Journal of the Marine Biological Association of the United Kingdom, 2014, 94(8), 1639–1649.
doi:10.1017/S0025315414001039

Plantigrade settlement of the mussel *Mytilus coruscus* in response to natural biofilms on different surfaces

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Surface properties affect the attachment of micro- and macroscopic marine organisms. The current study examined the settlement response of the mussel Mytilus coruscus *plantigrades to natural biofilms formed on surfaces of different wettability. The percentages of plantigrade settlement were not influenced by the biofilms formed on variously wettable surfaces in the short term, but after 10 days, the plantigrade settlement rates decreased on biofilms formed on lower wettability surfaces. In general, lower wettability of the surfaces resulted in the decrease of the dry weight, bacterial and diatom density and the thickness of natural biofilms when compared to high wettability surfaces. In contrast, chlorophyll-a concentration in biofilms was independent of the initial wettability of the surfaces. Comparative cluster analysis of bacterial denaturing gradient gel electrophoresis patterns revealed that high variability existed between the bacterial community on high wettability surfaces and that on low wettability surfaces. Thus, surface wettability affects the formation of natural biofilms, and this variation in biofilms developed on different wettability surfaces may explain the discrepancy in their corresponding inducing activities on* M. coruscus *plantigrade settlement. This finding provides new insight into interactions between mussel settlement, biofilm characteristics and surface properties.*

Keywords: *Mytilus coruscus*, plantigrade settlement, biofilms, surfaces

Submitted 7 January 2014; accepted 24 June 2014; first published online 30 July 2014

INTRODUCTION

Biofilms are ubiquitous in the marine world (García-Fernández *et al.*, 2013). Biofilms are composed of multiple species of microorganisms attached to the substratum and encased within a matrix of extracellular polymeric substance (Dobretsov *et al.*, 2013). Marine biofilms contain different species of heterotrophic bacteria, cyanobacteria, archaea, diatoms, protozoans and other microorganisms (Dobretsov, 2010; Callow & Callow, 2011; Wahl *et al.*, 2012). Biofilms can promote (Kirchman *et al.*, 1982; Bao *et al.*, 2007b; Yang *et al.*, 2013), inhibit (Holmström *et al.*, 1992; Dobretsov & Qian, 2004), or have no effect (Henschel & Cook, 1990; Wieczorek & Todd, 1998) on larval settlement and metamorphosis of many marine invertebrates. Thus, different species of marine invertebrates may exhibit varying responses to biofilms.

Despite the long-recognized role of biofilms as potential inducers of larval settlement and metamorphosis of marine invertebrates, knowledge about the interactions between marine biofilms and resettlement of metamorphosed plantigrades of mussels is limited. For example, biofilms enhanced or had no effect on settlement of juveniles of the brown mussel *Perna perna* (Ank *et al.*, 2009). Recently, Yang *et al.* (2014) demonstrated that natural biofilms could enhance settlement of plantigrades of the mussel *Mytilus coruscus*.

The formation of biofilms can be affected by the physical, chemical and biological factors (Dobretsov, 2009). Recently, effects of physical factors, especially the characteristic of the surface including wettability, roughness, colour and tension have received much attention (Terlizzi & Faimali, 2010). Wettability represents a fundamental property of any material; it reveals information about the chemical structure of the material (Genzer & Efimenko, 2006). Surface wettability may regulate patterns of cell accumulation and cell distribution during the early stages of biofilm development (Terlizzi & Faimali, 2010). In addition, surface wettability is one of the major factors influencing larval settlement of many marine invertebrates (Prendergast, 2010), e.g. the bryozoan, *Bugula neritina* (Rittschof & Costlow, 1989; Gerhart *et al.*, 1992), the mussel, *M. galloprovincialis* (Carl *et al.*, 2012), the barnacles *Balanus amphitrite* (Rittschof & Costlow, 1989) and *B. improvisus* (Dahlström *et al.*, 2004) and the ascidian, *Ascidia nigra* (Gerhart *et al.*, 1992). The studies described above focused on understanding the interaction between surface wettability and biofilm formation or the interaction between surface wettability and larval settlement of marine invertebrates. Although the interaction between surface wettability and the biofilm community is

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expected to play an important role in the larval settlement process (Maki *et al.*, 2000; Faimali *et al.*, 2004), knowledge of the interactions between surface wettability, biofilm characteristics and settlement of marine invertebrates is limited.

The mussel *M. coruscus* is an important fouling species in China (Cai *et al.*, 1994; Yang *et al.*, 2013), and inhabits the temperate zone along the coast of the East China Sea (Chang, 2007). In contrast with other marcofouling organisms, mussels are able to detach, cutting their byssal thread, to reattach on a new habit (Petrone, 2013). At the end of a planktonic larval phase, the competent pediveliger larvae of the mussels will settle and metamorphose in a suitable environment. The metamorphosed individuals are termed plantigrades (Bayne, 1964; Carl *et al.*, 2011, 2012), post-larvae (Cárceres-Martínez *et al.*, 1994; Yang *et al.*, 2008, 2011), spat, seed or juveniles (Alfaro *et al.*, 2004). Following the primary settlement, these plantigrades can detach from their byssal threads and resettle to a new location (Bayne, 1964; Kavouras & Maki, 2003; Petrone, 2013). In the present study, the authors investigated effects of surface wettability on the characteristics of natural biofilms, such as biofilm age, dry weight, bacterial and diatom densities, thickness, chlorophyll-*a* (chl *a*) and the biofilm community. Simultaneously, the effect of biofilms formed on surfaces of different wettabilities on the settlement of *M. coruscus* plantigrade was also examined. The purpose of this study was to determine whether the biofilm formation was influenced by surface wettability, and whether change of biofilm characteristics resulted in variance of the settlement of plantigrades of *M. coruscus*.

MATERIALS AND METHODS

Preparation and deployment of glass slides

In order to obtain the different wettabilities, microscopic glass slides (38 mm × 26 mm) were treated following the method of Gerhart *et al.* (1992). Briefly, surfaces of glass slides were prepared by heating at 500°C for 4 h, and stored in a desiccated cabinet at 100°C until used. Four modified surfaces were produced through silanisation using procedures summarized in Table 1. Two treatments, trimethylsilyl (TMS) and dimethyldichlorosilane (DMS), created low surface wettability. Two treatments, aminopropyltriethoxysilane (APS) and (3-chloropropyl)trimethoxysilane (CLPRS), created intermediate surface wettability. Non-treated glass slides (Glass) showed high surface wettability.

Drop spread measurement

The wettability of each surface was measured using the drop spread technique according to the procedures described by Gerhart *et al.* (1992). The spread of 25 µl drops of water (100, 80, 60, 40, 30, 20, 10, 0%) in methanol was measured to the nearest millimetre. The calculation of the standardized harmonic mean (SHM) of the drop spread data was conducted following the method of Gerhart *et al.* (1992). Six replicate measurements were used in the assays. Briefly, drop spread measurements were reduced to a single number, scaled from 0 to 100, by calculating a SHM of the drop spread measurements using the following equation (Gerhart *et al.*, 1992):

$$\mathrm{SHM} = \left[\left(\frac{8}{1/W_{100} + 1/W_{80} + 1/W_{60} + 1/W_{40} + 1/W_{30} + 1/W_{20} + 1/W_{10} + 1/W_{0}}\right) - 4\right] /16 \times 100$$

where W_{100} is mm drop spread at 100% water; W_{80} is mm drop spread at 80% water in methanol, etc; 8 indicates number of solvent concentrations used; 4 indicates minimum possible drop measurement in millimeters; 16 indicates maximum range of drop sizes in millimetres.

Development of natural biofilms

Biofilm slips were prepared by immersing clean all of the treated and untreated glass slides in coastal seawater at Gouqi Island (122°77′E 30°72′N), Zhejiang Province, China. All slips were placed on PVC holders and immersed at a depth of 0.5–1.0 m below the surface for 1–10 days in November 2012. The slips were brought back to the laboratory and thoroughly washed with autoclaved filtered seawater (AFSW) prior to use in biofilm collection for dry weight measurement, enumeration of diatom density, DNA extraction and mussel settlement bioassays on the same day.

Measurement of biofilm dry weight

Dry weight was measured by the method of Bao *et al.* (2007a). The biofilms on each glass slide were scraped off by using a sterile glass slide and separately suspended in AFSW. Each suspension was collected on a pre-weighed GF/C filter (Whatman glass fibre filter; pore size: 1.2 µm) by filtration. Each filter paper holding the biofilm was washed with 50 ml of 0.22 µm filtered distilled water, dried for 48 h in an oven at 80°C and cooled to room temperature in a desiccator before weighting. The dry weight of the biofilm was determined after subtracting the weight of the filter.

Table 1. Silanizing procedures used in the assay.

Surface	Silane (%)	Coupling solvent	Reaction interval	Rinse solvents	Dry interval
APS	2	95% EtOH	15–30 min	2 × EtOH, 1 × MeOH	15 min at 100°C
CLPRS	1	95% EtOH*	30 min	2 × EtOH, 1 × MeOH	15 min at 100°C
DMS	100	none	60 min	Drain	2 h at 100°C
TMS	1	CH_2Cl_2	60 min	1 × CH_2Cl_2	30 min at 100°C

*, sodium pyrophosphate buffer.

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Enumeration of densities of bacteria and diatoms

Biofilms were fixed in 5% formalin solution for a maximum duration of 3 weeks. Samples were washed with AFSW and stained by acridine orange (AO, 0.1%) for 5 min. Bacterial densities of stained samples were counted directly at 1000× magnification under an Olympus BX51 epifluorescence microscope. Densities of diatoms were counted directly at 200× magnification under a light microscope immediately after samples were brought back to the laboratory. The densities of bacteria and diatoms in each sample were enumerated from ten random fields of view.

Determination of biofilm thickness

Biofilm thickness was measured by the method of Yang *et al.* (2013). Biofilms of four ages were fixed in 5% formalin solution for 24 h. Biofilms were stained by propidium iodide (5 μg ml^{-1}) and incubated for 15 min in the dark. Slides were washed three times, and then were observed at 400× magnification under an Olympus FluoView™ FV1000 confocal laser scanning microscope (CLSM). Three replicate biofilms were examined for each age of biofilm. Ten random fields of view of each biofilm were selected for imaging and analysis. Thirty image stacks of varying thickness were generated to determine the full thickness of the biofilms in each field of view.

Chlα concentration in natural biofilms

The chlα concentration in biofilms was measured following the method of Wang *et al.* (2012). Briefly, natural biofilms were scraped from three replicate glass slides using sterile glass slides, filtered through membranes and preserved at −20°C. Chl *a* extraction was conducted at 4°C using 90% acetone for 14 h in darkness. To ensure complete extraction of chl *a*, samples were vortexed for 1 h at the end of the extraction period, then centrifuged for 10 min at 3000 rpm. The chl *a* concentration of the supernatants was determined spectrophotometrically (UNIC 2100 spectrophotometer). The wavelengths measured were 630, 647, 664 and 750 nm, respectively. The chl *a* concentrations were calculated using the equation of Ma *et al.* (2011).

DNA extraction

Biofilms were scraped from three replicate glass slides as above and centrifuged for 5 min at 10,000 g. The supernatant was discarded and genomic DNA was extracted using a 3S DNA Isolation Kit for Environmental Samples V2.2 following the manufacturer's instructions (Shenergy Biocolor Bioscience and Technology Company, Shanghai, China).

PCR amplification of 16S rDNA

Bacterial 16S rRNA genes were amplified using the primers 357F, which contains a GC clamp (5′-C GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG CCT ACG GGA GGC AGC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer *et al.*, 1993). PCR amplification was performed in a 25 μl reaction mixture containing 0.5 μl of each primer (10 μM), 1 μl of template DNA (40–80 ng), 0.25 μl of Ex Taq (5 U μl^{-1}), 2.5 μl of 10× PCR buffer, 2 μl of $MgCl_2$ (25 mM) and 0.5 μl of deoxynucleotide triphosphates (10 mM each) and sterile distilled water to a final volume of 25 μl. PCR cycling was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) thermocycler under the following conditions: an initial denaturing step at 94°C for 5 min, a touch-down thermal cycling of denaturation at 94°C for 1 min, annealing at 65–55°C for 1 min (reducing 0.5°C per cycle) and elongation at 72°C for 0.5 min. Then another fifteen PCR cycles were conducted, each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 0.5 min synthesis at 72°C. Finally, an extension step was carried out at 72°C for 8 min. PCR products were verified by agarose gel electrophoresis (1.2% weight/volume agarose) with ethidium bromide staining and visualized using an ultraviolet (UV) transilluminator.

Denaturing gradient gel electrophoresis analyses of bacterial community

Denaturing gradient gel electrophoresis analysis (DGGE) of the PCR amplified 16S rDNA was carried out using the D-Code™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). PCR products were resolved on a vertical gel containing 8% (w/v) polyacrylamide (arylamide:bisacrylamide, 37.5:1) and a denaturing gradient ranging from 40 to 70%. One hundred per cent denaturant is defined as a 7 M urea and 40% (v/v) deionized formamide. Electrophoresis (60 V, 14 h) was performed in 1 × TAE buffer (40 mM Tris-acetate, 20 mM acetate, 1 mM Na_2-EDTA) at 60°C. After electrophoresis, the gel was stained in ethidium bromide for 20 min and photographed under UV illumination. DGGE gel images were analysed using Quantity One analysis software (Bio-Rad). A similarity matrix was constructed based on the total number of bands observed in all biofilm bacterial communities and the presence or absence of these bands in each community. Agglomerative hierarchical clustering was performed using UPGMA (unweighted pair group method using arithmetic averages) and the similarities among communities were displayed as a dendrogram. Bacterial communities of three replicates within each treatment, at each time point were assessed using DGGE.

Mussel settlement bioassay

Plantigrades (shell length/height = 580 ± 39/431 ± 28 μm) of the mussel *M. coruscus* were obtained from the mussel hatchery farm of Dongtou, Zhejiang Province, China and carried, within 10 h, to Shanghai Ocean University, Shanghai. Prior to the settlement bioassay, plantigrades were maintained at 18 ± 1°C for more than 7 days and fed a diet of *Isochrysis galbana* at 1.0 × 10^5 cells ml^{-1} day^{-1}. In the settlement bioassay, ten plantigrades were transferred into individual glass Petri dishes (Ø64 mm × 19 mm height) containing 20 ml of AFSW and one biofilm slip. The inducing activity of biofilms was evaluated by the percentage of settled plantigrades. Settled plantigrades were verified after 12 h when they attached to the surface with byssal threads. Petri dishes, each containing 20 ml of AFSW, 10 plantigrades and a clean (non-biofilmed) glass slip were set up as negative controls (Blank) in all assays. Assays were conducted at 18 ± 1°C in darkness. Six replicates were used in assays.

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Statistical analysis

Settlement inducing activities of the biofilms were evaluated by the percentages of settled plantigrades. Prior to statistical analysis, all data expressed in percentages were arcsine-transformed and tested for normality and homogeneity. Normality of distribution was assessed using the Shapiro–Wilk test. Homogeneity of variance was verified using the O'Brien test. The effects of surface wettability on biofilm activity, dry weight, bacterial and diatom densities, biofilm thickness and chlα concentrations were analysed using the Kruskal–Wallis Test. All statistical computations were performed using JMP™ software (v.10.0.0). Differences were considered significant at $P < 0.05$. A one-way analysis of similarity (ANOSIM, PRIMER 6, Clarke & Warwick, 2001) was conducted with wettability and time point as the factors. ANOSIM computes a test statistics (R), where $R = 1$ if all replicates within a treatment are more similar to each other than any replicates from different treatments. R is approximately zero if the null hypothesis is true, that similarities between and within treatments are the same. Three replicates were conducted in the ANOSIM analysis. A Monte Carlo simulation where the Bray–Curtis matrix is randomly rearranged allows comparison between simulated and observed R-values and also determines the significance level at which the null hypothesis can be rejected.

RESULTS

Manipulation of surface hydrophobicity

The surface wettabilities of treated and untreated glass slides are shown in Figure 1. The surface SHM values ranged from 9.14 to 36.99. The SHM value was the highest in the untreated glass slides. The SHM values in DMS and TMS were lower than others, indicating that these two treatments resulted in most hydrophobic surfaces. The SHM values of APS and CLPRS were 20.57 and 35.29, respectively.

Plantigrade settlement in response to natural biofilms on manipulated surfaces

The percentages of settled plantigrades on natural biofilms formed on surfaces with different wettabilities are shown in Figure 2. In the negative controls, only 10% plantigrades settled on non-biofilmed glass slides. In general, the percentages of settlement significantly increased with the age of biofilms (Kruskal–Wallis: $P < 0.001$). After immersion for 1 and 3 days, no significant inhibiting effect on settlement rates was observed among the manipulated surfaces (Kruskal–Wallis: $P > 0.05$). After 6 days, no significant decrease in percentages of settlement was observed in surfaces treated by APS and TMS (Kruskal–Wallis: $P > 0.05$), whereas settlement rates increased significantly in surfaces treated by CLPRS and DMS (Kruskal–Wallis: $P < 0.05$) when compared to non-treated Glass. After 10 days, there were significantly lower settlement rates in surfaces treated by CLPRS and TMS (Kruskal–Wallis: $P < 0.05$), while no difference was observed in surfaces treated by APS and DMS (Kruskal–Wallis: $P > 0.05$).

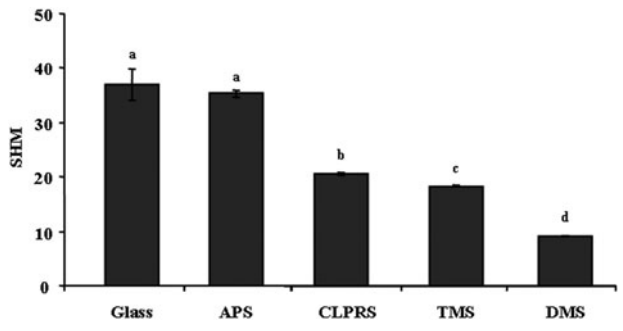

Fig. 1. The standardized harmonic mean values of various wettability surfaces. Data with significant difference are indicated by different letters. Data are means (±standard error) of six replicates. Glass, non-treated surface of glass slides. Treatments: APS, aminopropyltriethoxysilane; CLPRS, (3-chloropropyl)trimethoxysilane; TMS, trimethylsilyl; DMS, dimethyldichlorosilane.

Dry weight, bacterial and diatom densities on manipulated

The dry weights and the bacterial and diatom densities of biofilms at different wettabilities are shown in Figure 3. For the dry weight (Figure 3A), significant decrease was observed on surfaces treated by TMS after 1 and 10 days when compared with the untreated Glass. In contrast, there was no decrease in all treated surfaces after 3 and 6 days. In the bacterial densities (Figure 3B), the cell density only decreased in surfaces treated by DMS after 1 day (Kruskal–Wallis: $P < 0.05$). After 3 days, bacterial density increased compared with the untreated Glass (Kruskal–Wallis: $P < 0.05$). After 6 and 10 days, bacterial density decreased significantly in all treated surfaces except for that treated by CLPRS (Kruskal–Wallis: $P < 0.05$). In the case of diatom density (Figure 3C), no significant decrease was observed in all surfaces treated after 1 day (Kruskal–Wallis: $P > 0.05$) and 3 days (Kruskal–Wallis: $P > 0.05$). After 6 days, diatom density decreased significantly in surfaces treated by APS and DMS (Kruskal–Wallis: $P < 0.05$). After 10 days, there was significant decrease in surfaces treated by DMS and TMS (Kruskal–Wallis: $P < 0.05$).

Biofilm thickness

Confocal laser scanning microscope images of natural biofilms on surfaces of different wettabilities are shown in Figure 4. Significant difference was observed in surfaces treated with APS compared to Glass after 10 days, while both exhibited similar inducing activity on settlement of plantigrades. The thickness of biofilms on surfaces of different wettabilities is shown in Figure 5. Surface wettability affected significantly the thickness of natural biofilms. In addition, biofilm age significantly affected the thickness of the biofilm. The biofilm thickness on all surfaces increased with the increasing biofilm age (Kruskal–Wallis: $P < 0.05$), the maximum being obtained after 10 days.

Chlα analysis

The chl *a* concentrations in the biofilms on surfaces of different wettabilities are as shown in Figure 6. No significant difference was observed among all surfaces of different wettabilities even after 10 days (Kruskal–Wallis: $P > 0.05$). The

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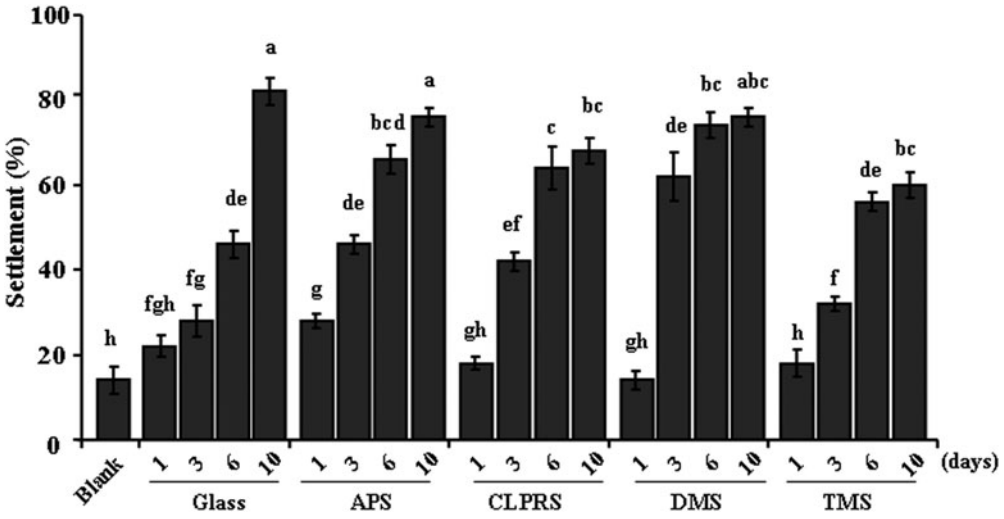

Fig. 2. Percentages of plantigrade settlement on natural biofilms formed on different wettability surfaces. Data with significant difference are indicated by different letters. Data are means (±standard error) of six replicates. Blank, non-biofilmed glass slides. Glass, non-treated glass slides. Treatments: APS, aminopropyltriethoxysilane; CLPRS, (3-chloropropyl)trimethoxysilane; TMS, trimethylsilyl; DMS, dimethyldichlorosilane.

minimum and maximum chl *a* concentrations were, respectively, 0.004 μg cm^{-2} on the surface treated by CLPRS after 1 day and 0.017 μg cm^{-2} on the surface treated by APS after 10 days.

Bacterial community analysis by DGGE

Comparative analysis of bacterial DGGE patterns using Bray–Curtis cluster analysis is shown in Figure 7. In general, Bray–Curtis cluster analysis showed that the variability between the various surface treatments was high. For 1 day-biofilms (Batch 1), cluster analysis revealed that the similarity between untreated Glass and surfaces treated by APS was 72%, and that two biofilms shared 65% with biofilms on other manipulated surfaces. A similar tendency was also found for 3 day-biofilms and 6 day-biofilms (Batch 1). For 10 day-biofilms (Batch 1), cluster analysis revealed that the variability between surfaces treated by DMS and TMS was the greatest (23%), and the variability between untreated Glass and surfaces treated by APS was the least (18%). For Batch 2, cluster analysis of bacterial communities in 1 day-biofilms revealed that the similarity between untreated Glass and surfaces treated by APS was 76%, and that two biofilms shared 72% with biofilms on other manipulated surfaces. For 3 day-biofilms and 6 day-biofilms, cluster analysis revealed that the variability between surfaces treated by CLPRS and TMS was the least (<20%). For 10 day-biofilms, cluster analysis revealed that the variability between untreated Glass and surfaces treated by APS was the greatest (24%), and the variability between surfaces treated by DMS and TMS was the least (18%). For Batch 3, cluster analysis of bacterial communities in 1 day-biofilms revealed that the similarity between untreated Glass and surfaces treated by APS was 76%, and that two biofilms shared 72% with biofilms on other manipulated surfaces. For 3 day-biofilms, cluster analysis revealed that the variability between surfaces treated by CLPRS and APS and between surfaces treated by DMS and TMS were the same (23%). For 6 day-biofilms, cluster analysis revealed that the variability between untreated Glass and surfaces treated by APS was the greatest (26%), and the variability between surfaces treated by TMS and CLPRS was the least (19%). For 10 day-biofilms, cluster analysis revealed that the variability between untreated Glass and surfaces treated by CLPRS was the greatest (23%), and the variability between surfaces treated by APS and TMS was the least (15%).

The ANOSIM test was performed to determine the similarity between treatments. *R*-values were interpreted as follows: $R > 0.75$ as well separated, $R > 0.5$ as overlapping, but clearly different, and $R < 0.25$ as barely separable at all, in accordance with the PRIMER-manual (Clarke & Warwick, 2001). For 1, 3 and 10 day-biofilms, difference between bacterial communities on the manipulated surfaces were observed, which all had an *R* value of >0.5. For 6 day-biofilms, significant difference between bacterial communities on the manipulated surfaces were observed, which both had an *R* value of >0.75. In addition, the ANOSIM test was also performed to determine the similarity between different-aged biofilms on each surface. For untreated Glass, no significant differences between bacterial communities on biofilms of four ages were observed; all had an *R* value of <0.25. For the treated surfaces a similar tendency was also observed in biofilms of four ages.

DISCUSSION

The effect of biofilms on the recruitment of marine invertebrates could depend on substrate characteristics (Terlizzi & Faimali, 2010). However, interaction between substrate and biofilm in affecting larval settlement remains little known (Faimali *et al.*, 2004). In the present study, the authors demonstrated that surface wettability affected the formation of natural biofilms, and this difference in biofilms might result in the variance of attracting settlement of plantigrades of *M. crouscus*.

Previous studies reported that there was no significant difference in the bacterial density between the surfaces with different wettabilities (Hung *et al.*, 2008). Similarly, Huggett *et al.* (2009) also demonstrated no difference between cell densities of bacteria in biofilms formed on treated surfaces of various

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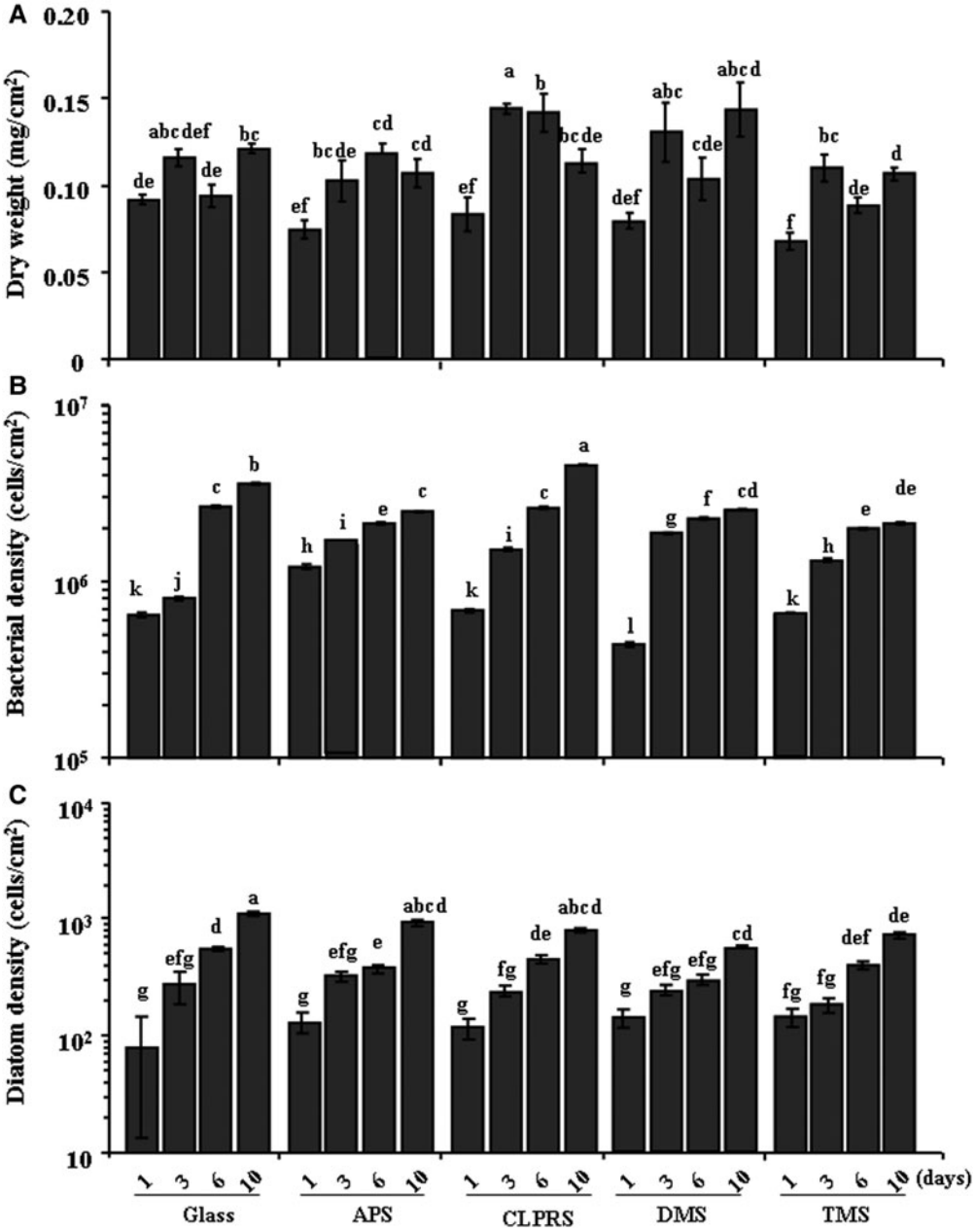

Fig. 3. Dry weights (A), bacterial densities (B) and diatom densities (C) of biofilms formed on surfaces of different wettabilities. Data with significant difference are indicated by different letters. Dry weights are means (±standard error) of six replicates. Bacterial and diatom densities are means (±SE) of 10 random fields of view. Glass, non-treated glass slides. Treatments: APS, aminopropyltriethoxysilane; CLPRS, (3-chloropropyl)trimethoxysilane; DMS, dimethyldichlorosilane; TMS, trimethylsilyl.

wettabilites. However, the results of the present study are not consistent with the above studies (Hung *et al.*, 2008; Huggett *et al.*, 2009), since significant decrease in bacterial densities was found on low wettability surfaces (TMS) when compared with the control after 10 days. Dalton *et al.* (1994) also suggested that surface wettability affects bacterial attachment. The possible explanation is that surface wettability may exert some control over the molecular rearrangements that occur on the surface of the bacteria causing different domains to be exposed (Maki *et al.*, 1990).

In the laboratory assays, some fouling diatom species adhered more strongly to hydrophobic surfaces than to hydrophilic surfaces (Finlay *et al.*, 2002; Holland *et al.*, 2004; Thompson *et al.*, 2008). In the field assays, diatom community composition was influenced by surface wettability (Hung *et al.*, 2008). The results of the present study demonstrated that surface wettability affected attachment of diatoms in natural biofilms, and that the diatom densities were reduced significantly on low wettability surfaces (DMS and TMS) when compared to the control. This indicates that hydrophobic surfaces may limit diatom attachment in the marine environment.

In the present study, surface wettability resulted in a decrease in the dry weight of 1 day-biofilms and 10 day-biofilms formed on surfaces treated by TMS, while no significant decrease was observed for other manipulated surfaces. On the other hand, surface wettabiltiy affected the biofilm thickness, and the thickness of biofilms, especially the

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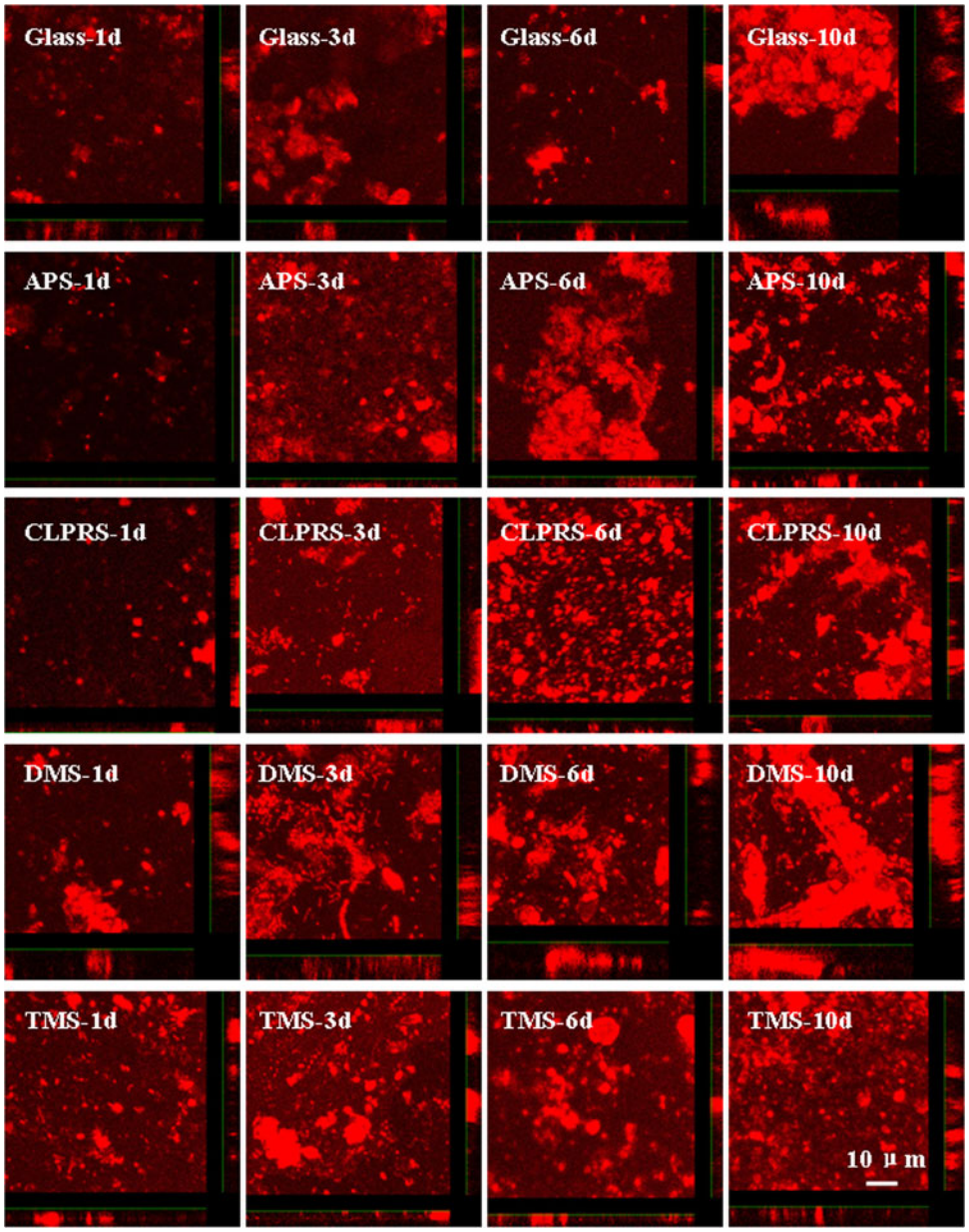

Fig. 4. Confocal microscopy micrographs of natural biofilms on different wettability surfaces. Glass-1d, 1 day-biofilms formed on non-treated glass slides; Glass-3d, 3 day-biofilms formed on non-treated glass slides; Glass-6d, 6 day-biofilms formed on non-treated glass slides; Glass-10d, 10 day-biofilms formed on non-treated glass slides. Treatments: APS-1d, 1 day-biofilms formed on surfaces treated with minopropyltriethoxysilane; APS-3d, 3 day-biofilms formed on surfaces treated with minopropyltriethoxysilane; APS-6d, 6 day-biofilms formed on surfaces treated with minopropyltriethoxysilane; APS-10d, 10 day-biofilms formed on surfaces treated with minopropyltriethoxysilane; CLPRS-1d, 1 day-biofilms formed on surfaces treated with (3-chloropropyl)trimethoxysilane; CLPRS-3d, 3 day-biofilms formed on surfaces treated with (3-chloropropyl)trimethoxysilane; CLPRS-6d, 6 day-biofilms formed on surfaces treated with (3-chloropropyl)trimethoxysilane; CLPRS-10d, 10 day-biofilms formed on surfaces treated with (3-chloropropyl)trimethoxysilane; DMS-1d, 1 day-biofilms formed on surfaces treated with dimethyldichlorosilane; DMS-3d, 3 day-biofilms formed on surfaces treated with dimethyldichlorosilane; DMS-6d, 6 day-biofilms formed on surfaces treated with dimethyldichlorosilane; DMS-10d, 10 day-biofilms formed on surfaces treated with dimethyldichlorosilane; TMS-1d, 1 day-biofilms formed on surfaces treated with trimethylsilyl; TMS-3d, 3 day-biofilms formed on surfaces treated with trimethylsilyl; TMS-6d, 6 day-biofilms formed on surfaces treated with trimethylsilyl; TMS-10d, 10 day-biofilms formed on surfaces treated with trimethylsilyl.

10 day-biofilm thickness, decreased significantly on surfaces of medium and low wettabilities in the present investigation. These findings for the first time demonstrate that surface wettability influences the abundance of natural biofilms, and that subsequently this variation may account for the discrepancy of mussel settlement rates.

Chlorophyll-*a* has been used as an indicator of biomass of photosynthetic organisms in biofilms (Thompson *et al.*, 1999; von der Meden *et al.*, 2010). The present results showed that chl *a* concentration was similar in the biofilms formed on the different wettability surfaces, indicating that chl *a* is independent of the initial wettability of a surface.

Recent studies suggested that no obvious difference was observed in biofilm bacterial community composition between low and high wettability substratum (Hung *et al.*, 2008). Huggett *et al.* (2009) also demonstrated that the community composition of marine biofilms on all surfaces was largely the same across all surfaces, indicating that the

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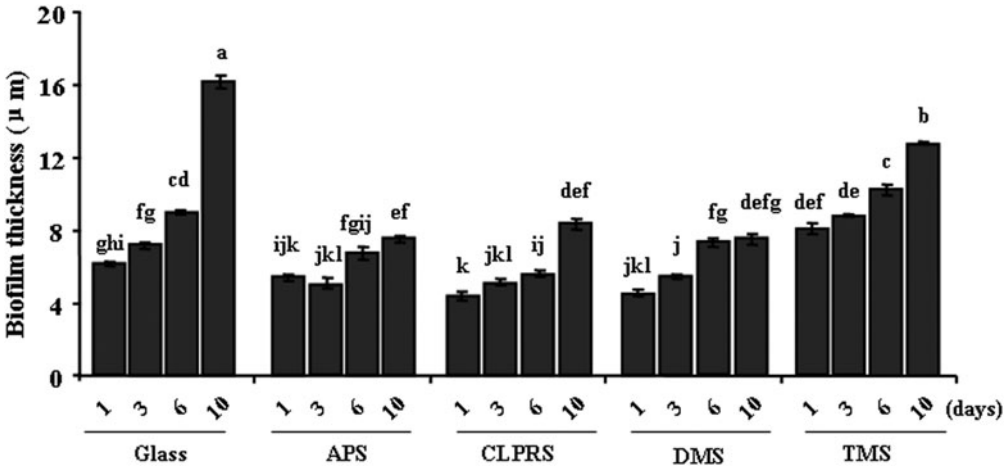

Fig. 5. The thickness of natural biofilms on different wettability surfaces. Data with significant difference are indicated by different letters. Data are means (±standard error) of 30 replicates. Glass, non-treated glass slides. Treatments: APS, aminopropyltriethoxysilane; CLPRS, (3-chloropropyl)trimethoxysilane; DMS, dimethyldichlorosilane; TMS, trimethylsilyl.

formation of a biofilm community on hard surfaces was not influenced by the initial wettability of that surface. However, in the present investigation, ANOSIM of DGGE profiles showed that the *R* values between the various surface treatments ranged from 0.63 to 1.00, suggesting that surface wettability influenced the bacterial community in biofilms formed on Gouqi Island, China. Subsequent investigation also demonstrated that mussel plantigrades preferred to settle on biofilms formed on high wettability surfaces. This finding indicates that the differences in the bacterial community may contribute to the variance of the settlement-inducing activities of these biofilms.

Biofilm age affects settlement and metamorphosis of larvae of many marine invertebrates (Huggett *et al.*, 2009; Yu *et al.*, 2010; Campbell *et al.*, 2011). For mussels, larval settlement in particular is positively correlated with the age of the biofilm, and enhanced larval settlement corresponds to the abundance and composition of the biofilm community (Bao *et al.*, 2007a; Toupoint *et al.*, 2012; Wang *et al.*, 2012). In the present study, plantigrade settlement of *M. coruscus* also increased with biofilm age. This finding on mussel plantigrade settlement is consistent with previous reports on mussel larval settlement (Bao *et al.*, 2007a; Toupoint *et al.*, 2012; Wang *et al.*, 2012). On the other hand, previous studies also suggested that the positive effects of aged biofilm could override the negative effect of low surface wettability on larval settlement (Hung *et al.*, 2008). Huggett *et al.* (2009) showed that bacterial community composition of biofilms that accumulated on surfaces over a 10-day period of immersion was similar, regardless of initial surface wettability. By contrast, the results of the present study demonstrated that the percentage of plantigrade settlement was reduced on the surfaces treated by TMS after 10 days when compared to the controls. The possible explanation is due to the difference of biofilms formed in the different sea areas or due to the variation among species tested. Further research is need.

In conclusion, this study demonstrated that surface wettability can affect the formation of natural biofilms, and the variation in biofilm characteristics, such as dry weight, bacterial and diatom densities, thickness and bacterial community for

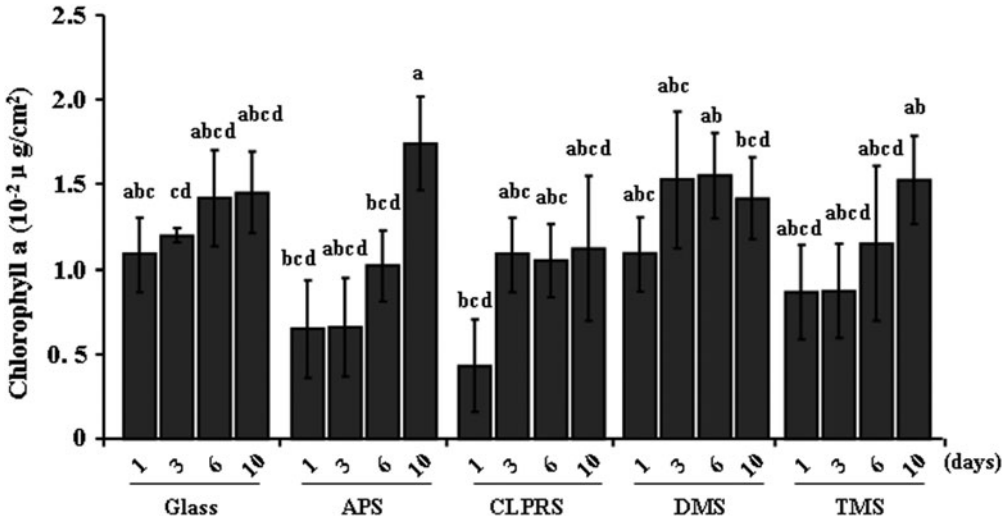

Fig. 6. Chlorophyll-*a* concentration in natural biofilms on different wettability surfaces. Data with significant difference are indicated by different letters. Data are means (±standard error) of six replicates. Glass, non-treated glass slides. Treatments: APS, aminopropyltriethoxysilane; CLPRS, (3-chloropropyl)trimethoxysilane; DMS, dimethyldichlorosilane; TMS, trimethylsilyl.

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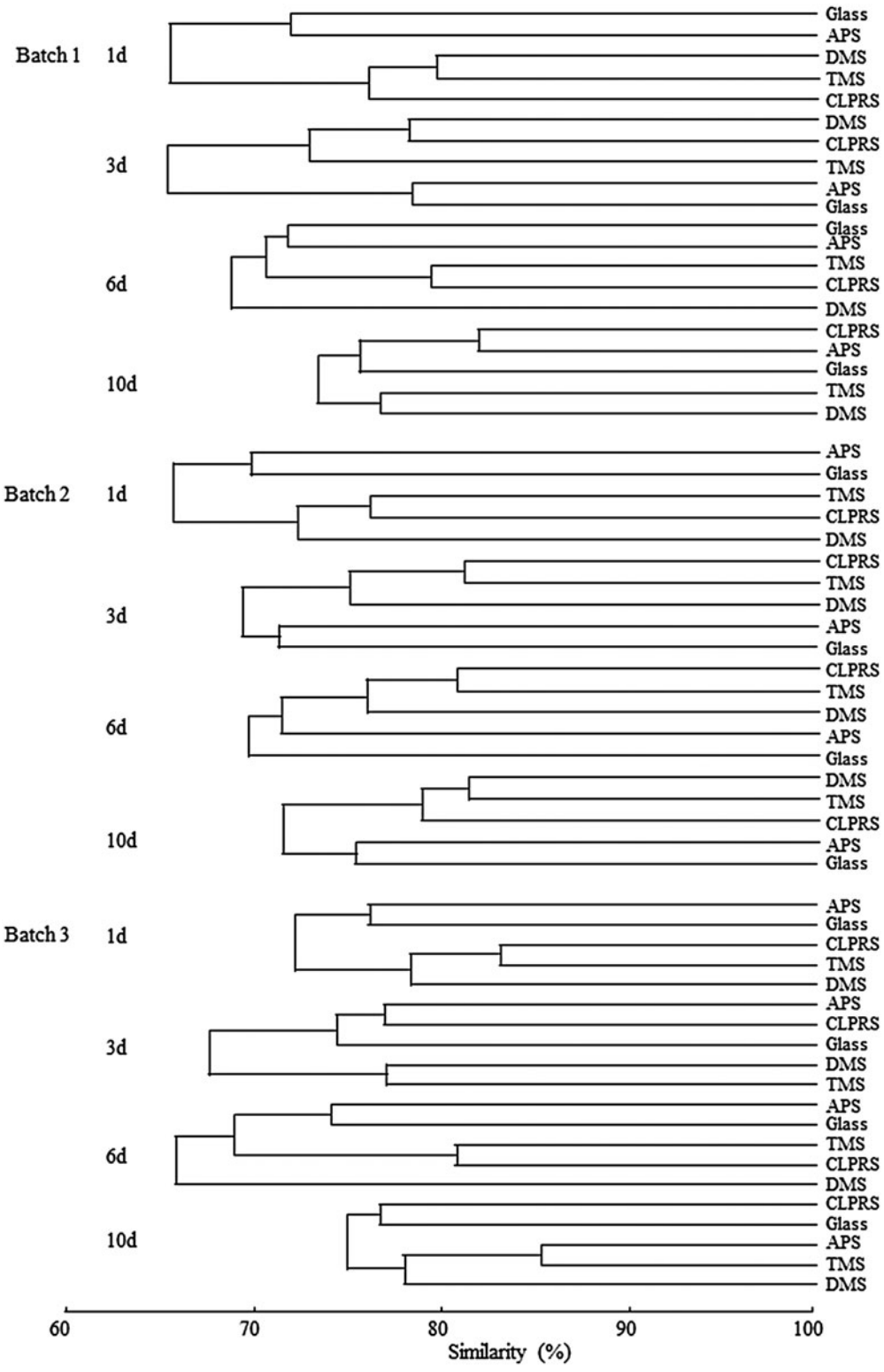

Fig. 7. Dendrogram generated from the denaturing gradient gel electrophoresis analysis profiles, showing the similarities of bacterial community composition of biofilms. Glass, non-treated glass slides. Treatments: APS, aminopropyltriethoxysilane; CLPRS, (3-chloropropyl)trimethoxysilane; DMS, dimethyldichlorosilane; TMS, trimethylsilyl.

different surface wettabilities may contribute to the discrepancy of their corresponding effect in inducing plantigrade settlement of the mussel *M. coruscus*. The present study provides novel information about the interactions between mussel settlement, biofilm characteristics and surface wettability.

FINANCIAL SUPPORT

This study was supported by the Shanghai Municipal Science and Technology Commission (12230502100), National Natural Science Foundation of China (No. 31101885),

https://doi.org/10.1017/S0025315414001039 Published online by Cambridge University Press

Innovation Program of Shanghai Municipal Education Commission (14ZZ143), Shanghai Universities First-class Disciplines Project of Fisheries to J-L Yang, by the Innovation Plan of Shanghai Graduate Education to Y-F Li and by Shanghai Outstanding Undergraduate Scholarship for Interdisciplinary Training to X-P Guo.

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