Natural antifouling defences of tropical sea stars

Thesis submitted by

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Abstract

Qualitative evidence suggests that sea stars have remarkably clean epidermal surfaces. To quantify surface-associated micro- and macroorganisms, field surveys were conducted in northern Queensland, Australia, during the wet and dry seasons. Mean bacterial abundances on 7 sea star species were approximately 10^4 to 10^5 cells cm^{-2} during both seasons. There were no consistent trends in bacterial abundances with season, species and aboral positions on sea star arms. Low numbers of parasitic and commensal macroorganisms were found on 6 sea star species, and no common generalist macrofouling organisms, such as algae, barnacles, serpulid polychaetes, bryozoans and ascidians, were discovered on any specimens. Sea stars therefore offered an excellent model to investigate the mechanisms driving fouling-resistant surfaces. Subsequently, 5 sea star species belonging to the order Valvatida were chosen for this study to investigate mechanical, physical and chemical fouling deterrence: *Acanthaster planci* (Family Acanthasteridae), *Linckia laevigata* and *Fromia indica* (both Family Ophidiasteridae), *Cryptasterina pentagona* (Family Asterinidae) and *Archaster typicus* (Family Archasteridae).

It has been proposed that mechanical antifouling defence mechanisms of sea stars are linked with pedicellariae, which are pincer-like appendages made of calcareous ossicles. In this study, the role of pedicellariae of the sea star *A. planci* in fouling control was investigated. The morphology and distribution of its pedicellariae were measured to determine if larvae or propagules of fouling organisms could settle between pedicellariae without being in their physical range. The elementary and straight pedicellariae of *A. planci* had a mean length of 0.7 mm and a mean distance of 2.6 mm. The total number of pedicellariae was proportional to the estimated surface area of *A. planci*. To determine how pedicellariae respond to tactile stimulation, pedicellariae were stimulated by touching either inner, outer or basal sites of pedicellariae with a hypodermic needle. Pedicellariae closed rapidly on touch and closed for significantly longer when touched at their inner sites (8.9 s) than outer (6.7 s) and basal (7.9 s) sites. Settling larvae were simulated by dropping silica beads (size: 50.2 μm, 181.5 μm, 255.7 μm and 510.7 μm, density: 2.5 g ml^{-1}) and zirconium/silica beads (size: 191.2 μm and 507.6 μm, density: 3.7 g ml^{-1}) over the pedicellariae. The percentage of responding pedicellariae increased proportionally with increasing size of the silica beads. However, the percentage also increased when zirconium/silica beads of similar size but higher density were used, demonstrating that the mass, not size, of the beads was the main driving factor for the closure of pedicellariae. Pedicellariae were also stimulated by placing larvae of the bryozoan *Bugula neritina* (250 μm)

and fragments of the alga *Chrysocystis fragilis* (150-1000 μm) over the pedicellariae. However, the response of the pedicellariae to the larvae of *B. neritina* was consistently low and none of the pedicellariae responded to the fragments of *C. fragilis*, demonstrating that for *A. planci* pedicellariae offer little or no defence against fouling.

Subsequently, physical antifouling defences of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were investigated. These sea stars have paxillae (modified ossicles with a median vertical pillar) on their aboral surfaces, which varied in diameter, height and distance depending on species and position on the aboral surface, providing unique and complex surface microtopographies for each species. The surfaces of the sea stars *L. laevigata*, *F. indica* and *A. typicus* were moderately wettable, with their mean seawater contact angles on the disk being 60.1º, 70.3º and 57.3º, respectively. The seawater contact angle of *C. pentagona* could not be measured. To evaluate the effectiveness of the surface microtopographies of sea stars in deterring the settlement of fouling organisms, field experiments with resin replicas of the 4 sea star species were conducted at 3 sites around Townsville, Australia, for 8 weeks during the dry and wet seasons. The fouling community and total fouling cover did not differ significantly between the replicas of sea stars and control surfaces at any site during the dry season. Significant differences between fouling communities on the replicas of the sea stars and control surfaces were detected at 2 sites during the wet season, however these differences were transitory and the total fouling cover also did not differ significantly between replicas of sea stars and control surfaces at 2 of the 3 sites. Despite a complex variety of structures, the surface microtopographies of these sea stars alone were not effective in deterring the settlement and growth of fouling organisms.

Finally, the role of natural products of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* in keeping the surface free of fouling organisms was investigated. Dichloromethane (non-polar), methanol (moderately polar) and water (polar) extracts of these sea stars all had concentration-dependant effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *Nitzschia closterium*, the serpulid *Hydroides elegans* and the bryozoan *B. neritina* in settlement assays. While all extracts at the highest concentration of 100 μg cm^{-2} significantly reduced the settlement of all fouling species, dichloromethane and methanol extracts at the lower concentrations of 10, 1 and 0.1 μg cm^{-2} were generally more effective than water extracts. The dichloromethane extract of the sea star *C. pentagona* was further fractionated using reversed-phase flash column chromatography and high performance liquid chromatography. Fractions at a concentration of 10 μg cm^{-2} were tested against the settlement of *N. closterium*.

Based on analysis with gas chromatography – mass spectrometry (GC-MS), the most bioactive fractions of *C. pentagona* contained several fatty acids and sterols.

To determine whether the compounds responsible for the observed antifouling effects were also present on the surface of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, surface-associated compounds were absorbed onto filter paper and tested against *Amphora* sp., *N. closterium*, *H. elegans* and *B. neritina* in settlement assays. These surface-associated compounds had species-specific effects on the settlement of fouling organisms. Subsequently, to demonstrate the presence and quantify the natural concentrations of the surface-associated compounds, surface extracts of the 4 sea star species were prepared by swabbing their surfaces with cotton wool, which was then extracted with dichloromethane. Using GC-MS, the fatty acids and sterols of each surface extract were identified and the surface concentrations of hexadecanoic acid, cholesterol, lathosterol and sitosterol quantified. Pure hexadecanoic acid, cholesterol, lathosterol and sitosterol as well as a mixture of these 4 compounds at 1000, 100, 10 and 1 ng cm^{-2} were tested against the settlement of *Amphora* sp., *N. closterium* and *B. neritina*. Hexadecanoic acid and cholesterol were the most effective compounds, significantly reducing the settlement of both *Amphora* sp. and *N. closterium* at concentrations of 1000 and ≥10 ng cm^{-2}, respectively. Lathosterol also significantly inhibited the settlement of *Amphora* sp. and *N. closterium* at the higher concentrations of 1000 and ≥100 ng cm^{-2}, respectively. In contrast, sitosterol was only effective against *N. closterium*, with significantly less settlement of this species at concentrations of ≥100 ng cm^{-2}. None of the 4 compounds had any effect on the settlement of *B. neritina*. The mixture of the compounds significantly reduced the settlement of only *N. closterium* at concentrations of ≥100 ng cm^{-2}.

In conclusion, this study has demonstrated that the surface chemistry, in particular the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol, of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* play a key role in deterring the settlement of fouling organisms. These compounds are therefore primary metabolites with a secondary function in fouling control. In contrast to the surface chemistry, the pedicellariae of *A. planci* and the surface microtopographies and wettabilities of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were ineffective.

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Chapter 1

General introduction

1.1 Biofouling in the marine environment

Marine biofouling is the formation of a complex layer of organisms on submerged solid, living or non-living, surfaces. When fouling organisms settle and grow on living surfaces, the non-symbiotic, often facultative, association between the fouling organism (epibiont) and the living surface (basibiont) is also termed epibiosis (Wahl 1989, Wahl and Mark 1999). Biofouling may have major economic impacts on aquaculture and shipping industries (Lodeiros and Himmelman 1996, Abbott et al. 2000, Champ 2000) and ecological impacts on living surfaces (reviewed by Wahl 1989, de Nys and Steinberg 1999).

The classical view of biofouling (Figure 1.1A) has been described as a sequence of (1) molecular fouling, when dissolved organic molecules adsorb to the surface and form a conditioning film, thereby changing physical and chemical surface properties, (2) microfouling, when bacteria, yeasts, protozoa and diatoms attach to the surface and form a biofilm, and (3) macrofouling, when algal spores and invertebrate larvae settle (reviewed by Little 1984, Wahl 1989, Maki and Mitchell 2002). This biofouling sequence implies some causality between the fouling stages and may only occur under certain conditions (Maki and Mitchell 2002). In contrast, the 'seasonal or probabilistic' view of biofouling (Figure 1.1B) is based on the probability that a molecule or fouling organism will encounter and attach to a substratum (Maki and Mitchell 2002). In this model, all 3 fouling stages take place concurrently and form a

complex web of interactions between the substratum and fouling organisms, and interspecifically between fouling organisms. Subsequent biofouling communities are dynamic both in their structure and function, growing continuously by mechanisms, such as disturbance, facilitation, inhibition and tolerance (Wahl 1989, Maki and Mitchell 2002).

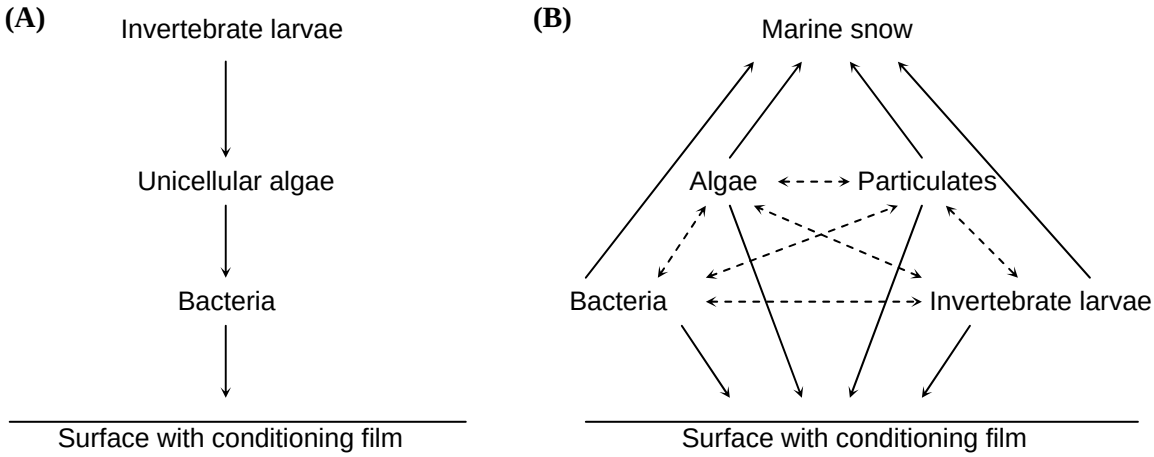

Figure 1.1 Diagrams of 2 models for biofouling: **(A)** the classical view of successional fouling on a substratum, and **(B)** the "seasonal or probabilistic" view of substratum fouling based on the probability that a particular fouling component will encounter the substratum. Adapted from Clare et al. (1992).

The settlement of fouling organisms is also influenced by the interaction of abiotic and biotic factors operating at different temporal and spatial scales (Rodríguez et al. 1993). Physical factors affecting the settlement of fouling organisms include hydrodynamics (Butman 1987), light (Crisp and Ritz 1973), gravity (Walt et al. 1985), salinity and temperature (Rodríguez et al. 1993) as well as surface characteristics, such as wettability (Callow et al. 2000, Finlay et al. 2002, Dahlström et al. 2004, Greer and Amsler 2004), topography (Andersson et al. 1999, Berntsson et al. 2000a,b, Callow et al. 2002) and colour (Yule and Walker 1984). Chemical factors include natural inducers or inhibitors associated with conspecifics (Knight-Jones and Crisp 1953, Burke 1986, Head et al. 2003, Head et al. 2004) and microbial films (Todd and Keough 1994, Wieczorek and Todd 1997, Lau et al. 2002, Patel et al. 2003). Finally, biological factors, such as larval age, size and behaviour may also influence the settlement of fouling organisms (Marshall and Keough 2003, Gribben et al. 2006).

A range of terms, such as 'adhesion', 'attachment' and 'settlement', have been used in the literature to describe the process of cells, propagules and larvae encountering and non-reversibly

attaching to a surface. In this study, the term 'settlement' refers to the adhesion and/or attachment of all fouling organisms, including subsequent metamorphosis where applicable.

1.2 Biological impacts of biofouling

Epibiosis can have significant consequences for the epibiont, the basibiont or both. The colonization of fouling organisms may be beneficial, because epibionts have a protective role for some basibionts. For example, the hydroid *Hydractinia* sp. on the shells of the hermit crab *Pagurus pollicaris* (Brooks and Mariscal 1985), and a range of epibionts on the carapace of the spider crab *Maja squinado* (Parapar et al. 1997) and the mussel *Mytilus edulis* (Laudien and Wahl 1999, Thieltges 2005), protect the basibionts from predation. Epiphytes on the intertidal seagrass *Zostera marina* also protect the seagrass from desiccation during low tide (Penhale and Smith 1977). Furthermore, hydroid colonies on the giant kelp *Macrocystis pyrifera* enhance frond growth during periods of low concentrations of inorganic nitrogen in seawater, due to the provision of ammonium excreted by the hydroid colonies (Hepburn and Hurd 2005, Hepburn et al. 2006).

In contrast to these positive impacts, epibiosis may also have major negative impacts on marine organisms. For marine algae, 2 of the most significant impacts of fouling are those on photosynthesis and growth. Epiphytes attenuate light reaching the surface of the seagrass *Z. marina* (Brush and Nixon 2002) and reduce the photosynthesis of the seagrasses *Thalassia testudinum* and *Z. marina* (Drake et al. 2003). Encrusting bryozoans also reduce the photosynthesis of *Fucus serratus* (Oswald et al. 1984) and *Gelidium rex* (Cancino et al. 1987). Contrary to the positive effects of hydroids on *M. pyrifera* during periods of low nitrogen concentrations (Hepburn and Hurd 2005), encrusting bryozoans can also reduce growth rates of this kelp (Dixon et al. 1984) and cause tissue damage, resulting in lower pigment concentration (Hepburn et al. 2006). Furthermore, the epiphyte *Polysiphonia lanosa* may also interfere with the reproductive output of the brown alga *Ascophyllum nodosum*, by reducing the receptacle biomass of its basibiont (Kraberg and Norton 2007).

A broad range of marine animals may also be negatively affected by epibionts. For bivalves, epibionts may negatively affect the shell condition, growth rate and survival of the pearl oysters *Pinctada fucata* (Mohammad 1976), *Pinctada margaritifera* (Mao Che et al. 1996, Pit and Southgate 2003) and *Pinctada maxima* (Taylor et al. 1997), the scallop *Euvola ziczac* (Lodeiros and Himmelman 1996) and the mussel *Perna perna* (Kaehler and McQuaid 1999). The endolithic boring sponge *Cliona* sp. and the polychaete *Polydora ciliata* also weaken the shells

of the gastropod *Littorina littorea* (Stefaniak et al. 2005, Thieltges and Buschbaum 2007), making them more prone to predation (Stefaniak et al. 2005). These boring species together with the barnacle *Balanus crenatus* and tissue-invading trematodes further reduce the fecundity, growth and survival of this gastropod (Thieltges and Buschbaum 2007). Furthermore, epibionts increase mass and drag (Wahl 1997) and restrict the mobility and lower the crawling speed of *L. littorea* (Buschbaum and Reise 1999) and *Batillaria zonalis* (Chan and Chan 2005).

Some epibionts may affect the biological function of a basibiont's appendages. For example, mussels attached to the branchial appendages of the horseshoe crab *Limulus polyphemus* can impair aeration of the crab's gills (Botton 1981). Encrusting bryozoans on the eyes and antennae of the crab *Carcinus maenas* can lead to the loss of function of these organs (Cadee 1991). Furthermore, epizoic barnacles, in particular the little known barnacle *Platylepas ophiophilus*, impair the ability of sea snakes, such as *Aipysurus laevis* and *Lapemis hardwickii*, to shed their skin (Zann 1975, Zann et al. 1975).

Finally, basibionts may be damaged by predators of epibionts. Kelp blades of *M. pyrifera* encrusted with the bryozoan *Membranipora membranacea* and the barnacle *Lepas pacifica* are more readily damaged by the labrid fish *Oxyjulis californica* (Bernstein and Jung 1979). The protozoan *Colacium vesiculosum* on the pelagic crustacean zooplankton *Daphnia* may also make the zooplankton more susceptible to planktivorous fish predation due to increased visibility (Chiavelli et al. 1993).

1.3 Defence mechanisms against biofouling

Given the ecological costs associated with epibiosis, many marine plants and animals have evolved defence mechanisms to reduce these disadvantages and ecological costs. Behavioural, mechanical, physical and chemical defence mechanisms of a range of marine organisms against the settlement of fouling organisms are introduced, with defence mechanisms of echinoderms as the focus of this thesis.

1.3.1 Behavioural defence mechanisms

A number of behavioural mechanisms have been identified that deter or control the settlement, growth and survival of fouling organisms. Some benthic motile species, such as isopods, crabs and gastropods, burrow into soft bottom substrates or hide in rock crevices, thereby abrading the surface and minimizing the exposure to fouling organisms (Glynn 1970, Becker and Wahl 1996,

Fernández-Leborans et al. 1997, Olafsdottir and Svavarsson 2002, Vasconcelos et al. 2007). The nocturnal activity of some crabs also restricts algal growth on their carapace (Becker and Wahl 1996). Furthermore, intertidal exposure to air leads to desiccation stress of fouling organisms on crabs and gastropods (Becker and Wahl 1996, Vasconcelos et al. 2007).

In high density populations of the gastropod *L. littorea*, specimens often move over one another, grazing and secreting mucus onto each others shells, all of which inhibit fouling (Wahl and Sönnichsen 1992). Similarly, the gastropod *Calliostoma zizyphinum* regularly wipes the surface of its shell with its foot, which has an important role for both feeding and antifouling (Holmes et al. 2001). When specimens of *C. zizyphinum* were prevented from wiping their shells, the fouling cover on their shells was 9 times higher than on wiped shells (Holmes et al. 2001).

While behavioural mechanisms against fouling organisms are important, they may not have evolved specifically as antifouling mechanisms. For example, crustaceans gain additional benefits from burrowing, such as reduced predation (Kuhlmann 1992), which may be of even greater importance than antifouling (Becker and Wahl 1996).

1.3.2 Mechanical defence mechanisms

Mechanical defence mechanisms may also reduce fouling on basibionts. Some marine organisms mechanically remove epibionts by shedding of surface layers (Keats et al. 1993). For example, the encrusting coralline alga *Spongites yengoi* sloughs off deep layers within the thallus (Keats et al. 1993) to remove associated micro- and macrofouling organisms, whereas *Sporolithon ptychoides*, *Neogoniolithon fosliei* and *Hydrolithon onkodes* only slough off surface layers (Keats et al. 1997), and the algae *Ascophyllum nodosum*, *Chondrus crispus* and *Dilsea carnosa* slough off the cuticle (Sieburth and Tootle 1981, Nylund and Pavia 2005). Sponges have a similar mechanical mechanism. When the ostia of the sponge *Halichondria panicea* get blocked with organic material and fouling organisms, the sponge sloughs off its complete outer tissue layer, starting at the rim of the oscula (Barthel and Wolfrath 1989). For higher organisms, mechanical defences can also be effective at removing epibionts. For example, the loggerhead sea turtle *Caretta caretta* sheds portions of their scutes, which also remove some sessile organisms (Caine 1986). Furthermore, some marine organisms remove epibionts by renewing their surfaces through continuous or periodical mucus secretion (Wahl et al. 1998, Bavington et al. 2004). Bavington et al. (2004) demonstrated that glycoproteins in the mucous of the sea star *Marthasterias glacialis* inhibit the adhesion of bacteria, by causing bacterial clumpings, which are subsequently washed away.

Mechanical removal of epibionts can also be achieved by friction between the surface of the basibiont and the sediment for burrowing species (Littler and Littler 1984, Wahl 1989, Svavarsson and Davidsdottir 1994) or between the surface and water for fast swimming species (Wahl 1989). Additionally, surface structures, such as spines of the bryozoans *Microciona atrasanguinea* and *Electra pilosa* (Stebbing 1973, Dyrynda 1986), and cleaning of the surface, such as the gills of the shrimps *Nihonotrypaea japonica* and *Upogebia major*, by active scraping with specialized appendages may reduce epibionts (Batang and Suzuki 2003). However, these mechanisms are of variable efficiency, depending on the local fouling pressure, the size of the epibionts and the proportion of the surface being cleaned (Wahl 1989).

Anecdotal evidence suggests that mechanical antifouling defence mechanisms of echinoderms are linked with pedicellariae, which are forcep- or pincer-like appendages made of calcareous ossicles and found in sea urchins and some sea stars (Chia and Amerongen 1975, Campbell and Rainbow 1977). Pedicellariae have reported functions in deterring predators and competitors (Jensen 1966, Lubchenco Menge and Menge 1974, van Veldhuizen and Oakes 1981), capturing mobile prey (Robilliard 1971, Campbell 1973, Campbell 1974, Chia and Amerongen 1975, Hendler and Franz 1982, Dearborn et al. 1991, Emson and Young 1994, Lauerman 1998) and deterring settling larvae of fouling organisms (Campbell and Rainbow 1977). In the only study of the antifouling role of pedicellariae, mobile pedicellariae and spines of the sea urchin *Echinus esculentus* prevented the settlement of cypris larvae of the barnacle *Balanus balanoides* (Campbell and Rainbow 1977). Pedicellariae of sea stars are proposed to also protect the surface from settling fouling organisms (Nichols 1966, Campbell and Rainbow 1977, Ruppert and Barnes 1994). However, the role of the pedicellariae of sea stars in keeping the surface free of any macrofouling organisms has not been rigorously examined and remains speculative.

1.3.3 Physical defence mechanisms

Research on physical defence mechanisms against the settlement of fouling organisms has focused on the wettability and microtopography of both natural and artificial surfaces. Surface wettability and surface tension influence the attachment and strength of attachment of fouling organisms to solid surfaces. While the effects of the surface wettability of artificial surfaces on the settlement of marine organisms are well documented (Callow et al. 2000, Finlay et al. 2002, Dahlström et al. 2004, Greer and Amsler 2004, Aldred et al. 2006), contact angle measurements of marine organism are scarce (Vrolijk et al. 1990, Becker et al. 2000) and completely lacking for echinoderms. Low surface energies of 23 to 27 mN m^{-1} of the gorgonians *Pseudopteragorgia americana* and *Pseudopteragorgia acerosa* (Vrolijk et al. 1990) have been correlated with reduced settlement of microfouling organisms.

Some surface microtopographies of artificial (Andersson et al. 1999, Berntsson et al. 2000a,b, Petronis et al. 2000, Callow et al. 2002, Hoipkemeier-Wilson et al. 2004, Scardino et al. 2006, Schumacher et al. 2007a,b) and natural surfaces (Baum et al. 2002, Scardino et al. 2003, Bers and Wahl 2004, Scardino and de Nys 2004, Guenther and de Nys 2006) also deter the settlement of fouling organisms. For example, artificial surfaces with microtopographies in the range of 50 to 100 μm (Andersson et al. 1999) and 30 to 45 μm (Berntsson et al. 2000a,b) reduce the settlement of the barnacle *Balanus improvisus*. However, the effect of these surfaces on settlement decrease with time and increasing recruitment intensity (Andersson et al. 1999, Berntsson et al. 2000b). This may be explained by a lack of preferred settlement substrata, reduced selectiveness for settlement cues with larval age or an increase in settlement cues associated with the presence of conspecifics (Berntsson et al. 2000b). Spores of the green macroalga *Enteromorpha linza* also respond to defined microtopographies, preferentially settling in valleys and against pillars (both 5 μm) (Callow et al. 2002). Fewer spores settled on the surfaces with lower profile features, probably due to higher energy requirements for settlement (Callow et al. 2002). Similarly, Schumacher et al. (2007a) demonstrated that surface microtopographies with a feature spacing of 2 μm reduced the settlement of spores of *Ulva* (syn. *Enteromorpha*). Effective surface microtopographies are often smaller than the size of larvae, propagules or cells of fouling organisms, thereby reducing the contact area available for the adhesion and attachment of larvae and propagules (Callow et al. 2002, Scardino et al. 2006).

One of the best examples of a natural surface with a physical defence mechanism is the skin of the pilot whale *Globicephala melas*, where 0.1-1.2 μm^2 pores enclosed in a network of nanoridges reduce the surface available for adhesion and attachment to the pore margins and tips of the nanoridges (Baum et al. 2002). Physical defence mechanisms of the periostracum of shell-bearing molluscs, which is a thin, flexible and sclerotinized protein layer covering calcified shells (Saleuddin and Petit 1983, Harper 1997), have also been investigated, because the periostracum has a secondary function in deterring epibionts (Bottjer and Carter 1980, Bottjer 1981, Kumar and Ayyakkannu 1991, Harper and Skelton 1993, Wahl et al. 1998, Scardino et al. 2003). Studies on the surface microtopographies of the periostracum show that micro-ripples with wavelengths of 1.8 to 1.9 μm on *Mytilus galloprovincialis* (Scardino et al. 2003, Scardino and de Nys 2004), 1.0 to 2.0 μm on *Mytilus edulis* (Bers and Wahl 2004, Bers et al. 2006a), 1.5 to 2.0 μm on *Perna perna* (Bers et al. 2006a) and 0.8 μm on *Pteria penguin* (Guenther and de Nys 2006) correlated with low fouling cover. Similarly, parallel ridges with irregular distances of 15 to 115 μm on the eggcase of the dogfish *Scyliorhinus canicula*, and evenly distributed knobbed surface structures, 10 μm in diameter, on the aboral skeleton plates of the brittle star *Ophiura texturata* had repellent effects on the ciliates *Zoothamnium commune*

and *Vorticella* sp. (Bers and Wahl 2004). Evenly distributed circular elevations, 200 μm in diameter, and spicule-like structures, 2 to 2.5 μm in length, between the elevations of the carapace of the crab *Cancer pagurus* repelled the barnacle *Balanus improvisus* (Bers and Wahl 2004).

Echinoderms have an epidermis, which covers an endoskeleton composed of a layer of collagenous connective tissue and small calcareous ossicles (Ruppert and Barnes 1994). In particular, sea stars of the orders Valvatida and Paxillosida have paxillae on their surfaces, which are modified ossicles with a median vertical pillar crowned with spinelets (Clark and Rowe 1971). The paxillae and spinelets provide complex and unique surface microtopographies with the potential to prevent the settlement of fouling organism. However, physical antifouling defence mechanisms regarding the wettability and surface microtopography of sea stars are unexplored.

1.3.4 Chemical defence mechanisms

Many studies have concentrated on the antifouling activity of surface-associated or exuded natural products extracted from marine algae and invertebrates (reviewed by Wahl 1989, Hay 1996, Steinberg et al. 2001, Steinberg et al. 2002). To unequivocally determine whether marine organisms use natural products to keep their surfaces free of fouling organisms, several criteria need to be fulfilled (Steinberg et al. 2001). Firstly, the marine organisms need to be generally free of fouling organisms in their natural environment. Secondly, the presence of putative compounds on or near the surface of the organism needs to be verified and their natural concentrations quantified. Thirdly, ecologically relevant concentrations of these compounds need to be tested in bioassays with ecologically relevant fouling species to determine whether these compounds have any antifouling effects (Schmitt et al. 1995, Hay 1996, Nylund and Pavia 2003, Dworjanyn et al. 2006, Nylund et al. 2007). Most studies on the antifouling role of surface-associated compounds have concentrated on algae (de Nys et al. 1998, Maximilien et al. 1998, Dworjanyn et al. 1999, Dobretsov et al. 2006, Dworjanyn et al. 2006, Nylund et al. 2007). In these studies, non-polar surface-associated compounds were extracted by dipping the algae in hexane or mixtures of hexane and dichloromethane, and compounds were then quantified (de Nys et al. 1998, Nylund et al. 2007). To date, only terpenoids from the brown alga *Dictyota menstrualis* (Schmitt et al. 1995), phlorotannins from the brown alga *Fucus vesiculosus* (Brock et al. 2007), furanones from the red alga *Delisea pulchra* (Dworjanyn et al. 1999, 2006) and triterpene glycosides from the sponges *Erylus formosus* and *Ectyoplasia ferox* (Kubanek et al. 2002) have been localized at or near the surface at biologically effective concentrations to deter fouling organisms.

Chemical antifouling defences of echinoderms have been investigated using crude extracts and isolated compounds. To identify crude extracts and compounds with antifouling activity, extracts and compounds from sea stars have commonly been tested at various concentrations against bacteria (Bryan et al. 1994, Iorizzi et al. 1995), algal spores (De Marino et al. 2000, Iken et al. 2001, 2003, Greer et al. 2003, Greer et al. 2006) and larvae of fouling organisms (Iorizzi et al. 1995, Bryan et al. 1996). For example, Bryan et al. (1994) showed that the ethanol body wall extracts of several sea star species at ecologically relevant concentrations inhibited the growth of marine microbial species. Similarly, the ethanol whole body extracts of the sea star *Luidia clathrata* inhibited the growth of the bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Iorizzi et al. 1995). Furthermore, aqueous and organic body wall extracts of the sea stars *Astropecten articulatus* and *Luidia clathrata* changed the normally fast and straight swimming movements of spores of the brown alga *Hincksia irregularis* to helical and erratic movements (Iken et al. 2003), had negative effects on the settlement and germination of *H. irregularis* (Greer et al. 2003), and reduced the settlement of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina* (Iorizzi et al. 1995). Finally, ethanol body wall extracts of several sea star species also reduced the settlement of the barnacle *B. amphitrite* and the bryozoan *B. neritina* (Bryan et al. 1996).

Although the results of bioassays with whole body or body wall extracts are useful to determine the presence of potential antifouling compounds in the tissues of sea stars, they are insufficient to unequivocally determine an antifouling role of compounds on the surface of sea stars. The presence and concentration of compounds on the surface of sea stars and their effectiveness at ecological relevant concentrations against the settlement of fouling organisms still have to be investigated.

1.3.5 Multiple defence mechanisms

Natural antifouling mechanisms are highly complex, with many marine species employing various combinations of behavioural, mechanical, physical and/or chemical defence mechanisms to deter the settlement of a wide range of fouling organisms. For example, the gorgonian *Pseudopterogorgia americana* uses both mechanical and physical defence mechanisms against fouling, by secreting large amounts of mucus and having low surface energies of 23 to 27 mN m^{-1} (Ciereszko et al. 1973, Vrolijk et al. 1990). The gorgonian *Leptogorgia virgulata* uses mechanical and chemical defences to deter fouling organisms, by shedding thin layers of spicule-containing material and possessing secondary metabolites (Patton 1972, Targett et al. 1983). Furthermore, the colonial ascidian *Polysyncraton lacazei* combines mechanical, chemical and extrinsic defences against a broad range of fouling

organisms, by sloughing surface layers, possessing secondary metabolites and having a surface-associated copepod and mite species grazing on its surface (Wahl and Banaigs 1991). Finally, examples of natural surfaces with both physical and chemical antifouling defence mechanisms are the mussel *Mytilus edulis* and the pilot whale *Globicephala melas*. The periostracum of the mussel *M. edulis* has micro-ripples with wavelengths of 1 to 2 μm (Bers and Wahl 2004, Bers et al. 2006a) and contains unidentified compounds, which deter the settlement of fouling organisms (Bers et al. 2006b). Similarly, the skin of the pilot whale *G. melas* has 0.1 to 1.2 μm^2 pores enclosed in a network of nanoridges and a zymogel with hydrolytic activities, both of which enhance the self-cleaning abilities of the skin (Baum et al. 2001, Baum et al. 2002).

1.4 Aims and chapter summaries

Qualitative evidence suggests that sea stars have epidermal surfaces free of fouling organisms. Therefore, the aims of this study are to (1) conduct field surveys of tropical sea stars in North Queensland, Australia, and quantify the abundance of surface-associated micro- and macro-organisms on these species and (2) investigate mechanical, physical and chemical antifouling defences of selected sea star species and, where effective, determine their mechanism of action. Understanding this mechanism of action will develop our understanding of the ecological importance of biofouling on benthic invertebrates. This study will also contribute to the development of novel physical and chemical antifouling technologies through the development of biomimics of natural antifouling processes, which have applications in aquaculture, marine transport and biotechnology industries. Novel antifouling technologies based on biomimics of natural antifouling processes have significant advantages over toxic chemicals currently used in antifouling paints, alleviating concerns about the leaching and biodegradation of active ingredients and non-target effects (de Nys and Steinberg 2002).

Five sea star species belonging to the order Valvatida (*Acanthaster planci* [Family Acanthasteridae], *Linckia laevigata* and *Fromia indica* [both Family Ophidiasteridae], *Cryptasterina pentagona* [Family Asterinidae] and *Archaster typicus* [Family Archasteridae]) were chosen for this study to investigate the natural antifouling defences of tropical sea stars. These tropical benthic species are widely distributed in the Indo-Pacific Ocean, with *A. planci*, *L. laevigata* and *F. indica* being found on coral reefs (Rowe and Gates 1995) and *C. pentagona* and *A. typicus* being found in inshore coastal environments (Rowe and Gates 1995, Dartnall et al. 2003).

In Chapter 2, field surveys were conducted to quantify surface-associated micro- and macro-organisms on tropical sea stars in both intertidal and subtidal zones in North Queensland, Australia, during the wet and dry seasons. Several sea star species with low bacterial abundances and without any common generalist macrofouling organisms were identified. Some surface-associated commensal and parasitic organisms were present.

In Chapter 3, the mechanical antifouling defences of one of these sea stars, the crown-of-thorns sea star *A. planci*, was explored. The morphology and distribution of its pedicellariae were examined and measured to determine if larvae or propagules of fouling organisms could settle between pedicellariae without being in their physical range. Furthermore, the responses of pedicellariae to tactile stimuli with a hypodermic needle, silica beads and larvae of the bryozoan *Bugula neritina* and fragments of the alga *Chrysocystis fragilis* were quantified.

In Chapter 4, the physical antifouling defences of the sea stars *L. laevigata*, *F. indica* and *A. typicus* were investigated. The wettabilities of these sea stars were measured and their surface microtopographies characterized. To evaluate the effectiveness of the surface microtopographies of these sea stars in deterring the settlement of fouling organisms, field experiments with resin replicas of the 4 sea star species were conducted at 3 sites during the dry and wet seasons, and the fouling community development was measured.

In Chapter 5, an alternative to the physical antifouling defences of the sea stars *L. laevigata*, *F. indica* and *A. typicus* was investigated. The role of natural products of these sea stars in deterring fouling organisms was determined by investigating the effects of dichloromethane, methanol and water extracts of these sea stars on the settlement of 4 common fouling species, the diatoms *Amphora* sp. and *Nitzschia closterium*, the serpulid *Hydroides elegans* and the bryozoan *B. neritina*, using laboratory based settlement assays. The dichloromethane extracts of the sea star *C. pentagona* were further separated and the most common fatty acids and sterols in the active fractions identified.

Based on the defined role of natural products of Chapter 5, the role of surface-associated compounds of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* in deterring the settlement of fouling organisms was further explored in Chapter 6. Surface-associated compounds of these sea stars were tested against the settlement of the diatoms *Amphora* sp. and *N. closterium*, the serpulid *H. elegans* and the bryozoan *B. neritina* in laboratory based settlement assays. Finally, surface-associated compounds of the 4 sea star species were chromatographed and identified. The major compounds hexadecanoic acid, cholesterol,

lathosterol and sitosterol were selected and tested against the settlement of fouling organisms in bioassays to provide an ecologically relevant context.

The results of this study are synthesized and discussed in Chapter 7. This Chapter also addresses directions for future research on antifouling defences of sea stars.

Chapter 2

Fouling-resistant surfaces of tropical sea stars [1]

2.1 Introduction

Epibiosis is a spatially close association between two or more organisms belonging to the same or different species (Wahl and Mark 1999). It may have major ecological impacts on the organisms involved, as epibionts may compete for food and space and reduce the growth and survival of basibionts (hosts) (reviewed by Wahl 1989, de Nys and Steinberg 1999). Most surfaces in an aquatic environment become rapidly fouled, whereas some marine organisms remain free of epibionts (Wahl 1989). These organisms have evolved defence mechanisms to reduce the disadvantages and ecological costs associated with epibiosis. These mechanisms include (1) mechanical defences, e.g. shedding of surface layers of crustose coralline algae (Keats et al. 1997), (2) physical defences, e.g. the surface microtopography of the mussels *Mytilus galloprovincialis* (Scardino and de Nys 2004) and *Mytilus edulis* (Bers and Wahl 2004) and (3) chemical defences, e.g. the release of metabolites by the macroalga *Delisea pulchra* (Maximilien et al. 1998, Dworjanyn et al. 2006), the sponges *Mycale adherens* (Lee and Qian 2003) and *Callyspongia* (*Euplacella*) *pulvinata* (Dobretsov et al. 2004), and the ascidian *Distaplia cylindrica* (McClintock et al. 2004).

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[1] Chapter 2 is adapted from Guenther J, Walker-Smith G, Waren A, de Nys R (2007) Fouling-resistant surfaces of tropical sea stars. Biofouling 23: 413-418

Qualitative evidence suggests sea stars have surfaces free of common generalist fouling organisms, such as algae, barnacles, serpulid polychaetes, bryozoans and ascidians (Bryan et al. 1996, Iken et al. 2003). The surface of sea stars consists of a thin cuticle overlying the epidermis (Holland and Nealson 1978), which covers an endoskeleton composed of a layer of collagenous connective tissue and small calcareous ossicles (Ruppert and Barnes 1994), but the presence of fouling-resistant surfaces of sea stars has not previously been documented. Therefore, the specific aim of this Chapter was to conduct field surveys of sea stars in the intertidal and subtidal zones in North Queensland, Australia, and quantify the abundance of surface-associated micro- and macroorganisms on these species with the broader goal of identifying natural models for antifouling mechanisms.

2.2 Materials and Methods

2.2.1 Collection sites

Field surveys were conducted in North Queensland, Australia, including intertidal zones at Lucinda (18°32'S, 146°20'E), Kissing Point and Rowes Bay in Townsville (19°14'S, 146°48'E) and Picnic Bay on Magnetic Island (19°11'S, 146°51'E) as well as subtidal zones at Mid Reef (19°04'S, 148°05'E), John Brewer Reef (18°38'S, 147°03'E) and Lizard Island (14°40'S, 145°27'E) on the Great Barrier Reef. The intertidal zones at Lucinda and Picnic Bay on Magnetic Island consist of sandy habitats, whereas those at Kissing Point and Rowes Bay in Townsville consist of rocky habitats. Surveys were conducted during the wet season in March/April 2005 and repeated during the dry season in September/October 2005.

2.2.2 Surface-associated microorganisms

To quantify surface-associated microorganisms on sea stars, live adult specimens (n = 5) of 7 sea star species (*Linckia laevigata*, *Fromia indica*, *Nardoa novaecaledoniae* and *Nardoa pauciforis* from Lizard Island, *Cryptasterina pentagona* from Kissing Point, *Archaster typicus* from Picnic Bay, *Nepanthia belcheri* from Rowes Bay) and reference substrata, i.e. small stones close to the sea stars (e.g. Dobretsov et al. 2005), at each site were collected with ambient seawater. Only specimens and reference substrata without surface-associated macroorganisms were collected. Surface samples (5 mm × 5 mm in size) were cut from proximal, middle and distal aboral positions of separate arms of each specimen to determine possible variation in bacterial abundance with arm length. Samples and reference substrata were dip-rinsed in sterile,

filtered (0.45 μm) seawater to remove unattached bacteria and subsequently stained with 4',6-diamidino-2-phenylindole, dilactate (DAPI, 10 μg ml^{-1} in phosphate buffered saline, Sigma-Aldrich) for 2 min. The numbers of bacteria in 5 random fields of view per sample were counted using a Leica DMLB fluorescence microscope at 400× magnification and the image analysis program Leica IM50 Image Manager. As sea stars have uneven and complex surface structures, the numbers of bacteria were counted in 2 superimposed planes of the same field of view to obtain an accurate count. The abundance of bacteria (number of cells cm^{-2}) was calculated from the projected surface area.

2.2.3 Surface-associated macroorganisms

Live adult specimens (n = 10, unless indicated otherwise) of 12 sea star species [*L. laevigata* and *F. indica* from both John Brewer Reef and Lizard Island, *N. novaecaledoniae*, *N. pauciforis*, *N. rosea* (n = 5 during the wet season only) and *Echinaster luzonicus* from Lizard Island, *C. pentagona* from Kissing Point, *A. typicus* from Picnic Bay, *N. belcheri* from Rowes Bay, *Acanthaster planci* from Mid Reef, *Astropecten velitaris* (n = 9 during the dry season only) and *Astropecten indicus* (n = 2 during the dry season only) from Lucinda] were collected. The aboral and oral surfaces as well as the ambulacral grooves of each specimen were visually examined in the field for the presence of surface-associated macroorganisms. All macro-organisms were identified to the lowest taxonomic level possible using relevant keys (e.g. Humes 1973, Humes 1976, Bruce 1978, Waren 1983) and counted.

2.2.4 Statistical analysis

To determine significant differences in bacterial abundance on sea stars between seasons (wet and dry seasons), sea star species (7 species) and position (proximal, middle and distal positions on arms), a partly nested analysis of variance (ANOVA, $\alpha = 0.05$) was performed with SYSTAT version 10 (Quinn and Keough 2002). Data on the bacterial abundance on the reference substrata were not included in the analysis. The factor 'position' was treated as an orthogonal factor, because bacteria separated by a few centimetres were assumed to be independent. The assumptions of homogeneity and normality of the data were checked with residuals versus predicted values plots and Q-Q plots of residuals, respectively. The data were square root transformed to meet these assumptions (Quinn and Keough 2002).

2.3 Results

2.3.1 Surface-associated microorganisms

The mean bacterial abundance at proximal, middle and distal positions on separate arms of the 7 sea star species ranged between 0.7 and 11.8 × 10^4 cells cm^{-2} during the wet season (Figure 2.1A) and between 0.5 and 11.3 × 10^4 cells cm^{-2} during the dry season (Figure 2.1B). There was a significant interaction term between 'Season' and 'Species' ($p = 0.002$, Table 2.1). This interaction was mainly driven by the sea stars *F. indica* and *C. pentagona* having fewer bacteria on their surfaces during the dry season than the wet season, whereas the other sea stars had similar bacterial abundances (Figure 2.1). There was also no consistent trend in bacterial abundance at proximal, middle and distal positions on sea star arms, because there was a significant interaction term between 'Species' and 'Position' ($p = 0.002$, Table 2.1). The bacterial abundances on all positions of *L. laevigata* were similar during the wet season, but the distal position had the most bacteria during the dry season. Similarly, the bacterial abundance on the distal position of *F. indica* was the highest during the wet season, but was very similar to those on proximal and middle positions during the dry season. In contrast, the bacterial abundances on all position of *N. belcheri* were similar during the wet season, but bacteria occurred in higher numbers on the distal position during the dry season. The bacterial abundances on the different positions of all other species were similar (Figure 2.1). The bacterial abundances on sea stars were consistently one order of magnitude higher than on the reference substrata. The mean bacterial abundances on the reference substrata during the wet and dry season were 4.2 × 10^3 and 7.2 × 10^3 cells cm^{-2} at Lizard Island, 5.3 × 10^3 and 4.8 × 10^3 cells cm^{-2} at Kissing Point, 1.7 × 10^3 and 4.0 × 10^3 cells cm^{-2} at Picnic Bay and 10.2 × 10^3 and 4.2 × 10^3 cells cm^{-2} at Rowes Bay, respectively.

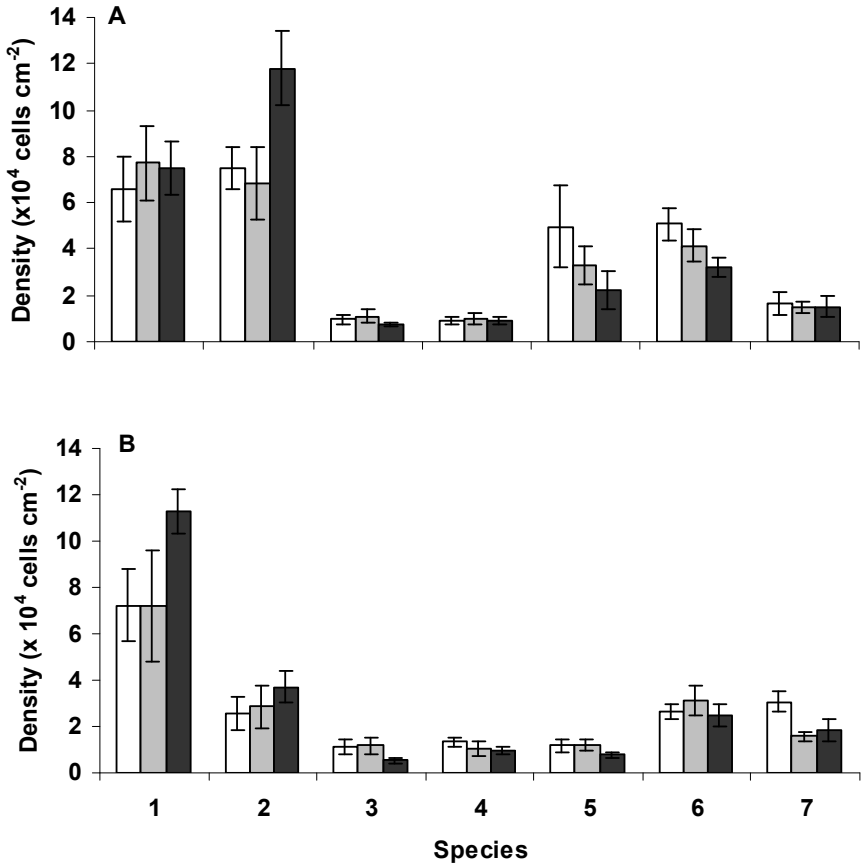

Figure 2.1 Bacterial abundance (× 10^4 cells cm^{-2}) at □ proximal, ■ middle and ■ distal positions on arms of 7 sea star species during the **(A)** wet season and **(B)** dry season. Species: 1 – *Linckia laevigata*, 2 – *Fromia indica*, 3 – *Nardoa novaecaledoniae*, 4 – *Nardoa pauciforis*, 5 – *Cryptasterina pentagona*, 6 – *Archaster typicus*, 7 – *Nepanthia belcheri*. Means ± SE are shown (n = 5 fields of view for each of 3 positions on each of 5 specimens of each species).

Table 2.1 Partly nested ANOVA of square root transformed bacterial abundance on proximal, middle and distal aboral positions on 7 sea star species during the wet and dry seasons (n = 5 fields of view for each of 3 positions on each of 5 specimens of each species).

	df	**MS**	**F**	**p**
Season	1	36158.407	2.35	0.131
Species	6	560679.000	36.37	<0.001
Season × Species	6	64487.997	4.18	0.002
Specimen (Season, Species)	56	15416.352	No test	
Position	2	5677.007	0.97	0.382
Season × Position	2	6367.867	1.09	0.340
Species × Position	12	16222.782	2.77	0.002
Season × Species × Position	12	5229.993	0.89	0.555
Specimen (Season, Species) × Position	112	5847.623	No test	
Error	840	2007.119		

2.3.2 Surface-associated macroorganisms

There were varying numbers of surface-associated parasitic or commensal gastropods, shrimps, polychaetes and copepods on 6 of the 12 sea star species (Table 2.2). The gastropods *Parvioris fulvescens*, *Asterolamia hians* and *Thyca* (*Granulithyca*) *nardoafrianti* were found exclusively on the aboral surface of the sea stars *A. typicus*, *A. indicus* and *N. pauciforis*, respectively, and the gastropod *Thyca crystallina* was found on the oral surface of *L. laevigata*. The commensal shrimp *Periclimenes soror* was found between spines of the aboral surface of *A. planci* and the polychaete *Ophiodromus* sp. in the ambulacral groove of *A. typicus*. The copepods *Stellicola illgi* and *Paramolgus* sp. were found on the aboral surface of *L. laevigata* and *E. luzonicus*, respectively. The remaining 6 sea star species did not have any surface-associated parasitic or commensal species on their surfaces and none of 12 sea star species had any common generalist macrofouling organisms, such as algae, barnacles, serpulid polychaetes, bryozoans and ascidians, on their surfaces.

2.4 Discussion

Our field observations have demonstrated that several tropical sea star species remain free of any common generalist macrofouling organisms, but some commensal and parasitic surface-associated organisms were present, and in most cases these associations were specific to one species. The bacterial abundance on the observed sea star species was approximately 10^4 to 10^5 cells cm^{-2}. It is possible, however, that our method of using live samples and staining surface-associated bacteria with DAPI visualised not only the bacteria on the outer side of the cuticle, but also subcuticular bacteria, which live in the space between the cuticle and epidermis. Previously, examinations of subcuticular bacteria included (1) staining pieces of tissue and squashing them with a coverslip, (2) removing larger portions of tissue and homogenising them (Kelly and McKenzie 1995), and (3) fixing tissues, critical point drying fixed tissues, using adhesive tape to pull away the adhering cuticle and expose underlying epidermal cell surfaces (Holland and Nealson 1978). Using our method, DAPI may have penetrated the permeable cuticle and also stained subcuticular bacteria if they were present in the observed species. Subsequently, the number of bacteria on the outer side of the cuticle may be even lower than reported.

Table 2.2 Taxonomic classifications of sea stars and their corresponding surface-associated macro-organisms as well as the total number of seas stars with surface-associated macro-organisms and the total number of surface-associated organisms found during the wet and dry seasons (n = 10 sea stars of each species for each season, except *Astropecten indicus* with n = 2 for the dry season only).

Sea star species	Surface-associated species	Location	Wet season		Dry season	
			Sea stars (n)	Surface-associated sp. (n)	Sea stars (n)	Surface-associated sp. (n)
Asteroidea Valvatida Acanthasteridae *Acanthaster planci*	Malacostraca Decapoda Palaemonidae *Periclimenes soror*	Mid Reef	1	1	0	0
Asteroidea Valvatida Archasteridae *Archaster typicus*	Gastropoda Caenogastropoda Eulimidae *Parvioris fulvescens*	Picnic Bay	3	4	0	0
	Polychaeta Aciculata Hesionidae *Ophiodromus* sp.	Picnic Bay	0	0	5	6
Asteroidea Valvatida Ophidiasteridae *Linckia laevigata*	Gastropoda Caenogastropoda Eulimidae *Thyca crystallina*	John Brewer Reef	1	1	0	0
	Maxillopoda Cyclopoida Lichomolgidae *Stellicola illgi*	Lizard Island	0	0	2	3
Asteroidea Valvatida Ophidiasteridae *Nardoa pauciforis*	Gastropoda Caenogastropoda Eulimidae *Thyca* (*Granulithyca*) *nardoafrianti*	Lizard Island	1	1	0	0
Asteroidea Paxillosida Astropectinidae *Astropecten indicus*	Gastropoda Caenogastropoda Eulimidae *Asterolamia hians*	Lucinda	-	-	2	4
Asteroidea Spinulosida Echinasteridae *Echinaster luzonicus*	Maxillopoda Cyclopoida Rhynchomolgidae *Paramolgus* sp.	Lizard Island	0	0	2	4

The bacterial abundances (excluding subcuticular bacteria) on the surface of echinoderms, in particular sea stars, are unexplored. Comparing the bacterial abundances on sea stars to those on other marine organisms is of limited use, because other marine organisms may not have a cuticle overlying the epidermis and therefore differ in their surface properties. Although the bacterial abundances on the observed sea stars were a magnitude higher than on the reference substrata, they were lower than on some other marine organisms. For example, the sponges *Haliclonia cymaeformis* and *Callyspongia* sp. (Dobretsov et al. 2005) as well as the algae *D. pulchra* (Maximilien et al. 1998) and *Ulva reticulata* (Dobretsov and Qian 2002) have bacterial densities in the order of approximately 10^6 to 10^7 cells cm^{-2}, despite having chemical defence mechanisms against the settlement of fouling organisms. The abundance and possibly composition of epibiotic bacterial communities as well as lack of macrofouling organisms on

sea stars may also be regulated by surface-associated bioactive compounds. Body wall extracts of sea stars have previously been tested for antifouling activities. Body wall extracts of a wide range of sea stars at ecologically relevant concentrations inhibit the growth of both marine and non-marine microbial species (Bryan et al. 1994, Iorizzi et al. 1995), inhibit the settlement of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina* (Iorizzi et al. 1995, Bryan et al. 1996) and change the swimming behaviour of the brown alga *Hincksia irregularis* (Iken et al. 2003, Greer et al. 2006). However, the presence of the bioactive compounds on the surface of sea stars still needs to be confirmed.

Sea stars may also have physical antifouling defences, working independently or synergistically with chemical antifouling mechanisms. Based on the structure and composition of the echinoderm cuticle, it has been suggested that the cuticle, which is a highly extended glycocalyx with chondroitin sulphate proteoglycan molecules, has an antifouling role by modulating the adhesive properties of the surface (McKenzie and Grigolava 1996). However, investigations on the effects of surface energy and surface microtopography of echinoderms on the settlement of fouling organisms have yet to be carried out.

The presence of surface-associated bacteria may also be beneficial for sea stars. These bacteria may have a symbiotic role, releasing compounds that help reduce or prevent the settlement of other fouling organisms. For example, bacterial communities on both the alga *U. reticulata* and the sponge *M. adhaerens* inhibit the settlement of the serpulid polychaete *Hydroides elegans* (Dobretsov and Qian 2002, Lee et al. 2006). The analyses of both the molecular and bacterial community compositions will offer an opportunity to determine whether similar mechanisms are present in sea stars.

Although no common generalist macro-fouling organisms were found on the surfaces of the sea stars, surface-associated commensal and parasitic organisms were present. The eulimid gastropod *T. nardoafrianti* and the copepod *Paramolgus* sp. were found for the first time associated with their respective host species. Few studies have investigated the ecological relationships between the host sea stars and their specialist commensals or parasites (Elder 1979, Janssen 1985, Egloff et al. 1988), and no research has been done on the mechanisms driving the selective settlement of these specialist invertebrates. Increased sampling of both sea star species and specimens at more locations will determine if the specialist commensals or parasites have a broader host range.

The key findings of this Chapter are the presence and quantification of surface-associated bacteria and some commensal and parasitic organisms and the complete lack of generalist

macrofouling organisms. Therefore, sea stars offer an excellent model to investigate and understand the mechanisms driving general fouling-resistant surfaces and the selective settlement of specialist invertebrates. Further research on the potential antifouling mechanisms of these sea stars will determine their biological significance and contribute to the increasing number of studies (e.g. Baum et al. 2002, Bers and Wahl 2004, Scardino and de Nys 2004) identifying marine organisms, which have evolved defences against the settlement of fouling organisms on their surfaces.

Chapter 3

Mechanical antifouling defences: Effects of the pedicellariae of the crown-of-thorns sea star *Acanthaster planci* [2]

3.1 Introduction

Pedicellariae are forcep- or pincer-like appendages made of calcareous ossicles and found in 2 echinoderm classes, Echinoidea and Asteroidea (Chia and Amerongen 1975, Campbell and Rainbow 1977). Echinoderm pedicellariae have reported functions in capturing particles and mobile prey, such as small crustaceans or fish (Robilliard 1971, Campbell 1973, Campbell 1974, Chia and Amerongen 1975, Hendler and Franz 1982, Dearborn et al. 1991, Emson and Young 1994, Lauerman 1998), and in deterring predators or competing sea stars (Jensen 1966, Lubchenco Menge and Menge 1974, van Veldhuizen and Oakes 1981). Furthermore, echinoid pedicellariae are proposed to protect the surface from settling fouling organisms. The only study on the protective role of echinoid pedicellariae showed that mobile pedicellariae and spines of the echinoid *Echinus esculentus* prevented the settlement of cyprid larvae of the barnacle *Balanus balanoides* as a defence against fouling (Campbell and Rainbow 1977). Although echinoid and asteroid pedicellariae do not share common traits (Jangoux and Lambert 1988),

[2] Chapter 3 is adapted from Guenther J, Heimann K, de Nys R (2007) Pedicellariae of the crown-of-thorns sea star *Acanthaster planci* are not an effective defence against fouling. Mar Ecol Prog Ser 340: 101-108

many articles and books suggest, or even state, that asteroid pedicellariae also prevent the settlement of fouling organisms (Nichols 1966, Ruppert and Barnes 1994) as well as foreign materials and organisms (Campbell 1971, Lambert et al. 1984, Roberts and Campbell 1988). However, the structure of asteroid pedicellariae combined with their proposed role in keeping the surface free of any macrofouling organisms has not been rigorously examined and remains speculative.

The crown-of-thorns sea star *Acanthaster planci* is widely distributed in Indo-Pacific coral reef communities, where it mainly feeds on staghorn and plate corals (Moran 1988). Of the 12 sea star species identified during the surveys in North Queensland (Chapter 2), the sea star *A. planci* was the only species with pedicellariae on the aboral surface. This species has large pedicellariae, but their morphology, distribution and role require more thorough investigations. Early studies described that the pedicellariae of *A. planci* consisted of 2 elongate valves that were variable in size and shape, and that they were abundant on the aboral surface (Campbell 1971, Caso 1974, Blake 1979). Campbell (1971) also showed that the pedicellariae of *A. planci* responded to tactile stimulation, such as a touch with a bristle or small spine, and movements of the commensal shrimp *Periclimenes soror*. However, no further studies on the functional role of the pedicellariae of *A. planci* have been conducted. The abundance of *A. planci* and its occurrence in the tropics, where fouling pressure is high, make it an ideal organism, with which to rigorously investigate the role of pedicellariae as a defence against fouling in sea stars.

The aims of this Chapter were to identify the role of the pedicellariae of *A. planci* in fouling control through quantifying (1) the morphology, distribution and abundance of the pedicellariae on the aboral surface of *A. planci* and (2) their response to tactile stimulation with a hypodermic needle, silica and zirconium/silica beads and to ecologically relevant fouling species, the bryozoan *Bugula neritina* and the chrysophyte alga *Chrysocystis fragilis*.

3.2 Materials and Methods

3.2.1 Collection and culture of *Acanthaster planci* and macrofouling organisms

Live specimens of *A. planci* were collected from the northern part of the Great Barrier Reef (GBR), Queensland, Australia: Little Broadhurst Reef (18°58'S, 147°41'E) in August 2004 (n = 10), Mid Reef (19°04'S, 148°05'E) in May 2005 (n = 13), Davies Reef (18°50'S, 147°38'E) in November 2005 (n = 7) and Bait Reef (19°48'S, 149°04'E) in February 2006 (n = 11). Following collection, they were visually inspected for any macrofouling organisms and their

size (R: major radius measured from centre to arm tip, r: minor radius measured from centre to interradial margin) was measured to the nearest mm using a vernier caliper. Specimens were kept temporarily in an outdoor recirculating tank (temperature: 26°C, salinity: 35 ppt) at the Marine and Aquaculture Research Facilities Unit at James Cook University.

Several colonies of the bryozoan *B. neritin*a were collected from pier pilings in Townsville (19.26°S, 146.80°E) and kept in constant darkness for 2 d in a recirculating tank. After exposing the colonies to bright light for approximately 30 min, larvae were released (Marshall and Keough 2003). Specimens of the alga *C. fragilis* were collected from Moore Reef (16.53°S, 146.12°E) located east of Fitzroy Island in the GBR in January 2004. Monoclonal cultures of *C. fragilis* were established and maintained in enriched seawater (ES) medium (Provasoli 1964) at the North Queensland Algal Identification/Culturing Facility at James Cook University (culture no. NQAIF037).

3.2.2 Morphology, distribution and abundance of pedicellariae

The morphology and distribution of pedicellariae were investigated to determine if larvae of fouling organisms could potentially swim amongst and settle between pedicellariae without being in their physical range. Live specimens were placed individually in a round plastic container (diameter: 160 mm), fully covered with sterile, filtered (0.45 μm) seawater and observed with a Leica MZ 125 dissection microscope. To determine the morphology and classify the pedicellariae of *A. planci* according to Jangoux and Lambert (1988), pedicellariae without organic tissues were examined. Selected pedicellariae were removed from the surface and immersed in a 3.5% sodium hypochlorite (NaOCl) solution until the organic tissue was cleared from the skeletal ossicles. The pedicellariae were then washed in distilled water, transferred to absolute ethanol and air-dried (Chia and Amerongen 1975, Roberts and Campbell 1988). Dried pedicellariae were gold sputter coated and inspected using a JEOL JSM-5410LV scanning electron microscope with an accelerating voltage of 10 kV.

To determine if the total number of pedicellariae on the aboral surface area of *A.planci* is proportional to the size of the specimens, the total number of pedicellariae on the aboral surface of each collected specimen (n = 41) was counted. The aboral surface area of each specimen was estimated by measuring the radius of the disk as well as the length (measured from the interradial margin to the tip of an arm) and width of each arm. Furthermore, the length of randomly selected pedicellariae (n = 10 on each of 10 specimens) and the distance between

randomly selected pedicellariae and their closest neighbouring pedicellaria (n = 10 on each of 10 specimens) were measured using the image analysis program Leica IM50 Image Manager.

Pedicellariae may be either open or closed for an extended period of time. Open pedicellariae are potentially able to catch larvae or propagules of fouling organisms, whereas closed pedicellariae are not. Therefore, randomly selected pedicellariae (n = 100 on each of 10 specimens) were observed after leaving the specimens in a container for 15 min without any disturbance. The percentage of open and closed pedicellariae of these specimens was then determined.

3.2.3 Tactile stimulation

The roles of pedicellariae in fouling control were investigated by observing their response to tactile stimulation with a hypodermic needle, silica and zirconium/silica beads, as well as to ecologically relevant fouling species, the bryozoan *B. neritina* and the chrysophyte alga *C. fragilis*. Live specimens of *A. planci* were individually placed in a round plastic container (160 mm in diameter) and fully covered with sterile, filtered (0.45 μm) seawater. After leaving the specimens in the container for 15 min without disturbance, open pedicellariae were mechanically stimulated by touching either the inner, outer or basal sites of pedicellariae once with a small (gauge 26) hypodermic needle (n = 20 pedicellariae for each site, randomly selected on each of 8 specimens, including n = 10 on central disk and n = 10 on arms). The closing responses of the pedicellariae to these stimuli were timed (i.e. the time from introduction of stimuli and closure of pedicellariae to the reopening of pedicellariae) using a Leica MZ 125 dissection microscope.

To simulate the effect of tactile stimulation by settling larvae and propagules of fouling organisms, 5 silica beads of 4 different mean sizes (± SE), 50.2 ± 1.4, 181.5 ± 1.3, 255.7 ± 2.3 and 510.7 ± 3.8 μm, with a density of 2.5 g ml^{-1} (Sigma-Aldrich) (Table 3.1) were dropped over each pedicellaria (n = 20 for each mean size, on the central disk of each of 8 specimens). The mass of these silica beads ranged between 0.2 and 174.4 μg (Table 3.1), simulating the mass of a variety of fouling organisms, including those used in this study. The tip of a 0.32 or 0.65 mm needle on a 250 μl gas tight syringe was positioned within 1 mm above each pedicellaria, and the beads were released, dropping onto the pedicellaria. The closing responses of the pedicellariae to these tactile stimuli were observed and timed. Furthermore, to test whether the size or mass of the artificial beads was important in triggering a closing response of the pedicellariae, zirconium/silica beads of similar mean size but of higher density than the silica

beads were used. Five zirconium/silica beads of 2 mean (± SE) sizes, 191.2 ± 1.9 and 507.6 ± 5.0 μm, with a density of 3.7 g ml^{-1} (Daintree Scientific) (Table 3.1), were dropped over each pedicellaria (n = 20 for each mean size, on the central disk of each of 8 specimens). Again, the closing responses of the pedicellariae to these tactile stimuli were observed and timed.

Table 3.1 Density (g ml^{-1}), mean size (± SE, diameter in μm) and mass per bead (μg) of silica and zirconium/silica beads used to stimulate the pedicellariae on the central disk of *Acanthaster planci*.

Type of bead	Density (g ml^{-1})	Mean size ± SE (μm)	Mass per bead (μg)
Silica	2.5	50.2 ± 1.4	0.2
		181.5 ± 1.3	7.8
		255.7 ± 2.3	21.9
		510.7 ± 3.8	174.4
Zirconium/silica	3.7	191.2 ± 1.9	13.5
		507.6 ± 5.0	253.4

To determine if pedicellariae are able to catch larvae and propagules of fouling organisms, randomly selected pedicellariae were stimulated with 2 ecologically relevant fouling species, the bryozoan *B. neritina* and the chrysophyte alga *C. fragilis*. The bryozoan *B. neritina* (Bryozoa: Gymnolaemata: Cheilostomatida: Bugulaidae) is a cosmopolitan fouling organism found throughout warmer waters, including tropical Australia. It also occurs in abundance in harbours on pier pilings, ship hulls and buoys (OECD 1965). In contrast to larvae of other fouling organisms, larvae of *B. neritina* are comparatively large, with a diameter of approximately 250 μm and a wet weight of approximately 5 μg. Using a 150 mm Pasteur glass pipette, 5 larvae of *B. neritina* of 2 ages, 0 to 2 h and 6 to 8 h (diameter: approximately 250 μm), were slowly released within 1 mm of a pedicellaria (n = 20 on the central disk of each of 6 specimens). These age ranges were chosen, because the non-feeding larvae of this species become less discriminatory in their choice of settlement substrate with larval age and, as such, older larvae may settle more rapidly (Marshall and Keough 2003, Gribben et al. 2006).

The benthic colonial chrysophyte alga *C. fragilis* (Chrysophyta: Pelagophyceae) is also found in the central GBR, where it colonises a variety of substrata, especially algal turf on rocks and dead standing corals, in water depths to at least 20 m (Lobban et al. 1995, Schaffelke et al. 2004). Mucilage fragments containing single cells (8 to 14 μm in diameter) of *C. fragilis* were dropped over the pedicellariae (n = 20 on the central disk of each of 6 specimens). Three

fragment lengths, 150 to 200, 550 to 600 and 950 to 1,000 μm with a wet weight of approximately 25, 94 and 163 μg, respectively, were examined, because fragmentation has been suggested to be an important means of dispersal for this species (Lobban et al. 1995). The interaction between pedicellariae and larvae or fragments was observed using a Leica MZ 125 dissection microscope. The number of pedicellariae that closed after tactile stimulation were counted and expressed as a percentage of the 20 pedicellariae of each specimen. The closing responses of the pedicellariae were also observed and timed. As a control for the effect of seawater movement alone on the closing responses of the pedicellariae, filtered seawater without beads, larvae or fragments was slowly released from the syringe or pipette onto the pedicellariae and the reaction of the pedicellariae was observed.

3.2.4 Statistical analysis

All statistical analyses were performed with SPSS version 12. A Pearson correlation analysis was used to determine a potential correlation between the total number of pedicellariae and the estimated aboral surface area of 41 specimens of *A. planci*. Closure times between inner, outer and basal sites of the pedicellariae were analysed with a 3-factor mixed-model ANOVA (factors: (1) position on specimen – arm or disk, (2) site on pedicellariae – inner, outer or basal, (3) specimens [blocked factor] – 8 specimens of *A. planci*) followed by Tukey's honestly significant difference (HSD) multiple comparison test (Quinn and Keough 2002). The assumptions of homogeneity and normality of the data were checked with standardized residuals versus predicted values plots and Q-Q plots of residuals, respectively. Because assumptions of homogeneity and normality were not met, the data were log-transformed. To calculate the minimum size and mass of silica beads required to trigger a closing response from the pedicellariae, a linear regression analysis was conducted, using the data on the 4 different silica bead sizes and their corresponding mean percentages of closed pedicellariae. A 1-factor ANOVA was used to determine significant differences between mean percentages of responding pedicellariae to *B. neritina* larvae of 2 different age ranges. Assumptions for this analysis were met.

3.3 Results

3.3.1 Surface-associated macrofouling organisms on *Acanthaster planci*

All observed specimens of *A. planci* (R = 59.1 ± 2.8 mm, r = 31.2 ± 1.4 mm, n = 41) were free of any macrofouling organisms on their surfaces. One specimen of the commensal shrimp

Periclimenes soror Nobili (Crustacea: Decapoda: Pontoniinae) was observed on the aboral surface of 1 *A. planci* (R = 52 mm, r = 28 mm) collected from Mid Reef in May 2005, whereas 2 specimens of the same shrimp species were observed on the aboral surface of 2 *A. planci* (R = 87 mm, r = 46 mm; R = 64 mm, r = 33 mm) collected from Bait Reef in February 2006.

3.3.2 Morphology, distribution and abundance of pedicellariae

Asteroid pedicellariae can be classified into 1 of the following 3 categories: elementary, alveolar and complex, depending on the articulation of the valves with the underlying skeletal plate (Jangoux and Lambert 1988). Scanning electron microscopy of pedicellariae without organic tissues demonstrated that all specimens of *A. planci* had only 1 type of pedicellariae: straight elementary. Each elementary pedicellaria consisted of 2 straight elongate valves (Figure 3.1A) that were supported by a basal ossicle (Figure 3.1B). When opening or closing, the 2 valves were able to move only in a linear plane with respect to each other, owing to the physical constraints imposed by the attachment of the valves to the basal ossicle.

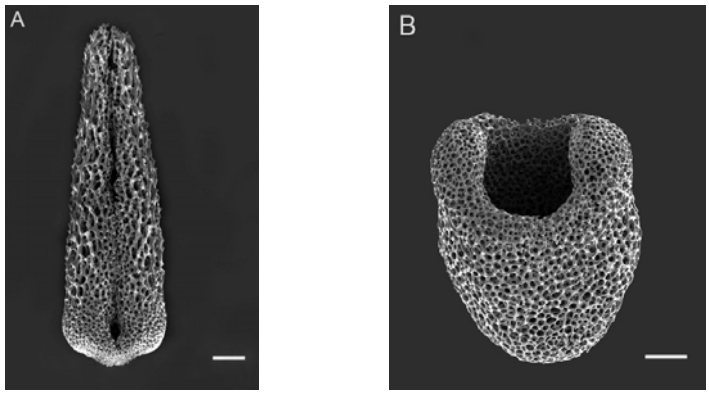

Figure 3.1 Scanning electron micrographs of **(A)** 2 straight valves and **(B)** the corresponding basal piece of a pedicellaria of *Acanthaster planci*, without organic tissues. Scale bar, 100 μm.

The pedicellariae on the aboral surface of all observed specimens occurred individually amongst spines and papulae. The abundance of pedicellariae on the aboral surface was variable (Figure 3.2). For example, 1 specimen of 37 cm^2 had 783 pedicellariae (corresponding to 21 pedicellariae cm^{-2}), whereas another with a similar surface area of 56 cm^2 had 26 pedicellariae (corresponding to 2 pedicellariae cm^{-2}). Despite this variation, the number of pedicellariae on the aboral surface generally increased with increasing surface area, because there was a weak but significant, positive correlation between the number of pedicellariae and the estimated aboral surface area of *A. planci* ($r = 0.587$, $p < 0.001$). The length of the pedicellariae ranged

between 0.2 and 1.5 mm with a mean (± SE) of 0.7 ± 0.1 mm and the distance between pedicellariae ranged between 0.7 and 5.8 mm with a mean (± SE) of 2.6 ± 0.3 mm. Based on mean distance between the pedicellariae, mean density was estimated to be 14.7 pedicellariae cm^{-2}. Because mean distance between pedicellariae was more than twice the mean length of pedicellariae, the pedicellariae were not able to physically cover and protect the entire aboral surface area from larvae or propagules of fouling organisms.

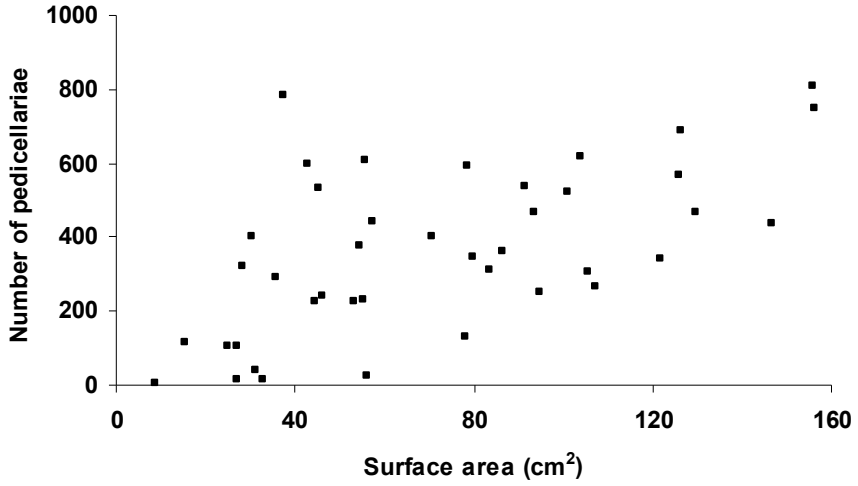

Figure 3.2 Correlation between the number of pedicellariae of *Acanthaster planci* and corresponding aboral surface area (cm^2) of each specimen (n = 41, Pearson correlation analysis: r = 0.587, p < 0.001).

When specimens were left in a container for 15 min without disturbance, the majority of the pedicellariae (79.5 ± 3.2%) were open, i.e. potentially prepared for immediate closure and defence against larvae or propagules of fouling organisms.

3.3.3 Tactile stimulation

When open pedicellariae were touched at either inner, outer or basal sites using a hypodermic needle, and all pedicellariae responded and closed. The pedicellariae touched at inner sites closed for significantly longer than the pedicellariae touched at outer and basal sites (p = 0.026, Table 3.2). Pedicellariae touched at inner sites closed for 8.9 ± 0.6 s, whereas pedicellariae touched at outer and basal sites closed for 6.7 ± 0.5 s and 7.9 ± 0.7 s (SE), respectively (Figure 3.3). While these differences are significant, the major outcome is that all pedicellariae closed for a period of time, during which they were unavailable to react to another stimulus.

Table 3.2 Three-factor mixed model ANOVA for log-transformed closure time of pedicellariae of *Acanthaster planci* after they were touched with a hypodermic needle at inner, outer or basal sites (Position: arm or disk; Site: inner, outer or basal site of pedicellariae; Specimen: 8 specimens).

	df	MS	*F*	p
Position	1	3.107	2.79	0.139
Site	2	3.388	4.80	0.026
Specimen	7	2.771	2.28	0.164
Position × Site	2	0.607	1.00	0.393
Position × Specimen	7	1.115	1.83	0.158
Site × Specimen	14	0.707	1.16	0.391
Position × Site × Specimen	14	0.608	1.14	0.323
Error	432	0.535		

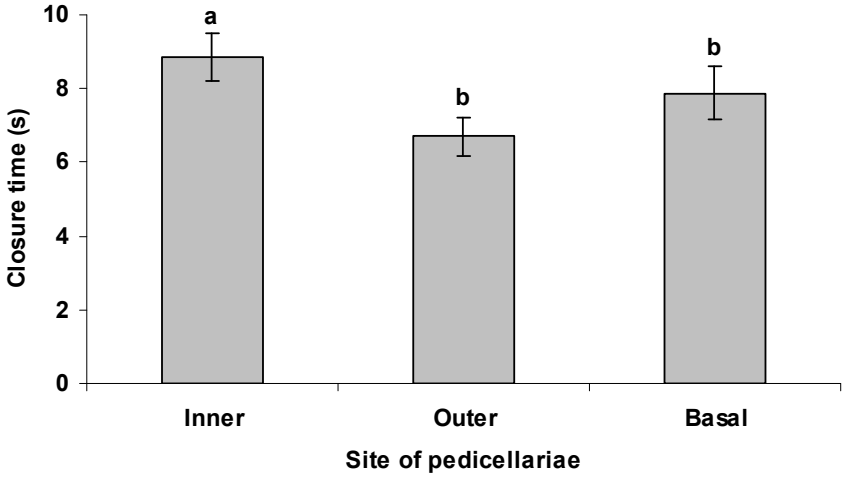

Figure 3.3 Closure time of pedicellariae of *Acanthaster planci* when touched with a hypodermic needle at inner, outer or basal sites Means ± SE are shown (n = 20 pedicellariae for each site on each of 8 specimens). Different letters indicate significant differences (Tukey's HSD multiple comparison test, $p < 0.05$ for log-transformed data).

The percentage of pedicellariae that responded to tactile stimulation with silica beads (2.5 g ml^{-1}) of varying sizes increased with increasing size of the beads, i.e. from 0.4 ± 0.4% for 50.2 μm silica beads to 82.5 ± 3.0% for 510.7 μm silica beads (Figure 3.4). However, the percentage of responding pedicellariae increased even further when zirconium/silica beads of similar size but higher density (3.7 g ml^{-1}) (Table 3.1) were used. The percentage of pedicellariae reacting to 191.2 μm zirconium/silica beads was twice as high as the percentage of pedicellariae reacting to 181.5 μm silica beads, i.e. 32.5 ± 3.2% compared with 16.1 ± 1.4%. Similarly, the percentage of pedicellariae reacting to 507.6 μm zirconium/silica beads reached the highest level of 100%, whereas only 82.5 ± 3.0% of the pedicellariae reacted to the 510.7 μm silica beads (Figure 3.4).

These results demonstrate that the mass, not size, of the beads was the main driving factor behind the proportional increase in responding pedicellariae. From the linear regression (r^2 = 0.983, p = 0.006), the minimum size of silica beads required to trigger a response from the pedicellariae was calculated to be >63.53 μm, which corresponds to a mass of >0.34 μg.

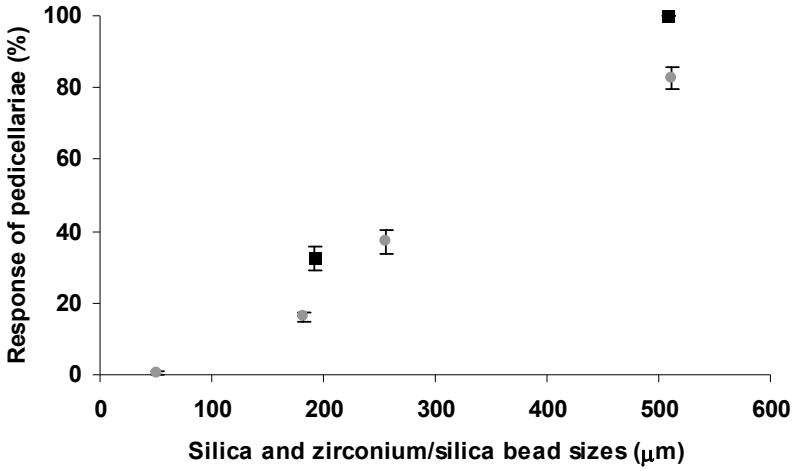

Figure 3.4 Percentage of responding pedicellariae of *Acanthaster planci* to silica (●) and zirconium/silica (■) beads of different sizes. Means ± SE are shown (n = 20 pedicellariae for each size on each of 8 specimens).

The response of pedicellariae to larvae of the bryozoan *B. neritina* of varying ages was consistently low, with only 15.0 ± 4.1% and 10.8 ± 2.0% of pedicellariae responding to 0 to 2 h and 6 to 8 h old larvae, respectively (Figure 3.5A). There was no significant difference between these 2 responses ($F_{1,10}$ = 0.839, p = 0.381). Those pedicellariae that responded to touch by the 0 to 2 h and 6 to 8 h old larvae closed quickly, with a mean closure time of 6.4 ± 1.2 s (n = 18) and 4.3 ± 1.0 s (n = 13), respectively (Figure 3.5B). However, most of the pedicellariae were not quick enough to actually catch the *B. neritina* larvae. During the experiment, only 1 larva was caught by a pedicellaria, which subsequently held on to the larva for 5 s, after which the valves of the pedicellaria opened again. The larva was released and was still able to swim off. The response to fragments of the fouling alga *C. fragilis* of varying lengths (150 to 200, 550 to 600 and 950 to 1000 μm) dropped over the pedicellariae was even less than that of *B. neritina*, with no fragments triggering any closing response of the pedicellariae. Slowly releasing filtered seawater without any beads, larvae or fragments did not stimulate a closing response by any pedicellariae.

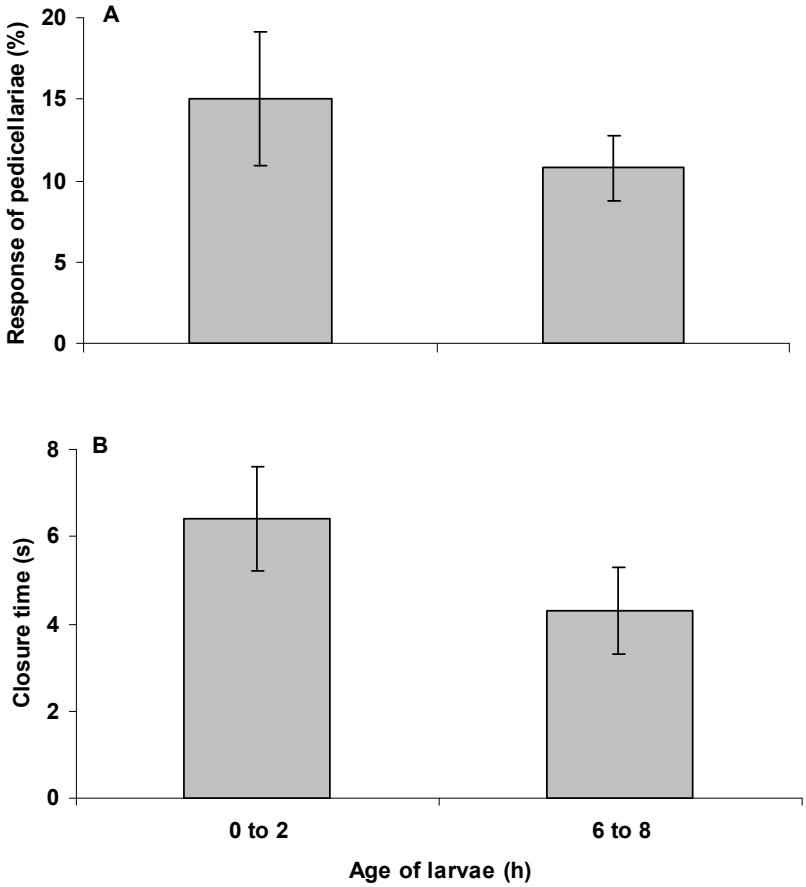

Figure 3.5 (A) Percentage of responding pedicellariae and **(B)** closure time of pedicellariae of *Acanthaster planci* when touched by 0 to 2 h old and 6 to 8 h old *Bugula neritina* larvae. Means ± SE are shown (n = 18 for 0 to 2 h old larvae and n = 13 for 6 to 8 h old larvae).

3.4 Discussion

This study showed that the pedicellariae of *A. planci* were sparsely distributed, required a high force to trigger a closing response, slowly reopened and did not effectively respond to ecologically relevant fouling organisms. These results suggest that elementary pedicellariae of *A. planci* are not as effective in fouling control as previously implied (Nichols 1966, Ruppert and Barnes 1994), if at all.

The elementary pedicellariae of *A. planci* of this study were straight and were neither long nor dense enough to physically cover and protect the entire aboral surface area, providing a significant defence-free zone for potential settlement of fouling organisms. The mean length of the pedicellariae of this study was less than previously described. Pedicellariae of *A. planci* have been reported to be 2 to 3 cm long (Campbell 1971) and 1.5 to 2.5 cm long (Caso 1974), but in both studies it was unclear how many pedicellariae and specimens of *A. planci* were observed

and how big the specimens were. The length of the pedicellariae may be a function of specimen size. However, the mean length of pedicellariae in this study was similar to the length of straight complex pedicellariae of other asteroid species. The straight complex pedicellariae on the aboral surface of the sea star *Marthasterias glacialis* (Asteroidea: Forcipulatida: Asteriidae) were approximately 0.5 to 2.0 mm long, with a variable density of more than 25 pedicellariae cm^{-2} (Lambert et al. 1984). Similarly, the straight complex pedicellariae of the sea star *Asterias rubens* (Asteroidea: Forcipulatida: Asteriidae) were also small, reaching a length of 0.5 to 0.6 mm; however, up to 3,000 pedicellariae per specimen (of unspecified size) were recorded (Roberts and Campbell 1988). In contrast to the pedicellariae of *A. planci*, the valves of the complex pedicellariae of *M. glacialis* and *A. rubens* are attached to the surface by a flexible stalk, which allows the valves to point in different directions (Lambert et al. 1984, Roberts and Campbell 1988) and cover a greater surface area surrounding each pedicellaria. Therefore, alveolar and complex pedicellariae may have a more effective role in protecting the surface from fouling organisms.

The responses of the pedicellariae of *A. planci* to the larvae of *B. neritina* were consistently low and none of the pedicellariae responded to the fragments of *C. fragilis*. Accordingly, there was also no response to any potential chemical stimulation associated with these organisms. Although comparatively large, the larvae of *B. neritina* weighed only 5 μg and when their swimming ability was taken into account, they were almost weightless in the water column. The larvae of *B. neritina* and fragments of *C. fragilis* clearly did not exert enough force onto the pedicellariae to trigger a response. Despite being free of any macrofouling organisms, the straight elementary pedicellariae of *A. planci* do not seem to be the driving mechanism of this antifouling defence. Furthermore, asteroid pedicellariae only occur in some families of the orders Paxillosida, Notomyotida, Valvatida and Forcipulatida, and all families in the order Spinulosida lack pedicellariae (Jangoux and Lambert 1988). Even at the species level, pedicellariae can be highly variable in numbers (Jangoux and Lambert 1988), which was also demonstrated for *A. planci* in this study. Given that fouling pressure is universal and asteroid pedicellariae are variable in numbers or even lacking in numerous species, they may not have evolved for a protective role against fouling organisms. Alternative mechanisms, including physical and chemical (Bryan et al. 1996) antifouling defences, may contribute to the lack of fouling on *A. planci* and other species. The commensal shrimp *P. soror* may also play a role in keeping the surface of *A. planci* free of fouling, but there are virtually no records on potential food sources of this species.

Previous studies demonstrated that asteroid pedicellariae have functions in capturing particles and mobile prey (Chia and Amerongen 1975) and deterring predators and competing sea stars

(van Veldhuizen and Oakes 1981). However, these functions may not apply to the straight elementary pedicellariae of *A. planci*. Specimens of *A. planci* feed mainly on staghorn and plate corals, rather than mobile prey, and also possess venomous spines to deter potential predators (Moran 1988). Nevertheless, the pedicellariae of *A. planci* may still have a limited protective role against small predators. Several species have been observed to feed on juvenile and adult *A. planci*, including the giant triton shell *Charonia tritonis*, the white-spotted pufferfish *Arothron hispidus*, 2 species of triggerfish *Balistoides viridescens* and *Pseudobalistes flavimarginatus*, two species of shrimp *Hymenocera picta* and *Neaxius glyptocerus* and the fire worm *Pherecardia striata* (reviewed by Moran 1986). Some predators may be smaller than *A. planci* and be able to move between the venomous spines without being affected by them. These predators are likely to exert a higher force onto the pedicellariae than the larvae or propagules of fouling organisms, and the resulting snapping action of the pedicellariae may deter them.

In conclusion, the proposal that pedicellariae defend against the settlement of fouling organism does not hold for all asteroid species and needs to be reconsidered. Future research will need to examine the diverse range of species with pedicellariae of many types, especially both alveolar and complex pedicellariae, to determine if there is in fact any antifouling role for asteroid pedicellariae.

Chapter 4

Physical antifouling defences: Effects of the wettability and surface microtopography of tropical sea stars [3]

4.1 Introduction

Research on physical defence mechanisms against the settlement of fouling organisms has focused on the wettability and microtopography of surfaces. Surface wettability and surface tension initially influence the attachment and strength of attachment of fouling organisms to solid surfaces. Surface wettability affects settlement, with many marine organisms preferring to settle on hydrophobic surfaces (Callow et al. 2000, Finlay et al. 2002, Dahlström et al. 2004, Greer and Amsler 2004, Aldred et al. 2006). Furthermore, low surface tensions of artificial surfaces (20-30 mN m^{-1}) (Dexter et al. 1975, Becker and Wahl 1991, Becker 1993, Becker et al. 1997) and natural surfaces, such as the gorgonians *Pseudopteragorgia americana* and *Pseudopterogorgia acerosa* (Vrolijk et al. 1990), have been correlated with reduced settlement and adhesiveness of both micro- and macrofouling organisms.

Similarly, some surface microtopographies of natural (Baum et al. 2002, Scardino et al. 2003, Bers and Wahl 2004, Scardino and de Nys 2004) and artificial surfaces (Andersson et al. 1999, Berntsson et al. 2000a,b, Petronis et al. 2000, Callow et al. 2002, Hoipkemeier-Wilson et

[3] Chapter 4 is adapted from Guenther J, de Nys R (2007) Surface microtopographies of tropical sea stars: Lack of an efficient physical defence mechanism against fouling. Biofouling 23: 419-429.

al. 2004, Scardino et al. 2006, Schumacher et al. 2007a,b) also deter the settlement of fouling organisms. These surface microtopographies are often smaller than the size of larvae, propagules or cells of fouling organisms, thereby reducing the contact area available for the adhesion and attachment of larvae and propagules (Baum et al. 2002, Callow et al. 2002, Scardino et al. 2006). An example is the skin of the pilot whale *Globicephala melas*, where 0.1 to 1.2 μm^2 pores enclosed in a network of nanoridges reduce the surface available for adhesion and attachment to the pore margins and tips of the nanoridges (Baum et al. 2002). Studies on the surface microtopographies of bivalves also show that micro-ripples with wavelengths of 1.8 to 1.9 μm on *Mytilus galloprovincialis* (Scardino et al. 2003, Scardino and de Nys 2004), 1.0 to 2.0 μm on *Mytilus edulis* (Bers and Wahl 2004, Bers et al. 2006a), 1.5 to 2.0 μm on *Perna perna* (Bers et al. 2006a) and 0.8 μm on *Pteria penguin* (Guenther and de Nys 2006) correlated with low fouling cover. Similarly, parallel ridges with a distance of 15 to 115 μm on the eggcase of the dogfish *Scyliorhinus canicula*, and knobbed surface structures 10 μm in diameter on the skeleton plates of the brittle star *Ophiura texturata* had repellent effects on microfouling organisms (Bers and Wahl 2004). Circular elevations, 200 μm in diameter, of the carapace of the crab *Cancer pagurus* also repelled macrofouling organisms (Bers and Wahl 2004).

In common with many of the model organisms investigated for natural antifouling defences, echinoderms generally have surfaces that are remarkably free of fouling organisms (McKenzie and Grigolava 1996). Echinoderms have an epidermis, which covers an endoskeleton composed of a layer of collagenous connective tissue and small calcareous ossicles (Ruppert and Barnes 1994). In particular, sea stars of the orders Valvatida and Paxillosida have paxillae on their surfaces, which are modified ossicles with a median vertical pillar crowned with spinelets (Clark and Rowe 1971). These provide complex and unique surface microtopographies with the potential to prevent the settlement of fouling organism. While chemical defence mechanisms of sea stars have been investigated (Iorizzi et al. 1995, Bryan et al. 1996, Iken et al. 2003, Greer et al. 2003, Greer et al. 2006), potential physical defence mechanisms are unexplored.

The aim of this Chapter was to determine the role of this complex surface microtopography of sea stars in keeping the surface free of fouling organisms, by measuring the seawater contact angles of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus*, identifying the scale of their surface microtopographies, and conducting field experiments with resin replicas of these sea stars.

4.2 Materials and Methods

4.2.1 Collection of specimens

Live specimens of four sea star species of the order Valvatida were collected from subtidal and intertidal zones in North Queensland, Australia. Specimens of *Linckia laevigata* and *Fromia indica* (both Family Ophidiasteridae) were collected from John Brewer Reef (18°38'S, 147°03'E), whereas *Cryptasterina pentagona* (Family Asterinidae) and *Archaster typicus* (Family Archasteridae) were collected from Kissing Point in Townsville (19°14'S, 146°48'E) and Picnic Bay on Magnetic Island (19°11'S, 146°51'E), respectively, in April and May 2005. Specimens were transported in ambient seawater and then kept temporarily in an outdoor recirculating tank (temperature: 26°C, salinity: 35 ppt) at the Marine and Aquaculture Research Facilities Unit at James Cook University. These sea star species are free of any macro-fouling organisms.

4.2.2 Surface wettability

The contact angles on the surface of *L. laevigata*, *F. indica* and *A. typicus* were measured using a modified captive bubble technique described by Thomason and Davenport (1995). Live specimens and control surfaces (glass slides covered with paraffin wax, melting point 60 to 62°C) (n = 5) were placed individually in a small glass aquarium filled with 15 l of filtered (0.45 μm) seawater at 25°C. A 1 ml syringe with a Whitacre spinal needle (22 gauge, length 8.9 cm) was pushed through a blind grommet on the side of the aquarium and used to insert a 2.7±0.4 mm air bubble (n = 3 on each of 5 specimens) from below a specimen (Thomason and Davenport 1995). Air bubbles were photographed with a Sony DSC-W5 digital still camera 10 s after the air bubble was placed on the specimen. The intermediate contact angle (θ) of each air bubble was measured using the imaging software Leica IM50 Image Manager. To validate this method and estimate the experimental error, contact angles of sessile seawater drops on the same glass slides covered with paraffin wax were measured with a goniometer (Rame-hart) at University of New South Wales, Australia. The water drop profiles were recorded and the images were processed using the imaging software RHI 2001 Imaging System, in which an advanced mathematical analysis of the true drop profile using a 5th order polynomial was utilised to ensure measurement reproducibility of ± 1°. The contact angles on the surface of *C. pentagona* were not measured, because the rapidly extended papulae of this species obscured the interface between the surface of the specimens and the air bubbles.

4.2.3 Surface microtopography

To determine the height and diameter of paxillae and the distance between paxillae, live tissue samples of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* from proximal, middle and distal positions on the aboral surface of separate arms were examined with a Leica DMLB compound microscope at ×50 and ×100 magnification and photographed. The height and diameter of paxillae and the distance between paxillae (n = 15 at each position on each of 3 specimens) were measured using the imaging software Leica IM50 Image Manager. To measure the height of the paxillae a cross section of each sample was made. When verifying the accuracy of the replicas of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, the height and diameter of paxillae and the distance between paxillae on the replicas were also measured using scanning electron microscopy (JEOL JSM-5410LV) and Leica IM50 Image Manager (n = 15 on each of 3 replicas per species). When verifying the accuracy of the replicas of the sea stars, the distance between spinelets covering the paxillae of the replicas of *L. laevigata*, *F. indica* and *C. pentagona* (n = 5 on each of 3 replicas) were also measured using scanning electron microscopy (JEOL JSM-5410LV) and the imaging software Leica IM50 Image Manager.

4.2.4 Fouling community development

To determine the role of the surface microtopographies of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* in fouling control (separate from other surface properties, such as surface-bound chemical effects that may interfere with the settlement responses of larvae of the fouling organisms), replicas of the sea stars as well as smooth and rough control surfaces (n = 10) were produced using resin-moulding materials. Marrs et al. (1995), Figueiredo et al. (1997), Scardino and de Nys (2004) and Bers and Wahl (2004) have previously used this method to produce replicas of natural surfaces with a demonstrated accuracy of 1 μm. Sea stars were blot-dried and then air-dried for 30 min to remove excess seawater. The disks, approximately 3 cm in diameter, of each specimen were covered with KERR Extrude Wash Type 3 polyvinylsiloxane material (KERR, USA) to form a mould, cured for 15 min and carefully peeled off the surface. Subsequently, DEVCON 2 Ton epoxy resin (DEVCON, USA) was poured into the mould and cured for 24 h, creating a replica of the natural surface. The heads of stainless steel screws (20 mm) were inserted into the backside of the replicas before the epoxy resin fully cured. Smooth control surfaces of similar surface area were produced from a smooth glass surface. Rough control surfaces were produced from the same glass surface and subsequently roughened with sandpaper (grade 80). Replicas produced with this method have an accuracy of 1 μm (Marrs et al. 1995). To verify the accuracy of these replicas, the surface microtopographies of both natural and replicated surfaces were visually compared using light microscopy (Leica MZ 125) and scanning electron microscopy (JEOL JSM-5410LV), respectively. All replicas were examined

for air bubbles on the surface using light microscopy. Replicas with air bubbles on the surface were discarded and replaced. Replicas of *L. laevigata*, *F. indica*, *C. pentagona*, *A. typicus* as well as smooth and rough control surfaces were screwed to three rigid opaque PVC sheets (50 cm × 90 cm) (n = 10 for each PVC sheet) in a randomised block design (6 treatments in each of 10 rows) to account for possible changes in fouling communities with the length of the PVC sheets. The PVC sheets were previously treated with Sigmaplane Ecol HA120 antifouling coating (Wattyl Protective and Marine Coatings) to reduce the settlement of fouling organisms on the sheets and minimise edge effects on the replicas. Treating the PVC sheets with this coating did not affect the settlement of fouling organisms on replicas (Scardino 2006).

To document the community development of fouling organisms on replicas, experiments were carried out concurrently *in situ* at 3 sites in Townsville, Australia: the Australian Institute of Marine Science (AIMS, 19°16'S, 147°04'E), the Townsville Yacht Club (19°15'S, 146°50'E) and the Breakwater Marina (19°15'S, 146°50'E). Experiments were carried out for 8 weeks in June 2005 (dry season) and December 2005 (wet season). Each panel was suspended to a water depth of 0.5 m. Replicas were photographed fortnightly with a Sony DSC-W5 digital still camera. Using the imaging software Leica IM50 Image Manager, the number and percentage cover of individual fouling organisms and the total percentage cover were quantified in the centre 1 cm^2 of each replica (Bers and Wahl 2004). The centre 1 cm^2 was chosen, since *C. pentagona* is a small species and possible edge effects were to be avoided. Fouling organisms were identified to the lowest taxonomic level possible using relevant keys (e.g. OECD 1965, OECD 1967, Zibrowius 1971, Underwood 1977).

4.2.5 Statistical analysis

One-factor analyses of similarities (ANOSIM, $\alpha = 0.05$), which tested for significant differences in fouling assemblages between the replicas, were calculated from Bray-Curtis similarity matrices on non-transformed numbers of individual fouling organisms (PRIMER version 5, Clarke and Warwick 1994). If fouling assemblages on the replicas were significantly different, pair-wise comparisons were performed. The significance level of these pair-wise comparisons was adjusted for multiple comparisons using the Bonferroni technique ($\alpha = 0.008$; Clarke and Warwick 1994, Sokal and Rohlf 2001). Data with significant results between replicas of sea stars and control surfaces were presented graphically using non-metric multi-dimensional scaling (nMDS) ordinations. Similarity percentages (SIMPER) identified the fouling species that contributed the most to the average dissimilarity between the replicas of sea star species and the control surfaces. To determine significant differences in the percentage

cover of these fouling species as well as the total fouling cover between the replicas after 8 weeks of submersion, the total percentage cover was analysed using one-factor analysis of variance (ANOVA, $\alpha = 0.05$), followed by Tukey's HSD multiple comparison tests (SPSS version 14). The assumptions of homogeneity and normality of the data were checked with residuals versus predicted values plots and Q-Q plots of residuals, respectively. To meet these assumptions, data on the total fouling cover at the Townsville Yacht Club during the dry season and the Breakwater Marina during the wet season were log-transformed and arcsine-transformed, respectively (Underwood 1981, Quinn and Keough 2002).

4.3 Results

4.3.1 Surface wettability

The surfaces of the sea stars *L. laevigata*, *F. indica*, *A. typicus* were moderately wettable. Using the captive bubble technique, the intermediate contact angles $\theta_{airbubble}$ on *L. laevigata*, *F. indica*, *A. typicus* and the control surface were 119.9 ± 2.1º, 109.7 ± 3.5º, 122.7 ± 2.1º and 84.3 ± 1.1º. These intermediate contact angles were converted to the contact angles $\theta_{seawater}$ using the formula: $\theta_{seawater} = 180º - \theta_{airbubble}$. The mean contact angles $\theta_{seawater}$ on the sea stars *L. laevigata*, *F. indica*, *A. typicus* and the control surface were 60.1º, 70.3º, 57.3º and 95.7º, respectively. When using the water droplet technique and the goniometer to estimate the experimental error of this experiment, the contact angle $\theta_{seawater}$ on the control surface was 93.8 ± 1.4º. The experimental error was estimated to be <3º, taking into consideration the difference between the converted contact angle $\theta_{seawater}$ of the control surface using the captive bubble technique (95.7º) and the contact angle $\theta_{seawater}$ using the water droplet technique (93.8º) as well as the measurement reproducibility of ± 1º of the goniometer.

4.3.2 Surface microtopography

The sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* had distinct surface microtopographies. The mean diameters and heights of paxillae and the mean distances between paxillae of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* differed considerably between species, but they were generally consistent between the proximal, middle and distal positions on the aboral surface of each species (Table 4.1, Figure 4.1). The paxillae of *L. laevigata* had intermediate diameters and heights and were very closely spaced (17.3 to 20.4 μm) (Table 4.1, Figure 4.1A). Similarly, the paxillae of *F. indica* had intermediate diameters, but they had the

smallest heights (50.6 to 53.6 μm) and the largest distances between them (108.2 to 112.4 μm) (Table 4.1, Figure 4.1B). The paxillae of *C. pentagona* had the smallest diameters (106.5 to 111.4 μm) and similar heights and diameters to *F. indica* (Table 4.1, Figure 4.1C). Finally, the paxillae of *A. typicus* had the largest diameters (191.1 to 229.4 μm) and heights (286.5 to 446.4 μm) of all four sea star species being 5 to 8 times higher than the paxillae of the other species, but only had intermediate distances between them (Table 4.1, Figure 4.1D).

Table 4.1 Diameter and height of paxillae as well as distance between paxillae in μm at proximal, middle and distal positions on the arms of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* and on replicas of the same sea star species. Means ± SE are shown (n = 15 for each position on each of 3 specimens).

	Natural Surface			Replica
	Proximal	**Middle**	**Distal**	
Diameter				
Linckia laevigata	116.9 ± 9.7	115.7 ± 2.5	114.1 ± 1.6	107.7 ± 5.3
Fromia indica	178.8 ± 7.9	172.3 ± 6.9	178.2 ± 6.1	161.0 ± 7.3
Cryptasterina pentagona	111.4 ± 1.2	108.4 ± 3.6	106.5 ± 5.2	107.5 ± 1.9
Archaster typicus	229.4 ± 21.1	203.6 ± 33.1	191.1 ± 9.0	202.9 ± 5.3
Height				
Linckia laevigata	104.8 ± 4.8	99.2 ± 4.5	98.5 ± 7.1	55.8 ± 1.7
Fromia indica	50.6 ± 1.8	52.1 ± 4.5	53.6 ± 3.7	69.8 ± 5.2
Cryptasterina pentagona	62.8 ± 8.5	50.4 ± 7.0	54.0 ± 1.6	68.3 ± 6.5
Archaster typicus	446.4 ± 2.6	378.8 ± 41.1	286.5 ± 11.4	287.0 ± 21.7
Distance				
Linckia laevigata	20.4 ± 2.0	17.3 ± 0.2	17.4 ± 0.4	22.2 ± 2.6
Fromia indica	110.7 ± 11.1	108.2 ± 10.6	112.4 ± 2.0	88.8 ± 3.6
Cryptasterina pentagona	94.0 ± 10.5	103.5 ± 5.0	93.9 ± 3.4	98.6 ± 6.8
Archaster typicus	90.8 ± 8.9	97.7 ± 9.2	81.5 ± 5.8	71.7 ± 3.7

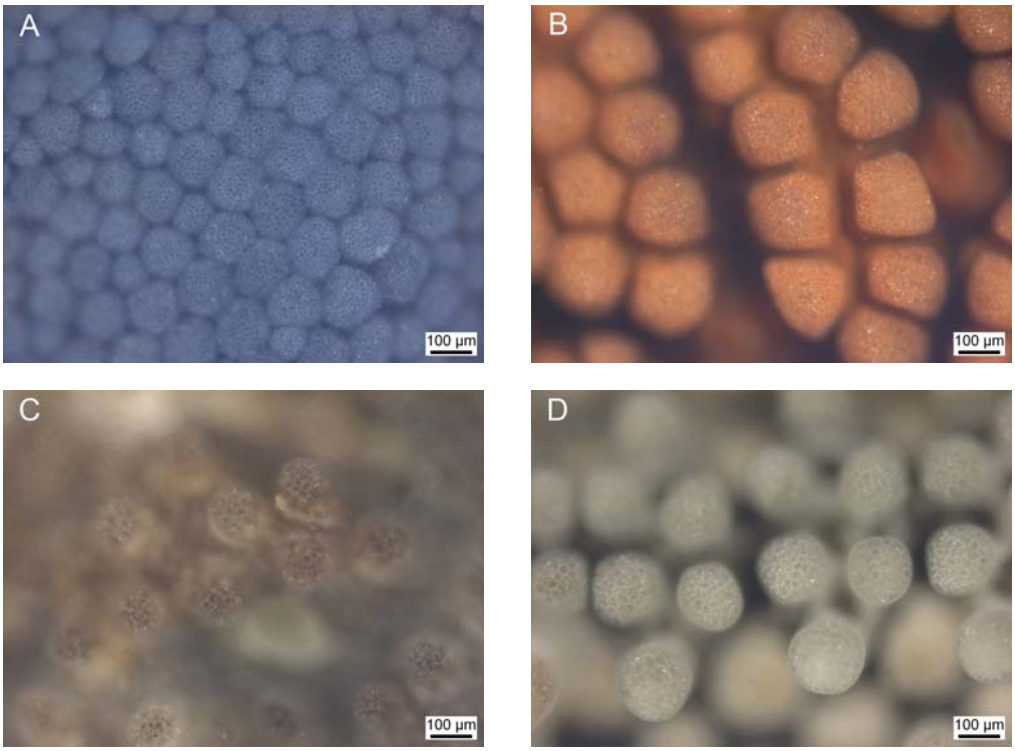

Figure 4.1 Aboral surface microtopographies of **(A)** *Linckia laevigata*, **(B)** *Fromia indica*, **(C)** *Cryptasterina pentagona* and **(D)** *Archaster typicus*. Scale bar, 100 µm.

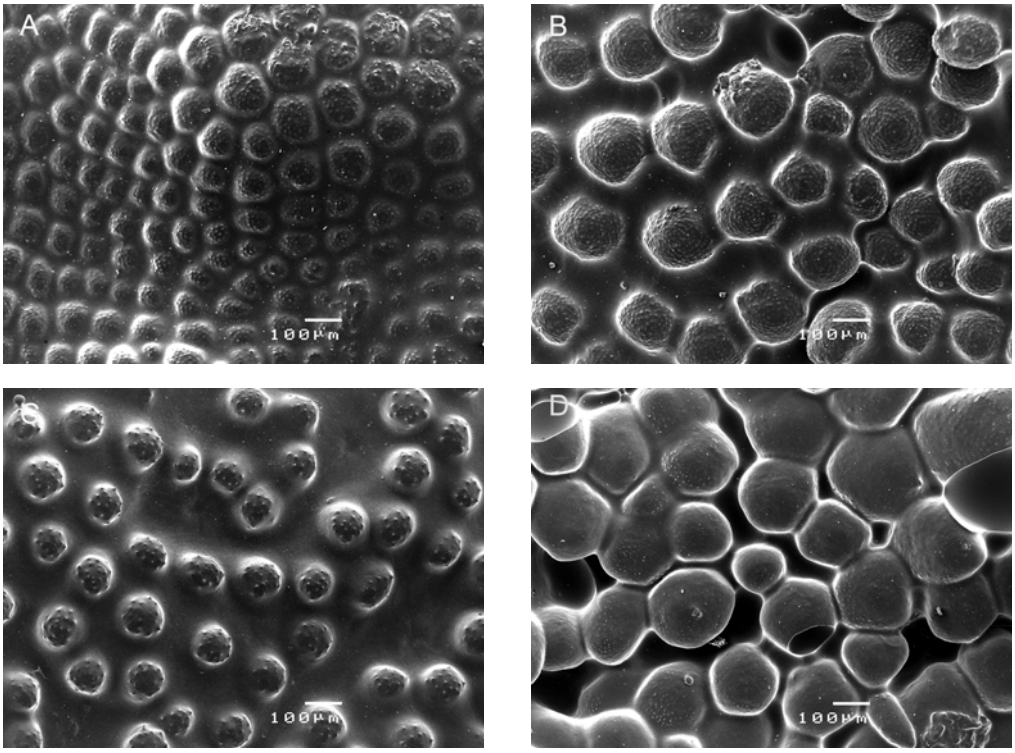

Figure 4.2 Scanning electron micrographs of replicas of **(A)** *Linckia laevigata*, **(B)** *Fromia indica*, **(C)** *Cryptasterina pentagona* and **(D)** *Archaster typicus*. Scale bar, 100 µm.

Replicas of the sea stars were accurate (Table 4.1), with the diameter and height of paxillae and the distance between paxillae on the replicas representing the paxillae on the natural surface of each sea star species. The only deviation from high resolution replication was the height of the paxillae of *L. laevigata*. Spinelets covering the paxillae of the sea stars *L. laevigata*, *F. indica* and *C. pentagona* were visible as small protrusions, which had a distance of 13.7 ± 0.6 μm, 12.2 ± 04 μm and 24.0 ± 0.9 μm, respectively (Figure 4.2A to C). The paxillae of the sea star *A. typicus* were smooth (Figure 4.2D).

4.3.3 Fouling community development

The surface microtopographies of all four sea star species did not have any effect on the fouling community composition and percentage cover on replicas at all sites during the dry season, whereas the surface microtopographies of *C. pentagona* and *A. typicus* only had transitory effects on the fouling community composition during the wet season. The replicas of the sea stars and control surfaces were most commonly fouled by the tube-building polychaetes *Hydroides elegans* and *Spirorbis* sp., the encrusting bryozoan *Watersipora subtorquata*, the tube-building amphipod *Corophium* sp., the barnacle *Balanus amphitrite*, the colonial ascidians *Botrylloides leachi* and *Diplosoma listerianum*, the brown alga *Rosenvingea intricata* and a filamentous brown alga with mean percentage covers of >5% at the three sites at different submersion times during the dry or wet season. Although the colonial bryozoan *Bugula neritina* occurred with mean percentage covers of <5%, it grew in abundance, forming large tufts with a height of several centimetres. Data on the fouling communities at AIMS after 6 weeks of submersion during the dry season is not available, as the site could not be accessed due to severe weather conditions and subsequent damage to the pontoon.

During the dry season, the ANOSIM analysis showed significant differences in fouling communities between replicas only at the Yacht Club after 6 weeks of submersion and at the Breakwater Marina after 4 and 6 weeks of submersion (Table 4.2A). The R-values indicate the extent of separation between the groups, $R > 0.75$ groups are well separated, $R > 0.50$ groups are overlapping but clearly different, $R < 0.25$ groups are barely separable (Clarke and Warwick 1994). Although the R-values were very small, some of these values were significantly different from zero (Table 4.2A). However, pair-wise comparisons demonstrated that these differences were due to significant differences between replicas of sea star species only, rather than between replicas of sea star species and the control surfaces. There were no significant differences in total fouling cover between the replicas of sea stars and the control surfaces at any of the three sites after 8 weeks of submersion (AIMS: $F_{5,52} = 0.807$, $p = 0.550$; Yacht Club: $F_{5,54} = 1.624$, $p = 0.169$ for log-transformed data; Breakwater Marina: $F_{5,54} = 1.451$, $p = 0.221$) (Figure 4.3A to C).

Table 4.2 Global R-values for fouling communities on replicated surfaces of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona*, *Archaster typicus*, smooth and rough control surfaces during the **(A)** dry season and **(B)** wet season (--- data not available).

(A) Dry season	**Week**	**Global R**	**p**
AIMS	2	---	---
	4	0.105	0.108
	6	---	---
	8	0.051	0.074
Yacht Club	2	0.042	0.161
	4	-0.010	0.567
	6	0.067	0.028
	8	0.026	0.176
Breakwater Marina	2	0.049	0.192
	4	0.069	0.040
	6	0.138	0.001
	8	0.048	0.057

(B) Wet season	**Week**	**Global R**	**p**
AIMS	2	---	---
	4	-0.006	0.461
	6	0.014	0.290
	8	0.001	0.487
Yacht Club	2	-0.025	0.724
	4	0.071	0.027
	6	0.076	0.017
	8	0.043	0.072
Breakwater Marina	2	0.112	0.001
	4	0.090	0.002
	6	0.113	0.003
	8	0.049	0.065

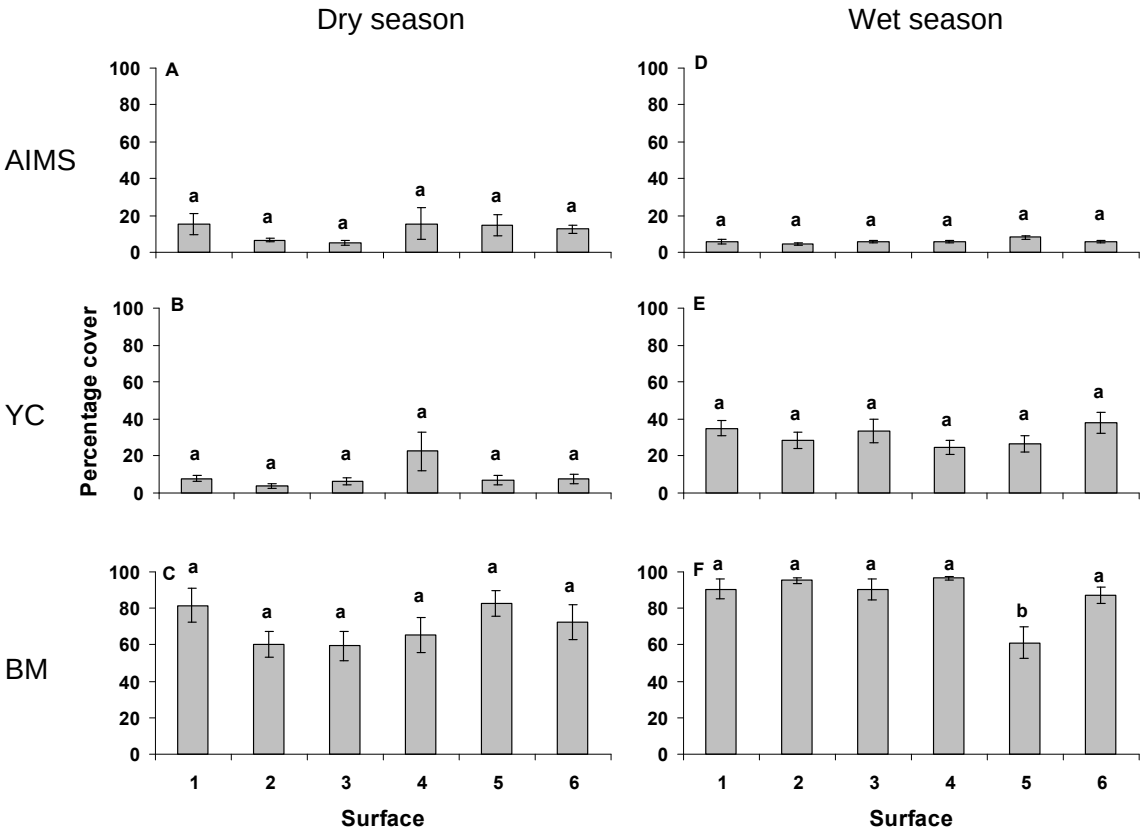

Figure 4.3 Total percentage cover of fouling organisms on replicas at **(A)** AIMS, **(B)** Yacht Club (YC) and **(C)** Breakwater Marina (BM) after 8 weeks of submersion during the dry season and **(D)** AIMS, **(E)** Yacht Club (YC) and **(F)** Breakwater Marina (BM) after 8 weeks of submersion during the wet season. Surface: 1 – *Linckia laevigata*, 2 – *Fromia indica*, 3 – *Cryptasterina pentagona*, 4 – *Archaster typicus*, 5 – Smooth control, 6 – Rough control. Means ± SE are shown (n = 10). Different letters indicate significant differences (Tukey's HSD multiple comparison test, $p < 0.05$).

During the wet season, the ANOSIM analysis detected significant differences in fouling communities only at the Yacht Club after 4 and 6 weeks of submersion and at the Breakwater Marina after 2, 4 and 6 weeks of submersion (Table 4.2B). Again, pair-wise comparisons did not detect any significant differences in fouling communities between replicas of sea stars and control surfaces at the Yacht Club after 4 and 6 weeks of submersion. At the Breakwater Marina after 2 weeks of submersion, the fouling communities on the replicas of *A. typicus* were significantly different from both the smooth ($p = 0.001$) and the rough ($p = 0.006$) control surfaces. After 4 weeks of submersion, the fouling communities on the replicas of *C. pentagona* were significantly different from both the smooth ($p = 0.001$) and the rough ($p = 0.002$) control surfaces. The differences between fouling communities on replicas of *A. typicus* and control surfaces after 2 weeks of submersion and on replicas of *C. pentagona* and control surfaces after 4 weeks of submersion are graphically presented with nMDS ordinations (Figure 4.4). The

separation of replicas into clusters of sea stars and control surfaces illustrate the distinction in their fouling community compositions (Figure 4.4). Furthermore, the SIMPER analysis showed that the fouling species *H. elegans*, *Corophium* sp. and *Spirorbis* sp. contributed the most to the dissimilarity between the replicas of sea stars and the control surfaces (Table 4.3). Based on these results, Figures 4.5A and B show the percentage cover of these fouling species on replicas of *A. typicus* and control surfaces after 2 weeks of submersion as well as on replicas of *C. pentagona* and control surfaces after 4 weeks of submersion. The percentage cover of *H. elegans* and *Corophium* sp. on replicas of *A. typicus* and control surfaces after 2 weeks of submersion were low. *H. elegans* had significantly less fouling cover on replicas of *A. typicus* than on rough control surfaces, whereas there were no significant differences in the percentage cover of *Corophium* sp. between replicas of *A. typicus* and control surfaces ($F_{2,27} = 1.649$, $p = 0.211$, Figure 4.5A). Differences in the percentage cover of fouling species were more evident on replicas of *C. pentagona* and control surfaces after 4 weeks of submersion, with significantly less fouling cover of *Corophium* sp. and *Spirorbis* sp. and significantly more fouling cover of *H. elegans* on replicas of *C. pentagona* than both smooth and rough control surfaces (Figure 4.5B). Notably, the observed significant differences in fouling communities at the Breakwater Marina were only transitory, since pair-wise comparisons did not detect any significant differences in fouling communities between replicas of sea stars and control surfaces after 6 weeks of submersion. Furthermore, there were no significant differences in total fouling cover between the replicas of sea stars and the control surfaces at both AIMS ($F_{5,51} = 2.004$, $p = 0.094$) and the Yacht Club ($F_{5,52} = 1.126$, $p = 0.358$) (Figure 4.3D and E). However, significant differences were detected at the Breakwater Marina ($F_{5,54} = 6.096$, $p < 0.0001$ for arcsine-transformed data). The Tukey's HSD multiple comparison tests showed that there was significantly less total fouling cover on the smooth control surface than on all other replicas of sea stars and the rough control surface ($p < 0.05$, Figure 4.3F), demonstrating a positive effect on fouling.

Table 4.3 SIMPER analysis identifying fouling species, in decreasing order of their importance, which contributed to the average dissimilarity between the replicas of sea star species and the control surfaces at the Breakwater Marina during the wet season, until 90% of the dissimilarity was accounted for.

Week	Replica pairs	Fouling species	Percentage
2	*Archaster typicus* and smooth control	*Hydroides elegans*	53.32
		Corophium sp.	38.62
2	*Archaster typicus* and rough control	*Hydroides elegans*	53.61
		Corophium sp.	39.83
4	*Cryptasterina pentagona* and smooth control	*Corophium* sp.	52.88
		Hydroides elegans	31.81
		Spirorbis sp.	8.86
4	*Cryptasterina pentagona* and rough control	*Corophium* sp.	54.70
		Hydroides elegans	28.05
		Spirorbis sp.	9.27

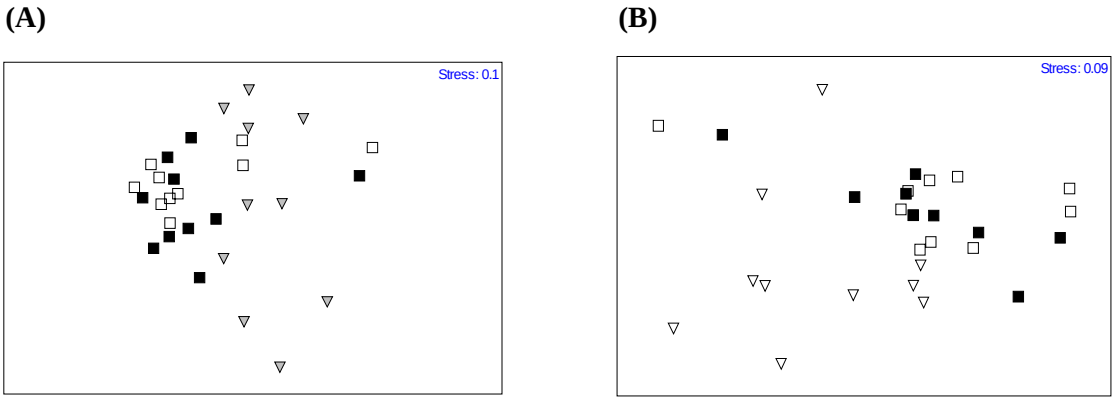

Figure 4.4 Two-dimensional MDS plot of fouling cover data for replicated surfaces at the Breakwater Marina after **(A)** 2 weeks of submersion and **(B)** 4 weeks of submersion during the wet season. Replicas: ▼ *Archaster typicus*, ▽ *Cryptasterina pentagona*, □ Smooth control, ■ Rough control. Stress values as indicated.

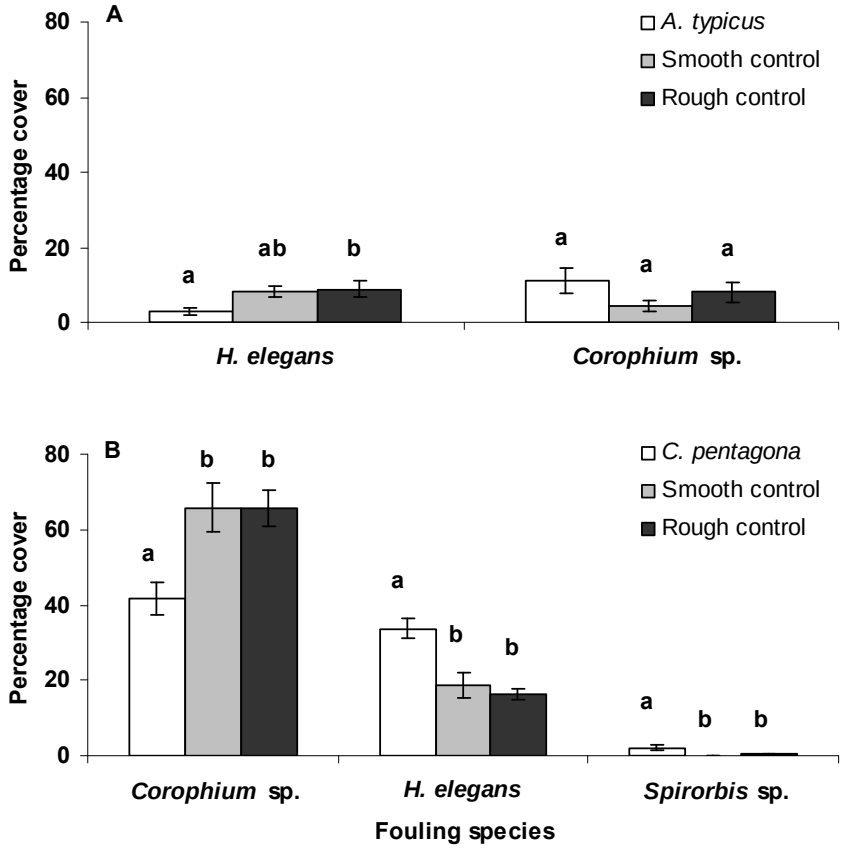

Figure 4.5 Percentage cover of fouling species on replicas of **(A)** *Archaster typicus* and control surfaces after 2 weeks of submersion and **(B)** *Cryptasterina pentagona* and control surfaces after 4 weeks of submersion at the Breakwater Marina during the wet season. Means ± SE are shown (n = 10). Different letters indicate significant differences (Tukey's HSD multiple comparison test, $p < 0.05$).

4.4 Discussion

The sea stars *L. laevigata*, *F. indica* and *A. typicus* were moderately wettable, with mean contact angles ranging between 57.3º and 70.3º. While the effects of the surface wettability of artificial surfaces on the settlement of marine organisms are well documented (Callow et al. 2000, Finlay et al. 2002, Dahlström et al. 2004, Greer and Amsler 2004, Aldred et al. 2006), contact angle measurements of marine organism are scarce (Vrolijk et al. 1990, Becker et al. 2000) and completely lacking for echinoderms. Becker et al. (2000) reported a wide range of wettabilities on the carapace of 45 decapod crustaceans, including contact angles between 60º and 70º for 11 species, and found no correlation between contact angles and the densities of fouling organisms.

The complex surface microtopographies of all 4 sea star species alone did not have any effect on the fouling community composition and percentage cover on replicas at all sites during the

dry season, whereas the surface microtopographies of *C. pentagona* and *A. typicus* only had transitory effects on the fouling community composition during the wet season. The non-differential fouling community development on replicas during the dry season may be explained by the scale of the surface microtopographies. The surface microtopographies of the 4 sea star species ranged between approximately 20 and 450 μm, which were similar to the larval sizes of the most common fouling organisms. For example, larvae of the serpulid *H. elegans* are 100 μm (Lam et al. 2003), larvae of the bryozoan *B. neritina* are 200 to 300 μm (Temkin and Zimmer 2002), larvae of the ascidian *D. listerianum* are 300 to 400 μm (Marshall and Keough 2003), and finally cyprid larvae of the barnacle *B. amphitrite* are 450 μm (Egan and Anderson 1986). The shape and size of the surface microtopographies of the replicas may have provided sufficient contact points for larvae to settle (Callow et al. 2002) and even attracted the settlement of fouling organism, which would explain the significantly higher percentage cover of fouling organisms on the replicas of sea stars and rough control surfaces than smooth control surfaces at the Breakwater Marina after 8 weeks of submersion during the wet season. Similar observations have been made with cyprid larvae of the barnacle *Semibalanus balanoides* and spores of the green alga *Enteromorpha* sp. preferring to settle on surfaces with a similar scale to their body size and having lowest levels of settlement on smooth surfaces (Hills and Thomason 1998a,b, Callow et al. 2002). Köhler et al. (1999) also found the mussel *M. edulis*, the barnacle *B. improvisus*, the polychaete *Polydora ciliata*, the diatom *Licmophora* sp. and the ciliate *Vorticella* sp., all common fouling species, preferred different textured surfaces to smooth surfaces. Furthermore, the scale of the surface microtopographies of sea stars was larger than the scale of surface microtopographies of both artificial (Andersson et al. 1999, Berntsson et al. 2000a,b, Petronis et al. 2000) and natural surfaces (Bers and Wahl 2004, Scardino and de Nys 2004) with demonstrated effects against the settlement of fouling organisms.

Transitory effects of the surface microtopography on the settlement of fouling organisms have also been observed during other field studies with natural (Guenther and de Nys 2006) and artificial surfaces (Andersson et al. 1999) as well as replicas of natural surfaces (Bers and Wahl 2004, Scardino and de Nys 2004). During any field study, a range of abiotic and biotic factors influence the fouling community composition and abundance, and these may have overshadowed the effects of the surface microtopography on the settlement of fouling organisms if there are any effects. First, biofilms may have partially masked the surface microtopography of the replicas and played an important role in providing settlement cues. Some larvae of macrofouling species are able to differentiate between biofilms of varying densities (Neal and Yule 1994a, Tsurumi and Fusetani 1998) and ages (Maki et al. 1988, Neal and Yule 1994b, Keough and Raimondi 1995), which either facilitate or inhibit larval settlement

(Todd and Keough 1994, Wieczorek and Todd 1997, Lau et al. 2002, Patel et al. 2003). Biofilms may have established on the surface of replicas due to the absence of natural products of sea stars. Bryan et al. (1994) and Iorizzi et al. (1995) have demonstrated that ethanol extracts of a range of sea stars inhibit the growth of marine microbial species. Second, gregarious behaviour of some fouling species, i.e. larva-larva and larva-adult interactions, may have influenced species composition. For example, the settlement of the barnacle cyprids of *B. amphitrite amphitrite* and *S. balanoides* (Bertness et al. 1992, Clare et al. 1994, Hills and Thomason 1996) and bryozoan larvae of *B. neritina* (Brancato and Woollacott 1982, Keough 1984a) increases with proximity to conspecifics. Third, biotic interactions between fouling species, such as competition and predation, play an important role in determining the development of a fouling community (Dayton 1971, Keough 1984b). Keough (1984b) demonstrated that the colonial ascidians *Didemnum patulum* and *Didemnum* sp. were capable of excluding other fouling species on the bivalve *Pinna bicolor*.

Regardless of possible effect mechanisms, the key finding of this Chapter is that the surface microtopography of tropical sea stars alone is not effective in deterring the settlement of common generalist fouling organisms. However, physical defence mechanisms of sea stars may work synergistically with behavioural, mechanical and/or chemical antifouling mechanisms, which have also been suggested for other marine organisms that remain remarkably clean (Vrolijk et al. 1990, Bers and Wahl 2004, Scardino and de Nys 2004, Bers et al. 2006b). Further research on other potential antifouling defence mechanisms of sea stars, working independently or synergistically, will elucidate how sea stars keep their surfaces free of fouling organisms.

Chapter 5

Chemical antifouling defences: Effects of crude extracts of sea stars

5.1 Introduction

In Chapter 4, the studies on potential physical antifouling defence mechanisms of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* demonstrated that wettabilities and surface microtopographies of these sea stars alone are not effective at deterring the settlement of fouling organisms. To further elucidate antifouling defence mechanisms of sea stars, the role of natural products in keeping the surface free of fouling organisms is investigated for *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* in this Chapter.

Chemical studies on crude extracts and isolated compounds (mainly steroid glycosides consisting of asterosaponins, steroidal cyclic glycosides, steroid monoglycosides and diglycosides) from echinoderm tissues have demonstrated that these extracts and compounds have cytotoxic (De Marino et al. 1998, Wang et al. 2002, Wang et al. 2003), haemolytic (Ivanchina et al. 2006), antiviral (Roccatagliata et al. 1996), antifungal (Chludil and Maier 2005), antimicrobial (Haug et al. 2002), feeding deterrent (McClintock and Vernon 1990, Iorizzi et al. 1995, McClintock et al. 2003) and antifouling (Iorizzi et al. 1995, Bryan et al. 1996, Greer et al. 2006) activities.

Testing whole-cell crude extracts of sea stars against the settlement of fouling organisms screens the widest possible range of compounds present within tissues, where synergism between compounds of an extract may be important (Teo and Ryland 1995), and gives a better understanding of the potential compounds responsible for the antifouling effects. To identify crude extracts and compounds with antifouling activity, extracts and compounds from sea stars have commonly been tested at various concentrations against selected fouling organisms using disc diffusion assays (Bryan et al. 1994, Iorizzi et al. 1995), motion analysis assays (Iken et al. 2001, Iken et al. 2003, Greer et al. 2006) or settlement assays (Iorizzi et al. 1995, Bryan et al. 1996, De Marino et al. 2000, Greer et al. 2003). In disc diffusion assays, extracts or compounds are applied to sterile filter paper discs, which are then placed on media inoculated previously with a microbial species. After incubation, the zones of microbial growth inhibition are measured (Bryan et al. 1994, Iorizzi et al. 1995). Bryan et al. (1994) showed that the ethanol body wall extracts of 13 of 15 tested sea star species at ecologically relevant concentrations inhibited the growth of at least 1 of 19 tested marine and non-marine microbial species. Similarly, the ethanol whole body extracts of the sea star *Luidia clathrata* inhibited the growth of the bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Iorizzi et al. 1995). Furthermore, the saponins certonardosides K-N (1-3,5) of the sea star *Certonardoa semiregularis* showed weak antibacterial activity against 3 of 20 isolated strains (Wang et al. 2003).

Motion analysis assays use swimming spore suspensions on glass slides to observe and record swimming behaviour, such as the rate of direction change and swimming speed, of spores exposed to extracts (e.g. Greer et al. 2006). Spores of the brown alga *Hincksia irregularis* exposed to low concentrations (<91 $\mu g\ ml^{-1}$) of aqueous and organic body wall extracts of the sea stars *Astropecten articulatus* and *L. clathrata* changed their normally fast and straight swimming movements to helical and erratic movements (Iken et al. 2003). At the highest concentration (909 $\mu g\ ml^{-1}$), spores were severely damaged or completely immobilized (Iken et al. 2003). Similarly, Greer et al. (2006) used motion analysis assays to identify bioactive fractions of both aqueous and organic body wall extracts of the same sea star species, *A. articulatus* and *L. clathrata*.

Finally, settlement assays use plastic surfaces as attachment substrata, and settlement rates and developmental stages of fouling organisms exposed to crude extracts or isolated compounds are quantified over time (e.g. Bryan et al. 1996, Iken et al. 2003). Isolated asterosaponins of the sea star *Goniopecten demonstrans* reduced the settlement of the brown alga *H. irregularis* (De Marino et al. 2000). Similarly, water and acetone body wall extracts of the sea stars *A. articulatus* and *L. clathrata* had significant effects on the settlement and germination of *H. irregularis* (Greer et al. 2003). Ethanol body wall extracts of 13 sea star species reduced the

settlement of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina* (Bryan et al. 1996). Of these, the extract of the sea star *Goniaster tesselatus* was the most active, reducing the settlement of *B. amphitrite* and *B. neritina* at concentrations of ≥4.8 μg ml^{-1} (Bryan et al. 1996). Similarly, the ethanol whole body extracts of the sea star *L. clathrata* inhibited the settlement of *B. amphitrite* and *B. neritina* at concentrations of ≥120 μg ml^{-1} and ≥4.8 μg ml^{-1}, respectively (Iorizzi et al. 1995).

Although the results of bioassays with whole body or body wall extracts are useful to determine the presence of potential antifouling compounds in the tissues of sea stars, they are insufficient to unequivocally determine an antifouling role of compounds on the surface of sea stars. When extracting whole body or body wall tissues, natural products, which are only found within cells and would never be found on exposed surfaces under natural conditions, may also be extracted and have an inhibitory effect on fouling organisms when tested in laboratory based settlement assays (Nylund and Pavia 2003). Therefore, the aim of the research presented in this Chapter was to identify the widest possible range of potential antifouling compounds present within tissues of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, by investigating the effects of whole body extracts of these sea stars on the settlement of 4 ecologically relevant fouling organisms. The research presented in Chapter 6 then demonstrates the ecological relevance of specific antifouling compounds from these sea stars, by identifying and quantifying selected surface-associated compounds and testing them at ecologically relevant concentrations against the settlement of fouling organisms.

5.2 Materials and Methods

5.2.1 Collection of specimens

Live specimens of the sea stars *L. laevigata* and *F. indica* were collected from John Brewer Reef (18°38'S, 147°03'E), whereas *C. pentagona* and *A. typicus* were collected from Kissing Point in Townsville (19°14'S, 146°48'E) and Picnic Bay on Magnetic Island (19°11'S, 146°51'E), respectively in February 2006. Specimens were transported in ambient seawater and then kept temporarily in an outdoor recirculating tank (temperature: 26°C, salinity: 35 ppt) at the Marine and Aquaculture Research Facilities Unit at James Cook University.

5.2.2 Preparation of crude extracts

Specimens of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* (n = 5) were measured, weighed, freeze-dried and reweighed. The freeze-dried tissues of each specimen were sequentially extracted 4 times with dichloromethane (non-polar), followed by methanol (moderately polar) and water (polar) at room temperature. Dichloromethane and methanol used were HPLC grade (Mallinckrodt) and water was obtained from a MilliQ water purification system (Millipore). The resulting crude extracts of each specimen were filtered through glass wool and concentrated in a rotary evaporator. Concentrated crude extracts were removed, and dichloromethane extracts were air-dried, whereas methanol and water extracts were dried under reduced pressure. Solvent controls were prepared by concentrating and drying the same amounts of dichloromethane, methanol and water used for the extractions. Dry extracts were stored at -18°C, until they were redissolved for use in settlement assays. Natural concentrations (mg g^{-1}) of dichloromethane, methanol and water extracts were estimated by dividing the final dry weight of each extract (mg) by the wet weight of whole sea stars (g) used to prepare the extracts.

To identify potential antifouling compounds of 1 of the 4 sea star species, the sea star *C. pentagona* was chosen, because its dichloromethane extract had strong effects against the settlement of the tested fouling organisms, and specimens of *C. pentagona* were abundant at Kissing Point in Townsville. To prepare bulk extracts of *C. pentagona*, the body walls of 44 specimens were dissected, weighed, freeze-dried and reweighed. The freeze-dried tissues of all specimens were combined and extracted 4 times with dichloromethane. The resulting crude extract was filtered through glass wool, concentrated using a rotary evaporator and air-dried. Dry extracts were stored at -18°C until further processing employing reversed-phase vacuum liquid column chromatography (VLC).

5.2.3 Vacuum liquid column chromatography and high performance liquid chromatography

The dichloromethane extract of *C. pentagona* (817 mg) was redissolved in dichloromethane, subjected to reversed-phase VLC (Phenomenex, Sepra C18-E, 50 µm, 65 Å) and eluted with a step gradient of methanol:water (80:20 to 100:0) to methanol:dichloromethane (80:20 to 50:50 to 0:100) to yield 12 fractions (Figure 5.1). Resultant fractions were dried and then stored at -18°C, until they were redissolved for use in settlement assays with the diatom *Nitzschia closterium* (CS-5, CSIRO) or for further fractionation with high performance liquid chromatography (HPLC). Fractions were also separated using reversed-phased thin-layer chromatography (TLC, Merck, RP-18), using methanol as solvent and visualised with a 10%

solution of sulphuric acid in ethanol (and 0.1% vanillin) and heating to identify sensitive compounds.

The most bioactive fractions 3-5 (216 mg) and 6-8 (64 mg) from the reversed-phase VLC were dissolved in dichloromethane:methanol (50:50) and further fractionated using semi-preparative HPLC (Shimadzu, LC-10AT pump coupled to a SPD M10A diode array detector and a Rheodyne 7725i injector, column Phenomenex Luna C18 (2), 5 μm, 100 Å, 150 × 10 mm). Compounds were eluted with an acetonitrile:water gradient (40:60 to 100:0) over 15 min with a flow rate of 3.5 ml min^{-1}. The combined bioactive fractions 3-5 and 6-8 were fractionated into a further 12 and 13 fractions, respectively (Figure 5.1), which were then tested in settlement assays with the diatom *N. closterium*.

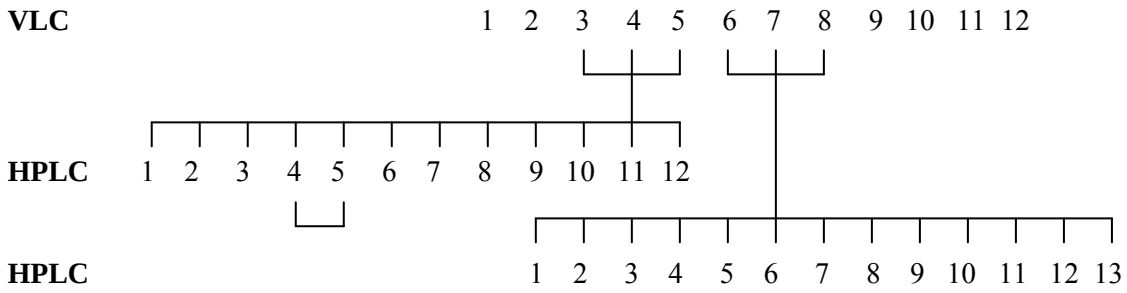

Figure 5.1 Flowchart of fractions obtained from the dichloromethane extract of *Cryptasterina pentagona* subjected to reversed-phase vacuum liquid column chromatography (VLC) and high performance liquid chromatography (HPLC).

5.2.4 Nuclear magnetic resonance

To identify the types of compounds in the bioactive fractions of the dichloromethane extract of *C. pentagona*, these fractions were analysed using nuclear magnetic resonance (NMR) spectroscopy. Each fraction was dissolved in deuterated chloroform and ^{1}H NMR spectra were recorded using a Bruker Advance 600 MHz NMR spectrometer with cryoprobe. Spectra were referenced to residual ^{1}H (δ 7.26) resonances in the deuterated solvent and recorded using standard Bruker pulse sequences. High-resolution mass spectra were measured employing a Bruker BioApex 47e FT-ICR mass spectrometer fitted with an, Analytica of Branford, electrospray source. Ions were detected in positive mode within a mass range of *m/z* 200-1000. Direct infusion of the sample (0.2 mg ml^{-1}) was carried out using a SGE 250 ul syringe and a Cole Palmer 74900 syringe pump at a flow rate of 100 μl h^{-1}.

5.2.5 Gas chromatography – mass spectrometry

Analysis with NMR demonstrated that mainly fatty acids and sterols were present in the most bioactive fractions of *C. pentagona*. Many of these fatty acids and sterols in these fractions were identified using gas chromatography – mass spectrometry (GC-MS). The fatty acids were converted to methyl esters by adding 100 µl of 5 N hydrochloric acid and 1 ml of purified water to each sample. Samples were then extracted with 1.8 ml of hexane:dichloromethane (4:1) 3 times and dried under nitrogen. Subsequently, 3 ml of methylation solution of methanol:hydrochloric acid:dichloromethane (10:1:1) were added and samples were incubated at 80°C for 1 h. After cooling, 1 ml of purified water was added and samples were extracted with 1.8 ml of hexane:dichloromethane (4:1) twice and the solvent fraction was run through a column packed with sodium sulphate to remove water and dried under nitrogen. Finally, 100 µl of dichloromethane were added and transferred to the GC-MS vials. Samples were analysed on a GC-MS system (Agilent 5973) with a 30 m DB5-MS column at a flow rate of 1.0 ml min^{-1} of helium as carrier gas and a temperature gradient of 50 to 310°C at 4°C min^{-1} and a hold time of 10 min. The mass spectrometer scanned m/z 50 to 550. A series of fatty acid methyl esters and sterol standards were also run. Compounds were identified based on retention time matching with standards, and mass spectra matching also with standards. Unknowns were identified employing the NIST and Wiley GC-MS databases.

5.2.6 Bioassays

To test the antifouling activity of the crude extracts of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* against ecologically relevant fouling organisms in laboratory based settlement assays, dichloromethane and methanol extracts were redissolved in ethanol and water extracts were redissolved in water. Extracts were applied to clear polystyrene Petri dishes (Sarstedt, diameter: 3.5 cm, base surface area: 9 cm^2) at concentrations of 100, 10, 1 and 0.1 µg cm^{-2} (n = 5) and placed on an orbital shaker at 75 rpm for 4 h to ensure even surface films and complete evaporation of the solvents. Fractions of the dichloromethane extraction of *C. pentagona* were applied to Petri dishes at a concentration of 10 µg cm^{-2} only. The concentrations are expressed in µg cm^{-2} rather than µg ml^{-1}, because most compounds in the dichloromethane and methanol extracts are non-polar or moderately polar, respectively, and are only partially soluble in seawater. They therefore adhere to the surface of the Petri dishes, giving approximate surface concentrations of 100, 10, 1 and 0.1 µg cm^{-2}. Water extracts are fully soluble in seawater and the surface concentrations of 100, 10, 1 and 0.1 µg cm^{-2} corresponded to approximate volumetric concentrations of 225, 22.5, 2.3 and 0.2 µg ml^{-1}, taking into consideration the

surface area of the base of the Petri dishes (9 cm^2) and the amount of seawater in each Petri dish during the bioassays (4 ml).

Settlement assays were run with the ecologically relevant diatoms *Amphora* sp. (CS-255, CSIRO) and *N. closterium* (CS-5, CSIRO), the serpulid *Hydroides elegans* and the bryozoan *Bugula neritina*. These fouling species were chosen for this study, because they are commonly found in tropical and subtropical waters, including the tropical waters off North Queensland (Hall 1984, Hallegraeff and Jeffrey 1984, Lewis et al. 2006).

The diatoms *Amphora* sp. and *N. closterium* were cultured in f2 media and kept in a temperature and light controlled room (25°C, 12 h light : 12 h dark, Sylvania Tri-phosphor fluorescent lamps, 5200 lumens). To run settlement assays with *Amphora* sp. and *N. closterium*, Petri dishes with extracts (n = 5) were prepared as described above and 4 ml of 0.45 µm filtered sea water (FSW) and 100 µl of a 2.5×10^5 algal cells ml^{-1} solution of either *Amphora* sp. or *N. closterium* were added to each dish. The dishes were kept in the same temperature and light controlled room for 4 h. Subsequently, Petri dishes were dip rinsed 3 times in 0.45 µm FSW to remove unattached diatoms. Attached diatoms were counted with a compound microscope at ×200 magnification in 5 fields of view per dish.

To run settlement assays with larvae of the serpulid *H. elegans*, adult specimens were collected from settlement plates at the Breakwater Marina in Townsville. Specimens were placed in Petri dishes containing 0.45 µm FSW. Their tubes were gently broken, after which gametes were released. Fertilized eggs were allowed to develop for 30 min and were then transferred to 2 l aerated glass beakers containing 0.45 µm FSW. Larvae were cultured at 10 larvae ml^{-1} and fed *Isochrysis* sp. (CS-177, CSIRO) at 6×10^4 algal cells ml^{-1} for 5 d, after which larvae were competent to settle. Because *H. elegans* larvae do not settle unless a settlement cue is provided, isobutyl methylxanthine (IBMX, Sigma-Aldrich, Catalogue number: I5879) was used to induce settlement (Bryan et al. 1997, Lau and Qian 1997). Petri dishes with extracts (n = 5) were prepared as described above and 4 ml of 10^{-4} M IBMX in 0.45 µm FSW and 20 larvae were added to each dish. FSW control dishes contained 4 ml of 10^{-4} M IBMX in 0.45 µm FSW only. The dishes were kept in the temperature and light controlled room for 48 h. Subsequently, settled and metamorphosed larvae having calcified tubes and tentacles were counted using a dissecting microscope.

Finally, to run settlement assays with larvae of the bryozoan *B. neritina*, several colonies of *B. neritina* were collected from settlement plates at the Yacht Club in Townsville and kept in

constant darkness in a recirculating tank at the Marine and Aquaculture Research Facilities Unit for 2 d. Colonies were then transferred to a 2 l glass beaker with 0.45 μm FSW and exposed to bright light for approximately 30 min, after which larvae were released (Marshall and Keough 2003). Petri dishes with extracts (n = 5) were prepared as described above and 4 ml of 0.45 μm FSW and 20 larvae were added to each dish. The dishes were kept in the temperature controlled room in the dark for 24 h. Subsequently, settled and metamorphosed larvae were counted using a dissecting microscope.

5.2.7 Statistical analysis

All statistical analyses were performed with SPSS version 14. To determine significant differences in the settlement of the diatoms *Amphora* sp. and *N. closterium* between treatments, the results were analysed with a 2-factor nested ANOVA (factors: (1) treatment – 4 different concentrations of the crude extract and controls, (2) dish nested in treatment – 5 replicate dishes for each treatment) followed by Tukey's HSD multiple comparison tests. Results on the metamorphosis of the serpulid *H. elegans* and the bryozoan *B. neritina* were analysed with a 1-factor ANOVA (factor: (1) treatment – 4 different concentrations of the crude extract and controls) followed by Tukey's HSD multiple comparison tests. The assumptions of homogeneity and normality of the data were checked with residuals versus predicted values plots and Q-Q plots of residuals, respectively. If assumptions of homogeneity and normality were not met, the data were square root transformed. (Underwood 1981, Quinn and Keough 2002). In settlement assays with the serpulid *H. elegans* and the bryozoan *B. neritina*, some experimental groups had mean metamorphosis of 0% or 100% and no variance; these groups were excluded from the statistical analyses.

5.3 Results

5.3.1 Natural concentrations of crude extracts

The mean natural concentrations of the dichloromethane, methanol and water extracts of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were similar between sea star species, except for the dichloromethane extract of *C. pentagona*, which was approximately 3 to 10× higher than the natural concentration of the dichloromethane extracts of the other 3 sea star species (Table 5.1). Overall, the mean natural concentration of the methanol extracts of the 4 sea star species was the highest of the 3 different extracts, ranging between 21.9 and 27.4 mg g^{-1} (ww) (Table 5.1).

Table 5.1 Natural concentrations in mg g^{-1} (ww) of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts for the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus*. Means ± SE are shown (n = 5).

	DCM	**MeOH**	**H_2O**
Linckia laevigata	2.41 ± 0.48	21.94 ± 1.21	6.41 ± 0.40
Fromia indica	6.63 ± 0.34	24.55 ± 0.75	9.13 ± 0.54
Cryptasterina pentagona	25.94 ± 4.98	27.42 ± 2.73	8.70 ± 0.70
Archaster typicus	8.51 ± 1.40	25.59 ± 1.64	8.87 ± 0.47

5.3.2 Bioassays with crude extracts

The dichloromethane, methanol and water extracts of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* had concentration-dependant effects on the settlement of the diatoms *Amphora* sp. and *N. closterium*, the serpulid *H. elegans* and the bryozoan *B. neritina*. The highest concentration (100 μg cm^{-2}) of the dichloromethane, methanol and water extracts of all sea star species significantly reduced the settlement of all fouling species. The effects of lower concentrations (10, 1 and 0.1 μg cm^{-2}) on the settlement of *Amphora* sp., *N. closterium*, *H. elegans* and *B. neritina* were species-specific.

For the sea star *L. laevigata* (Figure 5.2A to M), the dichloromethane extract was the most effective extract in reducing the settlement of fouling organisms, with concentrations of ≥1 μg cm^{-2} and ≥0.1 μg cm^{-2} significantly reducing the settlement of the diatom *N. closterium* (Figure 5.2D) and the serpulid *H. elegans* (Figure 5.2G), respectively. The methanol extract was also very effective, with concentrations of ≥1 μg cm^{-2} significantly reducing the settlement of *N. closterium* and *H. elegans* (Figure 5.2E and H). The water extracts at concentrations of ≥10 μg cm^{-2} significantly reduced the settlement of *N. closterium* (Figure 5.2F).

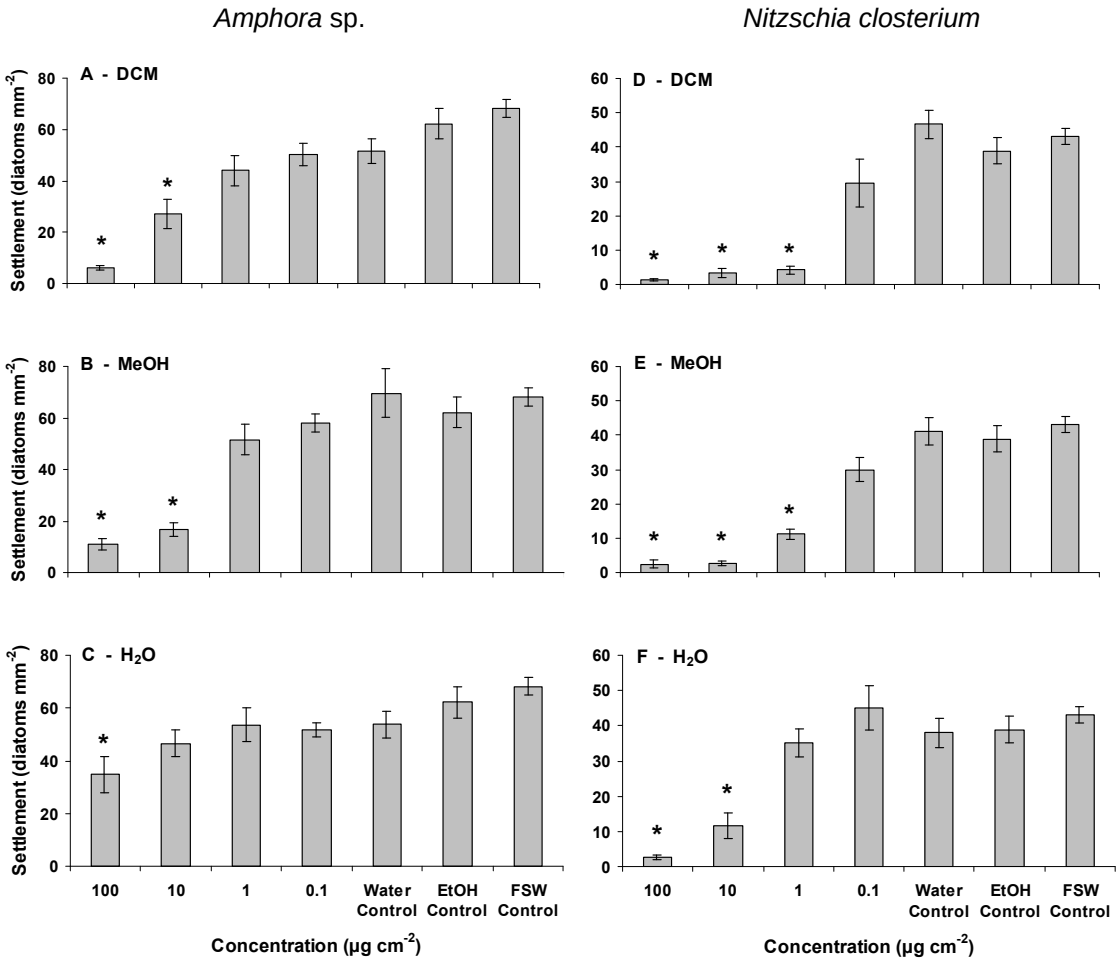

Figure 5.2 Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Linckia laevigata* on the settlement of the fouling species **(A)-(C)** *Amphora* sp. and **(D)-(F)** *Nitzschia closterium*. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

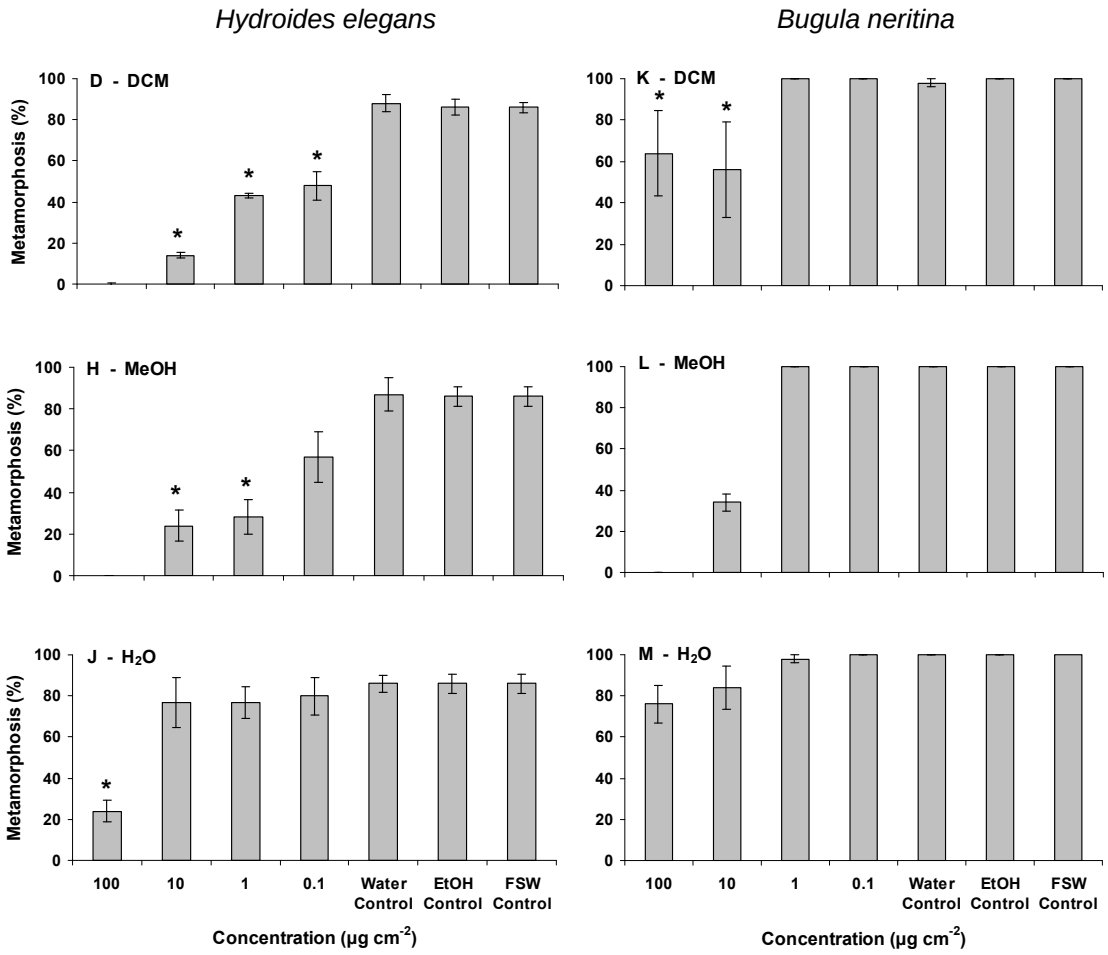

Figure 5.2 (continued) Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Linckia laevigata* on the metamorphosis of the fouling species **(G)-(J)** *Hydroides elegans* and **(K)-(M)** *Bugula neritina*. Means ± SE are shown (n = 5). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$, experimental groups with mean metamorphosis of 0% or 100% were excluded from the statistical analyses).

For the sea star *F. indica* (Figure 5.3A to M), very low concentrations of all crude extracts were effective in deterring settlement of most of the fouling species. The dichloromethane extract at a concentration of ≥0.1 μg cm^{-2} significantly reduced the settlement of the diatom *Amphora* sp. (Figure 5.3A), whereas methanol and water extracts at the same concentration significantly reduced the settlement of the serpulid *H. elegans* (Figure 5.3H and J). There was also no settlement of the bryozoan *B. neritina* larvae when exposed to the dichloromethane and methanol extracts at concentrations of ≥10 μg cm^{-2} (Figure 5.3K and L).

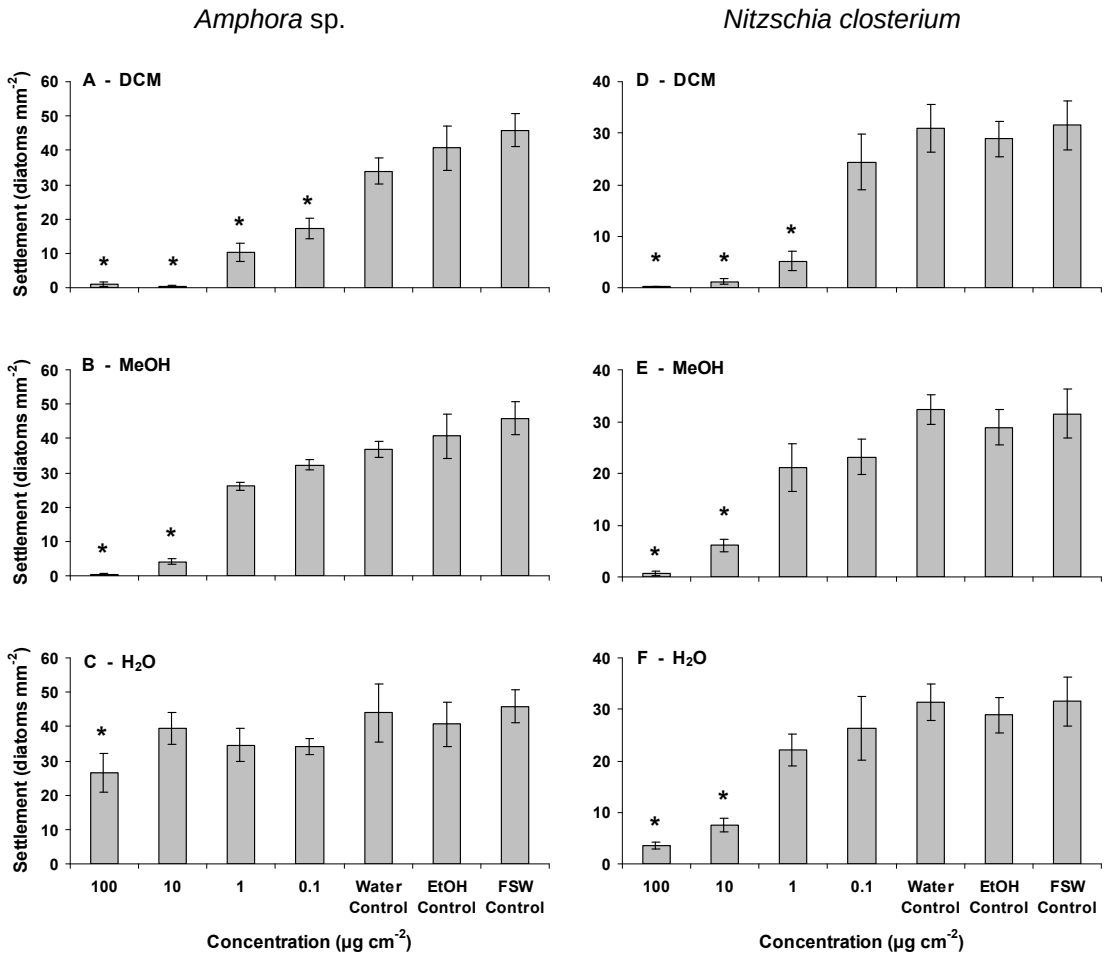

Figure 5.3 Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Fromia indica* on the settlement of the fouling species **(A)-(C)** *Amphora* sp. and **(D)-(F)** *Nitzschia closterium*. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

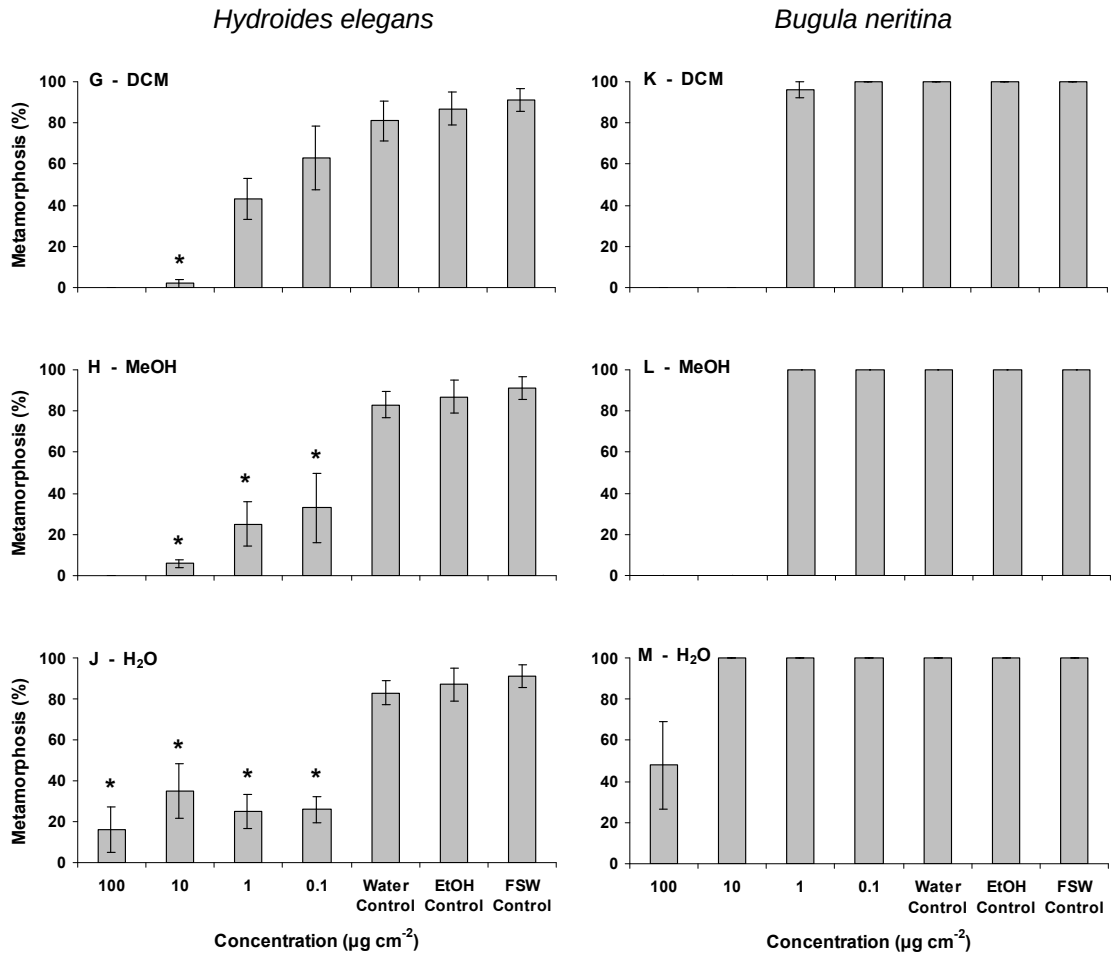

Figure 5.3 (continued) Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Fromia indica* on the metamorphosis of the fouling species **(G)-(J)** *Hydroides elegans* and **(K)-(M)** *Bugula neritina*. Means ± SE are shown (n = 5). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$, experimental groups with mean metamorphosis of 0% or 100% were excluded from the statistical analyses).

For the sea star *C. pentagona* (Figure 5.4A to M), the dichloromethane and methanol extracts were the most effective extracts at deterring the settlement of fouling species. The dichloromethane extract at concentrations of ≥1 μg cm^{-2} significantly reduced the settlement of *N. closterium* and *H. elegans* (Figure 5.4D and G). The methanol extract at concentrations of ≥1 and ≥0.1 μg cm^{-2} also significantly reduced the settlement of *N. closterium* and *H. elegans*, respectively (Figure 5.4E and H). The settlement of *B. neritina* larvae was completely inhibited when they were exposed to the methanol extract at a concentration of 100 μg cm^{-2} (Figure 5.4L). The water extract at concentrations of ≥10 μg cm^{-2} was effective against the settlement of *N. closterium* (Figure 5.4F).

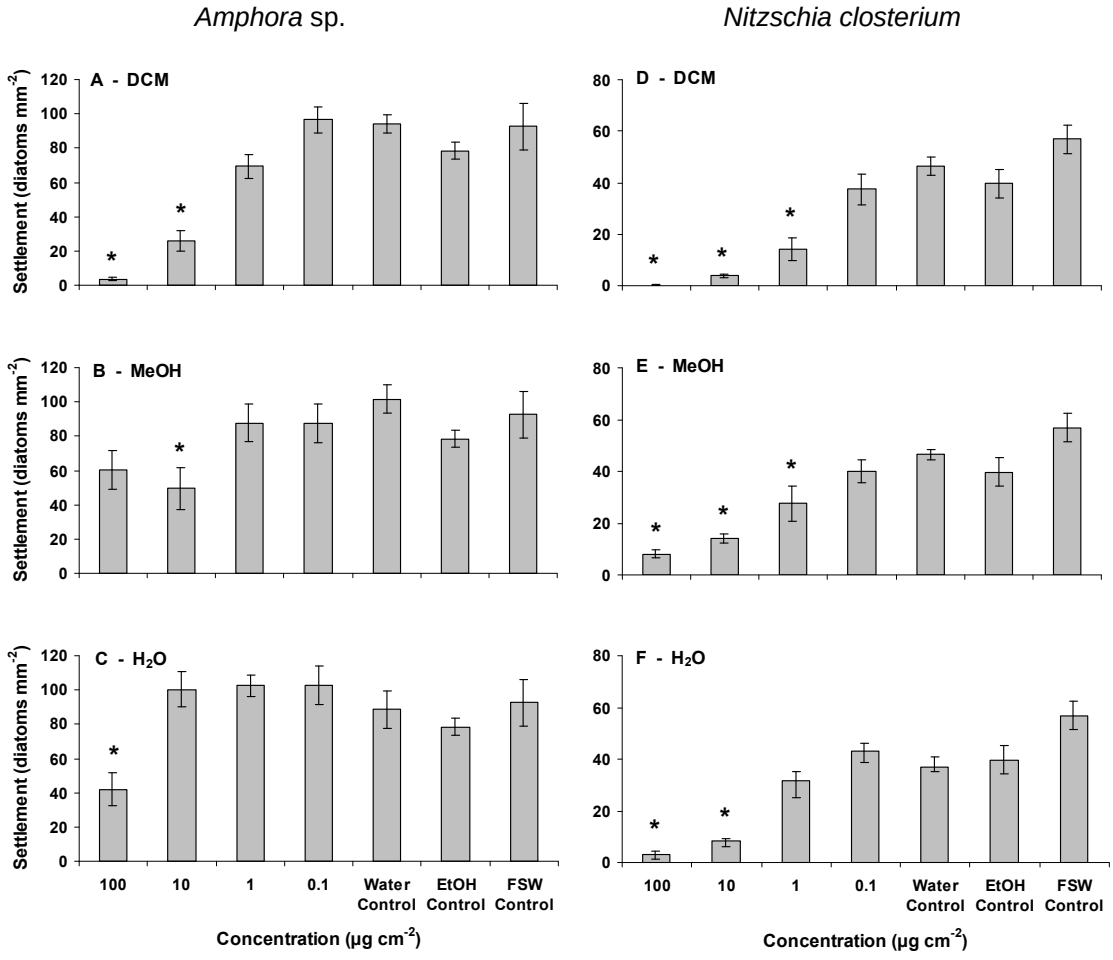

Figure 5.4 Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Cryptasterina pentagona* on the settlement of the fouling species **(A)-(C)** *Amphora* sp. and **(D)-(F)** *Nitzschia closterium*. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

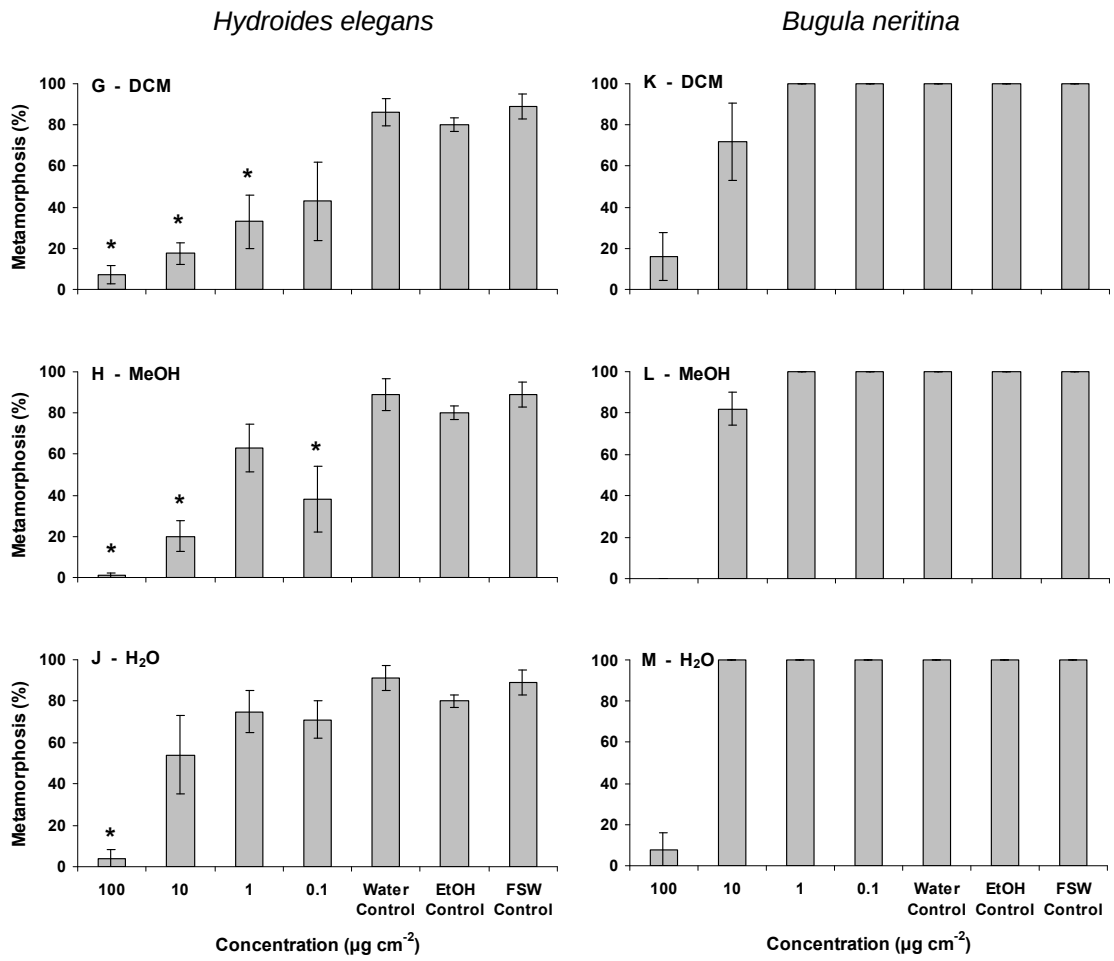

Figure 5.4 (continued) Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Cryptasterina pentagona* on the metamorphosis of the fouling species **(G)-(J)** *Hydroides elegans* and **(K)-(M)** *Bugula neritina*. Means ± SE are shown (n = 5). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$, experimental groups with mean metamorphosis of 0% or 100% were excluded from the statistical analyses).

Finally, for the sea star *A. typicus* (Figure 5.5A to M), the dichloromethane extract was also the most effective extract against the settlement of fouling species. The dichloromethane extract at concentrations of ≥0.1 μg cm^{-2} significantly reduced the settlement of *N. closterium* (Figure 5.5D) and at concentrations of ≥1 μg cm^{-2} significantly reduced the settlement of *Amphora* sp. (Figure 5.5A) and *H. elegans* (Figure 5.5G). Both the methanol and water extracts at concentrations of ≥10 μg cm^{-2} were effective against the settlement of *N. closterium* (Figure 5.5E and F), whereas the methanol extract at the same concentration also deterred the settlement of *H. elegans* (Figure 5.5H). There was no settlement of the *H. elegans* when a concentration of 100 μg cm^{-2} of both methanol and water extracts was used (Figure 5.5H and J).

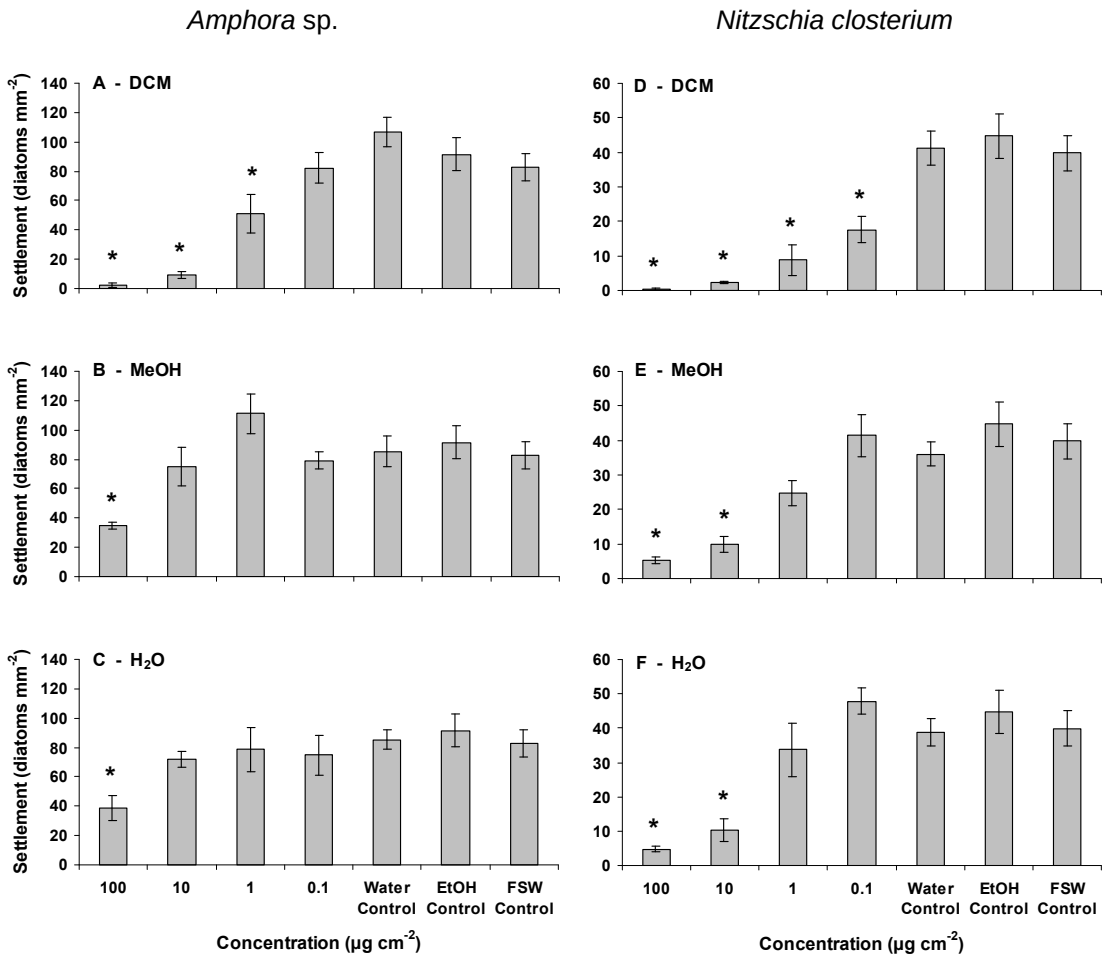

Figure 5.5 Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Archaster typicus* on the settlement of the fouling species **(A)-(C)** *Amphora* sp. and **(D)-(F)** *Nitzschia closterium*. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

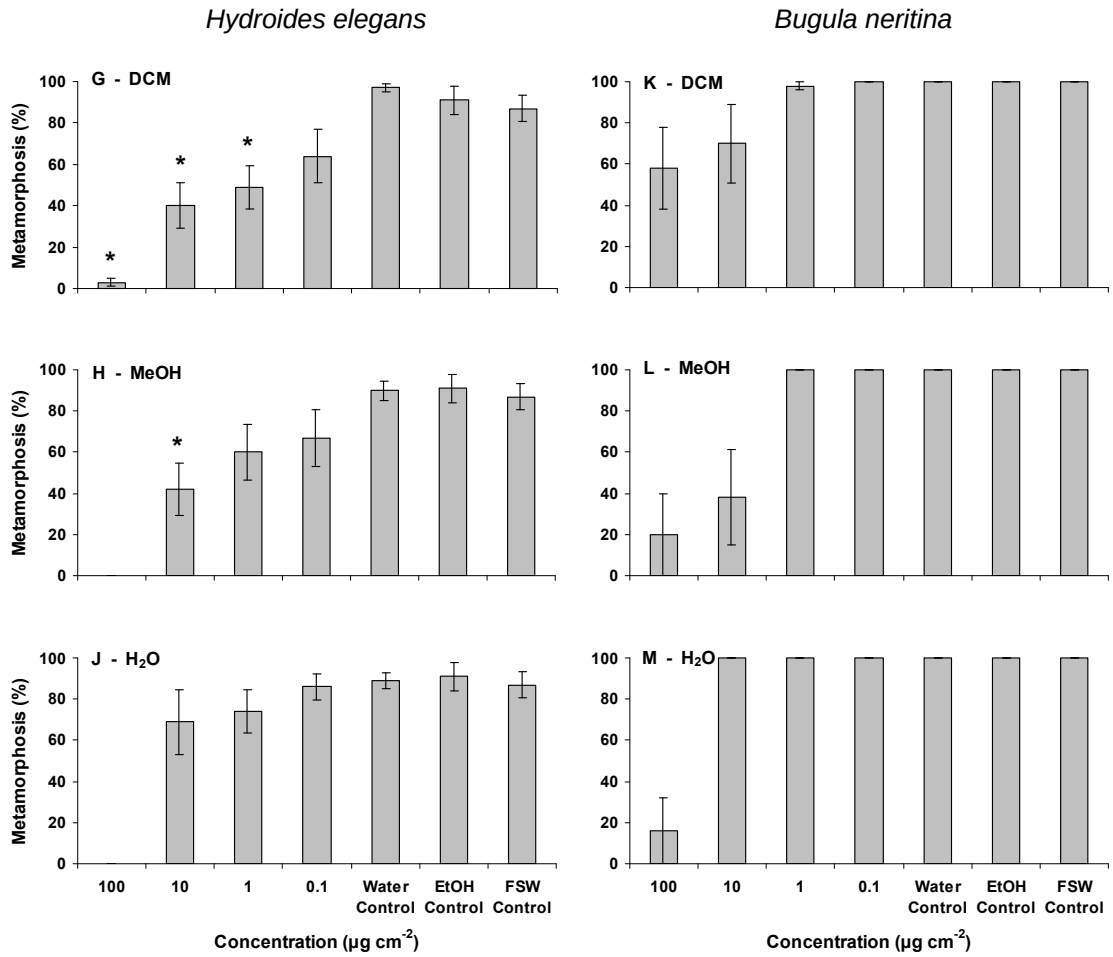

Figure 5.5 (continued) Effects of dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea star *Archaster typicus* on the metamorphosis of the fouling species **(G)-(J)** *Hydroides elegans* and **(K)-(M)** *Bugula neritina*. Means ± SE are shown (n = 5). Significant differences between the treatments and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$, experimental groups with mean metamorphosis of 0% or 100% were excluded from the statistical analyses).

5.3.3 Bioassays with fractions of the dichloromethane extract of *Cryptasterina pentagona*

The dichloromethane extract of *C. pentagona* was subjected to reversed-phase VLC to yield 12 fractions. The separation and visualization of compounds in each fraction using TLC showed that fractions 3 to 12 contained several compounds. Therefore, these fractions were selected and tested at a concentration of 10 µg cm^{-2} against the settlement of the diatom *N. closterium* as an indicator of overall activity. All fractions significantly reduced the settlement of *N. closterium* (Figure 5.6), with the fractions 5, 6, 7, 8, 9 and 12 being the most effective.

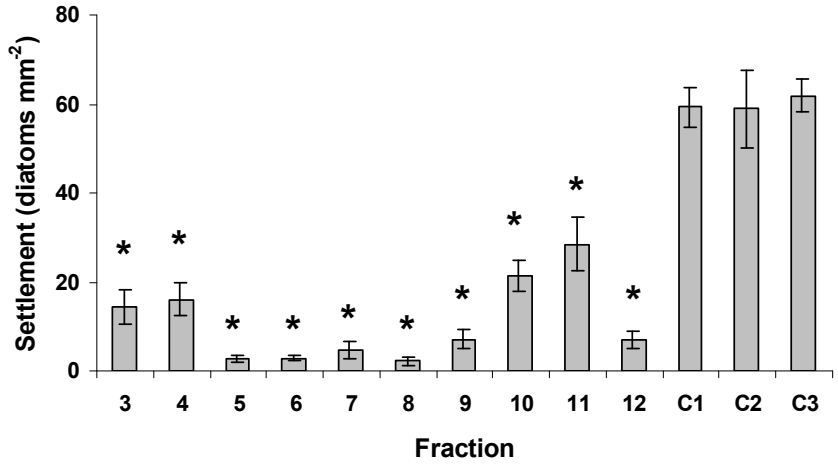

Figure 5.6 Effects of vacuum liquid column chromatography fractions of the dichloromethane extract of the sea star *Cryptasterina pentagona* at a concentration of 10 μg cm^{-2} on the settlement of the diatom *Nitzschia closterium.* C1 – DCM Control, C2 – EtOH Control, C3 – FSW Control. Means ± SE are shown (n = 5 fields of view in each of 4 dishes). Significant differences between the fractions and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

Based on the bioassays and TLC results, fractions 3, 4 and 5 (F3-5) as well as fractions 6, 7 and 8 (F6-8) of the dichloromethane extract of *C. pentagona* were combined and further fractionated using HPLC, and yielded 12 and 13 fractions, respectively (Figure 5.1). Based on the HPLC separation, selected fractions, which had dry weights of ≥2.2 mg, were tested at a concentration of 10 μg cm^{-2} against the settlement of *N. closterium*. Fractions 4+5, 8 and 10 of F3-5 and fractions 8, 12 and 13 of F6-8 significantly reduced the settlement of *N. closterium* (Figure 5.7).

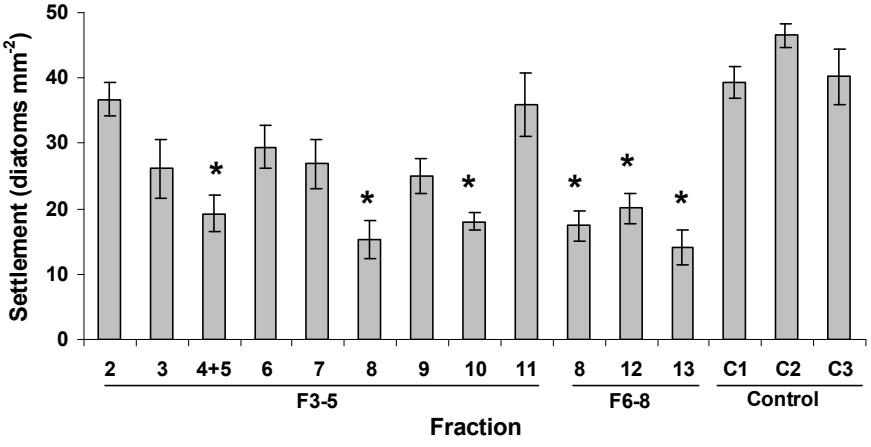

Figure 5.7 Effects of HPLC fraction of the dichloromethane extract of the sea star *Cryptasterina pentagona* at a concentration of 10 μg cm^{-2} on the settlement of the diatom *Nitzschia closterium.* C1 – DCM Control, C2 – EtOH Control, C3 – FSW Control. Means ± SE are shown (n = 5 fields of view in each of 4 dishes). Significant differences between the fractions and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

5.3.4 Nuclear magnetic resonance and gas chromatography – mass spectrometry

The ^{1}H NMR data on the active fractions of the dichloromethane extract of *C. pentagona* (Figure 5.7) showed that mixtures of fatty acids and sterols were present in all fractions. The fatty acids and sterols in each active fraction were further identified using GC-MS. A variety of long-chained unsaturated and saturated fatty acids, cholesterol and stigmasterol were identified as the most abundant compounds. The most common fatty acids and sterols in each active fraction are listed in Table 5.2. Cholesterol was present in all fractions, except for F3-5,8. Octadecanoic acid (C18:0) and hexadecanoic acid (C16:0) were present in 3 and 2 of the 6 active fractions, respectively, whereas other fatty acids only occurred in single fractions.

Table 5.2 Most common fatty acids and sterols in active fractions of the dichloromethane extract of the sea star *Cryptasterina pentagona*.

VLC fraction	HPLC fraction	Fatty acids and sterols
F3-5	4+5	C20:1
		Cholesterol
	8	C16:0
		C18:0
		C22:1
	10	C17:0
		C18:0
		C20:2
		C20:1
		Cholesterol
F6-8	8	C16:0
		C18:1
		C20:4
		Cholesterol
	12	C25:1
		Cholesterol
	13	C18:0
		Cholesterol
		Stigmasterol

5.4 Discussion

Dichloromethane (non-polar), methanol (moderately polar) and water (polar) extracts of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* all had concentration-dependant effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *N. closterium*, the serpulid *H. elegans* and the bryozoan *B. neritina*. While all extracts at the highest

concentration of 100 μg cm^{-2} significantly reduced the settlement of all fouling species, dichloromethane and methanol extracts at the lower concentrations of 10, 1 and 0.1 μg cm^{-2} were generally more effective than water extracts. In an attempt to identify potential antifouling compounds of 1 of the 4 sea star species, the sea star *C. pentagona* was chosen and analysis by NMR and GC-MS showed that bioactive fractions of the dichloromethane extract of this species contained several fatty acids and sterols, suggesting that some of these primary metabolites may be responsible for the observed antifouling effects of sea stars in their natural environment.

Previous studies on chemical antifouling defences of sea stars have focused on the antifouling role of crude extracts and isolated secondary metabolites, such as asterosaponins (Iorizzi et al. 1995, De Marino et al. 2000), but have not identified any fatty acids or sterols in crude extracts. Fatty acids and sterols are primary metabolites, which may also have a secondary function in fouling control for sea stars. Fatty acids of sponges and octocorals have previously been found to deter the settlement of fouling organisms. For example, the organic extract of the sponge *Phyllospongia papyracea* was composed of a mixture of fatty acids, of which C16:0 and C16:1 had antifouling activity against the byssus attachment of the mussel *Mytilus edulis* (Goto et al. 1992). Similarly, the organic extract of the octocoral *Dendronephthya* sp. contained a mixture of fatty acids and sterols, and was also effective against *M. edulis* (Mizobuchi et al. 1993).

Testing whole-cell crude extracts of sea stars, and in effect any other marine species, which remain relatively unfouled in their natural environments, against the settlement of ecologically relevant fouling organisms screens the widest possible range of compounds present within tissues, where synergism between compounds of an extract may be important (Teo and Ryland 1995). Furthermore, it gives a better understanding of the potential compounds responsible for the antifouling effects, providing an option to hone or select appropriate methods to move to the subsequent phase of isolating and confirming the ecologically relevant role of compounds present on the surface of the organisms. In this regard, a wide range of marine organisms have been screened and found to have potential antifouling compounds, including algae (Nylund and Pavia 2003, Bazes et al. 2006), sponges (Amsler et al. 2000, Kelly et al. 2005), bivalves (Bers et al. 2006b), soft corals (Rittschof et al. 1985, Slattery et al. 1997), ascidians (Wahl et al. 1994, Teo and Ryland 1995, Bryan et al. 2003, McClintock et al. 2004) and echinoderms (Iorizzi et al. 1995, Bryan et al. 1996, Greer et al. 2006).

However, using whole-cell crude extracts, as opposed to surface extracts, has a major disadvantage. When extracting whole tissues, natural products, which are only found within cells and would never be found on exposed surfaces under natural conditions, may also be extracted and have an inhibitory effect on fouling organisms when tested in laboratory based

settlement assays (Nylund and Pavia 2003). In contrast, the extraction, quantification and testing of surface-associated compounds is more useful in determining whether marine organisms use natural products to keep their surfaces free of fouling organisms (de Nys et al. 1998, Nylund et al. 2007). For example, crude extracts of the red algae *Chondrus crispus*, *Delesseria sanguinea*, *Osmundea ramosissima* and *Polyides rotundus* significantly inhibited the settlement of cyprid larvae of the barnacle *Balanus improvisus* (Nylund and Pavia 2003). However, in settlement preference assays with natural algal and control surfaces, only 1 species, *C. crispus*, significantly inhibited settlement of *B. improvisus*, suggesting that metabolites from inside the alga were extracted during the whole-cell extraction (Nylund and Pavia 2003). Similarly, Nylund et al. (2007) compared the effects of non-polar, whole-cell extracts of the algae *Delisea pulchra*, *Caulerpa filiformis*, *Dictyopteris acrostichoides*, *Dilophus marginatus*, *Soliera robusta* and *Pterocladia capillacea* to those of surface extracts of these 6 algal species. Whereas non-polar metabolites from the whole-cell extracts of all algal species inhibited algal settlement and germling development of *Polysiphonia* sp. and *Ulva australis*, surface extracts of only 2 of the 6 algal species, *D. pulchra* and *C. filiformis*, were inhibitory in settlement assays (Nylund et al. 2007). These 2 studies clearly demonstrate that results from settlement assays with whole-cell extracts are insufficient to predict an antifouling role of algal metabolites, and the same caution should be taken when interpreting the results from settlement assays with the whole-cell crude extracts of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* of this study.

It remains unclear how ecologically relevant the observed antifouling activity of the whole-cell crude extracts is to the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*. To further elucidate chemical antifouling defence mechanisms of these sea stars, the presence and concentration of fatty acids and sterols on the surface, and their role in deterring ecologically relevant fouling organisms, needs to be determined. The ecological relevance of surface-associated fatty acids and sterols of these sea stars will be addressed in the following Chapter.

Chapter 6

Chemical antifouling defences: Effects of surface extracts of sea stars

6.1 Introduction

Chapter 5 demonstrated that dichloromethane, methanol and water extracts of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* have concentration-dependant effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *Nitzschia closterium*, the serpulid *Hydroides elegans* and the bryozoan *Bugula neritina*, indicating that a range of potential antifouling compounds are present within the tissues of these sea stars. Further bioassays-guided fractionation of the dichloromethane extract of *C. pentagona* led to the identification of several fatty acids and sterols, which may be responsible for the observed antifouling effects.

Results from settlement assays with whole body extracts, however, are insufficient to predict an antifouling role of compounds of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* of this study. To unequivocally determine whether marine organisms use natural products to keep their surfaces free of fouling organisms, several criteria need to be fulfilled (Schmitt et al. 1995, Hay 1996, Nylund and Pavia 2003, Dworjanyn et al. 2006, Nylund et al. 2007). Firstly, the marine organisms need to be generally free of fouling organisms in their natural environment. Secondly, the presence of putative compounds on or near the surface of

the organism needs to be verified and their natural concentrations quantified. Thirdly, ecologically relevant concentrations of these compounds need to be tested in bioassays with ecologically relevant fouling species to determine whether these compounds have any antifouling effects. Most studies on the antifouling role of surface-associated compounds have concentrated on algae (de Nys et al. 1998, Maximilien et al. 1998, Dworjanyn et al. 1999, Dobretsov et al. 2006, Dworjanyn et al. 2006, Nylund et al. 2007). In these studies, non-polar surface-associated compounds were extracted by dipping the algae in hexane or mixtures of hexane and dichloromethane, and compounds were then quantified using GC-MS (de Nys et al. 1998, Nylund et al. 2007).

However, the 'dipping' technique is not appropriate for extracting surface-associated compounds of sea stars, because sea stars lack the protective cell walls of algae, and cells would most likely lyse during direct contact with organic solvents. Whereas cell damage of algae, caused by direct contact with organic solvents, can be quantified using fluorescence microscopy, techniques still have to be developed to be able to quantify the cell damage of animal tissues. Currently, an alternative to the 'dipping' technique is the 'swabbing' technique used by Schmitt et al. (1995) with the brown alga *Dictyota menstrualis* and by Kubanek et al. (2002) with the sponges *Erylus formosus* and *Ectyoplasia ferox*, whereby cotton wool or gauze pads were used to swab the surface and subsequently extracted with organic solvents.

The aims for this Chapter were to identify the surface-associated compounds responsible for the observed antifouling effects of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, by verifying the presence of active compounds on the surface of these sea stars, quantifying their natural concentrations and testing these compounds in settlement assays at ecologically relevant concentrations and with ecologically relevant fouling species.

6.2 Materials and Methods

6.2.1 Collection of specimens

Live specimens of the sea stars *L. laevigata* and *F. indica* were collected from John Brewer Reef (18°38'S, 147°03'E), whereas *C. pentagona* and *A. typicus* were collected from Kissing Point in Townsville (19°14'S, 146°48'E) and Picnic Bay on Magnetic Island (19°11'S, 146°51'E), respectively. Specimens were transported in ambient seawater and then kept

temporarily in an outdoor recirculating tank (temperature: 26°C, salinity: 35 ppt) at the Marine and Aquaculture Research Facilities Unit at James Cook University.

6.2.2 Bioassays with conditioned seawater

To test the antifouling activity of released (or water-borne) compounds of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* against ecologically relevant fouling organisms in laboratory based settlement assays, specimens of each species (n = 5) were kept individually in plastic containers (*L. laevigata*) or glass beakers (*F. indica*, *C. pentagona* and *A. typicus*) with 0.45 µm filtered seawater and aeration for 24 h. The amount of filtered seawater was based on their individual wet weights, with 75 ml of filtered seawater per 1 g of sea star being added to each container or beaker. Seawater controls were prepared by keeping filtered seawater in plastic containers and glass beakers for the same amount of time. The conditioned seawater was used to run bioassays with the diatoms *Amphora* sp. (CS-255, CSIRO) and *N. closterium* (CS-5, CSIRO) and the bryozoan *B. neritina*. Fouling organisms were cultured and bioassays were performed as described in Chapter 5, Section 5.2.6. Briefly, to perform settlement assays with *Amphora* sp. and *N. closterium*, 4 ml of conditioned seawater and 100 µl of a 2.5×10^5 algal cells ml^{-1} solution of either *Amphora* sp. or *N. closterium* were added to clear polystyrene Petri dishes (Sarstedt, diameter: 3.5 cm, surface area: 9 cm^2) (n = 5), which were then kept in a temperature and light controlled room for 4 h. Similarly, to perform settlement assays with *B. neritina*, 4 ml of conditioned seawater and 20 larvae were added to each Petri dish (n = 5), which were then kept in a temperature controlled room in the dark for 24 h.

6.2.3 Bioassays with surface-associated compounds

To test the antifouling activity of surface-associated compounds of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* against ecologically relevant fouling organisms in laboratory based settlement assays, surface-associated compounds of each specimen were absorbed using mixed cellulose ester filter paper (Advantec MFS). Filter papers were cut to fit into the clear polystyrene Petri dishes (diameter: 3 cm) and placed on the central aboral surface of each sea star to absorb the surface-associated compounds. Subsequently, these filter papers were placed in Petri dishes, 4 ml of 0.45 µm filtered seawater were added, and settlement assays with the diatoms *Amphora* sp. (CS-255, CSIRO) and *N. closterium* (CS-5, CSIRO), the serpulid *H. elegans* and the bryozoan *B. neritina* were run. Fouling organisms were cultured and bioassays were run as described in Chapter 5, Section 5.2.6, however attached diatoms were counted using a Leica DMLB fluorescence microscope, rather than a compound microscope.

6.2.4 Preparation of surface extracts

To identify surface-associated compounds of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, surface-associated compounds of each specimen (n = 3) were absorbed using cotton wool, which had been pre-washed twice with dichloromethane and twice with methanol. Small cotton balls were used to swab a projected surface area of 4 cm^2 on the aboral surface of each specimen. Each cotton ball with absorbed surface-associated compounds was then extracted with dichloromethane. Cotton ball controls were prepared by extracting the pre-washed cotton wool with the same amount of dichloromethane used for the extraction of surface-associated compounds. The extracts were air-dried and stored at -18°C until further processing for GC-MS analysis.

6.2.5 Analysis with gas chromatography – mass spectrometry

To prepare the surface extracts of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* for GC-MS analysis, the surface extracts were derivatized with a trimethyl silane (TMS) reagent, to ensure the crude extracts of *C. pentagona* that contained mainly fatty acids and sterols (Chapter 5, Section 5.3.4), of potentially low volatility, would be in the most suitable analysable form. Derivatizing extracts with a TMS reagent converts all compounds with OH functionality, including the fatty acids and sterols, to TMS derivatives and facilitates better peak resolution on the GC-MS due to the derivatives having increased volatility. The dry surface extracts were derivatized with 50 µl of bis(trimethylsilyl)trifluoro-acetamide (BSTFA, Sigma-Aldrich), incubated at 60°C for 2 h and dried under nitrogen. Subsequently, 100 µl of hexane were added and transferred to the GC-MS vials. The same GC-MS conditions were used as for the crude extract samples (Chapter 5, Section 5.2.5). A series of fatty acid methyl esters and sterol standards of known concentrations were also run. Compounds were identified based on retention time match with standards, and matching mass spectra also with standards. Natural concentrations of selected fatty acids and sterols in each sample were calculated by measuring peak areas for each compound and corresponding standard. The ratio of peak areas (compound/standard) was calculated for each compound and converted to concentration (ng μl^{-1}) by reference to standard curves. Surface concentrations of compounds on each sea star (ng cm^{-2}) were calculated, taking into consideration the swabbed surface area (4 cm^2).

6.2.6 Bioassays with hexadecanoic acid, cholesterol, lathosterol and sitosterol

Based on the chromatographic analyses of the surface extracts of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, hexadecanoic acid, cholesterol, lathosterol and sitosterol were selected for further testing against the settlement of fouling species. Hexadecanoic acid (Catalogue number: 76119), cholesterol (C8667), lathosterol (C3652) and sitosterol (C1270) were purchased from Sigma-Aldrich. These compounds as well as a mixture of the 4 compounds (1:1:1:1) were tested against *Amphora* sp., *N. closterium* and *B. neritina* at the concentrations of 1000, 100, 10 and 1 ng cm^{-2}, which includes the ecologically relevant concentrations of each compound (Table 6.1). Fouling organisms were cultured and bioassays were performed as described in Chapter 5, Section 5.2.6.

6.2.7 Statistical analysis

All statistical analyses were performed with SPSS version 14. The effects of conditioned seawater of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* on the settlement of the diatoms *Amphora* sp. and *N. closterium* were analysed with a 2-factor nested ANOVA (factors: (1) treatment – 4 different sea star species and controls, (2) dish nested in treatment – 5 replicate dishes for each treatment). The effects of conditioned seawater on the bryozoan *B. neritina* were analysed with a 1-factor ANOVA (factor: (1) treatment – 5 replicate dishes for each treatment).

To determine significant differences in the settlement of the diatoms *Amphora* sp. and *N. closterium* between filter papers with surface-associated compounds and controls, the results were analysed with a 2-factor nested ANOVA (factors: (1) treatment – filter paper with compounds and controls, (2) dish nested in treatment – 6 replicate dishes for each treatment) followed by Tukey's HSD multiple comparison tests. Results on the metamorphosis of the serpulid *H. elegans* and the bryozoan *B. neritina* were analysed with a 1-factor ANOVA (factor: (1) treatment – filter paper with extract and controls with 10 replicate dishes for *H. elegans* and 6 replicate dishes for *B. neritina*) followed by Tukey's HSD multiple comparison tests.

Similarly, the effects of hexadecanoic acid, cholesterol, lathosterol and sitosterol were analysed with a 2-factor nested ANOVA (factors: (1) treatment – 4 different concentrations of a compound and controls, (2) dish nested in treatment – 5 replicate dishes for each treatment) for *Amphora* sp. and *N. closterium* and a 1-factor ANOVA (factor: (1) treatment – 4 different concentrations of a compound and controls) for *B. neritina* followed by Tukey's HSD multiple comparison tests. For all statistical analyses, the assumptions of homogeneity and normality of the data were checked with residuals versus predicted values plots and Q-Q plots of residuals, respectively. If necessary, data were square root transformed to meet these assumptions (Underwood 1981, Quinn and Keough 2002).

6.3 Results

6.3.1 Bioassays with conditioned seawater

Conditioned seawater of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* was used to run settlement assays with the diatoms *Amphora* sp. and *N. closterium* and the bryozoan *B. neritina* to determine whether the sea stars release compounds into the water column to deter the settlement of fouling organisms. The conditioned seawater of all 4 sea star species did not have any significant effects on the settlement of *Amphora* sp. ($p = 0.324$), *N. closterium* ($p = 0.500$) and *B. neritina* ($F_{5,24} = 1.058$, $p = 0.407$) (Figure 6.1).

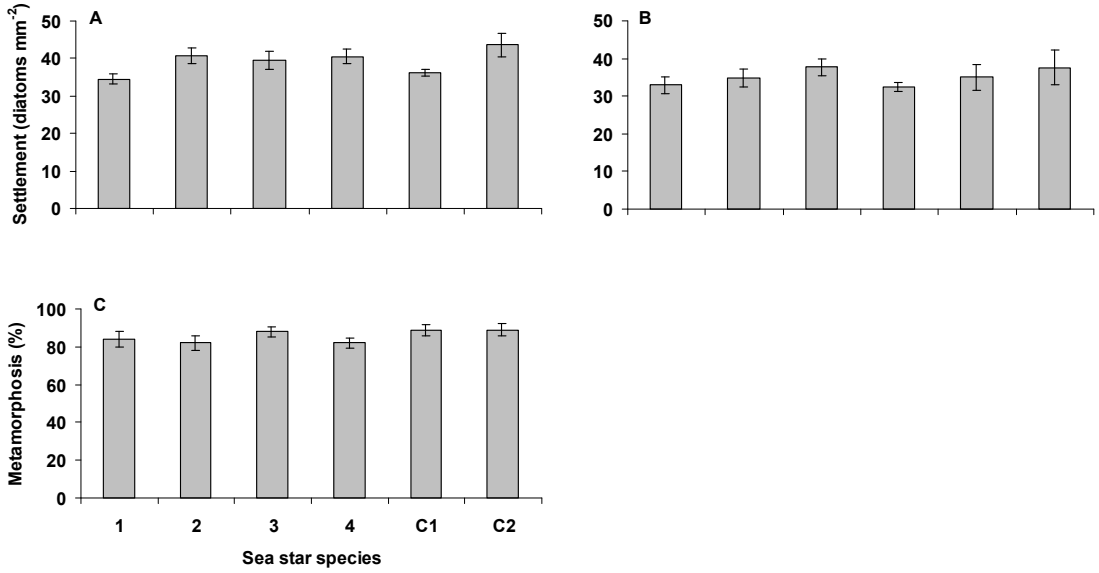

Figure 6.1 Effects of conditioned seawater of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* on the settlement (diatoms mm^{-2}) of **(A)** *Amphora* sp. and **(B)** *Nitzschia closterium* and the metamorphosis (%) of **(C)** *Bugula neritina.* 1 – *L. laevigata*, 2 – *F. indica*, 3 – *C. pentagona*, 4 – *A. typicus*, C1 – Control for *L. laevigata*, C2 – Control for *F. indica*, *C. pentagona* and *A. typicus*. Means ± SE are shown (n = 5 fields of view in each of 5 dishes for *Amphora* sp. and *N. closterium*; n = 5 for *B. neritina*).

6.3.2 Bioassays with surface-associated compounds

The surface-associated compounds of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were absorbed onto mixed cellulose filter papers, which were then used to run settlement assays with the diatoms *Amphora* sp. and *N. closterium*, the serpulid *H. elegans* and the bryozoan *B. neritina*. Overall, the surface-associated compounds of the sea stars *L.*

laevigata, *F. indica*, *C. pentagona* and *A. typicus* had species-specific effects on the settlement of *Amphora* sp., *N. closterium*, *H. elegans* and *B. neritina*. While the surface-associated compounds of the sea star *L. laevigata* significantly reduced the settlement of *N. closterium* by 42% to only 30.8 ± 3.7 diatoms mm^{-2} (Figure 6.2B), they had no significant effects on the settlement of *Amphora* sp., *H. elegans* and *B. neritina* (Figure 6.2A, C, D). The surface-associated compounds of the sea stars *F. indica* and *A. typicus* significantly reduced the settlement of both *Amphora* sp. and *N. closterium*, but not *H. elegans* and *B. neritina* (Figure 6.3 and 6.5). Surface-associated compounds of *F. indica* reduced the settlement of *Amphora* sp. and *N. closterium* by 24% (Figure 6.3A) and 58% (Figure 6.3B), respectively, whereas those of *A. typicus* reduced them by 23% (Figure 6.5A) and 31% (Figure 6.5B), respectively. In contrast, the surface-associated compounds of the sea star *C. pentagona* were the most effective compounds of the 4 sea star species, significantly reducing the settlement of 3 fouling species, *Amphora* sp., *N. closterium* and *H. elegans* by 25% (Figure 6.4A), 33% (Figure 6.4B) and 38% (Figure 6.4C), respectively. However, there was no significant difference between the settlement of *B. neritina* on filter papers with surface-associated compounds of *C. pentagona* and control surfaces (Figure 6.4D).

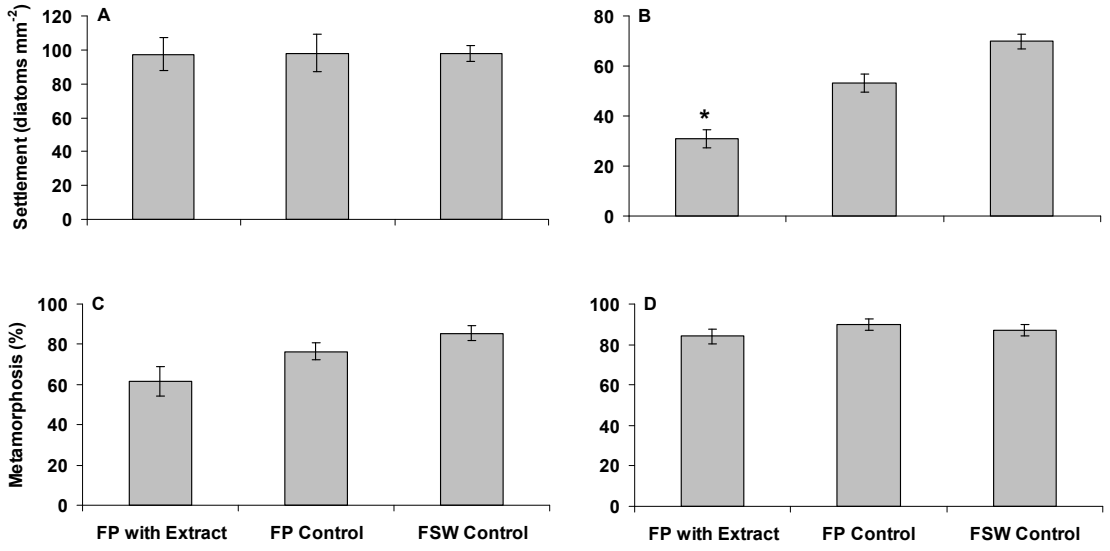

Figure 6.2 Effects of surface-associated compounds of the sea star *Linckia laevigata* on the settlement (diatoms mm^{-2}) of **(A)** *Amphora* sp. and **(B)** *Nitzschia closterium* and the metamorphosis (%) of **(C)** *Hydroides elegans* and **(D)** *Bugula neritina.* FP – filter paper, FSW – filtered seawater. Means ± SE are shown (n = 6 for *Amphora* sp., *N. closterium* and *B. neritina*; n = 10 for *H. elegans*). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

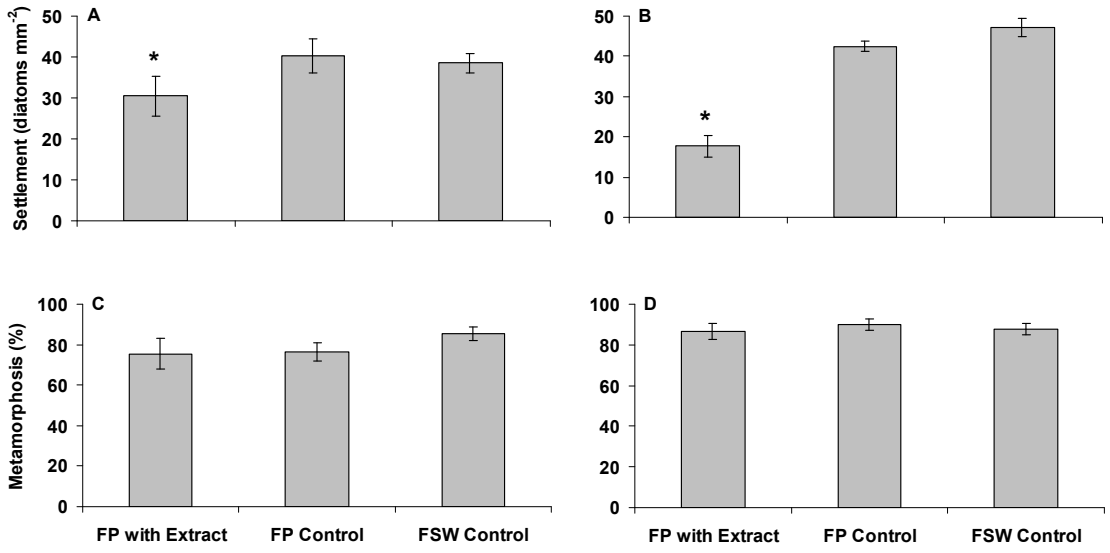

Figure 6.3 Effects of surface-associated compounds of the sea star *Fromia indica* on the settlement (diatoms mm^{-2}) of **(A)** *Amphora* sp. and **(B)** *Nitzschia closterium* and the metamorphosis (%) of **(C)** *Hydroides elegans* and **(D)** *Bugula neritina.* FP – filter paper, FSW – filtered seawater. Means ± SE are shown (n = 6 for *Amphora* sp., *N. closterium* and *B. neritina*; n = 10 for *H. elegans*). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

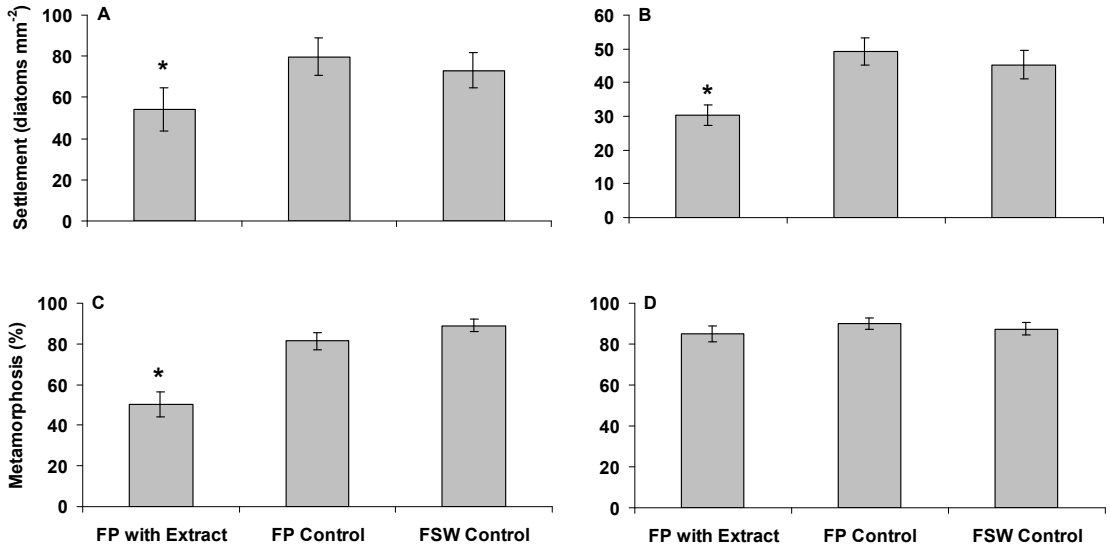

Figure 6.4 Effects of surface-associated compounds of the sea star *Cryptasterina pentagona* on the settlement (diatoms mm^{-2}) of **(A)** *Amphora* sp. and **(B)** *Nitzschia closterium* and the metamorphosis (%) of **(C)** *Hydroides elegans* and **(D)** *Bugula neritina.* FP – filter paper, FSW – filtered seawater. Means ± SE are shown (n = 6 for *Amphora* sp., *N. closterium* and *B. neritina*; n = 10 for *H. elegans*). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

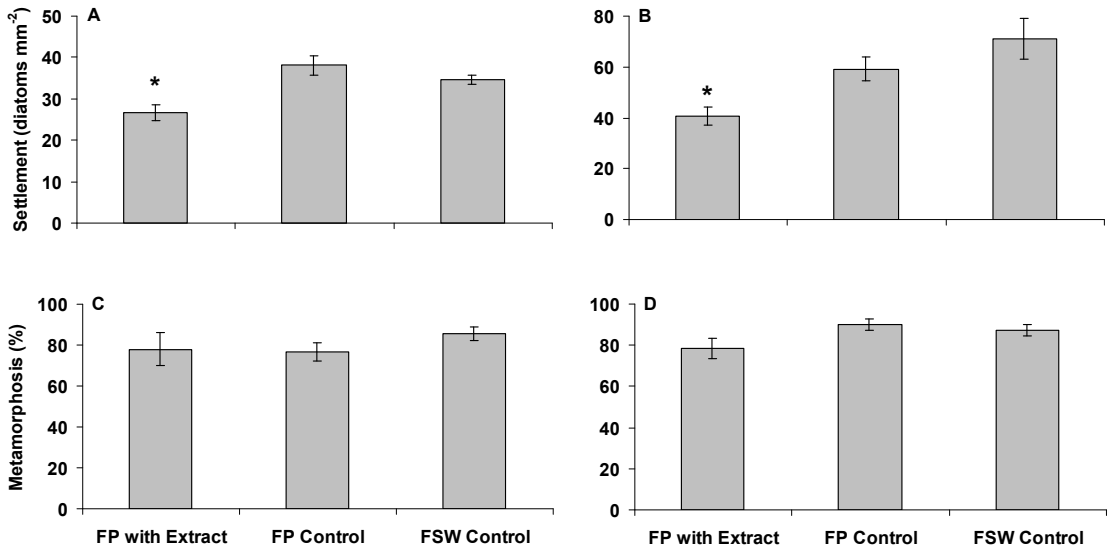

Figure 6.5 Effects of surface-associated compounds of the sea star *Archaster typicus* on the settlement (diatoms mm^{-2}) of **(A)** *Amphora* sp. and **(B)** *Nitzschia closterium* and the metamorphosis (%) of **(C)** *Hydroides elegans* and **(D)** *Bugula neritina.* FP – filter paper, FSW – filtered seawater. Means ± SE are shown (n = 6 for *Amphora* sp., *N. closterium* and *B. neritina*; n = 10 for *H. elegans*). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

6.3.3 Analysis with gas chromatography – mass spectrometry

The compounds of the surface extracts of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were identified employing GC-MS. Based on their high abundance, the fatty acid hexadecanoic acid and the sterols cholesterol, lathosterol and sitosterol (Figure 6.6) were selected and their natural concentrations on the aboral surface of each sea star species were quantified. While hexadecanoic acid and lathosterol were present on all 4 sea star species, cholesterol was present on only 3 sea star species (*L. laevigata*, *C. pentagona* and *A. typicus*) and sitosterol on only 2 species (*L. laevigata* and *F. indica*) (Table 6.1). The mean natural concentrations of hexadecanoic acid, cholesterol, lathosterol and sitosterol, as determined from the extraction of cotton balls used to swab a designated surface area on each sea star, ranged between approximately 2 and 286 ng cm^{-2}. While the surface concentration of sitosterol was low on both *L. laevigata* and *F. indica*, the concentrations of hexadecanoic acid, cholesterol and lathosterol were rather variable between sea stars species. In particular, lathosterol had a mean surface concentration of only 5.0 ng cm^{-2} on *C. pentagona*, but a concentration of 285.7 ng cm^{-2} on *L. laevigata* (Table 6.1).

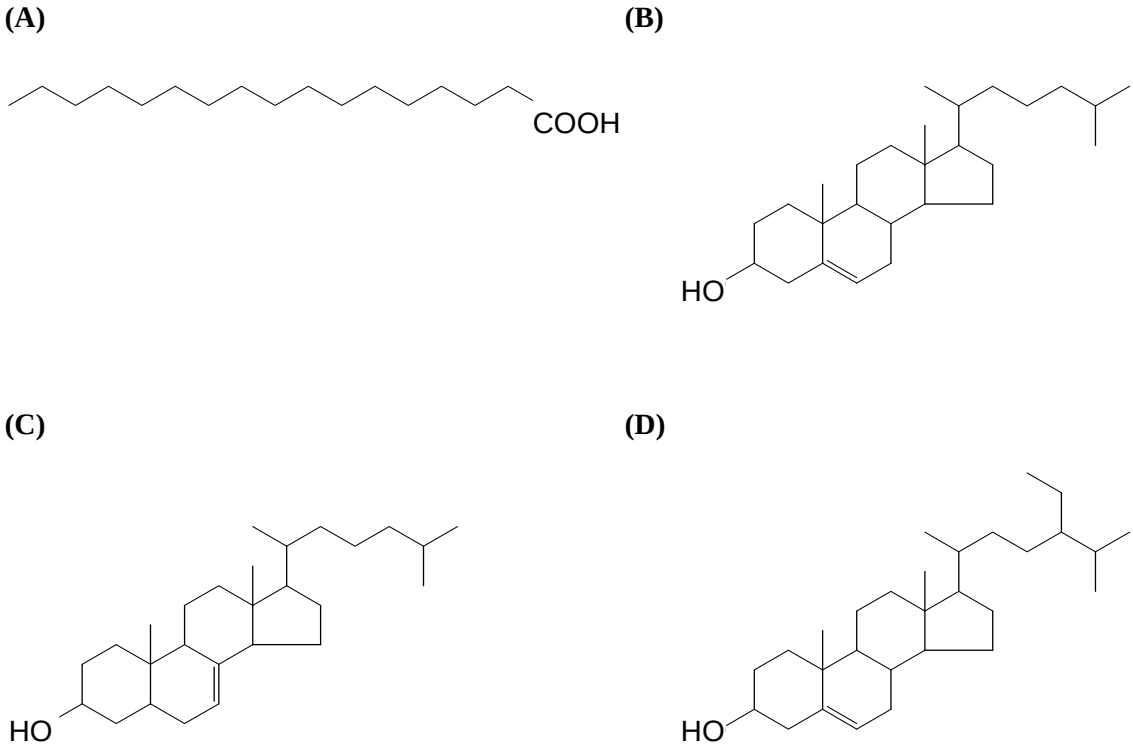

Figure 6.6 Chemical structures of **(A)** hexadecanoic acid, **(B)** cholesterol, **(C)** lathosterol and **(D)** sitosterol.

Table 6.1 Natural concentrations (ng cm^{-2}) of the surface-associated compounds hexadecanoic acid, cholesterol, lathosterol and sitosterol on the aboral surface of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus*. Means ± SE are shown (n = 3).

	Hexadecanoic acid	**Cholesterol**	**Lathosterol**	**Sitosterol**
Linckia laevigata	26.7 ± 16.3	65.2 ± 57.1	285.7 ± 140.9	3.6 ± 3.6
Fromia indica	34.7 ± 10.9	0.0 ± 0.0	137.5 ± 85.4	4.7 ± 2.9
Cryptasterina pentagona	42.3 ± 24.0	2.2 ± 1.1	5.0 ± 0.7	0.0 ± 0.0
Archaster typicus	6.4 ± 4.9	5.4 ± 1.7	7.5 ± 2.2	0.0 ± 0.0

6.3.4 Bioassays with hexadecanoic acid, cholesterol, lathosterol and sitosterol

To determine whether the surface-associated hexadecanoic acid, cholesterol, lathosterol and sitosterol are active against the settlement of fouling organisms, these pure compounds as well as a mixture of these 4 compounds (1:1:1:1) were tested against *Amphora* sp., *N. closterium* and

B. neritina at the ecologically relevant concentration range of 1000, 100, 10 and 1 ng cm^{-2}. Overall, hexadecanoic acid and cholesterol were the most effective compounds, significantly reducing the settlement of both *Amphora* sp. and *N. closterium* at concentrations of 1000 and ≥10 ng cm^{-2}, respectively (Figure 6.7A and B and Figure 6.8A and B). Even at the lowest concentration of 10 ng cm^{-2}, hexadecanoic acid and cholesterol reduced the settlement of *N. closterium* by 31% and 34%, respectively (Figure 6.8A and B). Lathosterol also significantly inhibited the settlement of the diatom species at concentrations of 1000 and ≥100 ng cm^{-2} for *Amphora* sp. and *N. closterium*, respectively (Figure 6.7C and Figure 6.8C). At a concentration of 1000 ng cm^{-2}, lathosterol significantly reduced the settlement of *Amphora* sp. by 44%; whereas the settlement of *N. closterium* was reduced by 81% to only 16.3 ± 2.6 diatoms mm^{-2}. In contrast to the 3 other compounds, sitosterol was only effective against *N. closterium*, with significantly less settlement of this species at concentrations of ≥100 ng cm^{-2} (Figure 6.8D). At concentrations of 1000 ng cm^{-2} and 100 ng cm^{-2}, the settlement of *N. closterium* was reduced by 29% and 35%, respectively. None of the 4 compounds had any effects on the settlement of *B. neritina* (Figure 6.9).

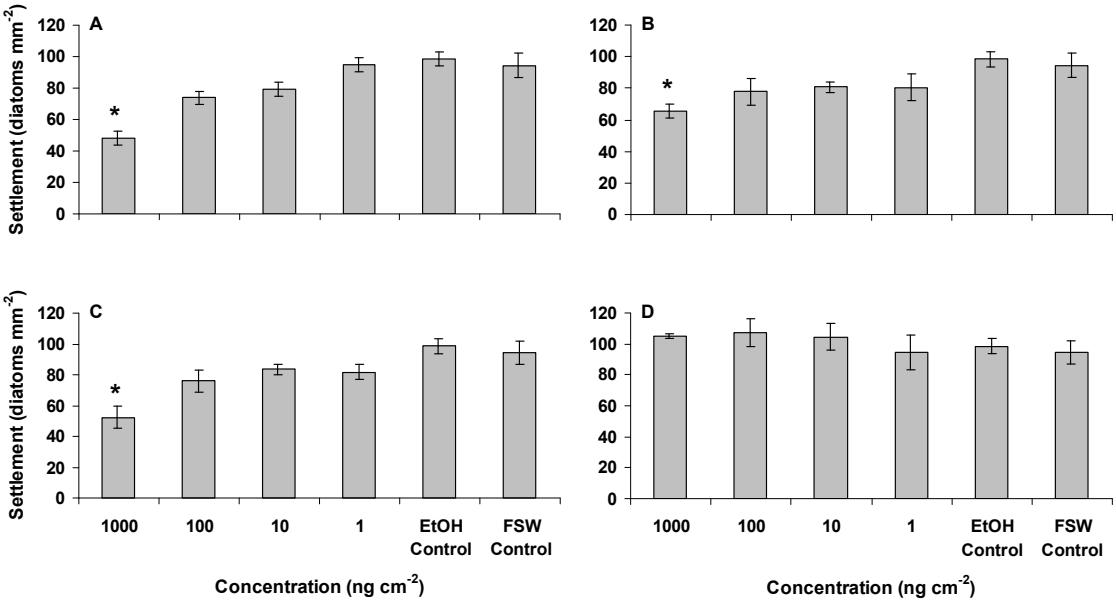

Figure 6.7 Effects of **(A)** hexadecanoic acid, **(B)** cholesterol, **(C)** lathosterol and **(D)** sitosterol on the settlement of *Amphora* sp. (diatoms mm^{-2}). EtOH – ethanol, FSW – filtered seawater. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

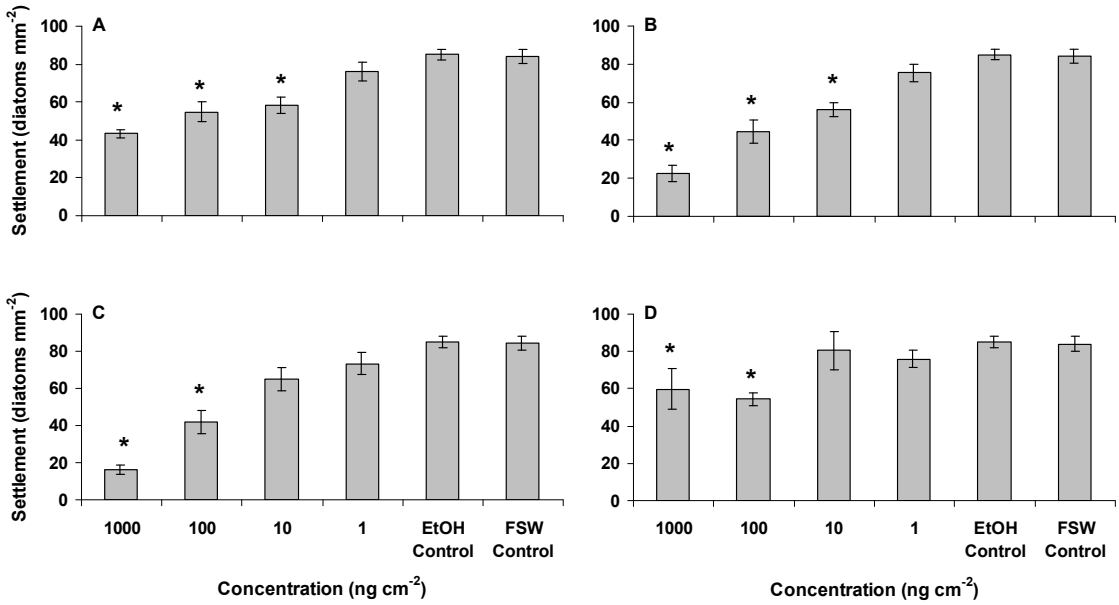

Figure 6.8 Effects of **(A)** hexadecanoic acid, **(B)** cholesterol, **(C)** lathosterol and **(D)** sitosterol on the settlement of *Nitzschia closterium* (diatoms mm^{-2}). EtOH – ethanol, FSW – filtered seawater. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

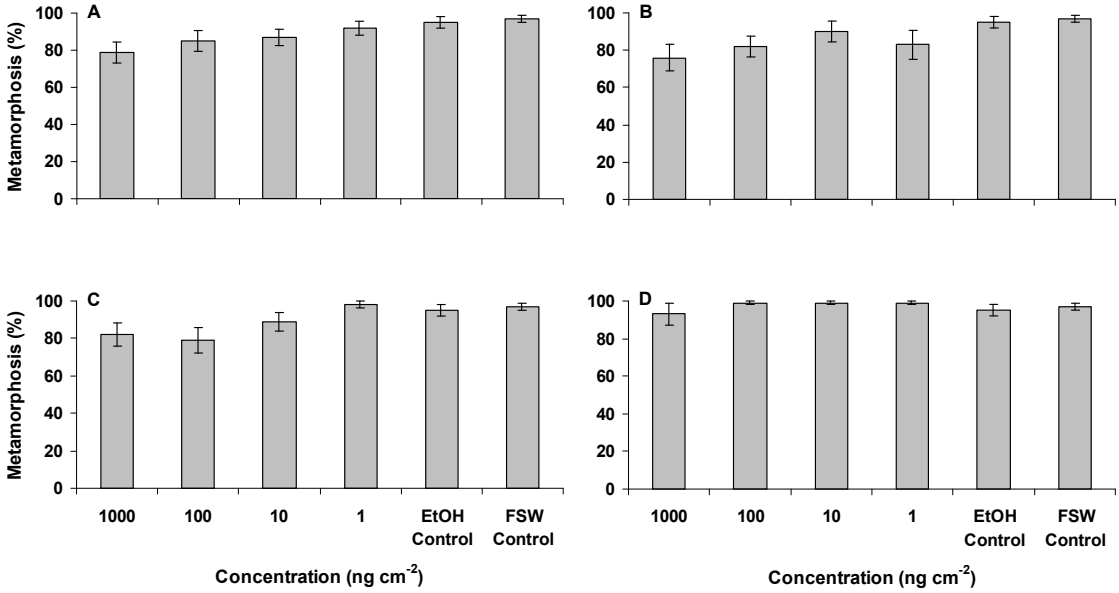

Figure 6.9 Effects of **(A)** hexadecanoic acid, **(B)** cholesterol, **(C)** lathosterol and **(D)** sitosterol on the metamorphosis of *Bugula neritina* (%). EtOH – ethanol, FSW – filtered seawater. Means ± SE are shown (n = 5). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

The mixture of hexadecanoic acid, cholesterol, lathosterol and sitosterol (1:1:1:1) significantly reduced the settlement of only *N. closterium* at concentrations of ≥100 ng cm^{-2} (Figure 6.10). At a concentration of 1000 ng cm^{-2}, the settlement of *N. closterium* was reduced by 77% to only 15.8 ± 4.9 diatoms mm^{-2}; whereas at a concentration of 100 ng cm^{-2}, the settlement was reduced by 61% to 27.4 ± 8.3 diatoms mm^{-2} (Figure 6.10B).

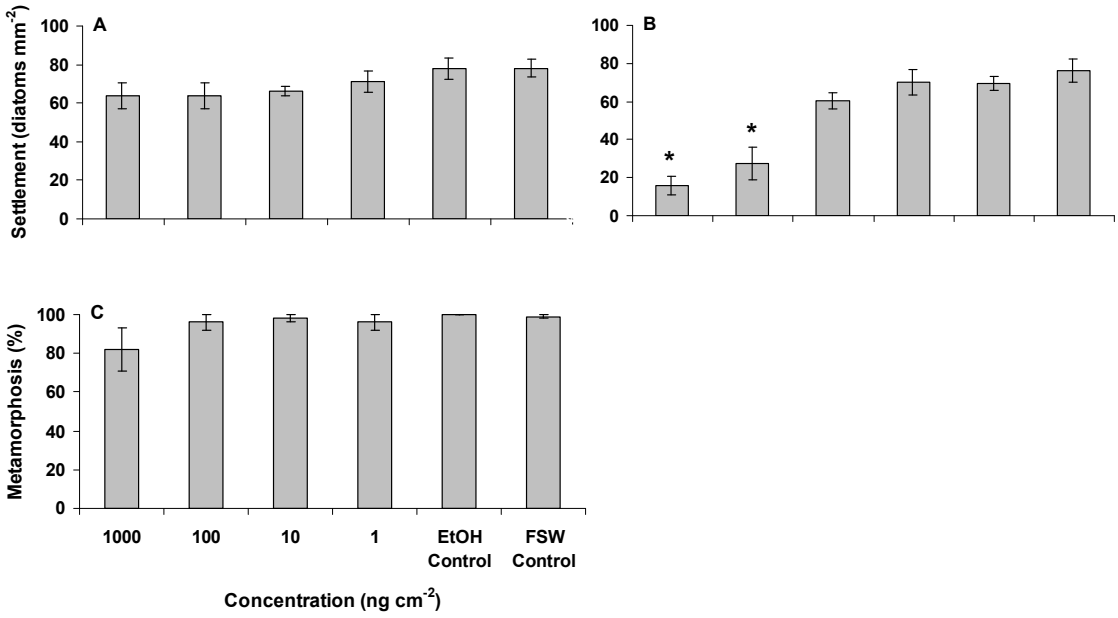

Figure 6.10 Effects of a mixture of hexadecanoic acid, cholesterol, lathosterol and sitosterol (1:1:1:1) on the settlement (diatoms mm^{-2}) of **(A)** *Amphora* sp., **(B)** *Nitzschia closterium* and the metamorphosis (%) of **(C)** *Bugula neritina*. EtOH – ethanol, FSW – filtered seawater. Means ± SE are shown (n = 5 fields of view in each of 5 dishes for *Amphora* sp. and *N. closterium*; n = 5 for *B. neritina*). Significant differences between the treatment and controls are indicated by * (Tukey's HSD multiple comparison test, $p < 0.05$).

6.4 Discussion

The conditioned seawater of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* had no effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *N. closterium* and the bryozoan *B. neritina*, demonstrating that these sea stars are unlikely to release antifouling compounds into the water column in sufficient quantities to deter fouling. In contrast, the surface-associated compounds of these sea stars had species-specific negative effects on the settlement of the diatoms *Amphora* sp. and *N. closterium* and the serpulid *H. elegans*, but not the bryozoan *B. neritina*. Subsequently, some surface-associated compounds

were identified, quantified and also tested at ecologically relevant concentrations. The surface-associated compounds hexadecanoic acid, cholesterol and lathosterol deterred the settlement of the diatoms *Amphora* sp. and *N. closterium*, but not the bryozoan *B. neritina*. Sitosterol also deterred the settlement of *N. closterium* at concentrations of ≥100 ng cm^{-2}, however these concentrations are higher than those found on the sea stars *L. laevigata* and *F. indica* and therefore not ecologically relevant. As non-polar compounds, hexadecanoic acid, cholesterol, lathosterol and sitosterol adhere to the surface and do not readily dissolve into the surrounding water.

Based on the results of this study, the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* are primary metabolites with a secondary function in fouling control. This finding is novel for sea stars, because previous studies have focused on the antifouling role of secondary metabolites, such as asterosaponins (Iorizzi et al. 1995, De Marino et al. 2000). As primary metabolites, fatty acids and sterols are a source of energy and are structural components of cell membranes that influence membrane properties, such as permeability (Papahadjopoulos et al. 1973, Voogt et al. 1993, Brown 2000). Previous studies on secondary functions of fatty acids and sterols have concentrated on sponges and octocorals. For example, the organic extract of the sponge *Phyllospongia papyracea* was found to be composed of a mixture of free fatty acids, including C16:0, C16:1 and C18:1 (Goto et al. 1992). Authentic free fatty acids were tested against the byssus attachment of the mussel *Mytilus edulis*, showing that C16:0 and C16:1 fatty acids had antifouling activities (Goto et al. 1992). Similarly, the organic extract of the octocoral *Dendronephthya* sp., containing a mixture of fatty acids (mainly C16 and C18) and sterols, was also effective against *M. edulis* (Mizobuchi et al. 1993). Furthermore, in settlement assays with crude extracts of the sponge *Crella incrustans* and cholesterol controls against larvae of the ascidian *Podoclavella moluccensis*, cholesterol controls also had a negative effect on the settlement of ascidian larvae (Davies et al. 1991). Finally, Lee et al. (2007) suggested that the species-specific, surface-associated bacterial communities and antifouling activities of the congeneric sponges *Mycale adhaerens* from Hong Kong and *M. laxissima* from the Bahamas might result from the differences in the fatty acid profiles of these sponges. *M. adhaerens* has a more diverse fatty acid profile, in terms of the type and relative abundance of fatty acids detected, than *M. laxissima* (Lee et al. 2007). Apart from antifouling activities, sterols may also deter predators. For example, the Antarctic soft coral *Alcyonium paessleri* produces, amongst other sterols, cholesterol, which is released into the surrounding water column and acts as a deterrent against predation by the sea star *Odontaster validus* (Slattery et al. 1997).

A mixture of hexadecanoic acid, cholesterol, lathosterol and sitosterol at the same concentrations were also tested against the settlement of the diatoms *Amphora* sp. and *N. closterium* and the bryozoan *B. neritina*. The mixture of compounds was only effective against the diatom *N. closterium* at concentrations of ≥100 ng cm^{-2}. Therefore, synergistic effects of the 4 compounds, i.e. stronger activity of the mixture compared to separate compounds, were not observed in any settlement assays with these fouling species at the ratio tested (1:1:1:1). Future experiments with mixtures of hexadecanoic acid, cholesterol lathosterol and sitosterol at ratios found on the surface of individual sea star species may provide a better understanding of the effects of natural surface concentrations of fatty acids and sterols on the settlement of fouling organisms.

The natural concentrations of surface-associated compounds of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were determined using the 'swabbing' technique. This technique may have compromised the accurate quantification of the natural concentrations of the surface-associated compounds. Firstly, swabbing the surface with cotton wool may have disrupted some cells and compounds from inside those cells may have been absorbed onto the cotton wool as well. Secondly, not all surface-associated compounds may have been absorbed, as Schmitt et al. (1995) noted that lipophilic compounds did not absorb well onto cotton wool. Thirdly, some surface-associated compounds may have been lost during the absorption and extraction process, followed by the preparation of samples for further analysis with GC-MS. These factors may explain some of the variation of the natural concentrations of hexadecanoic acid, cholesterol, lathosterol and sitosterol on *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*. Due to these compromising factors, a range of concentrations of pure hexadecanoic acid, cholesterol, lathosterol and sitosterol (1000, 100, 10 and 1 ng cm^{-2}) were tested, which included concentrations higher and lower than the mean natural concentrations found in this study. While the 'swabbing' technique was not optimal, it was suitable for this study, because it allowed the extraction of surface-associated compounds from animal tissues, while the extraction of compounds from within the cells was minimized. For future experiments, the 'dipping' technique (de Nys et al. 1998, Nylund et al. 2007), currently only used to extract surface-associated compounds on algae, may be adapted to make it suitable for the extraction of surface-associated compounds of animal tissues.

While the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol have antifouling activities against diatoms, it remains unclear whether these surface-associated compounds are produced by the sea stars or whether they are produced by surface-associated microorganisms. As demonstrated and discussed in Chapter 2, tropical sea stars also have bacteria living on their surfaces, with bacterial abundances being approximately 10^4 to 10^5 cells

cm^{-2}. These bacteria may have a symbiotic role, releasing compounds, such as fatty acids and sterols, that help reduce or prevent the settlement of other fouling organisms. For example, bacterial communities on both the alga *U. reticulata* and the sponge *M. adhaerens* inhibit the settlement of the serpulid *H. elegans* (Dobretsov and Qian 2002, Lee et al. 2006). The isolation and culture of surface-associated microorganisms of sea stars and subsequent identification and testing of their natural products will offer an opportunity to determine whether similar mechanisms are present in sea stars.

Chapter 7

Synthesis and discussion

Natural antifouling mechanisms are highly complex, with many marine species employing behavioural, mechanical, physical or chemical defence mechanisms, either singularly or in combination, to deter the settlement of the wide range of fouling organisms (Targett et al. 1983, Vrolijk et al. 1990, Wahl and Banaigs 1991, Baum et al. 2001, Baum et al. 2002, Bers et al. 2006a,b). While the antifouling defences of sea stars are generally poorly understood, they stand out in the marine environment with little or no fouling. Previous studies on antifouling defence mechanisms of sea stars have focused on potential chemical defences, testing crude extracts or isolated compounds of a range of sea star species against micro- and macrofouling organisms (Iorizzi et al. 1995, Bryan et al. 1996, De Marino et al. 2000, Greer et al. 2003, Iken et al. 2003, Greer et al. 2006). However, mechanical and physical antifouling defences remain unexplored and the ecological relevance of chemical antifouling defences has not been determined. Therefore, the results of this study contribute to an improved understanding of the antifouling defence mechanisms of the tropical sea stars *Acanthaster planci*, *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* and provide a number of novel findings in elucidating their mechanical, physical and chemical defences against fouling organisms. Furthermore, they contribute more broadly to the field of biofouling, exploring natural antifouling defence mechanisms.

The major findings of this study are:

1. Fouling-resistant surfaces (Chapter 2). Tropical sea star species in North Queensland, Australia, have low bacterial cover, and there are no common generalist macrofouling organisms on their epidermal surfaces.
2. Mechanical antifouling defences (Chapter 3). The elementary pedicellariae of the sea star *A. planci* do not effectively deter the settlement of the ecologically relevant bryozoan *Bugula neritina* and the chrysophyte alga *Chrysocystis fragilis*.
3. Physical antifouling defences (Chapter 4). The surface microtopographies of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, when reproduced with resin moulding materials, do not deter the settlement of fouling organisms *in situ* during both the dry and wet seasons. Furthermore, *L. laevigata*, *F. indica* and *A. typicus* are moderately wettable, with mean contact angles ranging between 57º and 70º.
4. Chemical antifouling defences (Chapter 5 and 6). Crude extracts of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* have concentration-dependent effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *Nitzschia closterium*, the bryozoan *B. neritina* and the serpulid *Hydroides elegans*. Furthermore, surface-associated compounds of these sea stars also deter the settlement of fouling organisms. Specifically, the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol deter the settlement of the diatoms *Amphora* sp. and *N. closterium* at ecologically relevant concentrations.

Field surveys conducted in North Queensland, Australia, showed that tropical sea stars have low bacterial abundances of 10^4 to 10^5 cells cm^{-2} and no common generalist macrofouling organisms, such as algae, barnacles, serpulid polychaetes, bryozoans and ascidians, on their epidermal surfaces. Sea stars therefore offer an excellent model to investigate the mechanisms driving fouling-resistant surfaces. Although no common generalist macrofouling organisms were found, some surface-associated parasitic or commensal macroorganisms were present, which were species-specific in most cases. The eulimid gastropod *Thyca* (*Granulithyca*) *nardoafrianti* and the copoepod *Paramolgus* sp. were associated with the sea stars *Nardoa pauciforis* and *Echinaster luzonicus*, respectively, for the first time. Further investigations of the ecological relationships between the host sea stars and their specialist parasites and commensals, and the mechanisms driving the selective settlement of these specialist invertebrates would be of interest.

Many articles and books suggest that pedicellariae of sea stars prevent the settlement of fouling organisms (Nichols 1966, Ruppert and Barnes 1994) and foreign materials and organisms (Campbell 1971, Lambert et al. 1984, Roberts and Campbell 1988), but their role in fouling

control had not been rigorously examined. This is the first study to demonstrate that the elementary pedicellariae of *A. planci* were sparsely distributed, required a high force to trigger a closing response, slowly reopened and did not effectively respond to ecologically relevant fouling organisms. Therefore, the proposal of pedicellariae defending against the settlement of fouling organism does not hold for all sea star species and needs to be reconsidered. Sea stars have different types of pedicellariae, which can be classified as elementary, alveolar or complex, depending on the articulation of the valves with the underlying skeletal plate (Jangoux and Lambert 1988). In contrast to elementary and alveolar pedicellariae, complex pedicellariae have valves, which articulate on a basal piece that is independent from the underlying skeleton, thereby increasing the efficiency of the appendages (Jangoux and Lambert 1988). There is scope to examine the diverse range of species with pedicellariae of different types, especially alveolar and complex pedicellariae, to determine if there is in fact any antifouling role for pedicellariae of sea stars. In particular, the valves of the straight complex pedicellariae on the aboral surface of the sea star *Marthasterias glacialis* can move in all directions, are longer (0.5 to 2.0 mm long) and have a higher density (more than 25 pedicellariae cm^{-2}) than the pedicellariae of the sea star *A. planci* of this study (Lambert et al. 1984), which may support a potential antifouling role. This is an obvious avenue to further uncouple the role of pedicellariae and fouling control, given if they are not effective in a sea star species such as *M. glacialis*, they are most likely ineffective as a general antifouling defence.

Subsequently, physical antifouling defences of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were investigated. Previous studies on physical antifouling defences of natural surfaces have shown that some surface microtopographies effectively deter the settlement of a range of fouling organisms (Baum et al. 2002, Scardino et al. 2003, Bers and Wahl 2004, Scardino and de Nys 2004). This is the first study to demonstrate that the paxillae of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* provide unique and complex surface microtopographies for each species. However, when decoupled from chemical and other surface properties, the surface microtopographies were not effective in deterring the settlement and growth of fouling organisms *in situ* during neither the dry nor wet season, when fouling communities were different. The non-differential fouling community development on replicas of sea stars and control surfaces could be explained by the scale of the surface microtopographies being similar to the larval sizes of the most common fouling organisms of this study and providing sufficient contact points for larvae to settle (Callow et al. 2002, Scardino et al. 2006). A range of abiotic and biotic factors may also have overshadowed any potential effects of the surface microtopography on the settlement of fouling organisms. For example, biofilms may have partially masked the surface microtopography of the replicas and played an important role in providing settlement cues (Todd and Keough 1994, Wieczorek and

Todd 1997, Lau et al. 2002, Patel et al. 2003). Biotic interactions between conspecifics (Brancato and Woollacott 1982, Keough 1984a, Bertness et al. 1992, Clare et al. 1994, Hills and Thomason 1996) and between fouling species, such as competition and predation, may also play an important role in determining the development of a fouling community (Dayton 1971, Keough 1984b). However, given the unique surface microtopographies of each studied sea star species, other species in the orders Valvatida and Paxillosida may have surface microtopographies that are more effective in deterring the settlement of fouling organisms. In addition, synergistic effects with behavioural, mechanical and/or chemical antifouling mechanisms are possible, and have also been suggested for other marine organisms that remain free of fouling organisms (Vrolijk et al. 1990, Bers and Wahl 2004, Scardino and de Nys 2004, Bers et al. 2006b).

To complete the suite of potential defensive mechanisms, chemical antifouling defences of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* were investigated and their ecological relevance determined. While physical antifouling defences of these sea stars alone are ineffective, this study has demonstrated unequivocally that chemical antifouling defences of *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* play a role in deterring the settlement of ecologically relevant fouling species, with the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol reducing the settlement of the diatoms *Amphora* sp. and *N. closterium*. While these compounds are primary metabolites (Papahadjopoulos et al. 1973, Voogt et al. 1993, Brown 2000), this is one of the few studies on primary metabilites with a secondary function in fouling control. Goto et al. (1992) and Mizobuchi et al. (1993) have also demonstrated that fatty acids, mainly C16:0, C16:1 and C18:0, and sterols of octocorals are effective against the mussel *Mytilus edulis*. Furthermore, Davies et al. (1991) showed that cholesterol inhibited the settlement of the ascidian *Podoclavella moluccensis*.

Previous studies have shown that the production of metabolites of marine organisms is dependant on both physical (e.g. temperature, light) and biological (e.g. life history, competition, size) variables (Harvell et al. 1993, Becerro et al. 1997, Hellio et al. 2004, Maréchal et al. 2004). The sterol composition of sea stars, such as *Asterias rubens*, can also be influenced by food availability, because most of the sterols are derived from the diet and only a few sterols are synthesized *de novo* (Voogt and Van Rheenen 1976). The effects of both physical and biological variables of the natural habitats of sea stars on the production of the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol are of interest, because metabolically stressed sea stars may have a compromised chemical antifouling defence mechanism and may eventually get fouled. Therefore, the interaction between the metabolic

stress of sea stars and the production of these surface-associated fatty acids and sterols may determine whether these compounds are part of a ubiquitous defence mechanism or not.

Of the 4 fouling species tested, the diatoms *Amphora* sp. and *N. closterium* were the only species, which were significantly inhibited by the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* at ecologically relevant concentrations, whereas previous studies have only investigated the effects of isolated compounds or crude extracts of sea stars on the settlement of spores of the alga *Hincksia irregularis* (De Marino et al. 2000, Greer et al. 2003), and larvae of the barnacle *Balanus amphitrite* (Bryan et al. 1996) and the bryozoan *B. neritina* (Bryan et al. 1996). From an ecological perspective, determining whether diatoms are the main fouling organisms in the natural habitats of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus*, will confirm the ecological relevance of these surface-associated compounds.

Hexadecanoic acid, cholesterol and lathosterol of the sea stars *L. laevigata*, *F. indica*, *C. pentagona* and *A. typicus* may not be the only compounds inhibiting the settlement of fouling organisms. Other compounds may have similar effects, because all crude extracts (dichloro-methane, methanol and water extracts) had concentration-dependant effects on the settlement of *Amphora* sp. *N. closterium*, *H. elegans* and *B. neritina*. Although hexadecanoic acid, cholesterol, lathosterol and sitosterol significantly reduced the settlement of the diatoms *Amphora* sp. and *N. closterium*, they did not completely inhibit their settlement, and synergism between the compounds studied and other compounds is likely.

Finally, the origin of the surface-associated compounds is of interest, because hexadecanoic acid, cholesterol and lathosterol may be produced by the sea stars or alternatively by their surface-associated microorganisms. These microorganisms may have a symbiotic role, releasing compounds, such as fatty acids and sterols that help reduce or prevent the settlement of other fouling organisms. Previous research has shown that bacterial communities on both the alga *Ulva reticulata* and the sponge *Mycale adhaerens* inhibit the settlement of the serpulid *H. elegans* (Dobretsov and Qian 2002, Lee et al. 2006). The isolation and culture of surface-associated microorganisms of sea stars and subsequent identification and testing of their natural products will offer an opportunity to determine whether similar mechanisms are present in sea stars.

References

Abbott A, Abel PD, Arnold DW, Milne A (2000) Cost – benefit analysis of the use of TBT: the case for a treatment approach. Sci Total Environ 258: 5-19

Aldred N, Ista LK, Callow ME, Callow JA, Lopez GP, Clare AS (2006) Mussel (*Mytilus edulis*) byssus deposition in response to variations in surface wettability. J R Soc Interf 3: 37-43

Amsler CD, Moeller CB, McClintock JB, Iken KB, Baker BJ (2000) Chemical defenses against diatom fouling in Antarctic marine sponges. Biofouling 16: 29-45

Andersson M, Berntsson K, Jonsson P, Gatenholm P (1999) Microtextured surfaces: towards macrofouling resistant coatings. Biofouling 14: 167-178

Barthel D, Wolfrath B (1989) Tissue sloughing in the sponge *Halochondria panicea*: a fouling organism prevents being fouled. Oecologia 78: 357-360

Batang SB, Suzuki H (2003) Gill-cleaning mechanisms of the burrowing thalassinidean shrimps *Nihonotrypaea japonica* and *Upogebia major* (Crustacea: Decapoda). J Zool 261: 69-77

Baum C, Meyer W, Roessner D, Siebers D, Fleischer LG (2001) A zymogel enhances the self-cleaning abilities of the skin of the pilot whale (*Globicephala melas*). Comp Biochem Physiol Part A 130: 835-847

Baum C, Meyer W, Stelzer R, Fleischer LG, Siebers D (2002) Average nanorough skin surface of the pilot whale (*Globicephala melas*, Delphinidae): considerations on the self-cleaning abilities based on nanoroughness. Mar Biol 140: 653-657

Bavington CD, Lever R, Mulloy B, Grundy MM, Page CP, Richardson NV, McKenzie JD (2004) Anti-adhesive glycoproteins in echinoderm mucus secretions. Comp Biochem Physiol Part B Biochem Mol Biol 139: 607-617

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

Becerro MA, Uriz MJ, Turon X (1997) Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida). Hydrobiologia 356: 77-89

Becker K (1993) Attachment strength and colonization patterns of two macrofouling species on substrata with different surface tension (*in situ* studies). Mar Biol 117: 301-309

Becker K, Wahl M (1991) Influence of substratum surface tension on biofouling of artificial substrata in Kiel Bay (Western Baltic): *in situ* studies. Biofouling 4: 275-291

Becker K, Wahl M (1996) Behaviour patterns as natural antifouling mechanisms of tropical marine crabs. J Exp Mar Biol Ecol 203: 245-258

Becker K, Siriratanachai S, Hormchong T (1997) Influence of initial substratum surface tension on marine micro- and macro-fouling in the Gulf of Thailand. Helgoländer Meeresunters 51: 445-461

Becker K, Hormchong T, Wahl M (2000) Relevance of crustacean carapace wettability for fouling. Hydrobiologia 426: 193-201

Bernstein BB, Jung N (1979) Selective pressures and coevolution in a kelp canopy community in southern California. Ecol Monogr 49: 335-355

Berntsson KM, Jonsson PR, Lejhall M, Gatenholm P (2000a) Analysis of behavioural rejection of micro-textured surfaces and implications for recruitment by the barnacle *Balanus improvisus*. J Exp Mar Biol Ecol 251: 59-83

Berntsson KM, Andreasson H, Jonsson PR, Larsson L, Ring K, Petronis S, Gatenholm P (2000b) 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

Bers AV, Prendergast GS, Zürn CM, Hansson L, Head RM, Thomason JC (2006a) A comparative study of the anti-settlement properties of mytilid shells. Biol Lett 2: 88-91

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

Bertness MD, Gaines SD, Stephens EG, Yund PO (1992) Components of recruitment in populations of the acorn barnacle *Semibalanus balanoides* (Linnaeus). J Exp Mar Biol Ecol 156: 199-215

Blake DB (1979) The affinities and origins of the crown-of-thorns sea star *Acanthaster* Gervais. J Nat Hist 13: 303-314

Bottjer DJ (1981) Periostracum of the gastropod *Fusitriton oregonensis*: natural inhibitor of boring and encrusting organisms. Bull Mar Sci 31: 916-921

Bottjer DJ, Carter JG (1980) Functional and phylogenetic significance of projecting periostracal structures in the Bivalvia (Mollusca). J Paleontol 54: 200-216

Botton ML (1981) The gill book of the horseshoe crab (*Limulus polyphemus*) as a substrate for the blue mussel (*Mytilus edulis*). Bull New Jersey Acad Sci 26: 26-28

Brancato MS, Woollacott RM (1982) Effect of microbial films on settlement of bryozoan larvae (*Bugula simplex*, *B. stolonifera* and *B. turrita*). Mar Biol 71: 51-56

Brock E, Nylund GM, Pavia H (2007) Chemical inhibition of barnacle larval settlement by the brown alga *Fucus vesiculosus*. Mar Ecol Prog Ser 337: 165-174

Brooks WR, Mariscal RN (1985) Protection of the hermit crab *Pagurus pollicaris* Say from predators by hydroid-colonized shells. J Exp Mar Biol Ecol 87: 111-118

Brown WH (2000) Introduction to organic chemistry, second edition. Saunders College Publishing, Orlando, Florida

Bruce AJ (1978) *Periclimenes soror* Nobili, a pontoniin shrimp new to the American fauna, with observations on its Indo-West Pacific distribution. Tethys 8: 299-306

Brush MJ, Nixon SW (2002) Direct measurements of light attenuation by epiphytes on eelgrass *Zostera marina*. Mar Ecol Prog Ser 238: 73-79

Bryan PJ, McClintock JB, Watts SA, Marion KR (1994) Antimicrobial activity of ethanolic body-wall extracts of echinoderms from the northern Gulf of Mexico. In: David B, Guille A, Féral JP, Roux M (eds) Echinoderms through time. A.A. Balkema, Rotterdam, pp 17-23

Bryan PJ, Rittschof D, McClintock JB (1996) Bioactivity of echinoderm ethanolic body-wall extracts: assessment of marine bacterial attachment and macroinvertebrate larval settlement. J Exp Mar Biol Ecol 196: 79-96

Bryan PJ, Qian PY, Kreider JL, Chia FS (1997) Induction of larval settlement and metamorphosis by pharmacological and conspecific associated compounds in the serpulid polychaete *Hydroides elegans*. Mar Ecol Prog Ser 146: 81-90

Bryan PJ, McClintock JB, Slattery M, Rittschof DP (2003) A comparative study of the non-acidic chemically mediated antifoulant properties of three sympatric species of ascidians associated with seagrass habitats. Biofouling 19: 235-245

Burke RD (1986) Pheromones and the gregarious settlement of marine invertebrate larvae. Bull Mar Sci 39: 323-331

Buschbaum C, Reise K (1999) Effects of barnacle epibionts on the periwinkle *Littorina littorea* (L.). Helgol Mar Res 53: 56-61

Butman CA (1987) Larval settlement of soft-sediment invertebrates - the spatial scales of pattern explained by active habitat selection and the emerging role of hydrodynamical processes. Oceanogr Mar Biol 25: 113-165

Cadee GC (1991) Carapaces of the shore crab *Carcinus maenas* as a substrate for encrusting organisms. In: Bigey FP (ed) Bryozoa: living and fossil. Société des Sciences Naturelles de l'Ouest de la France. Memoire hors serie, Nantes

Caine EA (1986) Carapace epibionts of nesting loggerhead sea turtles: Atlantic coast of U.S.A. J Exp Mar Biol Ecol 95: 15-26

Callow ME, Callow JA, Ista LK, Coleman SE, Nolasco AC, Lopex GP (2000) Use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. Appl Environ Microbiol 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

Campbell AC (1971) Studies on some effector organs in echinoderms. PhD thesis, University of Oxford, Oxford

Campbell AC (1973) Observations on the activity of echinoid pedicellariae: I. Stem responses and their significance. Mar Behav Physiol 2: 33-61

Campbell AC (1974) Observations on the activity of echinoid pedicellariae: II. Jaw responses of tridentate and ophiocephalous pedicellariae. Mar Behav Physiol 3: 17-34

Campbell AC, Rainbow PS (1977) The role of pedicellariae in preventing barnacle settlement on the sea-urchin test. Mar Behav Physiol 4:253-260

Cancino JM, Munoz J, Munoz M, Orellana MC (1987) Effects of the bryozoan *Membranipora tuberculata* (Bosc) on the photosynthesis and growth of *Gelidium rex* Santilices et Abbott. J Exp Mar Biol Ecol 113: 105-112

Caso ME (1974) Morfologia externa de *Acanthaster planci* (Linnaeus). J Mar Biol Assoc India 16: 83-93

Ciereszko LS, Mizelle JW, Schmidt RW (1973) Occurrence of taurobetaine in coelenterates and of polysaccharide sulfate in the gorgonian *Pseudopteragorgia americana*. In: Worthen LR (ed) Food drugs from the sea proceedings 1972. Marine Technology Society, Washington DC

Champ MA (2000) A review of organotin regulatory strategies, pending actions, related costs and benefits. Sci Total Environ 258: 21-71

Chan DHL, Chan BKK (2005) Effect of epibiosis on the fitness of the sandy shore snail *Batillaria zonalis* in Hong Kong. Mar Biol 146: 695-705

Chia FS, Amerongen H (1975) On the prey-catching pedicellariae of a starfish, *Stylasterias forreri* (de Loriol). Can J Zool 53: 748-755

Chiavelli DA, Mills EL, Threlkeld ST (1993) Host preference, seasonality, and community interactions of zooplankton epibiont. Limnol Oceanogr 38: 574-583

Chludil HD, Maier MS (2005) Minutosides A and B, antifungal sulfated steroid xylosides from the patagonian starfish *Anasterias minuta*. J Nat Prod 68: 1279-1283

Clare AS, Rittschof D, Gerhart DJ, Maki JS (1992) Molecular approaches to non-toxic antifouling. Invertebr Reprod Dev 22: 67-76

Clare AS, Freet RK, McClary M (1994) On the antennular secretion of the cyprid of *Balanus amphitrite amphitrite*, and its role as a settlement pheromone. J Mar Biol Assoc UK 74: 243-250

Clark AM, Rowe FEW (1971) Monograph of shallow-water Indo-West Pacific echinoderms. British Museum (Natural History), London

Clarke KR, Warwick RM (1994) Change in marine communities: an approach to statistical analysis and interpretation. Natural Environment Research Council, UK

Crisp DJ, Ritz DA (1973) Responses of cirripede larvae to light. I. Experiments with white light. Mar Biol 23: 327-335

Dahlström M, Jonsson H, Jonsson PR, Elwing H (2004) Surface wettability as a determinant in the settlement of the barnacle *Balanus improvisus* (Darwin). J Exp Mar Biol Ecol 305: 223-232

Dartnall AJ, Byrne M, Collins J, Hart MW (2003) A new viviparous species of asterinid (Echinodermata, Asteroidea, Asterinidae) and a new genus to accommodate the species of pan-tropical exiguoid sea stars. Zootaxa 359: 1-14

Davis AR, Butler AJ, van Altena I (1991) Settlement behaviour of ascidian larvae: preliminary evidence for inhibition by sponge allelochemicals. Mar Ecol Prog Ser 72: 117-123

Dayton PK (1971) Competition, disturbance and community organization: the provision and subsequent utilization of space in a rocky intertidal community. Ecol Monogr 41: 351-389

De Marino S, Iorizzi M, Palagiano E, Zollo F, Roussakis C (1998) Starfish saponins. 55. Isolation, structure elucidation, and biological activity of the steroid oligoglycosides from an Antarctic starfish of the family Asteriidae. J Nat Prod 61: 1319-1327

De Marino S, Iorizzi M, Zollo F, Amsler CD, Greer SP, McClintock JB (2000) Starfish saponins, LVI, three new asterosaponins from the starfish *Certonardoa demonstrans*. Eur J Org Chem 24: 4093-4098

de Nys R, Steinberg P (1999) Role of secondary metabolites from algae and seagrasses in biofouling control. In: Fingerman M, Nagabhushanam R, Thompson MF (eds) Recent advances in marine biotechnology, Volume 3. Science Publishers, Enfield, pp 223-244

de Nys R, Steinberg P (2002) Linking marine biology and biotechnology. Curr Opin Biotechnol 13: 244-248

de Nys R, Dworjanyn SA, Steinberg PD (1998) A new method for determining surface concentrations of marine natural products on seaweeds. Mar Ecol Prog Ser 162: 79-87

Dexter SC, Sullivan JD, Williams J, Watson SW (1975) Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Appl Microbiol 30: 298-308

Dearborn JH, Edwards KC, Fratt DB (1991) Diet, feeding behaviour, and surface morphology of the multi-armed Antarctic sea star *Labidiaster annulatus* (Echinodermata: Asteroidea). Mar Ecol Prog Ser 77: 65-84

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

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

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

Dobretsov S, Dahms HU, Tsoi MY, Qian PY (2005) Chemical control of epibiosis by Hong Kong sponges: the effect of sponge extracts on micro- and macrofouling communities. Mar Ecol Prog Ser 297: 119-129

Dobretsov S, Dahms HU, Harder T, Qian PY (2006) Allelochemical defense against epibiosis in the macroalga *Caulerpa racemosa* var. *turbinata*. Mar Ecol Prog Ser 318: 165-175

Drake LA, Dobbs FC, Zimmerman RC (2003) Effects of epiphyte load on optical properties and photosynthetic potential of the seagrasses *Thalassia testudinum* Banks ex Konig and *Zostera marina* L. Limnol Oceanogr 48: 456-463

Dworjanyn SA, de Nys R, Steinberg PD (1999) Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. Mar Biol 133: 727-736

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

Dyrynda PEJ (1986) Defensive strategies of modular organisms. Philos Trans R Soc Lond B Biol Sci 313: 227-243

Egan EA, Anderson DT (1986) Larval development of *Balanus amphitrite* Darwin and *Balanus variegatus* Darwin (Cirripedia, Balanidae) from New South Wales, Australia. Crustaceana 51: 188-207

Egloff DA, Smouse DT Jr, Pembroke JE (1988) Penetration of the radial hemal and perihemal systems of *Linckia laevigata* (Asteroidea) by the proboscis of *Thyca crystallina*, an ectoparasitic gastropod. Veliger 30: 342-346

Elder HY (1979) Studies on the host parasite relationship between the parasitic prosobranch *Thyca crystallina* and the asteroid starfish *Linckia laevigata*. J Zool 187: 369-391

Emson RH, Young CM (1994) Feeding mechanism of the brisingid starfish *Novodinia antillensis*. Mar Biol 118: 433-442

Fernández-Leborans G, Córdoba MJH, del Arco PG (1997) Distribution of ciliate epibionts on the portunid crab *Liocarcinus depurator* (Decapoda: Brachyura). Invertebr Biol 116: 171-177

Figueiredo MADO, Norton TA, Kain JM (1997) Settlement and survival of epiphytes on two intertidal crustose coralline algae. J Exp Mar Biol Ecol 213: 247-260

Finlay JA, Callow ME, Ista LK, Lopez GP, Callow JA (2002) The influence of surface wettability on the adhesion strength of settled spores of the green alga *Enteromorpha* and the diatom *Amphora*. Integr Comp Biol 42: 1116-1122

Glynn PW (1970) Growth of algal epiphytes on a tropical marine isopod. J Exp Mar Biol Ecol 5: 88-93

Goad LJ (1979) Sterols of marine invertebrates: composition, biosynthesis and metabolites. In: Scheuer PJ (ed) Marine natural products: chemical and biological perspectives, Vol. II. Academic Press, New York, pp 76-173

Goto R, Kado R, Muramoto K, Kamiya H (1992) Fatty acids as antifoulants in a marine sponge. Biofouling 6: 61-68

Greer SP, Amsler CD (2004) Clonal variation in phototaxis and settlement behaviors of *Hincksia irregularis* (Phaeophyceae) spores. J Phycol 40: 44-53

Greer SP, Iken KB, McClintock JB, Amsler CD (2003) Individual and coupled effects of echinoderm extracts and surface hydrophobicity on spore settlement and germination in the brown alga *Hincksia irregularis*. Biofouling 19: 315-326

Greer SP, Iken K, McClintock JB, Amsler CD (2006) Bioassay-guided fractionation of antifouling compounds using computer-assisted motion analysis of brown algal spore swimming. Biofouling 22: 125-132

Gribben PE, Marshall DJ, Steinberg PD (2006) Less inhibited with age? Larval age modifies responses to natural settlement inhibitors. Biofouling 22: 101-106

Guenther J, de Nys R (2006) Differential community development of fouling species on the pearl oysters *Pinctada fucata*, *Pteria penguin* and *Pteria chinensis* (Bivalvia, Pteriidae). Biofouling 22: 163-171

Hall DN (1984) Cheilostome bryozoa from Townsville Harbour and Picnic Bay, North Queensland. Proc R Soc Qd 95: 57-70

Hallegraeff GM, Jeffrey SW (1984) Tropical phytoplankton species and pigments of continental shelf waters of North and North-West Australia. Mar Ecol Prog Ser 20: 59-74

Harper EM (1997) The molluscan periostracum: an important constraint in bivalve evolution. Palaeontology 40: 71-97

Harper EM, Skelton PW (1993) A defensive value of the thickened periostracum in the Mytiloidea. Veliger 36: 36-42

Harvell CD, Fenical W, Roussis V, Ruesink JL, Griggs CC, Greene CH (1993) Local and geographic variation in the defensive chemistry of a West Indian gorgonian coral (*Briareum asbestinum*). Mar Ecol Prog Ser 93: 165-173

Haug T, Kjuul AK, Styrvold OB, Sandsdalen E, Olsen OM, Stensvag K (2002) Antibacterial activity in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea), and *Asterias rubens* (Asteroidea). J Invertebr Pathol 81: 94-102

Hay ME (1996) Marine chemical ecology: what's known and what's next? J Exp Mar Biol Ecol 200: 103-134

Head RM, Overbeke K, Klijnstra J, Biersteker R, Thomason JC (2003) The effect of gregariousness in cyprid settlement assays. Biofouling 19: 269-278

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

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

Hendler G, Franz DR (1982) The biology of a brooding seastar, *Leptasterias tenera*, in Block Island Sound. Biol Bull (Woods Hole) 162: 2273-2289

Hepburn CD, Hurd CL (2005) Conditional mutualism between the giant kelp *Macrocystis pyrifera* and colonial epifauna. Mar Ecol Prog Ser 302: 37-42

Hepburn CD, Hurd CL, Frew RD (2006) Colony structure and seasonal differences in light and nitrogen modify the impact of sessile epifauna on the giant kelp *Macrocystis pyrifera* (L.) C Agardh. Hydrobiologia 560: 373-384

Hills JM, Thomason JC (1996) A multi-scale analysis of settlement density and pattern dynamics of the barnacle *Semibalanus balanoides*. Mar Ecol Prog Ser 138: 103-115

Hills JM, Thomason JC (1998a) The effects of scales of surface roughness on the settlement of barnacle (*Semibalanus balanoides*) cyprids. Biofouling 12: 57-69

Hills JM, Thomason JC (1998b) On the effect of tile size and surface texture on recruitment pattern and density of the barnacle, *Semibalanus balanoides*. Biofouling 13: 31-50

Hoipkemeier-Wilson L, Schumacher JF, Carman ML, Gibson AL, Feinberg AW, Callow ME, Finlay JA, Callow JA, Brennan AB (2004) Antifouling potential of lubricious, micro-engineered, PDMS elastomers against zoospores of the green *Ulva* (*Enteromorpha*). Biofouling 20: 53-63

Holland ND, Nealson KH (1978) The fine structure of the echinoderm cuticle and the subcuticular bacteria of echinoderms. Acta Zool 59: 169-185

Holmes SP, Sturgess CJ, Cherrill A, Davies MS (2001) Shell wiping in *Calliostoma zizyphinum*: the use of pedal mucus as a provendering agent and its contribution to daily energetic requirements. Mar Ecol Prog Ser 212: 171-181

Humes AG (1973) Cyclopoid (Lichomolgidae) from octocorals at Eniwetok Atoll. Beaufortia 21: 135-151

Humes AG (1976) Distribution and hosts of *Stellicola* (Copepoda, Cyclopoida) associated with *Linckia* (Asteroidea) in the Indo-West Pacific. Beaufortia 25: 49-61

Iken K, Amsler CD, Greer SP, McClintock JB (2001) Qualitative and quantitative studies of the swimming behaviour of *Hincksia irregularis* (Phaeophyceae) spores: ecological implications and parameters for quantitative swimming assays. Phycologia 40: 359-366

Iken K, Greer SP, Amsler CD, McClintock JB (2003) A new antifouling bioassay monitoring brown algal spore swimming behaviour in the presence of echinoderm extracts. Biofouling 19: 327-334

Iorizzi M, Bryan P, McClintock J, Minale L, Palagiano E, Maurelli S, Riccio R, Zollo F (1995) Chemical and biological investigation of the polar constituents of the starfish *Luidia clathrata*, collected in the Gulf of Mexico. J Nat Prod 58: 653-671

Ivanchina NV, Kicha AA, Kalinovsky AI, Dmitrenok PS, Dmitrenok AS, Chaikina EL, Stonik VA, Gavagnin M, Cimino G (2006) Polar steroidal compounds from the far eastern starfish *Henricia leviuscula*. J Nat Prod 69: 224-228

Jangoux M, Lambert A (1988) Comparative anatomy and classification of asteroid pedicellariae. In: Burke RD, Mladenov PV, Lambert P, Parsley RL (eds) Echinoderm biology. Balkema, Rotterdam, pp 719-723

Janssen HH (1985) Three epizoic gastropods from Malaysia and the Philippines. Zeitschrift für Parasitenkunde 71: 553-560

Jensen M (1966) The response of two sea-urchins to the sea-star *Marthasterias glacialis* (L.) and other stimuli. Ophelia 3: 209-219

Kaehler S, McQuaid CD (1999) Lethal and sub-lethal effects of phototrophic endoliths attacking the shell of the intertidal mussel *Perna perna*. Mar Biol 135: 497-503

Keats DW, Groener A, Chamberlain YM (1993) Cell sloughing in the littoral zone coralline alga, *Spongites yengoi* (Foslie) Chamberlain (Corallinales, Rhodophyta). Phycologia 32: 143-150

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

Kelly MS, McKenzie JD (1995) Survey of the occurrence and morphology of sub-cuticular bacteria in shelf echinoderms from the north-east Atlantic Ocean. Mar Biol 123: 741-756

Kelly SR, Garo E, Jensen PR, Fenical W, Pawlik JR (2005) Effects of Caribbean sponge secondary metabolites on bacterial surface colonization. Aquat Microb Ecol 40: 191-203

Keough MJ (1984a) Kin-recognition and the spatial distribution of larvae of the bryozoan *Bugula neritina*. Evolution 38: 142-147

Keough MJ (1984b) Dynamics of the epifauna of the bivalve *Pinna bicolor*: interactions among recruitment, predation, and competition. Ecology 65: 677-688

Keough MJ, Raimondi PT (1995) Responses of settling invertebrate larvae to bioorganic films: effects of different types of films. J Exp Mar Biol Ecol 185: 235-253

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

Köhler J, Hansen PD, Wahl M (1999) Colonization patterns at the substratum-water interface: how does surface microtopography influence recruitment patterns of sessile organisms? Biofouling 14: 237-248

Kraberg Ac, Norton AT (2007) Effect of epiphytism on reproductive and vegetative lateral formation in the brown, intertidal seaweed *Ascophyllum nodosum* (Phaeophyceae). Phycol Res 55: 17-24

Kubanek J, Whalen KE, Engel S, Kelly SR, Henkel TP, Fenical W, Pawlik JR (2002) Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 131: 125-136

Kuhlmann ML (1992) Behavioural avoidance of predation in an intertidal hermit crab. J Exp Mar Biol Ecol 157: 143-158

Kumar SA, Ayyakkannu K (1991) Periostracum of the gastropod *Hemifusus pugilinus*: natural inhibitor of boring and encrusting organisms. J Mar Biol Assoc India 33: 374-378

Lam C, Harder T, Qian PY (2003) Induction of larval settlement in the polychaete *Hydroides elegans* by surface-associated settlement cues of marine benthic diatoms. Mar Ecol Prog Ser 263: 83-92

Lambert A, De Vos L, Jangoux M (1984) Functional morphology of the pedicellariae of the asteroid *Marthasterias glacialis* (Echinodermata). Zoomorphology 104: 122-130

Lau SCK, Qian PY (1997) Phlorotannins and related compounds as larval settlement inhibitors of the tube-building polychaete *Hydroides elegans*. Mar Ecol Prog Ser 159: 219-227

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

Laudien J, Wahl M (1999) Indirect effects of epibiosis on host mortality: seastar predation on differently fouled mussels. Mar Ecol 20: 35-47

Lauerman LML (1998) Diet and feeding behaviour of the deep-water sea star *Rathbunaster californicus* (Fisher) in the Monterey Submarine Canyon. Bull Mar Sci 63: 523-530

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

Lee OO, Lau SCK, Qian PY (2006) Defense against epibiosis in the sponge *Mycale adhaerens*: modulating the bacterial community associated with its surface. Aquat Microb Ecol 43: 55-65

Lee OO, Yang LH, Li X, Pawlik JR, Qian PY (2007) Surface bacterial community, fatty acid profile, and antifouling activity of two congeneric sponges from Hong Kong and the Bahamas. Mar Ecol Prog Ser 339: 25-40

Lewis JA, Watson C, ten Hove AH (2006) Establishment of the Caribbean serpulid tubeworm *Hydroides sanctaecrucis* Krøyer [in] Mörch, 1963, in northern Australia. Biol Invasions 8: 665-671

Little BJ (1984) Succession in microfouling. In: Costlow JD, Tipper RC (eds) Marine deterioration: an interdisciplinary study. E & FN Spon, London, pp 63-67

Littler M, Littler D (1984) Relationships between macroalgal functional form groups and substrata stability in a subtropical rocky intertidal system. J Exp Mar Biol Ecol 74: 13-34

Lobban CS, Honda D, Chihara M, Schefter M (1995) *Chrysocystis fragilis* gen. nov., sp. nov. (Chrysophyceae, Sarcinochrysidales), with notes on other macroscopic Chrysophytes (Golden algae) on Guam reefs. Micronesica 28: 91-102

Lodeiros CJM, Himmelman JH (1996) Influence of fouling on the growth and survival of the tropical scallop, *Euvola* (*Pecten*) *ziczac* (L. 1758) in suspended culture. Aquac Res 27: 749-756

Lubchenco Menge J, Menge BA (1974) Role of resource allocation, aggression and spatial heterogeneity in coexistence of two competing intertidal starfish. Ecol Monogr 44: 189-209

Maki JC, Mitchell R (2002) Biofouling in the marine environment. In: Bitton G (ed) Encyclopedia of environmental microbiology. John Wiley & Sons, New York, pp 610-619

Maki JS, Rittschof D, Costlow JD, Mitchell R (1988) Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. Mar Biol 97: 199-206

Mao Che L, Le Campion-Alsumard T, Boury-Esnault N, Payri C, Golubic S, Bezac C (1996) Biodegradation of shells of the black pearl oyster, *Pinctada margaritifera* var. *cumingii*, by microborers and sponges of French Polynesia. Mar Biol 126: 509-519

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

Marrs SJ, Thomason JC, Cowling MJ, Hodgkiess T (1995) A replica method for the study of marine biofilms. J Mar Biol Assoc UK 75: 759-762

Marshall DJ, Keough MJ (2003) Variation in the dispersal potential of non-feeding invertebrate larvae: the desperate larva hypothesis and larval size. Mar Ecol Prog Ser 255: 145-153

Maximilien R, de Nys R, Holmström C, Gram L, Givskov M, Crass K, Kjelleberg S, Steinberg PD (1998) Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga *Delisea pulchra*. Aquat Microb Ecol 15: 233-246

McClintock JB, Vernon JD (1990) Chemical defense in the eggs and embryos of Antarctic sea stars (Echinodermata). Mar Biol 105: 491-495

McClintock JB, Mahon AR, Peters KJ, Amsler CD, Baker BJ (2003) Chemical defences in embryos and juveniles of two common Antarctic sea stars and an isopod. Antarct Sci 15: 339-344

McClintock JB, Amsler MO, Amsler CD, Southworth KJ, Petrie C, Baker BJ (2004) Biochemical composition, energy content and chemical antifeedant and antifoulant defenses of the colonial Antarctic ascidian *Distaplia cylindrica*. Mar Biol 145: 885-894

McKenzie JD, Grigolava JV (1996) The echinoderm surface and its role in preventing microfouling. Biofouling 10: 261-272

Mizobuchi S, Shimidzu N, Katsuoka M, Adachi K, Miki W (1993) Chemical studies on antifouling substances in marine invertebrates. 1. Antifouling substances against the mussel in an octocoral *Dendronephthya* sp. Nippon Suisan Gakkaishi 59: 1195-1199

Mohammad MBM (1976) Relationship between biofouling and growth of the pearl oyster *Pinctada fucata* (Gould) in Kuwait, Arabian Gulf. Hydrobiologia 51: 129-138

Moran P (1986) The *Acanthaster* phenomenon. Oceanogr Mar Biol Annu Rev 24: 379-480

Moran P (1988) Crown-of-thorns starfish: questions and answers. Australian Institute of Marine Science, Townsville

Neal AL, Yule AB (1994a) The tenacity of *Elminius modestus* and *Balanus perforatus* cyprids to bacterial films grown under different shear regimes. J Mar Biol Assoc UK 74: 251-257

Neal AL, Yule AB (1994b) The interaction between *Elminius modestus* Darwin cyprids and biofilms of *Deleya marina* NCMB 1877. J Exp Mar Biol Ecol 176: 127-139

Nichols D (1966) Echinoderms. Hutchinson, London

Nylund GM, Pavia H (2003) Inhibitory effects of red algal extracts on larval extracts settlement of the barnacle *Balanus improvisus*. Mar Biol 143: 875-882

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

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

OECD (Organisation for Economic Co-operation and Development) (1965) Catalogue of main marine fouling organisms, Vol 2: Polyzoa. OECD Publications, Paris

OECD (Organisation for Economic Co-operation and Development) (1967) Catalogue of main marine fouling organisms, Vol 3: Serpulids. OECD Publications, Paris.

Olafsdottir SH, Svavarsson J (2002) Ciliate (Protozoa) epibionts of deep-water asselote isopods (Crustacea): Patterns and diversity. J Crustac Biol 22: 607-618

Oswald RC, Telford N, Seed R, Happey-Wood CM (1984) The effects of encrusting bryozoans on the photosynthetic activity of *Fucus serratus* L. Est Coast Shelf Sci 19: 697-702

Papahadjopoulos D, Cowden M, Kinelberg H (1973) Role of cholesterol in membranes. Effects on phospholipid-protein interactions, membrane permeability and enzymatic activity. Biochim Biophys Acta 330: 8-26

Parapar J, Fernandez L, Gonzalez-Guirriaran E, Muino R (1997) Epibiosis and masking material in the spider crab *Maja squinado* (Decapoda: Majidae) in the Ria de Arousa (Galicia, NW Spain). Cah Biol Mar 38: 221-234

Patel P, Callow ME, Joint I, Callow JA (2003) Specificity in the settlement – modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. Environ Microbiol 5: 338-349

Patton WK (1972) Studies on the animal symbionts of the gorgonian coral, *Leptogorgia virgulata* (Lamarck). Bull Mar Sci 22: 419-431

Penhale PA, Smith WO (1977) Excretion of dissolved organic carbon by eelgrass (*Zostera marina*) and its epiphytes. Limnol Oceanogr 22: 400-407

Petronis S, Berntsson K, Gold J, Gatenholm P (2000) Design and microstructuring of PDMS surfaces for improved marine biofouling resistance. J Biomat Sci-Poly Ed 11: 1051-1072

Pit JH, Southgate PC (2003) Fouling and predation; how do they affect growth and survival of the blacklip pearl oyster, *Pinctada margaritifera*, during nursery culture? Aquac Int 11: 545-555

Provasoli L (1964) Growing marine seaweeds. In: Davy de Virville AD, Feldmann J (eds) Proc 4th Int Seaweed Symp. Pergamon Press, New York, pp 9-17

Quinn GP, Keough MJ (2002) Experimental design and data analysis for biologists. Cambridge University Press, Cambridge

Rittschof D, Hooper IR, Branscomb ES, Costlow JD (1985) Inhibition of barnacle settlement and behavior by natural products from whip corals, *Leptogorgia virgulata* (Lamarck, 1815). J Chem Ecol 2: 551-562

Roberts MP, Campbell AC (1988) Functional anatomy of pedicellariae from *Asterias rubens* L. In: Burke RD, Mladenov PV, Lambert P, Parsley RL (eds) Echinoderm biology. Balkema, Rotterdam, pp 725-733

Robilliard GA (1971) Feeding behaviour and prey capture in an asteroid *Stylasterias forreri*. Syesis 4: 191-195

Roccatagliata AJ, Maier MS, Seldes AM, Pujol CA, Damonte EB (1996) Antiviral sulfated steroids from the ophiuroid *Ophioplocus januarii*. J Nat Prod 59: 887-889

Rodríguez SR, Ojeda FP, Inestrosa NC (1993) Settlement of benthic marine invertebrates. Mar Ecol Prog Ser 97: 193-207

Rowe FEW, Gates J (1995) Echinodermata. In: Wells A (ed) Zoological catalogue of Australia. Vol. 33, CSIRO Publishing, Melbourne

Ruppert EE, Barnes RD (1994) Invertebrate zoology. Saunders College Publishing, Fort Worth

Saleuddin ASM, Petit HP (1983) The mode of formation and the structure of the periostracum. In: Wilbur KM (ed) The Mollusca, Volume 4. Academic Press, New York, pp 199-234

Scardino AJ (2006) Biomimetic fouling control. PhD thesis, James Cook University, Townsville

Scardino AJ, de Nys R (2004) Fouling deterrence on the bivalve shell *Mytilus galloprovincialis*: A physical phenomenon? Biofouling 20: 249-257

Scardino A, de Nys R, Ison O, O'Connor W, Steinberg P (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctada imbricata*. Biofouling 19: 221-230

Scardino AJ, Harvey E, de Nys R (2006) Testing attachment point theory: diatom attachment on microtextured polyimide biomimics. Biofouling 22: 55-60

Schaffelke B, Heimann K, Marshall PA, Ayling AM (2004) Blooms of *Chrysocystis fragilis* on the Great Barrier Reef. Coral Reefs 23: 514

Schmitt TM, Hay ME, Lindquist N (1995) Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. Ecology 76: 107-123

Schumacher JF, Carman ML, Estes TG, Feinberg AW, Wilson LH, Callow ME, Callow JA, Finlay JA, Brennan AB (2007a) Engineered antifouling microtopographies – effect of feature size, geometry, and roughness on settlement of zoospores of the green alga *Ulva*. Biofouling 23: 55-62

Schumacher JF, Aldred N, Callow ME, Finlay JA, Callow JA, Clare AS, Brennan AB (2007b) Species-specific engineered antifouling topographies: correlations between the settlement of algal zoospores and barnacle cyprids. Biofouling 23: 307-317

Sieburth J, Tootle JL (1981) Seasonality of microbial fouling on *Ascophyllum nodosum* (L.) Lejol., *Fucus vesiculosus* L., *Polysiphonia lanosa* (L.) Tandy and *Chondrus crispus* Stackh. J Phycol 17: 57-64

Slattery M, Hamann MT, McClintock JB, Perry TL, Puglisi MP, Yoshida WY (1997) Ecological role for water-borne metabolites from Antarctic soft corals. Mar Ecol Prog Ser 161: 133-144

Sokal RR, Rohlf FJ (1995) Biometry: the principles and practices of statistics in biological research. Freeman, New York

Stebbing ARD (1973) Observations on colony overgrowth and spatial competition. In: Larwood GP (ed) Living and fossil Bryozoa. Academic Press, London, pp 173-183

Steinberg PD, de Nys R, Kjelleberg S (2001) Chemical mediation of surface colonization. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, Florida, pp 355-387

Steinberg PD, de Nys R, Kjelleberg S (2002) Chemical cues for surface colonization. J Chem Ecol 28: 1935-1951

Stefaniak LM, McAtee J, Shulman MJ (2005) The costs of being bored: Effects of a clinoid sponge on the gastropod *Littorina littorea* (L.). J Exp Mar Biol Ecol 327: 103-114

Svavarsson J, Davidsdottir B (1994) Foraminiferan (Protozoa) epizoites on Arctic isopods (Crustacea) as indicators of isopod behavior. Mar Biol 118: 239-246

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. J Chem Ecol 9: 817-829

Taylor JJ, Southgate PC, Rose RA (1997) Fouling animals and their effect on the growth of silver-lip pearl oysters, *Pinctada maxima* (Jameson) in suspended culture. Aquaculture 153: 31-40

Temkin MH, Zimmer RL (2002) Phylum Bryozoa. In: Young CM (ed) Atlas of marine invertebrate larvae. Academic Press, San Diego. pp 411-427

Teo SLM, Ryland JS (1995) Potential antifouling mechanisms using toxic chemicals in some British ascidians. J Exp Mar Biol Ecol 188: 49-62

Thieltges DW (2005) Benefit from an invader: American slipper limpet *Crepidula fornicata* reduces star fish predation on basibiont European mussels. Hydrobiologia 541: 241-244

Thieltges DW, Buschbaum C (2007) Vicious circle in the intertidal: Facilitation between barnacle epibionts, a shell boring polychaete and trematode parasites in the periwinkle *Littorina littorea*. J Exp Mar Biol Ecol 340: 90-95

Thomason JC, Davenport J (1995) The measurement of surface energy by video analysis of captive-bubble contact-angles. J Mar Biol Assoc UK 75: 763-766

Todd CD, Keough MJ (1994) Larval settlement in hard substratum epifaunal assemblages: a manipulative field study of the effects of substratum filming and the presence of incumbents. J Exp Mar Biol Ecol 181: 159-187

Tsurumi K, Fusetani N (1998) Effects of early fouling communities formed in the field on settlement and metamorphosis of cyprids of the barnacle, *Balanus amphitrite* Darwin. Biofouling 12: 119-131

Underwood AJ (1977) Barnacles: a guide based on the barnacles found on the New South Wales coast. Reed Education, Sydney

Underwood AJ (1981) Techniques of analysis of variance in experimental marine biology and ecology. Oceanogr Mar Biol Ann Rev 19: 513-605

van Veldhuizen HD, Oakes VJ (1981) Behavioural responses of 7 species of asteroids to the asteroid predator *Solaster dawsoni*. Oecologia 48: 214-220

Vasconcelos P, Cúrdia J, Castro M, Gaspar MB (2007) The shell of *Hexaplex* (*Trunculariopsis*) *trunculus* (Gastropoda: Muricidae) as a mobile hard substratum for epibiotic polychaetes (Annelida: Polychaeta) in the Ria Formosa (Algarve coast – southern Portugal). Hydrobiologia 575: 161-172

Voogt PA, Van Rheenen JWA (1976) On the origin of sterols in the sea star *Asterias rubens*. Comp Biochem Physiol 54B: 479-482

Voogt PA, Bissumbhar B, Idzinga TM, Wynne HJ, Jansen M (1993) Effects of cadmium on sterol composition in the aboral body-wall of the sea star *Asterias rubens* L. Comp Biochem Physiol 104C: 415-421

Vrolijk NH, Targett NM, Baier RE, Meyer AE (1990) Surface characterisation of two gorgonian coral species: implications for a natural antifouling defence. Biofouling 2: 39-54

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

Wahl M (1997) Increased drag reduces growth of snails: Comparison of flume and *in situ* experiments. Mar Ecol Prog Ser 151: 291-293

Wahl M, Banaigs B (1991) Marine epibiosis. III. Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). J Exp Mar Biol Ecol 145: 49-63

Wahl M, Sönnichsen H (1992) Marine epibiosis. IV. The periwinkle *Littorina littorea* lacks typical antifouling defences – why are some populations so little fouled? Mar Ecol Prog Ser 88: 225-235

Wahl M, Mark O (1999) The predominantly facultative nature of epibiosis: experimental and observational evidence. Mar Ecol Prog Ser 187: 59-66

Wahl M, Jensen PR, Fenical W (1994) Chemical control of bacterial epibiosis on ascidians. Mar Ecol Prog Ser 110: 45-57

Wahl M, Kröger K, Lenz M (1998) Non-toxic protection against epibiosis. Biofouling 12: 205-226

Walt DR, Smulow JB, Turesky SS, Hill RG (1985) The effect of gravity on initial microbial adhesion. J Colloid Interface Sci 107: 334-336

Wang W, Li F, Hong J, Lee CO, Cho HY, Im KS, Jung JH (2003) Four new saponins from the starfish *Certonardoa semiregularis*. Chem Pharm Bull 51: 435-439

Waren A (1983) A generic revision of the family Eulimidae (Gastropoda, Prosobranchia). J Molluscan Stud Suppl 13: 1-96

Wieczorek SK, Todd CD (1997) Inhibition and facilitation of bryozoan and ascidian settlement by natural multi-species biofilms: effects of film age and the roles of active and passive larval attachment. Mar Biol 128: 463-473

Yule AB, Walker G (1984) The temporary adhesion of barnacle cyprids: effects of some differing surface characteristics. J Mar Biol Assoc UK 64: 429-439

Zann LP (1975) Biology of a barnacle (*Platylepas ophiophilus* Lanchester) symbiotic with sea snakes. In: Dunson WA (ed) The biology of sea snakes. University Park Press, Baltimore, Maryland, pp 267-286

Zann LP, Cuffey RJ, Kropach C (1975) Fouling organisms and parasites associated with the skin of sea snakes. In: Dunson WA (ed) The biology of sea snakes. University Park Press, Baltimore, Maryland, pp 251-265

Zibrowius H (1971) The Mediterranean species of the genus *Hydroides* (Polychaeta, Serpulidae). Remarks on the so-called polymorphism of *Hydroides uncinata*. Tethys 2: 691-746

Appendix 1

Guenther J, Walker-Smith G, Waren A, de Nys R (2007) Fouling-resistant surfaces of tropical sea stars. Biofouling 23: 413-418

Guenther J, Heimann K, de Nys R (2007) Pedicellariae of the crown-of-thorns sea star *Acanthaster planci* are not an effective defence against fouling. Mar Ecol Prog Ser 340: 101-108

Guenther J, de Nys R (2007) Surface microtopographies of tropical sea stars: Lack of an efficient physical defence mechanism against fouling. Biofouling 23: 419-429