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Chemical antifouling defences of sea stars: effects of the natural products hexadecanoic acid, cholesterol, lathosterol and sitosterol

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ABSTRACT: The role of natural products in keeping the surfaces of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* free of fouling organisms was investigated. Conditioned seawater of these sea stars did not have any effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *Nitzschia closterium* and the bryozoan *Bugula neritina*. However, dichloromethane, methanol and aqueous extracts of whole sea stars at 100, 10, 1 and 0.1 µg cm^{-2} had concentration-dependent effects on the settlement of these fouling species and the polychaete *Hydroides elegans*. Based on bioassay-guided fractionation and analysis with proton nuclear magnetic resonance spectroscopy and gas chromatography-mass spectrometry (GC-MS), the most bioactive fractions contained several fatty acids and sterols. To determine whether the compounds responsible for the observed antifouling effects were present on the surface of all 4 sea star species, surface-associated compounds were tested against *Amphora* sp., *N. closterium*, *B. neritina* and *H. elegans*. These compounds reduced the settlement of at least 2 of the 4 fouling species for each sea star species. Using surface extractions and GC-MS analysis, the most abundant surface-associated fatty acids and sterols of each sea star species were identified and quantified. Hexadecanoic acid, cholesterol, lathosterol and sitosterol were tested at surface-associated concentrations, ranging from 1000 to 1 ng cm^{-2}, in settlement assays. Whereas the settlement of *B. neritina* was not affected by any of the compounds, hexadecanoic acid, cholesterol and lathosterol significantly reduced the settlement of *N. closterium* at surface-associated concentrations.

KEY WORDS: Biofouling · Sea star · Fatty acid · Sterol · Diatom

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INTRODUCTION

Marine biofouling, which is the formation of a complex layer of organisms on submerged surfaces, can have negative ecological impacts on living surfaces (reviewed by Wahl 1989, de Nys & Steinberg 1999). For example, biofouling may reduce the photosynthesis and growth of marine algae (Dixon et al. 1981, Drake et al. 2003), affect the shell condition, growth rate and survival of bivalves (Lodeiros & Himmelman 1996, Taylor et al. 1997, Kaehler & McQuaid 1999, Thieltges & Buschbaum 2007) and affect the condition and function of the appendages of crabs (Botton 1981, Cadee 1991). Many marine plants and animals have evolved behavioural, mechanical, physical and/or chemical defences to reduce these disadvantages and ecological costs (reviewed by Wahl 1989). In common with many organisms investigated for natural antifoul-

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ing defences, sea stars generally have surfaces that are remarkably free of fouling organisms (McKenzie & Grigolava 1996, Guenther et al. 2007a). However, mechanical antifouling defences associated with the pedicellariae of the crown-of-thorns sea star *Acanthaster planci* (Guenther et al. 2007b) and physical antifouling defences associated with the wettabilities and surface microtopographies of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* (Guenther & de Nys 2007) are inefficient and do not explain how these sea stars keep their surfaces free of fouling organisms. In contrast, crude extracts and isolated compounds from sea star tissues have commonly been tested against selected fouling organisms using disc diffusion assays (Bryan et al. 1994, Iorizzi et al. 1995), motion analysis assays (Iken et al. 2001, 2003, Greer et al. 2006) and settlement assays (Iorizzi et al. 1995, Bryan et al. 1996, De Marino et al. 2000, Greer et al. 2003). Iorizzi et al. (1995) demonstrated that the ethanol whole body extracts of the sea star *Luidia clathrata* inhibited the growth of the bacteria *Bacillus subtilis* and *Staphylococcus aureus*. Furthermore, spores of the brown alga *Hincksia irregularis* exposed to low concentrations (<91 µg 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 (Iken et al. 2003). At the highest concentration (909 µg ml^{-1}), spores were severely damaged or completely immobilized (Iken et al. 2003). Finally, isolated asterosaponins of the sea star *Goniopecten demonstrans* reduced the settlement of the brown alga *H. irregularis* (De Marino et al. 2000), whereas water and acetone body wall extracts of the sea stars *Astropecten 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 *Balanus amphitrite* and *Bugula 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 *Balanus amphitrite* and *Bugula neritina* at concentrations of ≥120 and ≥4.8 µg ml^{-1}, respectively (Iorizzi et al. 1995).

Testing whole-cell crude extracts of unfouled marine species 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 & Ryland 1995). Results from settlement assays with whole-cell extracts, however, are insufficient to predict any antifouling role of compounds, because 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 (Nylund & Pavia 2003, Nylund et al. 2007). To 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 & Pavia 2003, Dworjanyn et al. 2006, Nylund et al. 2007). First, the marine organisms need to be generally free of fouling organisms in their natural environment. Second, the presence of putative compounds on or near the surface of the organism needs to be verified and their natural concentrations quantified. Third, surface-associated concentrations of these compounds need to be tested in bioassays with ecologically relevant fouling species to determine whether these compounds have any antifouling effects.

The aim of the present study was to identify surface-associated chemical defences against fouling in the tropical sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* by investigating the effects of conditioned seawater, whole body extracts and selected surface-associated compounds of these sea stars on the settlement of the ecologically relevant diatoms *Amphora* sp. and *Nitzschia closterium*, the serpulid polychaete *Hydroides elegans* and the bryozoan *Bugula neritina*, which are commonly found in tropical and subtropical waters (Hall 1984, Hallegraeff & Jeffrey 1984, Lewis et al. 2006, Gottschalk et al. 2007).

MATERIALS AND METHODS

Collection of specimens. Live specimens of the sea stars *Linckia laevigata* and *Fromia indica* were collected from John Brewer Reef (18° 38′ S, 147° 03′ E), *Cryptasterina pentagona* from Kissing Point in Townsville (19° 14′ S, 146° 48′ E) and *Archaster typicus* from Picnic Bay on Magnetic Island (19° 11′ S, 146° 51′ E), Queensland, Australia, in February 2006. Specimens were transported in ambient seawater and kept temporarily in an outdoor recirculating tank (26°C, 35 ppt) at the Marine and Aquaculture Research Facilities Unit at James Cook University.

Conditioned seawater. To test the antifouling activity of waterborne compounds of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* using conditioned seawater, specimens of each species (n = 5) were blot-dried, weighed and then kept individually in plastic containers (*L. laevigata*) or glass beakers (*F. indica*, *C. pentagona* and *A. typicus*) with 0.45 µm filtered seawater (FSW) (75 ml FSW per 1 g of

sea star) and aerated for 24 h. Seawater controls were prepared by keeping FSW in plastic containers and glass beakers for the same amount of time.

Bioassays with conditioned seawater The conditioned seawater was used to run settlement assays with the diatoms *Amphora* sp. (CS-255, CSIRO) and *Nitzschia closterium* (CS-5, CSIRO) and the bryozoan *Bugula neritina*. The polychaete *Hydroides elegans* was not tested, because it was not present in sufficient numbers at our collection sites when the settlement assays were carried out. 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 cycle, Sylvania Tri-phosphor fluorescent lamps, 5200 lumens). To run 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 the 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. Furthermore, 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 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. To run 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 the temperature-controlled room in the dark for 24 h. Subsequently, settled and metamorphosed larvae were counted using a dissecting microscope.

Preparation of crude extracts. Specimens of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* (n = 5) were weighed, freeze-dried and reweighed. The freeze-dried tissues of each specimen were sequentially extracted 4 times with dichloromethane, followed by methanol and water (solvents of increasing polarity) at room temperature. Dichloromethane and methanol were HPLC grade (Mallinckrodt), and water was from a MilliQ water purification system (Millipore). The crude extracts of each specimen were filtered through glass wool and concentrated using a rotary evaporator. Concentrated crude extracts were removed and dichloromethane extracts air-dried, whereas methanol and water extracts were dried under reduced pressure with a speed vac concentrator. Solvent controls were prepared by concentrating and drying the same amounts of dichloromethane, methanol and water used for the extractions. Dry extracts were kept individually and 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, the sea star *Cryptasterina pentagona* was selected, because the dichloromethane crude extract of this species performed well in the settlement assays and this species was highly abundant at the collection site. The objective of this approach was to identify the structural characteristics of active compounds, and to guide quantification of active compounds from surface extracts. The body walls of 40 specimens were dissected, weighed, freeze-dried, reweighed and then extracted 4 times with dichloromethane. The resulting crude extract was filtered, 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).

VLC and HPLC. The dichloromethane extract of *Cryptasterina 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. Resultant fractions were dried and stored at –18°C until redissolved for use in settlement assays with the diatom *Nitzschia closterium* or for further fractionation by HPLC. Fractions were also separated using reversed-phase thin-layer chromatography (RP-TLC; Merck, RP-18), using methanol as a solvent and visualised with a 10% solution of sulphuric acid in ethanol, and 0.1% vanillin, followed by heating to identify sensitive compounds.

The most bioactive fractions, F3-5 (216 mg) and F6-8 (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 employing 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 F3-5 and F6-8 were fractionated into a further 12 and 13 fractions, respectively. Based on the HPLC separation, selected fractions with dry weights ≥2.2 mg were tested at a concentration of 10 µg cm^{-2} against the settlement of *Nitzschia closterium*.

Bioassays with crude extracts. To test the antifouling activity of individual crude extracts of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and

Archaster typicus, dichloromethane and methanol extracts were redissolved in ethanol, and water extracts were redissolved in water. Extracts and solvent controls were applied to clear polystyrene Petri dishes (n = 5) and placed on an orbital shaker at 75 rpm for 4 h to ensure even surface films at concentrations of 100, 10, 1 and 0.1 μg cm^{-2} and complete evaporation of the solvents. Fractions of the bulk 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 to moderately polar and are only partially soluble in seawater, giving approximate surface concentrations of 100, 10, 1 and 0.1 μg cm^{-2}. Water extracts are completely 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}, considering the surface area of the base of the Petri dishes (9 cm^2) and the amount of FSW in each Petri dish (4 ml).

The settlement assays with *Amphora* sp., *Nitzschia closterium* and *Bugula neritina* were performed as described above, using 0.45 μm FSW instead of conditioned seawater. To run settlement assays with larvae of *Hydroides elegans*, adult specimens were collected from settlement plates at the Breakwater Marina in Townsville. 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 no. I5879) was used to induce settlement (Bryan et al. 1997, Lau & Qian 1997). Petri dishes with extracts and solvent controls (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.

Identification of potential antifouling compounds. To identify the types of compounds in the bioactive fractions of the dichloromethane extract of *Cryptasterina pentagona*, the bioactive 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 using 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 to 1000. Direct infusion of the sample (0.2 mg ml^{-1}) was carried out using a SGE 250 μl syringe and a Cole Palmer 74900 syringe pump at a flow rate of 100 μl h^{-1}.

Analysis with NMR demonstrated that mainly fatty acids and sterols were present in the most bioactive fractions of *Cryptasterina pentagona*; subsequently, many of these were identified using gas chromatography-mass spectrometry (GC-MS). The fatty acids were converted to their 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 a methylation solution consisting 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 twice with 1.8 ml of hexane:dichloromethane (4:1); the solvent fraction was eluted through a column packed with sodium sulphate to remove water and dried under nitrogen. Finally, 100 μl of hexane were added and the resultant sample transferred to GC-MS vials. Samples were analysed on a GC-MS system (Agilent 5973) equipped with a 30 m DB5-MS column, a sample flow rate of 1.0 ml min^{-1} of helium as carrier gas and an oven temperature gradient of 50 to 310°C at 4°C min^{-1}; a hold time of 10 min was employed. The mass spectrometer scanned within a mass range of m/z 50 to 550. A series of fatty acid methyl esters and sterol standards were also analysed. Compounds were identified based on their retention time and mass spectra matching those of the standards. Unknowns were identified employing National Institute of Standards and Technology (NIST) and Wiley GC-MS databases.

Extraction and bioassays with surface-associated compounds. To test the antifouling activity of the surface-associated compounds of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus*, mixed cellulose ester filter papers (Advantec MFS, diameter: 3 cm, n = 6 for *Amphora* sp., *Nitzschia closterium* and *Bugula neritina*, n = 10 for *Hydroides elegans*) were placed on the central aboral surface of each sea star for 10 s to absorb any surface-associated compounds. These filter papers were subsequently placed in the Petri dishes, and 4 ml of 0.45 μm FSW were added. The settlement assays with *Amphora* sp., *N. closterium*, *H. elegans* and *B. neritina* were run as

described above, however, attached diatoms were counted using a Leica DM LB fluorescence microscope rather than a compound microscope.

Identification of surface-associated compounds. The surface-associated compounds of each specimen of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* (n = 3) were absorbed using cotton wool (Schmitt et al. 1995, Kubanek et al. 2002) that had been pre-washed twice with dichloromethane and twice with methanol. Cotton wool was used as the solid extraction medium because attempts to use mixed cellulose ester filter paper proved problematic. Small cotton balls were used to swab a projected surface area of 4 cm^2 on the aboral surface of each specimen. These were 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. To identify the compounds using GC-MS, the surface extracts were derivatized with a trimethyl silane (TMS) reagent, converting all compounds with OH functionality, including the fatty acids and sterols, to their TMS derivatives, and so facilitating better peak resolution on the GC-MS. 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 analysis conditions were used for these samples as for the crude extract samples. A series of fatty acid methyl esters and sterol standards of known concentrations were also run. Compounds were identified based on retention time and mass spectra match 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).

Bioassays with hexadecanoic acid, cholesterol, lathosterol and sitosterol. Based on the GC-MS analyses of the surface extracts, hexadecanoic acid, cholesterol, lathosterol and sitosterol were selected for further testing against the settlement of *Amphora* sp., *Nitzschia closterium* and *Bugula neritina*. *Hydroides elegans* was not tested because it was not present in sufficient numbers at our collection sites when the settlement assays were carried out. Hexadecanoic acid (catalogue no. 76119), cholesterol (C8667), lathosterol (C3652) and sitosterol (C1270) were purchased from Sigma-Aldrich. These compounds were dissolved in ethanol and applied to clear polystyrene Petri dishes (Sarstedt, diameter: 3.5 cm, base surface area: 9 cm^2) at surface-associated concentrations of 100, 10, 1 and 0.1 ng cm^{-2} (n = 5). Settlement assays were performed as described above.

Statistical analysis. All statistical analyses were performed with SPSS version 14. To determine significant differences in the settlement of the diatoms *Amphora* sp. and *Nitzschia closterium* between treatments, the results were analysed with a 2-factor nested ANOVA followed by Tukey's honestly significant difference (HSD) multiple comparison test. Results on the metamorphosis of *Hydroides elegans* and *Bugula neritina* were analysed with a 1-factor ANOVA followed by Tukey's HSD multiple comparison test. 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). In settlement assays with *H. elegans* and *B. neritina*, some experimental groups had mean metamorphosis of 0% or 100% and no variance; these groups were excluded from the statistical analyses.

RESULTS

Bioassays with conditioned seawater

To determine whether sea stars release compounds into the water column to deter fouling, conditioned seawater of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* was used to run settlement assays with the diatoms *Amphora* sp. and *Nitzschia closterium* and the bryozoan *Bugula neritina*. The conditioned seawater of all 4 sea star species did not have any significant effects on the settlement of *Amphora* sp. (F = 1.178, p = 0.324), *N. closterium* (F = 0.876, p = 0.500) or *B. neritina* (F = 1.058, p = 0.407).

Natural concentrations of crude extracts

The mean natural concentrations of the dichloromethane, methanol and water extracts of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* were similar between sea star species, except for the dichloromethane extract of *C. pentagona*, which was approximately 3 to 10 times higher than that of the other 3 sea star species (Table 1). Overall, the mean natural concentration of the methanol

Table 1. Natural concentrations in mg g^{-1} (dry weight:wet weight) 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
L. laevigata	2.41 ± 0.48	21.94 ± 1.21	6.41 ± 0.40
F. indica	6.63 ± 0.34	24.55 ± 0.75	9.13 ± 0.54
C. pentagona	25.94 ± 4.98	27.42 ± 2.73	8.70 ± 0.70
A. typicus	8.51 ± 1.40	25.59 ± 1.64	8.87 ± 0.47

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} (dry weight:wet weight) (Table 1).

Bioassays with crude extracts

The dichloromethane, methanol and water extracts of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* had concentration-dependent effects on the settlement of *Amphora* sp., *Nitzschia closterium*, *Hydroides elegans* and *Bugula 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 ($p < 0.05$). 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 (Table 2).

For *Linckia laevigata*, the dichloromethane extract was the most effective extract in reducing the settlement of fouling organisms, with concentrations of ≥1 and ≥0.1 µg cm^{-2} significantly reducing the settlement of *Nitzschia closterium* (F = 38.452, $p < 0.001$) and *Hydroides elegans* (F = 17.283, $p < 0.001$), respectively (Table 2). The methanol extract was also very effective, with concentrations of ≥1 µg cm^{-2} significantly reducing the settlement of *N. closterium* (F = 33.375, $p < 0.001$) and *H. elegans* (F = 13.802, $p < 0.001$). The water extract at concentrations of ≥10 µg cm^{-2} significantly reduced the settlement of *N. closterium* (F = 22.971, $p < 0.001$), whereas concentrations of ≥100 µg cm^{-2} significantly reduced the settlement of *Amphora* sp. (F = 6.042, $p < 0.001$), *H. elegans* (F = 9.178, $p < 0.001$) and *Bugula neritina* (F = 3.476, $p < 0.05$).

For *Fromia indica*, very low concentrations of all crude extracts were effective in deterring settlement of most of the fouling species (Table 2). The dichloromethane extract at a concentration of ≥0.1 µg cm^{-2} significantly reduced the settlement of *Amphora* sp. (F = 41.817, $p < 0.001$), whereas methanol and water extracts at the same concentrations significantly reduced the settlement of *Hydroides elegans* (methanol: F = 20.066, $p < 0.001$; water: F = 14.375, $p < 0.001$). There was also no settlement of *Bugula neritina* larvae when exposed to the dichloromethane and methanol extracts at concentrations of ≥10 µg cm^{-2}.

For *Cryptasterina pentagona*, the dichloromethane and methanol extracts were the most effective extracts at deterring the settlement of fouling species (Table 2). The dichloromethane extract at concentrations of ≥1 µg cm^{-2} significantly reduced the settlement of *Nitzschia closterium* (F = 73.600, $p < 0.001$) and *Hydroides elegans* (F = 12.116, $p < 0.001$). The methanol extract at concentrations of ≥1 and ≥0.1 µg cm^{-2} also significantly reduced the settlement of *N. closterium* (F = 31.653, $p < 0.001$) and *H. elegans* (F = 15.585, $p < 0.001$), respectively. The settlement of *Bugula neritina* larvae was completely inhibited when they were exposed to the methanol extract at a concentration of 100 µg cm^{-2}. The water extract at concentrations of ≥10 µg cm^{-2} was effective against the settlement of *N. closterium* (F = 48.232, $p < 0.001$).

Finally, for *Archaster typicus*, the dichloromethane extract was also the most effective extract against the settlement of fouling species (Table 2). The dichloromethane extract at concentrations of ≥0.1 µg cm^{-2} significantly reduced the settlement of *Nitzschia closterium* (F = 58.915, $p < 0.001$), and at concentrations of ≥1 µg cm^{-2} significantly reduced the settlement of *Amphora* sp. (F = 42.676, $p < 0.001$) and *Hydroides elegans* (F = 16.595, $p < 0.001$). Both the methanol and water extracts at concentrations of ≥10 µg cm^{-2} were effective against the settle-

Table 2. Lowest concentrations (µg cm^{-2}) of the dichloromethane (DCM), methanol (MeOH) and water (H_2O) extracts of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus*, which significantly deterred the settlement of *Amphora* sp., *Nitzschia closterium*, *Hydroides elegans* and *Bugula neritina*. Concentrations tested: 100, 10, 1 and 0.1 µg cm^{-2}

		Amphora sp.	*N. closterium*	*H. elegans*	*B. neritina*
L. laevigata	DCM	10	1	0.1	10
	MeOH	10	1	1	10
	H_2O	100	10	100	100
F. indica	DCM	0.1	1	10	10
	MeOH	10	10	0.1	10
	H_2O	100	10	0.1	100
C. pentagona	DCM	10	1	1	10
	MeOH	10	1	0.1	10
	H_2O	100	10	100	100
A. typicus	DCM	1	0.1	1	10
	MeOH	100	10	10	10
	H_2O	100	10	100	100

ment of *N. closterium* (methanol: $F = 37.157$, $p < 0.001$; water: $F = 33.825$, $p < 0.001$), whereas the methanol extract at the same concentration also deterred the settlement of *H. elegans* ($F = 12.020$, $p < 0.001$). There was no settlement of *H. elegans* when a concentration of 100 µg cm^{-2} of both methanol and water extracts was used.

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

Due to the strong and broad spectrum activity of the dichloromethane extract of *Cryptasterina pentagona*, this extract 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 *Nitzschia closterium* as an indicator of overall activity. All fractions significantly reduced the settlement of *N. closterium* ($p < 0.05$) (Fig. 1), with the fractions 5, 6, 7, 8, 9 and 12 being the most effective. Furthermore, 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* (Fig. 2).

Identification of potential antifouling compounds

The ^{1}H NMR data on the active fractions of the dichloromethane extract of *Cryptasterina pentagona* showed that mixtures of fatty acids and sterols were

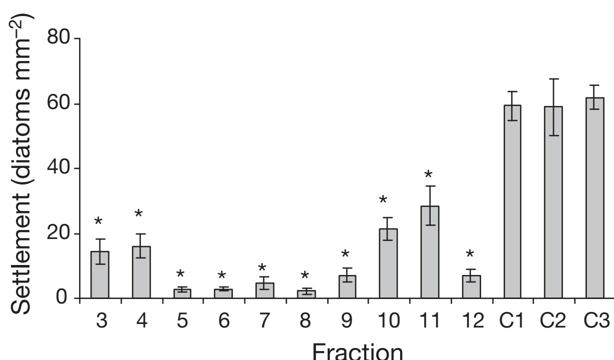

Fig. 1. *Nitzschia closterium*. Effects of vacuum liquid column chromatography fractions of the dichloromethane extract of the sea star *Cryptasterina pentagona* at a concentration of 10 µg cm^{-2} on the settlement of the diatom *N. closterium*. C1: dichloromethane control; C2: EtOH control; C3: filtered seawater control. Means ± SE are shown (n = 5 fields of view in each of 4 dishes). *: significant differences between the fractions and controls (Tukey's HSD multiple comparison test, $p < 0.05$). For details of fractions see 'Materials and methods, VLC and HPLC'

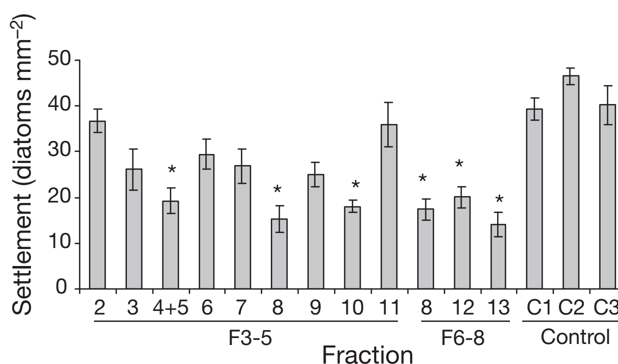

Fig. 2. *Nitzschia closterium*. Effects of HPLC fractions of the dichloromethane extract of the sea star *Cryptasterina pentagona* at a concentration of 10 µg cm^{-2} on the settlement of the diatom *N. closterium*. C1: dichloromethane control; C2: EtOH control; C3: filtered seawater control. Means ± SE are shown (n = 5 fields of view in each of 4 dishes). *: significant differences between the fractions and controls (Tukey's HSD multiple comparison test, $p < 0.05$). For details of fractions see 'Materials and methods, VLC and HPLC'

present in all fractions. The fatty acids and sterols in each active fraction were further identified using GC-MS. A variety of medium-chain length 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 3. Cholesterol was present in all fractions, bar one. Octadecanoic acid (C18:0) and hexadecanoic acid (C16:0) were present in many of the active fractions, while other fatty acids only occurred in single fractions (Table 3).

Bioassays with surface-associated compounds

The surface-associated compounds of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* were absorbed onto mixed cellulose filter papers, which were then used to perform settlement assays with *Amphora* sp., *Nitzschia closterium*, *Hydroides elegans* and *Bugula neritina*. The extraction of the filter paper (FP control) had no significant effect on the settlement of any organism tested in FSW, validating the method used in identifying the effects of surface-associated compounds (Fig. 3). Overall, the surface-associated compounds of *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 *L. laevigata* significantly reduced the settlement of *N. closterium* by 42% to only 30.8 ± 3.7 diatoms mm^{-2} ($F = 67.652$, $p < 0.001$) (Fig. 3B), they had no significant effects on the settlement of *Amphora* sp., *H. elegans* or *B. neritina*

Table 3. Most common fatty acids and sterols in active fractions of the dichloromethane extract of the sea star *Cryptasterina pentagona*. VLC: vacuum liquid column chromatography

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

(Fig. 3A,C,D). The surface-associated compounds of *F. indica* and *A. typicus* significantly reduced the settlement of both *Amphora* sp. (*F. indica*: $F = 3.681$, $p = 0.03$; *A. typicus*: $F = 5.111$, $p = 0.008$) and *N. closterium* (*F. indica*: $F = 38.377$, $p < 0.001$; *A. typicus*: $F = 29.370$, $p < 0.001$), but not *H. elegans* or *B. neritina* (Fig. 3E–H,M–P). Surface-associated compounds of *F. indica* reduced the settlement of *Amphora* sp. and *N. closterium* by 24% (Fig. 3E) and 58% (Fig. 3F), respectively, whereas those of *A. typicus* reduced them by 23% (Fig. 3M) and 31% (Fig. 3N), respectively. In contrast, the surface-associated compounds of *C. pentagona* were the most effective compounds of the 4 sea star species, significantly reducing the settlement of 3 fouling species: *Amphora* sp. ($F = 7.595$, $p = 0.001$), *N. closterium* ($F = 16.020$, $p < 0.001$) and *H. elegans* ($F = 19.343$, $p < 0.001$) by 25% (Fig. 3I), 33% (Fig. 3J) and 38% (Fig. 3K), 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 (Fig. 3L).

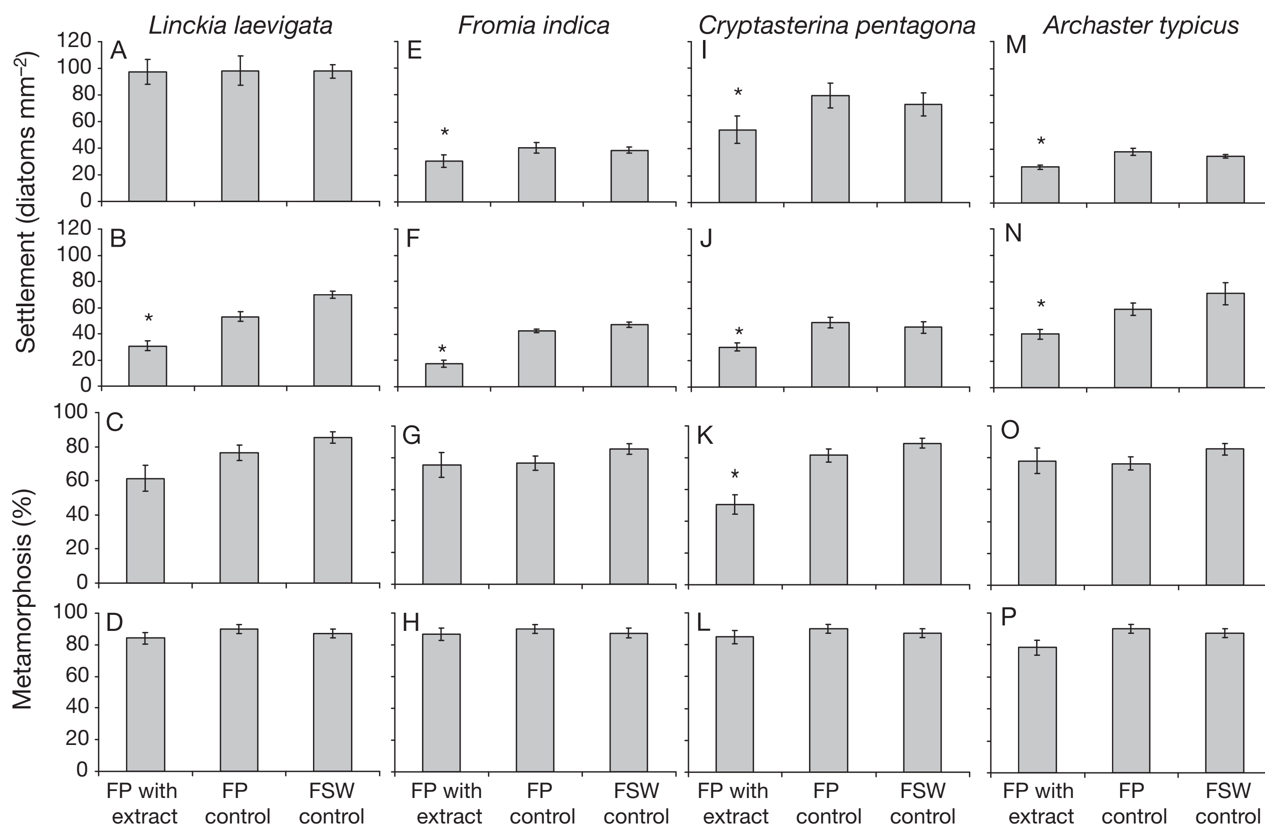

Fig. 3. *Amphora* sp., *Nitzschia closterium*, *Hydroides elegans* and *Bugula neritina*. Effects of surface-associated compounds of the sea stars *Linckia laevigata* (A–D), *Fromia indica* (E–H), *Cryptasterina pentagona* (I–L) and *Archaster typicus* (M–P) on the settlement (diatoms mm^{-2}) of *Amphora* sp. (A,E,I,M) and *N. closterium* (B,F,J,N) and the metamorphosis (%) of *H. elegans* (C,G,K,O) and *B. neritina* (D,H,L,P). 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 (Tukey's HSD multiple comparison test, $p < 0.05$)

Analysis with GC-MS

The compounds of the surface extracts of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* were identified employing GC-MS. Based on their high abundance, the fatty acid hexadecanoic acid and the sterols cholesterol, lathosterol and sitosterol 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 species (*L. laevigata*, *C. pentagona* and *A. typicus*) and sitosterol on only 2 species (*L. laevigata* and *F. indica*) (Table 4). 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 star 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 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 were tested against *Amphora* sp., *Nitzschia closterium* and *Bugula neritina* at the surface-associated concentrations 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. (hexadecanoic acid: $F = 8.983$, $p < 0.001$; cholesterol: $F = 3.762$, $p = 0.003$) and *N. closterium* (hexadecanoic acid: $F = 11.261$, $p < 0.001$; cholesterol: $F = 20.141$, $p < 0.001$) at concentrations of 1000 and ≥10 ng cm^{-2}, respectively (Figs. 4A,B & 5A,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 (Fig. 5A,B). Lathosterol also significantly inhibited the settlement of the diatom species at concentrations of 1000 and ≥100 ng cm^{-2} for *Amphora* sp. ($F = 8.636$, $p < 0.001$) and *N. closterium* ($F = 29.128$, $p < 0.001$), respectively (Figs. 4C & 5C). 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* ($F = 6.146$, $p < 0.001$), with significantly less settlement of this species at concentrations of ≥100 ng cm^{-2} (Fig. 5D). At concentrations of 1000 ng cm^{-2} and 100 ng cm^{-2}, the settlement of *N. closterium* was reduced by 29 and 35%, respectively. The settlement of *B. neritina* was not significantly affected by any of the 4 compounds.

Table 4. 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

DISCUSSION

The conditioned seawater of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* had no effects on the settlement of the ecologically relevant diatoms *Amphora* sp. and *Nitzschia closterium* and the bryozoan *Bugula 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 effects, deterring the settlement of *Amphora* sp., *N. closterium* and the serpulid polychaete *H. elegans*, but not *B. neritina*. Subsequently, surface-associated compounds were identified, quantified and also tested at these concentrations. The surface-associated compounds hexadecanoic acid, cholesterol and lathosterol deterred the settlement of *N. closterium* at concentrations measured on the surface of sea stars, but not *Amphora* sp. or *B. neritina*. Sitosterol also deterred the settlement of *N. closterium* at concentrations of ≥100 ng cm^{-2}; however, these concentrations are considerably higher than those found on the sea stars *L. laevigata* and *F. indica* and are therefore not ecologically relevant. As a caveat, the compounds hexadecanoic acid, cholesterol, lathosterol and sitosterol adhere to the surface and do not read-

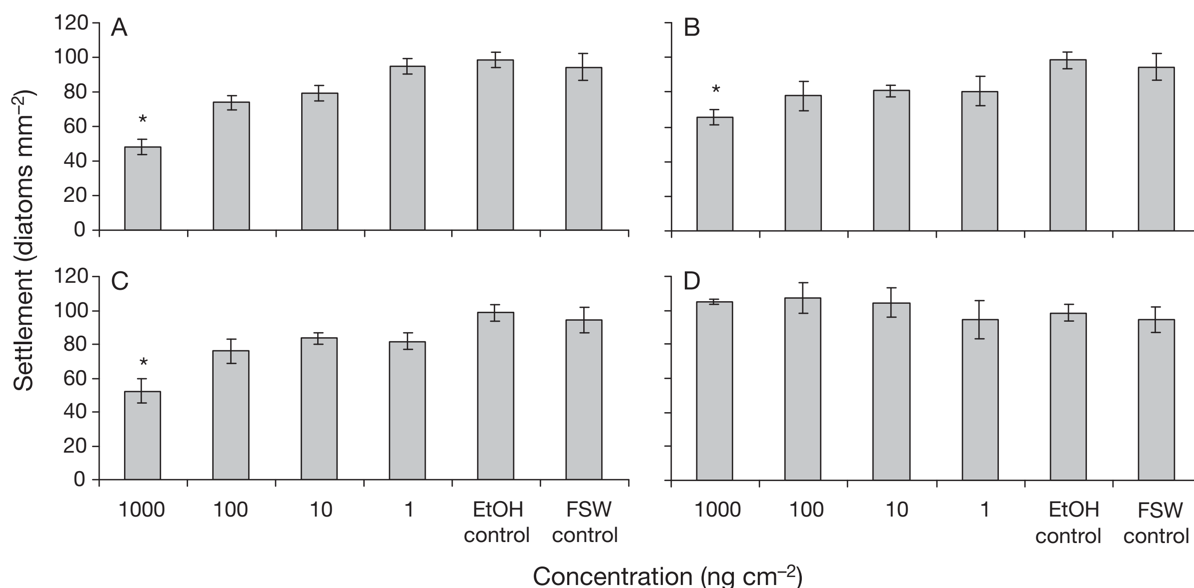

Fig. 4. *Amphora* sp. Effects of (A) hexadecanoic acid, (B) cholesterol, (C) lathosterol and (D) sitosterol on the settlement of *Amphora* sp. (diatoms mm^{-2}). FSW: filtered seawater. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). *: significant differences between the treatment and controls (Tukey's HSD multiple comparison test, $p < 0.05$)

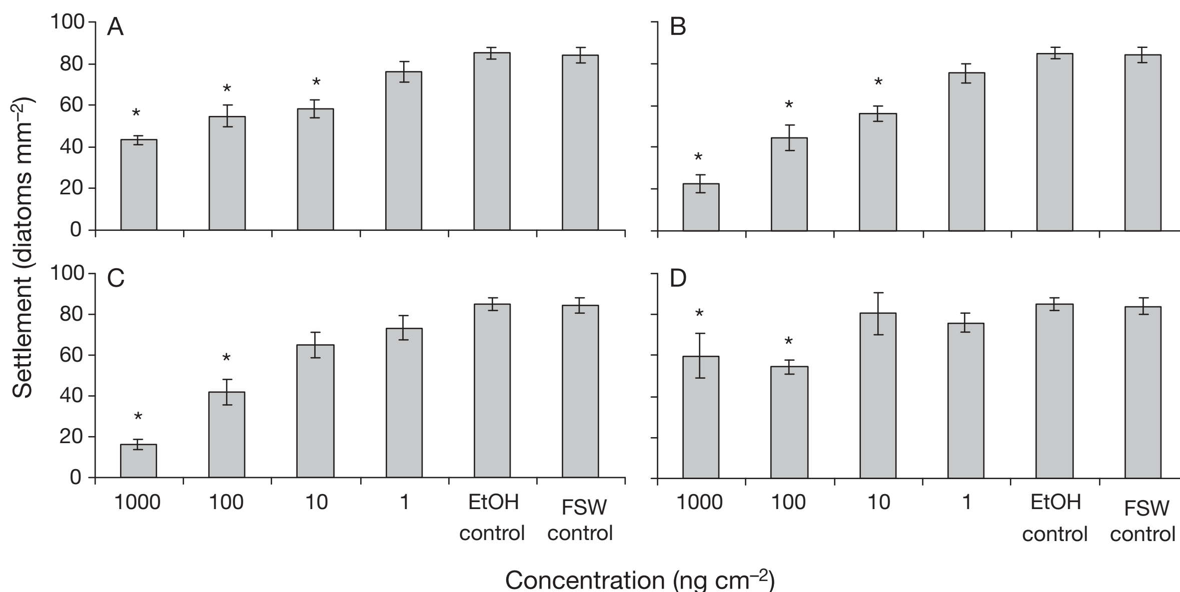

Fig. 5. *Nitzschia closterium*. Effects of (A) hexadecanoic acid, (B) cholesterol, (C) lathosterol and (D) sitosterol on the settlement of *N. closterium* (diatoms mm^{-2}). FSW: filtered seawater. Means ± SE are shown (n = 5 fields of view in each of 5 dishes). *: significant differences between the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

ily dissolve into the surrounding water. We have been able to extract these from the surface of the sea stars, but the extraction efficiency using the methods employed has not been determined, and if anything they will underestimate surface concentrations.

Based on the results of the present study, the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol of *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus* are primary metabolites with a secondary function in fouling control. Previous studies on chemical antifouling defences of sea stars have identified the antifouling role of crude extracts and 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. 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). However, the literature documenting the secondary function of primary metabolites in marine organisms is growing. 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* is 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*, with C16:0 and C16:1 fatty acids having 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 *Mytilus edulis* (Mizobuchi et al. 1993). Furthermore, crude extracts of the sponge *Crella incrustans* and cholesterol controls had a negative effect on the settlement of larvae of the ascidian *Podoclavella moluccensis* (Davis 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 *Mycale laxissima* from the Bahamas might result from the differences in the fatty acid profiles of these sponges. *Mycale 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).

The natural concentrations of surface-associated compounds of the sea stars *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster 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 the present study. While the swabbing technique was not optimal, it was suitable for the present study because it allowed the extraction of surface-associated compounds from animal tissues, while the extraction of compounds from within the cells was minimized. We acknowledge that not all active metabolites will be removed, identified, or quantified using this technique, but this method provides a first step to understanding the complexity of antifouling defences for benthic invertebrates.

The production of metabolites of marine organisms is dependent 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, since most of the sterols are derived from the diet and only a few sterols are synthesized de novo (Voogt & 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 become 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.

While the surface-associated compounds hexadecanoic acid, cholesterol and lathosterol are a chemical defence against the diatom *Nitzschia closterium*, it remains unclear whether these surface-associated compounds are produced by the sea stars or by their surface-associated micro-organisms. Tropical sea stars, including *Linckia laevigata*, *Fromia indica*, *Cryptasterina pentagona* and *Archaster typicus*, have bacteria living on their surfaces, with bacterial abundances being approximately 10^4 to 10^5 cells cm^{-2} (Guenther et al. 2007a). These bacteria may have a symbiotic role, releasing compounds, such as fatty acids and sterols, which help reduce or prevent the settlement of other fouling organisms. For example, bacterial communities on both the alga *Ulva reticulata* and the sponge *Mycale adhaerens* inhibit the settlement of the serpulid polychaete *Hydroides elegans* (Dobretsov & Qian 2002, Lee et al. 2006). The isolation and culture

of surface-associated micro-organisms 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.

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