Micro-fabricated polydimethyl siloxane (PDMS) surfaces regulate the development of marine microbial biofilm communities

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This study explored an antifouling (AF) concept based on deployment of microfabricated polydimethyl siloxane (PDMS) surfaces with 1–10 μm periodicity corrugated topographies in temperate marine waters. The effect of the surfaces on the development of microbial biofilms over 28 days and during different seasons, including both summer and winter, was examined using confocal laser scanning microscopy (CLSM) as well as terminal restriction fragment (T-RF) analysis for phylogenetic fingerprinting. The microscale topography significantly impacted biofilm development by altering the attachment pattern and reducing microcolony formation on the 1, 2 and 4 μm PDMS surfaces. Also, field deployments over 28 days showed a significant reduction in biovolume on the 4 and 10 μm PDMS surfaces despite altered environmental conditions. The microfabricated PDMS surfaces further significantly impacted on the community composition of the biofilms, as revealed by changes in T-RF profiles, at different stages of development. Moreover, altered biofilm resistance was demonstrated by exposing pre-established biofilms on 10 μm micro-fabricated surfaces to enhanced flagellate predation by a heterotrophic protist, Rhynchomonas nasuta. Pronounced changes in the overall marine microbial biofilm development as well as community composition warrant exploring substratum modification for marine AF applications.

Key words: marine biofouling; surface modification; biofilms; microbial community analysis; terminal restriction fragments analysis

Introduction

Manmade surfaces submerged in marine waters are rapidly colonised by fouling organisms, resulting in attachment and developmental processes collectively known as biofouling (Nakasono et al. 1993; Carl et al. 2011; Briand et al. 2012). Multiple and potentially interdependent stages of development are involved in microfouling or biofilm development, which includes microbial colonisation and proliferation, aggregation and microcolony formation (Flemming 2002; Monds & O’Toole 2009), as well as macrofouling, where surfaces are colonised by barnacles, molluscs and various other macroorganisms (Characklis & Cooksey 1983; Callow & Callow 2002). These biofouling processes negatively impact on a range of maritime industries (Townsin 2003; Fitridge et al. 2012) resulting in, for example, increased hydrodynamic drag (Schultz et al. 2011) and microbially induced corrosion (Otero et al. 2004).

Novel environmentally benign technologies aimed at reducing marine biofouling include the use of fouling-release (FR) coatings (Mieszkin et al. 2012), non-toxic detergents (Steinberg et al. 1997; Murugan & Ramasamy 2003; Kristensen et al. 2008), biodegradable polymer coatings (Yu 2003; Faï et al. 2007), fibre flocking (Phillippi et al. 2001,) and inorganic coatings of biocidal agents (Rajagopal et al. 2006; Hajkova et al. 2007; Carl, Poole, Sexton et al. 2012; Vucko et al. 2012). Several technologies based on observations on the varying degrees of biofouling on natural marine surfaces and progress made in research on biomimicry have also been explored (Armstrong et al. 2001; Carl, Poole, Sexton et al. 2012; Carl, Poole, Vucko et al. 2012; Vucko et al. 2014). For example, antifouling (AF) strategies have been adopted from living marine organisms that deter biofouling on their surfaces (Clare 1996), including the use of bioactive AF compounds isolated from a marine seaweed (Maneeld et al. 2006; Paul et al. 2006) and surface microtopography mimicking the surface features of sharkskin (Schumacher et al. 2007). For surface topography modifications, substratum structures such as channels (Hoipkemeier-Wilson et al. 2004), micro-ripples (Bers & Wahl 2004) and longitudinal ridges can all influence the settlement of various marine macrofouling organisms, including algal spores (Carman et al. 2006)

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and diatoms (Sweat & Johnson 2013). In addition, surface topography-dependent attachment was reported for the model biofilm forming bacterium Pseudomonas aeruginosa (Kaufmann et al. 2002; Whitehead et al. 2005). Proposed mechanisms for reducing biofouling by such technologies include changes in surface free energy (Decker et al. 2013), alteration in the attachment points and adhesion strength of the fouling organisms (Scardino et al. 2008), which facilitate easy removal of surface associated biota. A previous report on the surface topography-dependent impacts on specific macrofouling events suggests that the resulting decrease in biofouling may be due to reduced colonisation and biofilm formation by microorganisms rather than, or in addition to, a direct effect on the settlement of higher sessile organisms (Schumacher et al. 2007; Mieszkin et al. 2013).

Here, microfabricated polydimethyl siloxane (PDMS) surfaces were employed to study the effect of surface patterning on the development of natural microbial biofilms on submerged surfaces in marine waters over a period of 28 days, and during different seasons. The attachment pattern of microbial fouling organisms, microcolony formation, and the established biofilm biomass were observed by confocal laser scanning microscopy (CLSM). The microbial community composition of established biofilms on the surfaces was assessed by a molecular based phylogenetic fingerprinting method in order to reveal surface topography-dependent changes. Further, the resistance of established marine biofilms on microfabricated surfaces was challenged by protist predation to highlight topography-dependent effects. An assessment of the relative contributions of intrinsic (surface modification) and extrinsic (environmental parameters) factors on biofilm formation during different seasons was enabled by the sampling design and information available on environmental conditions that could impact on the microbial fouling processes examined in this study.

Material and methods

Surface preparation and pretreatment for field deployment

The tested surfaces were designed with corrugated microscale topographies, containing a square wave profile of groove widths of 1, 2, 4 and 10 μm, which resulted in long trenches (Supplementary Figure S1; Supplementary material is available via a multimedia link on the online article webpage.) (Vucko et al. 2014). The textures were produced by casting to avoid the stresses and associated irregularities that could occur during the alternative process of stamping. The 1 and 2 μm surfaces were created using 125 × 125 mm template casting photopolymer plates containing microscale groove widths of 1 and 2 μm respectively; a groove depth of 0.5 μm was made using a Leica EBMF 10 Electron Beam Lithography (EBL; Leica EBMF 10.5, Wetzlar, Germany) writer. The 4 and 10 μm surfaces were made using grating samples with groove widths of 4 and 10 μm, with a grating ratio of 1:1 in the grating width:depth, on 75 × 75 mm plates using spin on photoresists (maP-1200 series; Micro Resist Technology GmbH, Berlin, Germany) technique. Master plates were made by copying EBL and spin resist plates into nickel electroform. Surfaces used in this study were prepared using Dow Corning Sylgard 184 PDMS (Dow Corning, Midland, USA) in a 10:1 ratio of base elastomer:curing agent. The two components were mixed thoroughly and degassed in a vacuum desiccator. A thin layer of release agent, CRC 5–56 (Castle Hill, Australia), was sprayed onto the nickel shim before PDMS was poured onto the grating surface and cured for 30 min at 75°C. PDMS and epoxy surfaces without corrugated topography were created as control (0 μm) surface treatments and surface material respectively (Bloecher et al. 2013).

The PDMS surfaces with 0, 1, 2, 4 and 10 μm microscale topography used in this study were cut into circular coupons, with a radius of 13 mm using an industrial cutter. Circular microfabricated surfaces were treated using 3% v/v mild liquid detergent (Pyronog, Suma, Manukau, New Zealand), followed by rinsing with sterile deionised water (MilliQ; Thermo-Fisher Scientific, Scoresby, Australia) and followed by ethanol (70% v/v). The circular microfabricated surfaces were placed at the bottom of individual wells in 24-well microtitre plates (Falcon ® Becton Dickinson, North Ryde, Australia) in triplicate for each type of surface topography for field deployment. These pre-treatment steps were carried out one day prior to field deployment.

Marine field experiments using microfabricated PDMS surfaces

The microfabricated PDMS surfaces were deployed at the Sydney Institute of Marine Science (SIMS) at Chowder Bay, Mosman Sydney (latitude: 34°S and longitude: 151°E). The microfabricated surface coupons with 15 mm diameter were fitted tightly to the base of the 24-well microtitre plate wells (BD), which were covered with 1 cm × 1 cm nylon mesh secured to the plate using fishing line. Triplicates of the microfabricated PDMS surfaces were randomly arranged within each plate, with individual plates representing a single time point. The plates were deployed on Chowder Bay wharf, secured by nylon rope at ~ 2 m depth at low tide. The depth ensured samples remained immersed at low tides. The plates were collected on days 2, 5, 7, 14, 21 and 28 and transported in calcium-magnesium-free seawater (CMFSW) (0.45 M NaCl, 10 mM KCl, 7 mM Na2SO4 and 0.5 mM NaHCO3, all chemicals were purchased from UNIVAR,
Ingleburn, Australia), with reduced ionic concentration to maintain microbial viability. The surfaces were processed within 1 h of collection to reduce changes in microbial community composition and structure. The field experiment was repeated three times, with three replicates for each surface topography (Table 1).

**Flagellate predation experiments.**

Two types of Table 1. Time and duration of community composition and structure. The within 1 h of collection to reduce changes in microbial viability. The surfaces were processed to maintain microbial viability. The surfaces were processed within 1 h of collection to reduce changes in microbial community composition and structure. The field experiment was repeated three times, with three replicates for each surface topography (Table 1).

**Predation experiments in environmental diffusion chambers.**

The resistance to grazing of established biofilms on microfabricated surfaces was examined using the heterotrophic flagellate, *Rhynchosoma nasuta*, in the field during the period from 15 March to 30 May 2011 (Table 1). *R. nasuta* cultures were routinely passaged in 15 ml of half strength marine salt solution (0.5 × NSS: 8.8 M NaCl, 0.935 M MgCl₂·6H₂O, 0.735 M Na₂SO₄, 0.205 M CaCl₂·2H₂O, 0.125 M KCl, 0.04 M NaHCO₃, 0.02 M KBr, 4 mM SrCl₂·6H₂O and 4 mM H₂BO₃; Mårdén et al. 1985) supplemented with 600 µl of heat-killed *Pseudomonas aeruginosa* PAO1 (overnight culture grown to OD₆₀₀ 1.5, treated at 65°C for 2 h) in 0.1 strength M9 medium (4.78 mM Na₂HPO₄, 2.2 mM K₂PO₄, 0.86 mM NaCl, 1.87 mM (NH₄)₂SO₄, 0.2 mM MgSO₄ and 0.01 mM CaCl₂). Cultures were stored in tissue culture flasks (Sarstedt Belmore, Australia) at room temperature (c.19°C) without shaking. Four to seven day old *R. nasuta* cultures were used in the grazing assays. Prior to experiments, the flagellates were enumerated using a haemocytometer viewed under a light microscope (Leica DMLB, Leica Microsystems, Wetzlar, Germany) at 400 × magnification.

Microfabricated PDMS surfaces were mounted onto glass slides fixed in environmental diffusion chambers (McFeters & Stuart 1972) sealed with 1.2 µm nitrocellulose membrane filters (MF-Millipore™ Membrane Filters, Millipore, Kilsyth, Australia) to allow establishment of natural microbial biofilm communities and exclude larger predators. In brief, the environmental diffusion chamber is a unit of three perspex pieces sandwiched using membrane filters commonly used to cultivate environmental microorganisms *in situ*, with a number of injection ports installed for biofilm studies (Kaeberlein et al. 2002). Each chamber housed 0, 1, 2, 4 and 10 µm micro-fabricated surfaces. The chambers were secured in a crate with 1.25 mm thick cable and nylon mesh (3 mm × 4 mm spacings) to protect the chambers from fish and other marine macroorganisms. The chambers were deployed at the same site and depth as the samples for the 28-day biofilm development field experiments (see previous section). After 15 days, half the chambers were inoculated with ~ 5.0 × 10⁵ *R. nasuta* ml⁻¹ via the injection ports (Erken et al. 2011). The biofilms were exposed to the elevated grazing for 5 days prior to sample collection.

**CLSM imaging of biofilms and image analysis.**

Marine biofilms established on the microfabricated PDMS surfaces were stained using Live/Dead® BacLight™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA) by submerging the samples in Syto® 9 (1 µM) and propidium iodide (1 µM) solution for 20 min prior to microscopic examination. CLSM imaging of the biofilms was performed using an upright CLSM Zeiss 710 microscope (Carl Zeiss, Oberkochen, Germany). Five random fields of view were imaged for each sample covering a surface area of 212.3 × 212.3 µm and Z-stack images were obtained using the ZENS2010 software package with a W Plan 40 × Dic M27 (Zeiss Microscopy, Thornwood, NY, USA) objective lens. The number of Z-stack slices taken varied depending on the biofilm height in each image, with each slice corresponding to 0.75 µm. Imaging software ZENS2010 was employed and standard settings included: the pinhole for the imaging set at 70.6 µm; the two lasers Alexa Fluor 488 (499/519) and pI (536/617) (Ex/Em wavelength in nm respectively; Carl Zeiss, Oberkochen, Germany); default laser intensity (2.0%); default gain set at 750; and 8-bit image resolution set at 1,024 × 1,024 image size.

The 3D CLSM images were analysed using IMARIS (Bitplane Scientific Software, Belfast, UK) image analysis software. The 3D images containing green fluorescent signals indicating live cells and red fluorescent signals indicating membrane-compromised cells were analysed using built-in algorithms (surface smoothing: 0.400 µm) to create artificial surfaces representing bacterial cells, presented in voxel (volume pixel) and expressed as ‘biovolume’. A smoothing radius equal to ~1 voxel was used to avoid any impact on volume measurements resulting

Table 1. Time and duration of field experiments and surfaces involved.

| Date (DD/MM) | Season/year | Duration (days) | Surfaces involved (µm) |
|--------------|-------------|-----------------|------------------------|
| 25/04–26/05  | Winter 2010 | 28              | 0, 1, 2, 4, 10         |
| 22/11–11/12  | Summer 2010 | 28              | 0, 1, 2, 4, 10         |
| 15/03–30/05**| Autumn 2011 | 20              | 0, 1, 2, 4, 10         |
| 9/11–7/12*   | Summer 2011 | 28              | 0, 1, 2, 4, 10         |

*Two types of 0 µm surfaces involved including PDMS and EPOXY.
**Flagellate predation experiments.
from using a high smoothing radius on small objects. An alternative channel for the combined green and red signals was created to calculate the total biovolume from overlapping individual fluorescent channels that would otherwise cause an overestimation of the biovolume. Information including microcolony number (clumps of 20 or more cells per 150 μm² of surface area) and biovolume were recorded.

**DNA extraction of attached microbial communities on microfabricated PDMS surfaces**

The microfabricated PDMS surfaces with established marine biofilms were submerged in 1 ml of CMSFW supplemented with 10 mM EDTA and 1% v/v 3 M™ rapid enzyme cleaner (3M) to dislodge and lyse attached cells. The solution was incubated at room temperature for 2 h in the dark on a platform orbital shaker (Ratek, Boronia, Australia; 80 rpm). The samples were subsequently mixed with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Fluka, Sigma-Aldrich, St Louis, MO, USA) and ~ 0.2 g of poly(vinylpyrrolidone) (PVPP) to remove excess proteins and humic acids. The samples were centrifuged at 10,000 × g (BOECO U-32R) at 4°C for 10 min and the aqueous phase was removed and treated with one volume of chloroform:isoamyl alcohol (24:1) (Fluka). The mixture was centrifuged for 10 min and the aqueous phase removed as above and 0.1 volume of sodium acetate (3 M NaOAc) and 0.6 volume of isopropanol were added to precipitate the genomic DNA as described by Wilson (2001). The extracted DNA was resuspended in 50 μl of TE buffer with 2 μl of RNase (Sigma, USA) and stored at −20°C until T-RF PCR amplification.

**Generation of T-RFfs from 16S rRNA genes of the attached microbial communities**

Segments of the 16S rRNA gene (V1–V3 regions) were amplified using the DNA extracted from the biofilms formed on microfabricated PDMS surfaces using modified primer sequences: forward primer 27F 6FAM, 5′-FAM AGA GTT TGA TCM TGG CTC AG-3′; and reverse primer 519R, 5′-GWA TTA CCG CGG CKG CTG-3′. The PCR reactions contained 5 nmol of forward and reverse primers, 15 μl of EconoTaq™ Plus Green 2 × Master Mix (Lucigen, Middleton, WI, USA), ~ 0.5 ng of DNA template, and molecular grade water to 30 μl. Positive and negative controls contained *P. aeruginosa* PA01 (Holloway 1955) DNA and sterile molecular grade water respectively. The PCR reactions were performed using a PCR Thermocycler (Eppendorf, Hamburg, Germany) with the cycle conditions as follows: initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products were electrophoresed using sodium borate (SB) buffer (pH 7.8) and DNA concentration determined using spectrophotometry (Nanodrop ND-1000, ThermoScientific, Wilmington, DE, USA).

PCR products were purified using DNA Clean & Concentrator™ Kit (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer’s instructions. The elution steps were modified by including 5 min incubation after the inoculation of 22 μl of molecular grade water (note: 2 μl is absorbed by the silica column with 20 μl in actual eluted solution). The samples were eluted by centrifugation at 13,000 × g for 30 s. DNA samples were quantified by spectrophotometry (Nanodrop, Biolab) to calculate the volume required to provide ~ 200 ng of DNA for the restriction enzyme digestion.

Restriction enzyme digest reactions contained 2 μl of 10 × NEB 4 Buffer, 5 U of *Hae*III restriction endonuclease (NEB Biolab), ~ 200 ng of purified T-RFLP PCR product, and sterile molecular grade water to 20 μl. The reaction mix was incubated at 37°C for 4 h followed by incubation at 65°C for 20 min to denature the restriction enzyme. Digested T-RF samples were purified using DNA Clean & Concentrator™ Kit (Zymo, USA) by eluting 20 μl of the final solution to give 10 ng μl⁻1 of T-RFs. Purified T-RF samples were subjected to fragment analysis.

**T-RF fragments analysis**

T-RF files were analysed using Peak Scanner™ (version 1.0; Applied Biosystems, Forster City, CA, USA). The T-RFs were identified by comparison to a GSLIZ 500 fragment ladder. T-RFs < 30 bp and >550 bp were eliminated from analysis as they were outside the size standard range. All peaks above a fluorescence unit of 1 were exported into the online T-RF analysis pipeline T-REX (T-RFLP analysis Expedited; http://trex.biolpce.org/) (Culman et al. 2009). In T-REX, ‘true’ T-RFs were filtered from background noise (Abdo et al. 2006) and aligned using a T-Align algorithm (Smith et al. 2006). The peak areas in each sample were standardised by the total peak area to provide the relative abundance of each T-RF. This resulted in a ‘species by sample’ data matrix that was used for multivariate statistical analysis.

**Data processing and statistical analysis of established biofilms and community composition**

The number of microcolonies and biovolume of biofilms on each surface topography, for each day and experiment were plotted using PRISM 5 (GraphPad Software, San Diego, CA, USA). The datasets were checked for
Different surface topographies yielded significant differences that were independent of the sampling time. Pairwise comparison of each microfabricated surface and control surfaces revealed that each of the four microfabricated PDMS surfaces (1, 2, 4 and 10 μm) caused a significant reduction in microcolony formation in the early stages (day 2) of biofilm formation ($t_{4,16} > 3.224, p < 0.006$ in all cases). There was a significant reduction in microcolony formation on the 1, 2, and 4 μm PDMS surfaces for late stage (day 28) biofilms ($t_{4,16} > 3.515, p < 0.004$ in all cases). Pairwise comparisons of biovolumes of day 2 biofilms revealed a significant reduction on both the 4 and 10 μm PDMS surfaces ($t_{4,16} = 1.813, p = 0.0483$ and $t_{4,6} = 2.238, p = 0.013$, respectively), while at day 28, the 1 and 4 μm PDMS surfaces also contained a significantly reduced biovolume ($t_{4,16} = 2.245, p = 0.027$ and $t_{4,16} = 2.418, p = 0.015$, respectively).

Surface topography-dependent biofilm resistance was assessed by exposing established biofilms to predation by a heterotrophic protist. Biofilm biovolumes averaged from three field deployments revealed a surface-dependent reduction on the 10 μm PDMS surfaces only (Figure 4). PERMANOVA analysis of biovolumes showed no significant impact of increased grazing pressure on the different surface topographies. Differences in biovolumes were possibly obscured by the significant variations in biovolumes across the three experiments ($F_{4,8} = 2.944, p = 0.095$). Two of the three experiments (three replicates for each), showed significant reductions in biovolumes for the 10 μm PDMS surfaces, as compared to the ungrazed control ($F_{2,96} = 10.71, p < 0.001$).

**Surface associated marine microbial community composition altered by surface topography and predation**

The development of the attached marine microbial communities on the different microfabricated PDMS surfaces was monitored by the *Hae*III-digested terminal restriction fragment patterns. A non-parametric multidimensional scaling (nMDS) ordination plot displayed very different patterns for the clustering of community composition of microorganisms from seawater compared to surface-associated microorganisms, on both the PDMS and EPOXY surfaces ($F_{1,28} = 8.622, p < 0.001$, Figure 5a).

In addition, surface-associated community analysis revealed differences in community phylogenetic composition on the different microfabricated PDMS surfaces (Figure 5b), as well as experiments performed across different periods (Figure 5c). The community composition of biofilms attached to the microfabricated PDMS differed from that of biofilms formed on smooth PDMS surfaces. Fourth root transformed T-RF data, with a reduced emphasis on relative abundance values,
Figure 1. Representative CLSM images of the 14-day-old established marine biofilms, stained using the live/dead BacLight viability kit (images taken from the Winter 2010 experiment) on different microfabricated PDMS surfaces (top from left: 0, 1 and 2 μm; bottom from left: 4 and 10 μm). Images contain green fluorescent signals indicating live cells and red fluorescent signals indicating membrane-compromised cells. Each image is 212.3 μm x 212.3 μm in X–Y plane with two corresponding X–Z planes. Scale bars (grey bands at the bottom left corner of each image) represent 30 μm.

Figure 2. Microcolony formation analysis for marine biofilms established on the 0 μm (control), 1 μm (••), 2 μm (•••), 4 μm (••••), and 10 μm (•••••) microfabricated PDMS surfaces up to a period of 28 days. The graph was plotted based on the average (number of microcolonies 150 μm²) from all three experiments; n = 9. Error bars represent the SE of the mean.

Figure 3. Biovolume analysis for the marine biofilms established on the 0 μm (control), 1 μm (••), 2 μm (•••), 4 μm (••••), and 10 μm (•••••) microfabricated PDMS surfaces up to a period of 28 days. The graph is based on the average (μm³) from all three experiments; n = 9. Error bars represent the SE of the mean.
indicated that community compositions on the 4 and 10 μm PDMS surfaces were significantly different from the control and the smooth PDMS surfaces at days 7–14 ($t_{4,27} = 1.603$, $p = 0.011$ and $t_{4,24} = 1.573$, $p = 0.021$, respectively) and days 14–21 ($t_{4,27} = 1.533$, $p = 0.027$ and $t_{4,20} = 2.253$, $p = 0.002$, respectively). T-RFs transformed using presence/absence analysis showed that the differences in the community compositions observed on the 10 μm PDMS surface diminished on both days 7–14 and days 14–21 ($t_{4,24} = 1.145$, $p = 0.270$ and $t_{4,20} = 1.234$, $p = 0.280$), demonstrating the impact of the relative abundance values on the analysis. The factors of surface topography and sampling time (day) were each significant (ST: $F_{4,12} = 1.504$, $p = 0.030$; day: $F_{4,23} = 4.325$, $p = 0.001$) as was the interaction between the factors (ST × Da: $F_{16,235} = 1.2159$, $p = 0.010$) on the resulting community composition.

Figure 4. Biovolume analysis of R. nasuta-treated (Grazed) and non-treated (Ungrazed) microbial communities on different microfabricated surfaces (0, 1, 2, 4 and 10 μm) averaged across three experiments. Error bars represent the SE of the mean.

Figure 5. nMDS plot of microbial community derived from HaeIII digested terminal restriction fragments. (a) Seawater samples (SW) and surface associated community compositions (PDMS and epoxy respectively); (b) samples of different microfabricated PDMS surface topographies (0 μm, 1 μm, 2 μm, 4 μm, and 10 μm); (c) sample, either PDMS or SW, presented with three experimental runs (SW1, SW2, SW3, PDMS1, PDMS2, and PDMS3) respectively; (d) two different surface materials (PDMS and EPOXY respectively). The text (2, 7, 14, 21, and 28 days) indicates the stage of biofilm establishment at which the community compositions were extracted.
As a result of elevated flagellate predation within the environmental chambers following the inoculation of the flagellate *R. nasuta*, significant variations were observed in the composition of the bacterial biofilm communities across the three experiments ($F_{2,96} = 10.71$, $p < 0.001$). Significant interactions between the individual experiments and grazing ($Ex \times Gr$: $F_{2,96} = 2.41$, $p < 0.001$) were observed, but not between experiments and surface topography ($Ex \times ST$: $F_{8,96} = 0.850$, $p = 0.9305$), suggesting that elevated predation pressure induced community shifts independently from the surface topography.

**Influence of surface material on marine microbial community composition**

The impact of surface material on attached marine microbial communities was assessed using non-textured PDMS and epoxy surfaces. The biofilm community (fourth root transformed) showed significant differences based on the surface material used ($F_{1,28} = 8.622$, $p < 0.001$), time ($F_{4,28} = 9.641$, $p < 0.001$), as well as the interaction of the two factors ($F_{1,28} = 3.218$, $p < 0.001$). The success of the biofilm community composition was significantly different on the two different surface materials (Figure 5d).

**Discussion**

In this study, the potential control of biofilm formation, i.e. the microbial stage of fouling, was explored using microfabricated PDMS surfaces. Microscopic examination of the established biofilms revealed significant changes in biofilm development in conjunction with changes in the community composition of attached biofilms on different microfabricated surfaces. Several factors, including surface topography, predation resistance, surface material, microbial community composition and environmental parameters contributed to the altered microbial biofilm development.

**Surface topography influences biofilm development**

The microfabricated PDMS material used in this study featured a corrugated surface topography. Surface topography has often been correlated with the extent of bacterial adhesion (An et al. 1995; Anselme et al. 2010; Ploux et al. 2010; Ponche et al. 2010; Bazaka et al. 2011). Different types of surface patterning addressed in the literature include (i) irregular or random surface topographies (Harris & Richards 2004; Singh et al. 2011); (ii) regularly patterned surfaces (Scheuerman et al. 1998; Chung et al. 2007); and (iii) hierarchical surface structures (Crick et al. 2011; Ma et al. 2011). These different patterns can both induce as well as inhibit bacterial attachment (Scheuerman et al. 1998; Chung et al. 2007). While the mechanisms leading to changes in cell–substratum interactions on the different surfaces were not explored, this study demonstrated how surface topography mediates very distinct attachment patterns during the microbial fouling stage. Microscopic examination of the exposed surface samples showed that marine planktonic bacteria preferentially attached within the grooves in the initial stages of colonisation and biofilm formation (Figure 1). This effect may resemble the recently described ‘shelter’ effect (Scardino et al. 2008), where grooved surfaces or pits have been postulated to be important for providing protection against external stresses, such as surface hydrodynamic forces (Scheuerman et al. 1998). Indeed, etched topography with 0.2–2 μm pits can provide shelter for bacteria, with smaller cells being retained in higher numbers than larger rod-shaped cells (Whitehead et al. 2005). In the present study, chains of coccioid bacterial cells were arranged in parallel lines following the surface topography, within the grooves of 1 and 2 μm modified surfaces, further supporting the shelter effect model. This finding suggests that a unique surface topography can inhibit or attract bacterial attachment based on cellular traits such as shape and size.

In addition, minimum bacterial attachment on substrata with roughness both smoother and rougher than 0.16 μm suggests that bacterial adhesion may be regulated by the presence of sub-microcentric grooves (Kaufmann et al. 2002). The present study revealed that a groove width of 4 μm resulted in parallel attachment of microbial cells on both sides of the groove wall. Gaps observed between cells within the groove suggest that direct cell–cell contact was not occurring. This outcome reflects a reduction in microcolony formation of marine biofilms on microfabricated surfaces. Interestingly, surface topography that can influence metabolic activities of bacteria has also been suggested. Some illustrations of this include induced production of extracellular polysaccharides (EPS) on smooth rather than on rough glass surfaces (Mitik-Dineva 2009; Mitik-Dineva et al. 2009), and different ultrafine-grained titanium surfaces induced differential EPS production and attachment (Truong et al. 2009). Such observations highlight specific and complex bacterial responses during colonisation on microfabricated surfaces and may align with the findings reported here.

**Flagellate predation as a measure of biofilm resistance on microfabricated surfaces**

The impact of surface topography on biofilm resistance was explored by measuring the ability of the biofilm to resist flagellate grazing. The field deployments using environmental diffusion chambers showed that the biovolume
was reduced on 10 μm PDMS surfaces under elevated grazing pressure compared with the non-inoculated chambers (Figure 4). The findings for the 10 μm PDMS surfaces suggest that the larger groove width allowed the access of flagellates to the bacterial cells. Thus, microfabricated topographies with grooves smaller than predators (1, 2 and 4 μm) can provide micro-refuges from grazing predation (Scardino et al. 2006). Although there was no significant difference in biovolume between different microfabricated surfaces and the control, variations in biovolumes were significant across experiments. This variation may result from changes in a range of conditions, such as increased marine plankton following rainfall events, potentially leading to rapid growth of the bacteria, offsetting losses due to predation (Sherr & Sherr 2002), as well as the proliferation of nearby heterotrophic and autotrophic bacteria through grazing mediated nutrient remineralisation (Azam et al. 1983; Pernthaler 2005). Community analysis of established marine biofilms within the environmental chambers revealed significant changes due to elevated grazing pressure, which is in agreement with many studies detailing protozoan grazing-induced bacterial community shifts based on taxonomic composition analysis (Simek et al. 1997; Jurgens et al. 1999). This community shift, however, was not mediated by a particular micro-scale surface topography.

Marine microbial community composition as a factor regulating biofouling on different substrata

One major challenge for the development of marine AF surfaces is the constantly changing community compositions of potential marine microbial colonisers displaying a range of different colonisation and fouling phenotypes. Indeed, the surface attached communities investigated here showed high bacterial community variation. Many organisms are able to attach to immersed surfaces, and fouling has been generally considered an inevitable event (Flemming & Ridgway 2008).

However, the fouling process may also exhibit specificity. In the present study, coccoid-shaped bacteria were commonly present on 1 and 2 μm PDMS surfaces, while rod-shaped bacteria were the major fouling organisms on the 4 and 10 μm PDMS surfaces. Filamentous bacteria and some larger fouling organisms with higher organisation were observed after exposure for >14 days in marine waters. Selection of bacterial microbial fouling organisms based on surface topography as well as surface material was clearly evident in the T-RF community composition assessment (Figure 5a–d). The communities observed on different microfabricated PDMS surfaces were distinct from the seawater community composition at all times (Figure 5a). The lottery hypothesis proposed by Sale (1976) to explain the coexistence of different species in the same niche, and supported in the context of biofilm communities by Burke et al. (2011), fits the observations of the present study. Here the initial community composition, on day 2, showed significant differences compared to the established communities on day 28 (Figure 5b). The early recruitment of microbial communities relies in part on the competition for space and stochasticity, ie whoever can get to the surface first wins the ‘lottery’ of colonisation (Robertson 1995). However, while the field deployment was conducted on three separate occasions across an extended period of time (November 2010–December 2011), an apparent convergence to community compositions sharing higher similarity was recorded after exposure for 28 days in the field for corresponding surfaces in each of the separate experiments (Figure 5c). Such an outcome was obtained despite fluctuating environmental conditions, including temperature differences across seasons and significant weather events such as storms during the period of field deployments. This suggests that a strong correlation exists between the substratum surface and the established community composition.

Marine microorganisms may adapt to a sessile lifestyle to enhance their survival during harsh environmental conditions (Videla 1996; Morton et al. 1998). Pronounced specificity in such surface colonisation responses has been reported (Lachnit et al. 2011). In particular, the original bacterial community associated with the temperate marine red alga Delisea pulchra was restored following the removal/disturbance of epiphytic bacteria, suggesting this microbial community was highly resilient (Longford 2008). The high degree of community similarity and resilience in marine microbial biofilm communities might be attributed to host specific effects as well as mechanisms such as colonisation-mediated chemical compounds, cell–cell interactions and aggregation, as facilitated by extracellular signalling communication (Wieczorek & Todd 1998; Qian et al. 2007; Dobretsov et al. 2013). However, the significantly different community composition on the PDMS and epoxy surfaces could reflect the different physical-chemical characteristics of the surface materials (Figure 5d). For example, the differences in surface free energy and hydrophobicity between PDMS and epoxy surfaces could attract specific bacteria, hence promoting the colonisation of significantly different communities (Paguirigan & Beebe 2009).

Environmental parameters affecting microbial attachment in the marine environment

Biofouling in marine waters is affected by a range of potentially highly variable environmental parameters, including season (Wieczorek et al. 1996), sunlight (Maruthamuthu et al. 1993), temperature (Fletcher 1977), flow rates (Hunt & Parry 1998) and salinity (Anil &
Kurian 1996). In this study, temperature and rainfall were recorded across the seasonal sampling (BOM 2013). The higher temperature recorded during summer compared to winter correlated with a two-fold increase in accumulated biomass. Such a season-dependent increase in biofouling pressure has been observed in various studies, including the temperature-regulated attachment of microorganisms to inanimate surfaces, first reported by Fletcher (1977). Moreover, temperature-linked seasonal conditions regulate bacterial metabolism and differentially influence microbial fouling processes (Fera et al. 1989).

In addition, rainfall events during the 28-day field trial coincided with a distinct reduction in biovolume on day 14 of the summer 2010 field trial and day 28 of the winter 2011 field trial, respectively. Major rainfall can introduce higher flow conditions and hence higher shear stress on surface attached organisms (Lau & Liu 1993; Blenkinsopp & Lock 1994; Neal & Yule 1994). Furthermore, rainfall events can also create an influx of nutrients and decrease in salinity from run-off (Hoover et al. 2006). In this study, increased levels of nutrients due to rainfall on day 5 may explain the pronounced increase in biovolume recorded on day 7 (summer 2010).

The environmental conditions reported here, as well as other parameters such as salinity, osmotic stress and sunlight, could greatly impact on the establishment of surface-associated microbial communities (Dexter & Zhang 1991; Maruthamuthu et al. 1993; Wai et al. 1998). However, the different microfabricated surfaces included in the field trials were exposed to the same conditions, at any given time. Therefore, the distinct effects on the biofilm communities caused by changes in environmental factors, while impacting on different stages of the microbial fouling process, did not mask the regulatory effect on microbial attachment and biofilm formation mediated by specific microfabricated surfaces.

Conclusion
As reported here, microfabricated surfaces exposed to marine waters acquire a microbial biofilm for which microcolony formation is differentially inhibited and the biovolume reduction varies. The community composition became altered during the microbial fouling process over 28 days of field deployment. Given these outcomes, it is likely that macrofouling development on microfabricated surfaces, with altered microbial communities and hence potentially different settlement cues for larger fouling organisms (Qian et al. 2007; Dobretsov et al. 2013; Mieszkin et al. 2013), would also be impacted (Wang et al. 2012). The consequences of microcolony inhibition, biovolume reduction and community composition shifts on subsequent marine biofouling remain to be addressed, however postulated outcomes include disrupting the development of a mature complex biofouling community and extending the period required for a mature macrofouling community to become established (Zardus et al. 2008). Positive downstream outcomes of such effects may extend the use of short-term or intermittent applications such as underwater sensors (Delauney et al. 2010). The promising findings reported here may carry significant advantages in a range of marine AF applications.

Acknowledgement
The work was funded by the Centre for Marine Bio-Innovation, the CSIRO Wealth from Ocean Flagship Program, the Australian Research Council and the National Research Foundation and Ministry of Education Singapore under its Research Centre of Excellence Program. The authors thank Gary Housley for microscopy imaging support and the Sydney Institute of Marine Science for use of its facilities.

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