin Wahl für seine Betreuung, seine Unterstützung bei der Durchführung dieser Arbeit und seine stete Diskussionsbereitschaft.

Dr. Florian Weinberger danke ich für die Einführung in die chemischen Fragestellungen und seine ungewöhnlichen Ideen und Begeisterungsfähigkeit, die erheblich zur Komplettierung dieser Arbeit beigetragen haben.

Meinen Projektpartnern danke ich sehr herzlich, denn ohne ihre Hilfe, ihren Einsatz und ihr Interesse wäre ein solch globales Experiment nicht durchführbar gewesen: Prof. Bernardo da Gama und Felipe da Silva (Brasilien), Dr. Martin Thiel und Nelson Valdivia (Chile), Prof. Christopher McQuaid und Eliecer Diaz (Südafrika), Dr. Jeremy Thomason und Heather Sugden (Großbritannien), Andrew Scardino (Australien), Prof. Peter Petraitis und Erika Carlson Rhile (USA) und Dr. Sergey Dobretsov (erst Russland, dann Hong Kong, und jetzt Kiel).

Den Mitarbeitern von TNO Science und Industry, Dr. Peter Willemsen, Dr. Job Klijnstra und Dr. Fraddy D'Souza danke ich für die wiederholte Unterstützung bei der Durchführung der chemischen Untersuchungen.

Nadja Stärck, Frauke Symanowski, Claas Hiebenthal, Gabrielle Prendergast, Constanze Zürn, Sven Rohde, Gitta Spindler und Peter Deines danke ich für ihre Unterstützung im Labor, bei den Feldversuchen und beim bereitwilligen Korrekturlesen.

Mein ganz besonderer Dank gilt meiner lieben Freundin Ute Kossak, die in den vergangenen Jahren nicht nur mit mir das Arbeitszimmer geteilt hat und jederzeit zu (außer-)fachlichen Diskussionen bereit war, sondern die es auch wie kein zweiter verstanden hat, meine Launen zu ertragen, mich trotzdem immer wieder zum Lachen zu bringen und mich zu ermutigen.

Der Studienstiftung des deutschen Volkes danke ich herzlich für die unkomplizierte, großzügige Unterstützung meiner Arbeit, der Deutschen Forschungsgemeinschaft sei gedankt für die Sachmittelbeihilfe (WA 708/13 1+2).

Margarete und Peter Främcke danke ich für ihre Fürsprache.

Meinen Eltern und meinem Bruder danke ich von Herzen für ihre umfassende Unterstützung in allen Lebenslagen, und für ihr mir immer gegenwärtiges und gewisses Vertrauen.

Curriculum Vitae

Anna Valeria Bers

geboren am 12.11.1976
in Köln
Staatsangehörigkeit: deutsch

Studium

- 05/2004 - derzeit Promotion im Fachbereich Marine Ökologie am IfM-Geomar
Leibnizinstitut für Meereswissenschaften an der CAU Kiel
- 03/2003 Diplom in Biologie (CAU Kiel)
Hauptfach: Zoologie
Nebenfächer: Biologische Meereskunde, Geologie
- 01/2002 - 01/2003 Diplomarbeit am Institut für Meereskunde Kiel
Thema: Surface structure of marine organisms and its influence on fouling
- 02/2000 - 02/2001 Studium der Meereswissenschaften an der Otago University in
Dunedin, NZ; Abschluß mit dem Postgraduate Diploma in Marine
Sciences; *Abschlußarbeit: Observations on induced spawning in
the euryalid snake star Astrobrachion constrictum*
- 02/1999 Vordiplom Biologie
- 09/1996-01/2003 Studium der Biologie (Diplom) an der Christian-Albrechts-
Universität zu Kiel (CAU)

Berufliche Tätigkeiten

- 10/2005 - 01/2006 Biodiversity Consultant für UNDP Maldives
- 11/2003 - 05/2004 Meeresbiologin auf Kuramathi, Rasdhoo Atoll, Malediven

Stipendien

- 2004-2006 Promotionsstipendium der Studienstiftung des deutschen Volkes
- 2005 Stipendium des Carlo-Schmid-Programms für Praktika in
Internationalen Organisationen

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Dissertation ist nach Form und Inhalt meine eigene Arbeit. Sie wurde weder ganz noch zum Teil einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein erstes und bisher einziges Promotionsverfahren. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

Kiel, 1. November 2006

Teile dieser Dissertation wurden bereits wie folgt veröffentlicht bzw. eingereicht:

Bers AV, Prendergast GS, Zürn CM, Hansson L, Head RM and Thomason JC (2006): A comparative study of the antissettlement properties of mytilid shells. *Biology Letters* 2, 88-91

Bers AV, D'Souza F, Klijnsstra JW, Willemsen PR and Wahl M (2006): Chemical defence in mussels - antifouling effects of crude extracts of the periostracum of the blue mussel *Mytilus edulis*. *Biofouling* 22(4), 251-259

Bers, AV, da Gama BAP, da Silva FV, Petraitis PS, Dobretsov S, Thiel M, Valdivia N, Scardino AJ, McQuaid C, Diaz E, Sudgen H, Thomason JC and Wahl M: Relevance of mytilid microtopographies for antifouling defence and invasiveness - a global comparison. Eingereicht bei *Marine Ecology Progress Series*

Bers AV, Weinberger F, D'Souza F, Klijnsstra JW, Wahl M: Bioassay guided investigation of one periostracal extract of *Mytilus edulis* with regard to its antifouling potential. Wird eingereicht beim *Journal of Chemical Ecology*