The effect of surface colour on the formation of marine micro and macrofouling communities

Sergey Dobretsov*, Raeid M.M. Abed and Christian R. Voolstra

Department of Marine Science and Fisheries, College of Agricultural and Marine Sciences, Sultan Qaboos University, Muscat, Oman; Department of Biology, College of Science, Sultan Qaboos University, Muscat, Oman; Red Sea Research Center, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia

(Received 11 September 2012; final version received 26 February 2013)

The effect of substratum colour on the formation of micro and macrofouling communities was investigated. Acrylic tiles, painted either black or white were covered with transparent sheets in order to ensure similar surface properties. All substrata were exposed to biofouling at 1 m depth for 40 d in the Marina Bandar al Rowdha (Muscat, Sea of Oman). Studies were conducted in 2010 over a time course of 5, 10 and 20 d, and in 2012 samples were collected at 7, 14 and 21 d. The densities of bacteria on the black and white substrata were similar with the exception of day 10, when the black substrata had a higher abundance than white ones. Pyrosequencing via 454 of 16S rRNA genes of bacteria from white and black substrata revealed that Alphaproteobacteria and Firmicutes were the dominant groups. SIMPER analysis demonstrated that bacterial phylotypes (uncultured Gammaproteobacteria, Actibacter, Gaetbulicola, Thalassobius and Silicibacter) and the diatoms (Navicula directa, Navicula sp. and Nitzschia sp.) contributed to the dissimilarities between communities developed on white and black substrata. At day 20, the highest amount of chlorophyll a was recorded in biofilms developed on black substrata. SIMPER analysis showed that Folliculina sp., Ulva sp. and Balanus amphitrite were the major macrofouling species that contributed to the dissimilarities between the communities formed on white and black substrata. Higher densities of these species were observed on black tiles. The results emphasise the effect of substratum colour on the formation of micro and macrofouling communities; substratum colour should to be taken into account in future studies.

Keywords: colour; biofouling community; bacteria; diatoms; biofilms; pyrosequencing

Introduction

Biofouling, defined in the framework of this study as unwanted recruitment and growth of organisms on submerged surfaces, is a multi-stage process, which includes adsorption of dissolved organic molecules, colonisation by prokaryotes, eukaryotes and recruitment of invertebrate larvae and algal spores (Wahl 1989). These stages can overlap, be sequential or occur in parallel (Dobretsov et al. 2006). Many biological (ie presence of fouling species) and physical factors (ie current, type of substratum including colour, texture, orientation) influence the settlement of propagules and the formation of biofouling communities (Qian et al. 2007) by attracting some species and repelling others (Hills & Thomason 1996; Glasby 1999; Hodson et al. 2000; Bers & Wahl 2004; Swain et al. 2006). The presence of biofilms, their composition and substratum properties are among the most important factors that determine the settlement (ie attachment) of propagules of biofouling species (see reviews by Dobretsov et al. 2006; Qian et al. 2007).

Information regarding the effect of substratum colour on the settlement of larvae of fouling organisms is limited. However, larvae of most invertebrates, such as barnacles (Yule & Walker 1984), abalone (Horiguchi 1984), pearl oysters (Monteforte & Garcia-Gasca 1994), tube worms (James & Underwood 1994), ascidians (Hurlbut 1993; Svane & Dolmer 1995) and hydrozoa (Guenther et al. 2009) all show a preference for dark substrata. In addition, the settlement rates of algal spores are higher and the adhesion strength of the spores is stronger on darker substrates (Swain et al. 2006; Finlay et al. 2008; Shine et al. 2010). This phenomenon was explained as a result of the negative phototaxis of settling propagules (Svane & Dolmer 1995) and/or by the differential adhesion of larvae to different colours of Intersleek® 700 (Robson et al. 2009). Previous studies have investigated the settlement of a single species in response to different colours (Finlay et al. 2008; Robson et al. 2009) and complex macrofouling communities (Swain et al. 2006; Satheesh & Wesley 2010). Community-based studies on the formation of macrofouling communities in tropical waters clearly demonstrated an effect of substratum colour; however, these studies were conducted over a short period of time (maximum 16 d) (Satheesh & Wesley 2010). It was not clear if the observed effect would disappear or become more
prominent in longer-term (>16 d) studies. Furthermore, there was no information about the effect of substratum colour on the formation of microbial communities, which can markedly influence the formation of macrofouling communities (Hadfield 2011). Knowledge of the impact of substratum colour on the formation of biofouling communities is important for the development of anti-fouling coatings and the protection of industrial equipment in seawater.

In this study, the effect of the substratum colour (black or white) on the formation of microfouling and macrofouling communities in tropical waters was investigated. While the development of microfouling communities was followed over 21 d, macrofouling communities were followed over 40 d. The hypothesis that substratum colour affects the formation of microbial communities was tested. In addition, the premise that the effect of surface colour becomes less prominent with increasing length of immersion was also evaluated.

Material and methods

Experimental design

The formation of microfouling communities was studied on acrylic plastic slides (76 × 26 × 0.8 mm) painted with either black or white spray enamel (AGP-2-AER and AGP-1-AER, China). The formation of macrofouling communities was studied on plastic tiles (200 × 150 × 3 mm) painted with the same black or white spray enamel. All the surfaces of the black and white slides and tiles were covered with overhead transparency sheets (3M, China) in order to maintain similar physical and chemical properties. A similar approach was used previously (Swain et al. 2006) and laboratory experiments with larvae of Balanus amphitrite demonstrated that these sheets were not toxic and did not affect barnacle settlement (data are not shown). The surface of the sheets was hydrophilic with a contact angle of 82°.

The study was conducted at a depth of 1 m in the semi-closed Marina Bandar Al Rawdha (Muscat, Oman 23° 34′ 55″N 58° 36′ 27″E), which has the highest level of biofouling observed in the region. During the experiment, the average water temperature was 28.8 °C, salinity was 35.5 ppt and O₂ concentration was 6.67 ppm.

Biofouling by diatoms and bacteria was investigated on plastic slides that were deployed vertically in racks for 20 d (Dobretsov & Thomason 2011) from 8 to 28 April 2010. The experiment was terminated after 20 d due to high levels of settlement of macrofouling species, which could have had their own associate microbiota (Wahl et al. 2012) with the potential to camouflage differences in the microfouling community. Triplicate black and white slides were retrieved from the water after 5, 10 and 20 d, placed into slide boxes (Fisher Scientific, USA) filled with freshly filtered (0.2 μm) seawater from the experimental site and fixed with formaldehyde immediately (4% final v/v). Fixed slides were brought to the laboratory and processed within a few weeks (see below). To study macrofouling communities, plastic tiles were deployed individually for 40 d in 2010 (8 April–18 May) at the same time, location and direction as the plastic slides. Four replicate black and white tiles were retrieved from the water after 5, 10, 20, 30 and 40 d and brought to the laboratory where they were processed immediately (see below).

Analysis of biofouling communities

For the plastic slides, the number of diatoms in each identifiable genus and the number of bacteria was estimated. The attached bacteria were visualized after staining with the DNA-binding fluorochrome 4,6-diamidino-2-phenyldindole (DAPI, Fluka Ltd, Switzerland) at 0.5 μg ml⁻¹ according to Dobretsov and Thomason (2011). Briefly, the slides were stained with DAPI for 15 min and the density of bacteria in 20 randomly selected fields of view (0.001 mm²) was then counted via epifluorescence microscopy (Axioskop, Zeiss, Germany; magnification 1000×; λ_ex = 359 nm, λ_em = 441 nm). The density of diatoms in 25 randomly selected fields of view (3.5 mm²) was counted via microscopy (Nikon Eclipse, USA; magnification 400×). The main genera of diatoms were determined, and the mean densities were calculated. Values are presented as individuals mm⁻².

The biomass of photosynthetic organisms on the slides was analysed by quantification of chlorophyll a. The biofilm on each slide was scrapped from a 2 cm² area using a sterile scalpel. This biofilm was extracted in 1 ml of 90% acetone (Fisher Chemicals, USA) in the dark for 24 h. The amount of chlorophyll a was determined spectrophotometrically (Lorenzen 1966).

Macrofouling species were identified under a dissecting microscope (magnification = 20×, Olympus, USA) and the number of individuals was counted on each tile. Photographs of dominant species were made and samples were taken for taxonomic identification.

Pyrosequencing of bacterial communities

Individual plastic tiles (200 × 150 × 3 mm) were deployed vertically for 21 d in the same marina in 2012 (10–31 March). Three replicate black and white tiles were retrieved from the water after 7, 14 and 21 d. This experiment employed the larger tiles as the smaller slides were found to yield an insufficient amount of DNA for sequencing. Preliminary experiments suggested that there was no measurable effect of substratum size on the formation of microbial communities. The biofilm from the entire surface of a tile was scraped off into a sterile Eppendorf® tube using a sterile scalpel and immediately transferred to the laboratory on ice, where the samples...
were stored at −20°C. After 14 d, the area around macrofouling organisms was not sampled as these species could have introduced their own associated microorganisms. DNA was extracted from all samples using a Power Biofilm MoBio Kit (MOBIO, Germany) following the manufacturer instructions. The DNA of triplicate samples at each sampling time was pooled. For amplification of the 16S rRNA gene for pyrosequencing, the DNA concentration was adjusted to 30 ng μl⁻¹ and PCRs were performed in 30 μl reactions using the Multiplex PCR Kit (Qiagen, Hilden, Germany). To obtain an amplicon suitable for sequencing, the primers 784F (5′CTATGCGCCTTGCCAGCCCGCTCAGTAAGGATTAGATACCGCTGTA3′) and 1061R (5′CGTATC CGCTCC-CTCGGCGCATCAGN NNNNNNCTCRRCACGAGCTGACGAC3′) were used (Andersson et al. 2008). These primers amplified consistently well with only minor amplification of the chloroplast 16S sequences, which would present a confounding factor in this pyrosequencing study. The reverse primer included the 454 adapter sequences, a barcode (shown as N) and a linker sequence (CT). The forward primer did not contain a barcode sequence. The temperature cycling for amplification was 1 cycle at 95 °C for 15 min, 30 cycles at 95 °C for 30 s, single cycles at 55 and 72 °C for 40 s each, respectively, and one final extension at 72 °C for 10 min. All PCRs were performed in triplicate and products were pooled in equal amounts after purification with a Qiagen PCR purification kit. Sequencing was performed from the reverse primer on a 454 sequencer (Roche, USA) using the Titanium GS FLX chemistry on a quarter of a PicoTiterPlate. For data analysis steps, Mothur software version 1.16.1 was used (Schloss et al. 2009). For phylogenetic assessment, sequences were aligned against the SILVA database release 108 alignment (Pruesse et al. 2007). Rarefaction curves, operational taxonomic unit (OTU) number and diversity estimates (Chao 1, Abundance-based Coverage Estimators (ACE)) were calculated using the software ESPRIT (Sun et al. 2009) available at http://www.biotech.ufl.edu/people/sun/esprit.html.

**Statistical analysis**

The densities of bacteria and diatoms on the slides were square root transformed in order to ensure normality of variance for subsequent statistical analysis. The data presented in all the figures are not transformed. The assumption of normality was verified with the Shapiro–Wilk’s test (Shapiro & Wilk 1965). Two-way ANOVA was used to test the effects of sampling time and colour on the densities of bacteria and diatoms and the total abundance of macrofouling organisms. *Post hoc* multiple comparisons were performed using the LSD test. In all cases the threshold for significance was 5%. The effects of colour and sampling time on the biofouling community structure were analysed using ANOSIM (analysis of similarity) and SIMPER (similarity percentage) procedures (PRIMER software, Plymouth Marine Laboratory), which were based on multi-dimensional scaling (MDS) of the Bray–Curtis similarity index (Clarke 1993). For the construction of a similarity matrix, the data were square root transformed. As a measure of macrofouling species diversity, the Shannon diversity and Margalef’s species richness and evenness

![Figure 1](image)

**Figure 1.** The total density of bacteria developed on black and white slides during the 20 d course of the experiment. The data are the means ± SD (n = 3). Data that are significantly different according to an LSD test (ANOVA: p < 0.05) are indicated by different letters above the bars.

**Table 1.** The effect of colour and time of sampling on the total density of bacteria, amount of chlorophyll a, the density of diatoms in biofilms and the total number of invertebrate macrofouling species.

| Factor                        | df | MS   | F     | p     |
|-------------------------------|----|------|-------|-------|
| Total density of bacteria     |    |      |       |       |
| Colour                        | 1  | 33.65| 0.7   | 0.42  |
| Time                          | 2  | 4986.2 | 104.02| >0.0001|
| Colour × time                 | 2  | 44.8 | 0.93  | 0.42  |
| Error                         | 12 | 47.93|       |       |
| Amount of chlorophyll a       |    |      |       |       |
| Colour                        | 1  | 0.4  | 3.61  | 0.08  |
| Time                          | 2  | 0.49 | 4.41  | 0.03  |
| Colour × time                 | 2  | 0.21 | 1.09  | 0.19  |
| Error                         | 12 | 0.11 |       |       |
| Density of diatoms             |    |      |       |       |
| Colour                        | 1  | 3.17 | 4.96  | 0.05  |
| Time                          | 2  | 2.54 | 3.98  | 0.05  |
| Colour × time                 | 2  | 0.4  | 0.63  | 0.55  |
| Error                         | 12 | 0.64 |       |       |
| Total number of invertebrates  |    |      |       |       |
| Colour                        | 1  | 4023 | 14.21 | 0.002 |
| Time                          | 3  | 1653 | 5.84  | 0.007 |
| Colour × time                 | 3  | 793  | 2.8   | 0.07  |
| Error                         | 16 | 283  |       |       |

Note: Results of 2-way ANOVA. Statistically significant (p < 0.05) values are highlighted in bold text.
indices were calculated. In order to assess the occurrence of associations between the bacteria and the diatoms on the white and black substrata, Spearman’s correlation coefficients (ρ) were calculated for all pairwise associations between the numbers of diatoms and bacteria, the diatom species richness and the amount of chlorophyll a.

Results

Microfouling communities

The presence of biofilms was observed visually after 10 d by the change in background colour from black or white to light brown (Supplementary information, Figure S1). There were no visual differences between the colour of the biofilm on the black and white substrata.

The densities of bacteria

The total number of bacteria in biofilms increased 7–10-fold during the 20 d of immersion of the plastic slides (Figure 1). Statistical analysis showed that the duration of the experiment significantly influenced the total densities of the bacterial populations (ANOVA; p < 0.0001; Table 1). Bacterial densities on black and white slides were similar except at day 10, when black slides had a higher abundance of bacteria than white ones (ANOVA, LSD, p = 0.04).

Pyrosequencing of bacterial communities

Sequencing resulted in a total number of 28,851 sequences of the 16S rRNA gene. The reads were split according to barcodes and quality trimmed to an average quality of 27 in a 50 base pairs (bp) window, resulting in a set of 12,230 (black tiles) and 8,715 (white tiles) sequences. The data were analyzed using QIIME software. The relative abundances of the major bacterial groups are shown in Figure 2. The proportions of the major bacterial groups were as follows: Alpha–Alphaproteobacteria, Beta–Betaproteobacteria, Gamma–Gammaproteobacteria, Delta–Deltaproteobacteria, Epsilon–Epsilonproteobacteria, Bacteroidetes, Chlamydiae, Planctomycetes, Firmicutes, Acidobacteria, Actinobacteria.

Figure 2. Relative abundances (%) of the major groups of bacteria present in biofilms developed on black (B) and white (W) substrata. 1B-3B and 1W-3W represent the time of sampling in weeks (eg 1B = sampling of the black substratum after 1 week). Alpha – Alphaproteobacteria, Beta – Betaproteobacteria, Gamma – Gammaproteobacteria, Delta – Deltaproteobacteria, Epsilon – Epsilonproteobacteria, Bacter – Bacteroidetes, Chlam – Chlamydiae, Planct – Planctomycetes, Firm – Firmicutes, Acido – Acidobacteria, Actino – Actinobacteria.
sequences with identifiable barcode and sufficient length (278 bp). The number of detected OTUs in biofilms from black and white slides was comparable and did not change with time. Analysis of rarefaction curves revealed that the curves did not level off at the 97% cut-off level, and more sequences are needed to cover the whole community diversity (Figure S2, Supplementary information). OTU richness, as determined by the Chao1 index based on a 97% sequence similarity threshold to define OTUs, ranged from 2216 to 4482 in biofilms from white slide samples and 3462–4800 in biofilms from black samples (Table S1, Supplementary information).

More than 32% of the obtained sequences were affiliated to the Alphaproteobacteria (Figure 2). The second dominant group of bacteria was that belonging to the phylum Firmicutes (16–30% of total sequences). The taxa Gammaproteobacteria, Actinobacteria and Chlamydiae were each responsible for 2–4% of the obtained sequences, whereas the rest were distributed among other taxa, such as Deltaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Planctomycetes, Acidobacteria, Verrucomicrobia, Fusobacteria, Nitrospira and Chloroflexi (each <1% of total sequences) (Figure 2). The most abundant group in all samples was the undescribed and novel species of bacteria (Figure S3, Supplementary information). For example, one of the most dominant bacteria on all substrata belonged to an uncultured Gammaproteobacteria; its sequence did not match any described species. Other dominant species included bacteria belonging to the genera Gaethulicola and Winogradskyella. Whilst the quantity of different species varied on the different substrata during this study, the species composition of major (Figure S3A, Supplementary information) and rare (Figure S3B, Supplementary information) bacterial groups remained largely similar. Nevertheless, cluster analysis showed that bacterial communities formed after 2 and 3 weeks on black and white slides, respectively, were different; communities developed and changed more quickly on white slides than on black ones (Figure 3A). ANOSIM analysis revealed that the bacterial species composition on white and black substrata differed significantly (ANOSIM, $r = 0.94$, $p = 0.006$). SIMPER analysis revealed that the major species contributing to the dissimilarity between communities developed on the white and black substrata belonged to the following taxa: uncultured Gammaproteobacteria, Actibacter, Gaethulicola, Thalassobium and Silicibacter (Table 2). The contribution for any single species did not exceed 12%. The contribution of some species (eg Gaethulicola) increased with time; others (eg Actibacter) decreased their contribution in terms of the dissimilarity between communities developed on the white and black substrata. Generally, the number of species contributing more than 60% of the dissimilarity between communities decreased over time from 17 (at the beginning) to 13 (at the end of the study).

The abundance of phototrophs as measured by chlorophyll $a$

The amount of chlorophyll $a$ obtained from white slides covered with biofilm was similar throughout the experiment (Figure 4A). At 20 d, the amount of chlorophyll $a$
on biofilms developed on black slides was the highest recorded, and was significantly different to other samples (ANOVA, LSD, \( p < 0.05 \)). Statistical analysis showed that only the duration of the experiment significantly (ANOVA, \( p < 0.05 \)) influenced the amount of chlorophyll \( a \) in the biofilms (Table 1).

### Diatoms

The density of diatoms increased over the 20 d incubation period for black slides, whereas the increase was over a period of 10 d for white slides (Figure 4B). The density of diatoms was significantly influenced both by the duration of the experiment and the colour of the substratum (ANOVA, \( p < 0.05 \)) (Figure 4B, Table 1). Six species of diatoms (Navicula sp., \( N. \) directa, Nitzschia sp., Cylindrotheca sp., Amphora costata and Diploneis sp.) were detected in the biofilms. \( N. \) directa dominated on all slides (Table S2, Supplementary information). Some species of diatom, eg Nitzschia sp., were found predominantly on the white substrata, while others, eg Navicula sp., predominated on the black substrata. The MDS ordination of abundances of different diatom species showed that the communities developed after 20 d were different from the communities developed after 10 d (Figure 3B). The diatom communities developed at 5 d were different from ones developed after 10 d except for one replicate that shared some similarities. Diatoms communities on white slides were slightly different to those on black slides at 5 and 10 d, but by 20 d they formed one aggregated group, reflecting higher similarities among them (lower part of the MDS plot). SIMPER analysis demonstrated that Navicula directa, Navicula sp. and Nitzschia sp. contributed 36.47, 19.24 and 16.79%, respectively, to the dissimilarity between the communities developed on the white and black substrata. At 10 d on white substrata, the number of bacteria and diatoms showed a significant (\( \rho = -0.9; \ p < 0.05 \)) negative correlation (Table S3, Supplementary information). There was a strong positive correlation (\( \rho = 0.9; \ p < 0.05 \)) between the amount of chlorophyll \( a \) and the diatom species richness at 5 d on the black substratum.

The density of diatoms increased over the 20 d incubation period for black slides, whereas the increase was over a period of 10 d for white slides (Figure 4B). The density of diatoms was significantly influenced both by the duration of the experiment and the colour of the substratum (ANOVA, \( p < 0.05 \)) (Figure 4B, Table 1). Six species of diatoms (Navicula sp., \( N. \) directa, Nitzschia sp., Cylindrotheca sp., Amphora costata and Diploneis sp.) were detected in the biofilms. \( N. \) directa dominated on all slides (Table S2, Supplementary information). Some species of diatom, eg Nitzschia sp., were found predominantly on the white substrata, while others, eg Navicula sp., predominated on the black substrata. The MDS ordination of abundances of different diatom species showed that the communities developed after 20 d were different from the communities developed after 10 d (Figure 3B). The diatom communities developed at 5 d were different from ones developed after 10 d except for one replicate that shared some similarities. Diatoms communities on white slides were slightly different to those on black slides at 5 and 10 d, but by 20 d they formed one aggregated group, reflecting higher similarities among them (lower part of the MDS plot). SIMPER analysis demonstrated that Navicula directa, Navicula sp. and Nitzschia sp. contributed 36.47, 19.24 and 16.79%, respectively, to the dissimilarity between the communities developed on the white and black substrata. At 10 d on white substrata, the number of bacteria and diatoms showed a significant (\( \rho = -0.9; \ p < 0.05 \)) negative correlation (Table S3, Supplementary information). There was a strong positive correlation (\( \rho = 0.9; \ p < 0.05 \)) between the amount of chlorophyll \( a \) and the diatom species richness at 5 d on the black substratum.

### Table 2. The contribution of particular bacteria to total dissimilarity (as percentages) between bacterial communities developed on the black and white substrata using SIMPER (similarity of percentage) analysis.

| Species contributing to dissimilarity | 1 week | 2 weeks | 3 weeks |
|--------------------------------------|--------|---------|---------|
| Taxon                                | Contribution (%) | Taxon | Contribution (%) | Taxon | Contribution (%) |
| Alteromonas                          | 6.45 | Uncultured Gammaproteobacteria | 9.01 | Gaethulicola | 12.03 |
| Uncultured Sphingomonas              | 5.80 | Winogradskyella | 7.07 | Thioclava | 9.40 |
| Actibacter                           | 5.67 | Uncultured Rhizobiales | 7.01 | Iamia | 5.74 |
| Uncultured                           | 5.44 | Gaethulicola | 6.55 | Winogradskyella | 5.73 |
| Gaethulicola                         | 4.60 | Uncultured Ruegeria | 6.24 | Tenacibaculum | 5.49 |
| Uncultured                           | 4.01 | Psychoserpens | 5.99 | Thalassobius | 4.17 |
| Alteromonadaceae                     | 3.98 | Roseovarius | 4.20 | Uncultured Actinobacteria | 4.10 |
| Hellea                               | 3.92 | Thalassobius | 3.81 | Silicibacter | 3.06 |
| Uncultured Saprospiraceae            | 3.47 | Hellea | 3.60 | Vibrio | 2.93 |
| Psychoserpens                        | 3.47 | Uncultured | 2.61 | Uncultured Flammeovirgaceae | 2.81 |
| Roseovarius                          | 3.07 | Flavobacteriales | 2.31 | Uncultured | 2.58 |
| Uncultured                           | 2.93 | Actibacter | 2.11 | Gammaproteobacteria | 2.54 |
| Oceanspirillales                     | 2.82 | Uncultured Oceanspirillales | 2.05 | Uncultured Bacteroidetes | 2.26 |
| Uncultured Chromatiales              | 2.60 | Brumimicrobium | 2.00 | Other species | 37.16 |
| Thalassobius                         | 2.21 | Other species | 35.44 | Other species | 37.16 |
| Silicibacter                         | 2.11 | Other species | 35.44 | Other species | 37.16 |
| Ruegeria                             | 2.07 | Other species | 35.44 | Other species | 37.16 |
| Rhodovulum                           | 2.02 | Other species | 35.44 | Other species | 37.16 |
| Lacinutrix                           | 33.36 | Other species | 35.44 | Other species | 37.16 |

Note: The contributions are averaged over all significant pair-wise treatment comparisons. Bacteria that contributed less than 2% are not shown.
Analysis of macrofouling communities

After 20 d, all substrata had many visible white and orange-yellow bryozoan colonies (Figure S1, Supplementary information). A total of 13 macrofouling species were found on black and white tiles including: the green alga Ulva (Enteromorpha) sp. (Chlorophyta: Ulvophyceae), the protozoan Folliculina sp. (Infusoria: Heterotricha), an unidentiﬁed sponge (Porifera: Demospongiae), hydroid polyps of Obelia sp. and an unidentiﬁed Leptomedusa (Cnidaria: Hydrozoa), the barnacles B. amphitrite and B. trigonus (Crustacea: Cirripedia), the bryozoans Bugula cf. neritina, Celleporaria cf. aperta, C. vermiformis, Schizoporella sp. (Bryozoa: Cheilostomata) and the tunicates Ciona sp. and an unidentiﬁed species (Chordata, Ascidiaeae: Phlebobranchia). Although the alga Ulva sp. (54% of individuals), the infusorium Folliculina sp. (41.3% of all individuals) and the bryozoan Schizoporella sp. (1.3% of all individuals) dominated on all substrata throughout the experiments, their densities were different on white and black tiles (see below).

The total number of Ulva sp. decreased during the experiment (Figure 5A, Table 1) and was only signiﬁcantly inﬂuenced by the duration of the study (ANOVA, df = 3, f = 35.14, p = 0.01). The effect of substratum colour on the density of Ulva sp. was observed only after 30 and 40 d, there being a signiﬁcantly higher density of Ulva sp. on black tiles (ANOVA, LSD, p < 0.05).

The density of macrofouling invertebrate species increased during the course of the experiment (Figure 5B). Macrofouling was more abundant on black tiles, although the effect of colour disappeared after 30 d. Statistical analysis demonstrated that both the duration of the experiment and the colour of the substratum signiﬁcantly (ANOVA, p < 0.05) inﬂuenced the densities of invertebrates species (Table 1). The MDS ordination
of abundances of invertebrate species demonstrated that the structure of the macrofouling community was different at different sampling points (Figure 3C). Communities formed after 10 d and after 40 d were different from those formed after 20–30 d, the latter of which showed some similarities (lower part of the MDS plot). In addition, a MDS plot showed that invertebrate communities on black and white tiles were different throughout the experiment, except at 40 d when both communities appeared as one cluster (Figure 3C).

ANOSIM results demonstrated that the colour of the substratum significantly affected the species composition of macrofouling communities (ANOSIM, $r = 0.14$, $p = 0.02$). SIMPER analysis showed that the species that contributed most to the dissimilarity between the communities were the green alga Ulva sp. (contributed 41.1%), the infusorium Folliculina sp. (39.6%) and the barnacle B. amphitrite (12.7%). Other species contributed 6.6% of the dissimilarities among the communities developed on the white and black substrata.

Major differences in the Margalef’s species richness, Shannon diversity and evenness indices were observed for macrofouling communities developed on black and white substrata at 10 d (Figure 6). The lowest species richness, diversity and evenness were observed on the white tiles at 10 d. There were no differences between the species richness, diversity and evenness of macrofouling communities developed on black and white substrata after 10 d.

**Discussion**

This study shows that the colour of the substratum affected the formation of microfouling as well as macrofouling communities. Previous reports demonstrated an effect of background colour on macrofouling, but during short-term (<16 d) experiments (Swain et al. 2006; Satheesh & Wesley 2010). In the present study, the density of macrofouling was higher on the black substratum up to 30 d. However, the impact of substratum colour on the formation of biofouling communities diminished over time. Thus, most likely, the colour of the substratum would have no effect on biofouling communities in a long-term (several months) exposure.

This study demonstrates for the first time that the colour of the substratum can also affect microfouling communities. Usually, the black substratum had a higher amount of chlorophyll $a$ and a higher density of bacteria and diatoms, but this effect was not constant over time. The absence of a simple general trend may be attributed to the differences in species composition of the microbial communities during the period of the investigation.

Pyrosequencing showed that the bacterial communities that developed on the white and black substrata were dominated by bacteria belonging to the class Alphaproteobacteria and the phylum Firmicutes (Figure 2). A high density of Alphaproteobacteria in marine biofilms was recorded in a number of studies that used different molecular techniques (Jones et al. 2007; Huggett et al. 2009; Trindade-Silva et al. 2011; Toupoint et al. 2012). Alphaproteobacteria and bacteria belonging to the phyla Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria dominated in 6 and 12 d intertidal biofilms from Hong Kong waters as shown by the PhyloChip method (Chung et al. 2010). In contrast, the majority of bacteria settled on glass slides in different Arctic sites were affiliated to the Gammaproteobacteria, the Cytophaga–Flavobacteria cluster of Bacteroidetes, Verrucomicrobia and Planctomycetales (Webster & Negri 2006).

In the present study, Gaetbulicola and Winogradskyella were among the most dominant bacterial phylotypes. The
bacterium *Gaethulicola byunsanensis* was isolated from a tidal flat sample collected in Korea (Yoon et al. 2010). Species of the genus *Winogradskyella* have been isolated frequently from different substrata in the marine environment (Nedashkovskaya et al. 2005). The data presented here suggest that some of the dominant bacterial species (eg uncultured Gammaproteobacteria) were unclassified and novel species. A high number of OTUs in the biofilms formed on black and white slides suggested that these communities are quite diverse. These findings highlight the fact that more research is needed in order to identify and investigate the diversity of bacteria present in marine biofilms.

The diatoms *Navicula* sp., *N. directa* and *A. costata* dominated on slides during the period of this study. In contrast, the diatom *Cylindrotheca* sp. dominated on non-biocidal coatings in a study conducted at the same location (Dobretsov & Thomason 2011). The genera *Amphora, Navicula, Nitzschia, Licmophora* and *Cylindrotheca* were dominant in marine biofilms developed on different substrata (Patil & Anil 2005; Zargiel et al. 2011; Briand et al. 2012).

Pyrosequencing of bacterial communities and analysis of diatom communities supported the conclusion that background colour affected the formation of microbial communities. While the composition of the microbiota remained similar in all treatments, the diversity of bacterial and diatom species was different on white and black substrata. Differences in surface wettability of substrata did not affect bacterial composition (Huggett et al. 2009). In the present study, SIMPER analysis suggested that the bacterial phylotypes (uncultured Gammaproteobacteria, *Actibacter, Gaethulicola, Thalassobius* and *Silicibacter*) and the diatoms (*Navicula directa, Navicula* sp. and *Nitzschia* sp.) contributed to dissimilarity between communities on the white and black substrata. The question why particular bacterial and diatom species were present on substrata varying in colour alone should be a subject for future investigations.

In the present investigation, the green alga *Ulva* sp., the infusorium *Folliculina* sp. and the barnacle *B. amphitrite* contributed to dissimilarity between the communities developed on the white and black substrata. These organisms were observed at higher densities on the black substratum. It was demonstrated that the percentage cover of *Ulva* sp. was greater on black surfaces (Swain et al. 2006). The strength of attachment of sporelings (young plants) of *U. linza* was shown to be stronger on darker backgrounds (Finlay et al. 2008). Similarly, *B. amphitrite* was preferentially recruited on red and blue coloured panels (Satheesh & Wesley 2010). Several authors have also reported that the adhesion of the cypris larvae of barnacles was stronger to dark coloured surfaces (Yule & Walker 1984; Robson et al. 2009).

There are several reasons that could explain why the structure of macro and microfouling communities is affected by the colour of the substratum. Some invertebrate larvae and spores of algae prefer darker, less reflective substrata due to their negative phototaxis (Svane & Dolmer 1995). It has been shown that the adhesion strength of barnacles and algal sporelings is higher on dark substrata (Yule & Walker 1984; Finlay et al. 2008; Robson et al. 2009). These reasons may explain why higher densities of these species were found on the black surfaces in the present experiments. Other reasons could include the amount of radiant energy adsorbed or reflected and the temperature of the substrata. Differential predation and grazing may be another reason (Swain et al. 1998).

The current study showed that the density of bacteria and diatoms were negatively correlated on white substrata after 10 d (Table S3). The presence of a negative relationship between diatoms and bacteria on antifouling coatings has been reported previously (Dobretsov & Railkin 1994; Dobretsov & Thomason 2011). Conversely, beneficial interactions between bacteria and diatoms have also been shown (Head et al. 2004; Wigglesworth-Cooksey & Cooksey 2005; Wang et al. 2012). The different microbial communities formed on the black and white substrata could influence the species composition, as well as the settlement, of invertebrate larvae and algal cells. It has been shown that chemical and physical cues from bacteria and diatoms can either induce or inhibit larval and spore settlement (see reviews: Dobretsov et al. 2006; Hadfield 2011). The relationship between the surface properties of the substratum and formation of microbial communities is well known (Jones et al. 2007; Briand et al. 2012; Mieszkin et al. 2012). Changes in biofilm structure can lead to the formation of different macrofouling communities (Lau et al. 2005; Dobretsov & Qian 2006). It is probable that the observed differences between the communities on the black and white substrata were not the consequence of a single reason, but due rather to a combination of several factors. More comprehensive experiments are needed to evaluate and test these hypotheses.

In conclusion, this study demonstrates that the colour of the substratum can affect the formation of biofouling communities in short-term experiments. The effect of substratum colour on microbial communities has been shown for the first time. These findings have serious implications for future ecological experiments, as well as for the development of antifouling technology. Researchers should employ experimental substrata and controls of similar colour; otherwise the observed differences in biofouling communities may be attributed to the colour of the substratum and not to the chemical/physical properties being tested. For antifouling screening, darker substrata are recommended, as they promote a higher
density of fouling organisms. However, white or light coloured coatings would be preferable to maximise the protection of structures against marine fouling.

Acknowledgements
The authors acknowledge Dr Andrew N. Ostrovsky (Vienna University) for the identification of bryozoan species and Ms Annika Vaksmaa (Roskilde University) for her help with the experiments and data analysis. The authors thank Mr Khalid Al-Hashmi for his help in the identification of diatoms. 454 pyrosequencing was funded by King Abdullah University of Science and Technology (KAUST), Saudi Arabia. The authors wish to thank Dr Till Bayer for raw data collection and the Bioscience Core Lab at KAUST for preparation and sequencing of 454 libraries. The work of SD was supported by a Sultan Qaboos University internal grant IG/AGR/FISH/12/01 and by a HM Sultan Qaboos Research Trust Fund SR/AGR/FISH/10/01. SD acknowledged the help of Professor R. Coutinho (IEAPM, Arraiol do Cabo, Brazil) and the programme science without frontiers (CNPq). RA would like to thank the Hanse-Wissenschaftskolleg (HWK), Institute for Advanced Study, Germany for their support.

References
Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyren P, Engstrand L. 2008. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS ONE. 3:e2836.
Bers AV, Dahlberg M. 2004. The influence of natural surface microtopographies on fouling. Biofouling. 20:43–51.
Briand J-F, Djeridia I, Jamet D, Coupe S, Bressy C, Molmeret A, Le Berre B, Coto M, Le Berre B, Rimet F, Bouchez A, Blach Y. 2012. Pioneer marine biofilms on artificial surfaces including antifouling coatings immersed in two contrasting French Mediterranean coast sites. Biofouling. 28:453–463.
Chung HC, Lee OO, Huang YL, Mok SY, Kolter R, Qian PY. 2010. Bacterial community succession and chemical profiles of subtidal biofilms in relation to larval settlement of the polychaete Hydroides elegans. ISME J. 4:817–828.
Clarke KR. 1993. Nonparametric multivariate analyses of changes in community structure. Aust J Ecol. 18:117–143.
Dobretsov S, Dahms H-U, Qian PY. 2006. Inhibition of biofouling by marine microorganisms and their metabolites. Biofouling. 22:43–54.
Dobretsov S, Qian PY. 2006. Facilitation and inhibition of larval attachment of the bryozoan Bugula neritina in association with monospecies and multispecies biofilms. J Exp Mar Biol Ecol. 333:263–274.
Dobretsov S, Thomason JC. 2011. The development of marine biofilms on two commercial non-biocidal coatings: a comparison between silicone and fluoropolymer technologies. Biofouling. 27:869–880.
Dobretsov SV, Rallkin AJ. 1994. Correlative relationships between marine microfouling and macrofouling. Russ J Mar Biol. 20:87–90.
Finlay JA, Fletcher BR, Callow ME, Callow JA. 2008. Effect of background colour on growth and adhesion strength of Ulva sporelings. Biofouling. 24:219–225.
Glasy TM. 1999. Interactive effects of shading and proximity to the seafloor on the development of subtidal epibiotic assemblages. Mar Ecol Prog Ser. 190:113–124.
Guenther J, Carl C, Sunde LM. 2009. The effects of colour and copper on the settlement of the hydroid Ectopleura larynx on aquaculture nets in Norway. Aquaculture. 292:252–255.
Hadfield M. 2011. Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites. Annu Rev Mar Sci. 3:453–470.
Head RM, Davenport J, Thomason JC. 2004. The effect of depth on the accrual of marine biofilms on glass substrata deployed in the Clyde Sea, Scotland. Biofouling. 20:177–180.
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.
Hodson SL, Burke CM, Bissett AP. 2000. Biofouling of fish-cage netting: the efficacy of a silicone coating and the effect of netting colour. Aquaculture. 184:277–290.
Horiguchi YN. 1984. Settlement behavior of young abalone Haliotis discus to chromatic and achromatic plates made of methacrylate resin. Bull Faculty Fish – Mie Univ. 11:219–226.
Huggett MJ, Nedved BT, Hadfield MG. 2009. Effects of initial surface wettability on biofilm formation and subsequent settlement of Hydroides elegans. Biofouling. 25:387–399.
Hurlbut CJ. 1993. The adaptive value of larval behavior of a colonial ascidian. Mar Biol. 115:253–262.
James RJ, Underwood AJ. 1994. Influence of colour of substratum on recruitment of spirorb tubeworms to different types of intertidal boulders. J Exp Mar Biol Ecol. 181:105–115.
Jones P, Cottrell M, Kirchman D, Dexter S. 2007. Bacterial community structure of biofilms on artificial surfaces in an estuary. Microb Ecol. 53:153–162.
Lau SCK, Thiagarajan V, Cheung SCK, Qian P-Y. 2005. Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates. Aquat Microb Ecol. 38:41–51.
Lorenzen CJ. 1966. A method for the continuous measurement of in vivo chlorophyll concentration. Deep Sea Res Oceanographic Abstracts. 13:223–227.
Mieszkina S, Martin-Tanchereau P, Callow ME, Callow JA. 2012. Effect of bacterial biofilms formed on fouling-release coatings from natural seawater and Cobetta marina, on the adhesion of two marine algae. Biofouling. 28:953–968.
Monterofte M, Garcia-Gasca A. 1994. Spat collection studies on pearl oysters Pinctada mazatlanica and Pteria sterna (Bivalvia, Pteriidae) in Bahia de La Paz, South Baja California, Mexico, Hydobiologia. 291:21–34.
Nedashkovskaya O, Kim SB, Han SK, Snauwaert C, Vancanneyt M, Swings J, Kim KO, Lysenko AM, Rohde M, Frolova GM, et al. 2005. Winogradskyella thalassocola gen. nov., sp. nov., Winogradskyella epiphytica sp. nov. and Winogradskyella extima sp. nov., marine bacteria of the family Flavobacteriaceae. Intern J Syst Evol Microb. 55:49–55.
Patil JC, Anil AC. 2005. Biofilm diatom community structure: influence of temporal and substratum variability. Biofouling. 21:189–206.
Pruess E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, GLOCKNER FO. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.
Qian PY, Lau SCK, Dahms H-U, Dobretsov S, Harder T. 2007. Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture. Mar Biotechnol. 9:399–410.
Robson MA, Williams D, Wolff K, Thomason JC. 2009. The effect of surface colour on the adhesion strength of *Elminius modestus* Darwin on a commercial non-biocidal anti-fouling coating at two locations in the UK. Biofouling. 25:215–227.

Satheesh S, Wesley GS. 2010. Influence of substratum colour on the recruitment of macrofouling communities. J Mar Biol Assoc UK. 90:941–946.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microb. 75:7537–7541.

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

Shine R, Brischoux F, Pile AJ. 2010. A seasnake’s colour affects its susceptibility to algal fouling. Proc Royal Soc B: Biol Sci. 277:2459–2464.

Sun Y, Cai Y, Liu L, Yu F, Farrell ML, Farmerie W. 2009. ESPRIT: estimating species richness using large collections of 16S rRNA pyrosequences. Nucleic Acids Res. 37:e75.

Svane I, Dolmer P. 1995. Perception of light at settlement: a comparative study of two invertebrate larvae, a scyphozoan planula and a simple ascidian tadpole. J Exp Mar Biol Ecol. 187:51–61.

Swain G, Herpe S, Ralston E, Tribou M. 2006. Short-term testing of antifouling surfaces: the importance of colour. Biofouling. 22:425–429.

Swain GW, Nelson WG, Preedeekanit S. 1998. The influence of biofouling adhesion and biotic disturbance on the development of fouling communities on non-toxic surfaces. Biofouling. 12:257–269.

Toupoint N, Mohit V, Linossier I, Bourgougnon N, Myrand B, Olivier F, Lovejoy C, Tremblay R. 2012. Effect of biofilm age on settlement of *Mytilus edulis*. Biofouling. 28:985–1001.

Trindade-Silva AE, Rua C, Silva GGZ, Dutillh BE, Moreira AP, Edwards RA, Hajdu E, Lobo-Hajdu G, Vasconcelos AT, Berling RGC, Thompson FL. 2011. Taxonomic and functional microbial signatures of the endemic marine sponge *Arenosclera brasiliensis*. PLoS ONE. Available from: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0039905

Wahl M. 1989. Marine Epibiosis.1. Fouling and antifouling – some basic aspects. Mar Ecol Prog Ser. 58:175–189.

Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F. 2012. The second skin: ecological role of epibiotic biofilms on marine organisms. Front Microbiol. 3:292.

Wang C, Bao W-Y, Gu Z, Li Y-F, Liang X, Ling Y, Cai S-H, Shen H-D, Yang J-L. 2012. Larval settlement and metamorphosis of the mussel *Mytilus coruscus* in response to natural biofilms. Biofouling. 28:249–256.

Webster NS, Negri AP. 2006. Site-specific variation in Antarctic marine biofilms established on artificial surfaces. Environ Microbiol. 8:1177–1190.

Wigglesworth-Cooksey B, Cooksey KE. 2005. Use of fluorophore-conjugated lectins to study cell-cell interactions in model marine biofilms. Appl Environ Microbiol. 71:428–435.

Yoon J-H, Kang S-J, Jung Y-T, Oh T-K. 2010. *Gaethulicola byunsanensis* gen. nov., sp. nov., isolated from tidal flat sediment. Intern J Syst Evol Microb. 60:196–199.

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.

Zargiel KA, Coogan JS, Swain GW. 2011. Diatom community structure on commercially available ship hull coatings. Biofouling. 27:955–965.