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Comparative analysis of biofilm community on different coloured substrata in relation to mussel settlement

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Mussels are typical macrofouling organisms in the world. In this study, the interaction between the settlement of Mytilus coruscus *plantigrades and bacterial community on coloured substrata was determined. Bacterial communities in biofilms developed on seven coloured substrata were analysed by Illumina Miseq sequencing. The mussel settlement response to coloured substrata with no biofilms was also examined.* Flavobacteria, Alphaproteobacteria *and* Gammaproteobacteria *were the first, second and third most dominant groups in seven biofilm samples. The results suggest that the inducing activities of these biofilms on plantigrade settlement varied with coloured substrata and the lowest percentage of settlement was observed on biofilms on the green substratum. High-throughput sequencing showed that bacterial community in biofilms also changed with the substratum colour. No significant difference in the inducing activity on plantigrade settlement was observed between the coloured substrata with no biofilms. Thus, difference in plantigrade settlement response may be correlated to the changes in bacterial community on coloured substrata. This finding extends current knowledge of interaction among mussel settlement and bacterial community variability.*

Keywords: *Mytilus coruscus*, mussel settlement, bacterial community, coloured substrata, Miseq sequencing

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INTRODUCTION

Mussels secrete byssal threads and attach themselves to underwater structures, such as coastal power stations, ships' hulls, aquaculture net cages and other maritime systems, with costly consequences (Dobretsov & Qian, 2003; Ank *et al.*, 2009; Briand, 2009; Carl *et al.*, 2011). According to the numbers of published papers, mussels have been viewed as important macrofouling organisms (Briand, 2009). Understanding the environmental factors controlling mussel settlement is therefore imperative in the fields of biofouling and antifouling research.

In contrast to other many marine invertebrates, the settlement of mussels is not permanent (Bayne, 1964; Carl *et al.*, 2012). Initially, the mussel pediveligers can make a settle response to various substrata (Satuito *et al.*, 1995; Dobretsov, 1999; Bao *et al.*, 2007; Alfaro *et al.*, 2011; Yang *et al.*, 2013b). These above pediveligers metamorphose and are described as post-larvae (Bayne, 1971; Cárceres-Martínez *et al.*, 1994; Satuito *et al.*, 1995; Yang *et al.*, 2008, 2011), plantigrades (Bayne, 1964; Seed, 1969; Carl *et al.*, 2011, 2012) or juveniles (Alfaro *et al.*, 2004). Subsequently, plantigrades can cut the byssal threads, detach and reattach on the appropriate substrata (Kavouras & Maki, 2003; Petrone, 2013). A number of researchers have focused on larval settlement of many species of mussels, such as *Mytilus edulis* (Dobretsov & Qian, 2003), *M. galloprovincialis* (Satuito *et al.*, 1995; Bao *et al.*, 2007; Yang *et al.*, 2007, 2011), *M. coruscus* (Wang *et al.*, 2012; Yang *et al.*, 2013a), *Perna canaliculus* (Young *et al.*, 2011; Ganesan *et al.*, 2012) and *Aulacomya maoriana* (Alfaro *et al.*, 2011). However, the interaction between environmental factors and mussel plantigrade settlement has hardly been investigated.

Biofilms exist on nearly all submerged natural and artificial surfaces (Palmer & White, 1997; Thiyagarajan *et al.*, 2006; Nasrolahi *et al.*, 2012; Wahl *et al.*, 2012). Marine biofilms are assemblages of microbes along with the exopolysaccharide matrix (Decho, 2000; Flemming & Wingender, 2010; Shikuma & Hadfield, 2010). The roles of biofilms in biofouling have been increasingly recognized, and some studies have shown that biofilms influence the settlement of larvae of many invertebrates (Wieczorek & Todd, 1998; Dobretsov, 2009; Qian & Dahm, 2009; Hadfield, 2011). However, knowledge of biofilms as well as of the interaction between biofilms and fouling organisms is still limited (Fusetani, 2011).

Bacterial communities in marine biofilms are correlated to the settlement process of larvae of many species of marine invertebrate (Huang & Hadfield, 2003; Qian *et al.*, 2003; Lau *et al.*, 2005; Chung *et al.*, 2010). For mussels, Wang *et al.* (2012) reported that the biofilm composition change may impact on larval settlement of *M. coruscus*. Although the

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role of bacterial community in biofouling by macroorganisms has been recognized, knowledge of the interaction between the bacterial community and plantigrade settlement of mussel is limited.

Substratum properties including surface colour (Swain *et al.*, 2006; Robson *et al.*, 2009; Dobretsov *et al.*, 2013), orientation (Connell, 1999; Glasby, 2000; Bellou *et al.*, 2012), depth (Bellou *et al.*, 2012), substratum type (Jones *et al.*, 2007; Bellou *et al.*, 2012; Li *et al.*, 2014), roughness (Verran & Boyd, 2001; Allion *et al.*, 2006), wettability (Vrolijk *et al.*, 1990; Gerhart *et al.*, 1992; Carl *et al.*, 2012; Yang *et al.*, 2014), topography (Scardino & de Nys, 2011; Carl *et al.*, 2012) and surface energy (Allion *et al.*, 2006; Finlay *et al.*, 2010) influence the biofouling communities. To date, surface colours have received some attention with regard to their effects on larval settlement of macro-fouling organisms (Prendergast, 2010; Satheesh & Wesley, 2010), especially in barnacles (Pomerat & Reiner, 1942; Yule & Walker, 1984; Kon-ya & Miki, 1994; Prendergast, 2010). For example, Pomerat & Reiner (1942) reported that the barnacle *Balanus eburneus* prefers to settle on black glass. Robson *et al.* (2009) investigated the interaction between surface colour and barnacle settlement, and found that black has the highest recruitment density. However, little is known about interaction between substratum colour and microfouling, especially in biofilm communities, with the exception of recent work on microfouling formed on black and white coloured surfaces (Dobretsov *et al.*, 2013). To date, no study has documented the three-way relationship between substratum colours, biofilm community and mussel settlement. Therefore, we hypothesized that biofilm communities formed on different coloured substrata in the marine environment would impact mussel settlement behaviour.

The mussel, *M. coruscus*, which inhabits the East China Sea, is a typical macrofouling organism in China (Cai *et al.*, 1994; Yang *et al.*, 2013b). In the present study, the authors explored the mechanism of bacterial community assembly in the seven coloured substrata, and investigated the subsequent plantigrade settlement response of the mussel *M. coruscus* corresponding to changes of bacterial community.

MATERIALS AND METHODS

Development of natural biofilms

To obtain different surface colours, the emulsion varnish of seven colours was spray-coated over only one side of clean glass slides (38 × 26 mm^2). To avoid the chemical and physical properties of coloured coatings, we kept these coloured substrata in flowing water for 7 days, inserted the side of slides containing coatings into PVC holders, and then immersed them at 0.5–1.0 m for 28 days in coastal seawater at Gouqi Island (122°77′E 30°72′N, Zhejiang Province, China) in September 2014. The side of each slide with no coating was exposed to form the natural biofilms.

Cell density of bacteria in biofilms

Cell density of bacteria in biofilms was evaluated according to the modified method (Yang *et al.*, 2007). Biofilms were scraped off, kept in autoclaved filtered seawater (AFSW) of 10 mL, and fixed with 10 mL of 10% formalin solution (5% final concentration). To ensure the well distribution of bacteria, the bacterial suspension was vortexed prior to enumerating. Acridine orange (AO 0.1%) was used to stain the suspended bacteria, and 0.2 μm pore size polycarbonate Nuclepore (Whatman 4.9 cm^2) filters was used to collect the stained suspension and to count bacterial numbers via an Olympus BX-51 epifluorescence microscope. The bacterial number was counted randomly from 10 fields of view.

DNA extraction and MiSeq sequencing

Total DNA was extracted from each biofilm sample with three replicates using the method of Li *et al.* (2014) and purified by the EZNA® Soil DNA Kit (Omega Bio-tek, Norcross, GA). Amplicons were extracted from 2% agarose gels and purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) following the manufacturer's protocols. The purified amplicons were quantified by QuantiFluor™ -ST (Promega, USA), and the equimolar amplicons were paired-end sequenced (2 × 250) on the Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd, Shanghai, China. The original paired Illunina Miseq reads were submitted to the NCBI sequence Read Archive (SRA) database (accession number: SRP049006).

Settlement bioassay

Plantigrades of the mussel *M. coruscus* were provided by Shengsi Service Center of Marine Science and Technology Development, Zhejiang province, China. *Isochrysis galbana* was provided for plantigrades at 1.0–2.0 × 10^5 cells mL^{-1} day^{-1}. In the plantigrade settlement assay, 20 ml AFSW, one biofilm slip and 10 plantigrades were added into each glass Petri dish (Ø64 × 19 mm^2 height). Settlement-inducing activities of these biofilms on different coloured substrata were determined by settled plantigrades (%). When plantigrades secreted the byssus and attached on the substrata, they were viewed as settled plantigrades. A Petri dish containing a non-biofilmed glass slip of seven coloured substrata, 10 plantigrades and 20 ml AFSW was designed as the negative control. Assays were performed at 18°C with six replications and no light.

Statistical and bioinformatics analysis

Data of settlement rates (%) were arcsine-transformed and tested for normality with JMP™. The effects of colour on biofilm activity were assessed using Kruskal–Wallis and Steel–Dwass All Pair test. Correlations between bacterial densities and biofilm settlement inducing activity were conducted with a Spearman's rank correlation test. Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.17) (Caporaso *et al.*, 2010) with the following criteria (Li *et al.*, 2014). The phylogenetic affiliation of each 16S rRNA gene sequence was analysed by RDP Classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU115) 16S rRNA database with confidence threshold of 70% (Amato *et al.*, 2013). A one-way analysis of similarity (ANOSIM, PRIMER 6, Clarke & Warwick, 2001) was conducted with substratum colour as the factor. ANOSIM computes a test statistic (R), where $R = 1$ if all replicates within a treatment are more similar to each other than any replicates from

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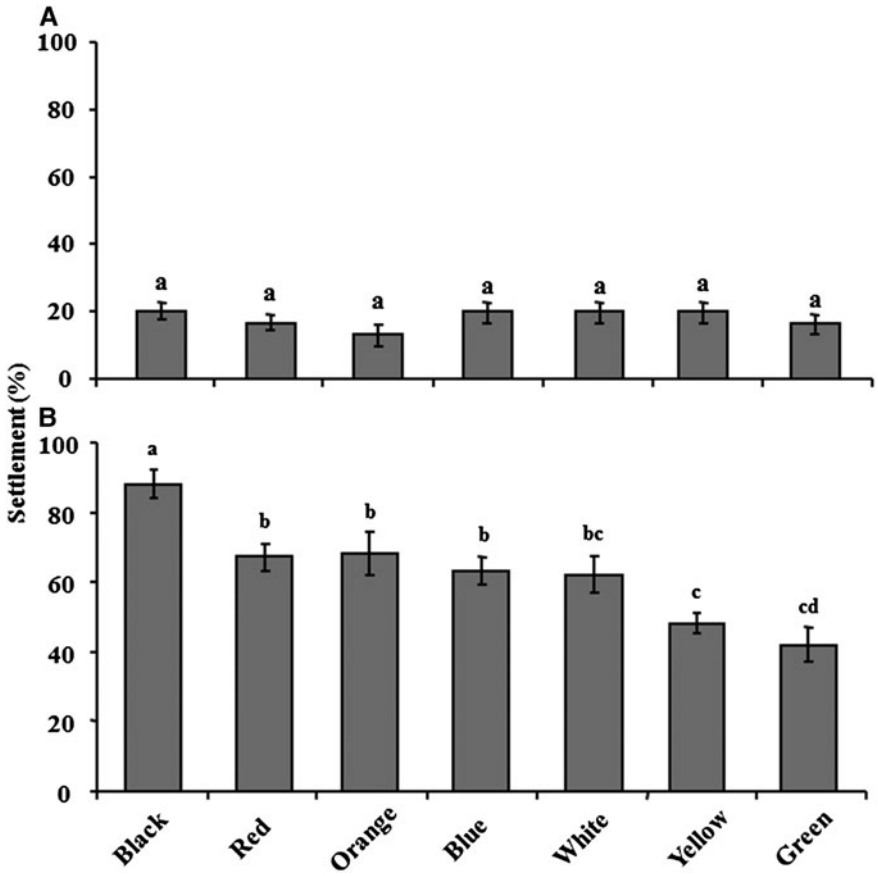

Fig. 1. Percentages of plantigrade settlement of *M. coruscus* on non-biofilmed surfaces (A) and biofilms (B) developed on seven coloured substrata. Data with significant difference are indicated by different letters. Means ± SE (N = 6). A, percentages of plantigrade settlement on non-biofilmed substrata with seven different colours. B, percentages of plantigrade settlement on marine biofilms formed on coloured substrata.

different treatments. *R* is ~0 if the null hypothesis is true that similarities between and within treatments are the same. 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

Settlement bioassay

The percentages of plantigrade settlement on non-biofilmed and biofilmed substrata are shown in Figure 1. The plantigrades showed no significant preference to settle on the non-biofilmed substrata (Kruskal–Wallis test, $P > 0.05$; Figure 1A). The biofilms developed on the black substrata showed the strongest inducing activities (Kruskal–Wallis test, $P < 0.05$; Figure 1B), reaching a maximum of 88%. The biofilms developed on the substrata of red, orange, blue and white showed a moderate (60–70%) inducing activity, while other biofilms formed on yellow and green substrata exhibited low (<60%) inducing activities (Figure 1B).

Bacterial densities of biofilms

The bacterial densities varied in biofilms formed on seven coloured substrata (Kruskal–Wallis test, $P < 0.05$; Figure 2). The highest bacterial density ($3.4 \times 10^7 \pm 5.5 \times 10^5$ cells cm^{-2}) in biofilms was observed on the blue substrata, while the lowest bacterial density ($5.2 \times 10^6 \pm 4.7 \times 10^5$ cells cm^{-2}) in biofilms was observed on the red substrata. The bacterial density in biofilms was negatively correlated to the settlement inducing activity of biofilms (Spearman's rank correlation, $P < 0.01$, Table S1).

Diversity and composition of biofilm bacterial community by pyrosequencing

Pyrosequencing analysis showed a total of 627,335 valid reads and 17,807 OTUs from biofilm samples on black, red, orange, blue, white, yellow and green substrata. At a 3% dissimilarity level, 98.9–99.3% of the species were obtained in seven biofilm samples through Good's coverage estimations. Based on OTUs at 3% dissimilarity, Rarefaction analysis showed that the rarefaction curves of all biofilm samples tended to approach the saturation plateau (Fig. S1A). Rank-abundance curves suggested that highly abundant bacteria accounted for relatively low proportions in seven biofilm samples and sequences that belonged to rare organisms were dominant (Fig. S1B).

Twenty-seven different phyla were determined from seven biofilm samples. Among all seven biofilm samples, 55–59% of the sequences belonged to *Bacteroidetes*, which was the most abundant phylum (Figure 3, Table S2). *Proteobacteria* was

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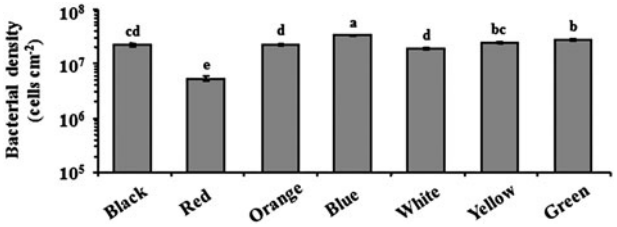

Fig. 2. Bacterial densities of biofilms formed on seven coloured substrata. Data are reported as the mean (±SE) of 10 random fields of view. Different letters indicate significant differences.

the second most dominant group and accounted for 38, 37, 40, 41, 41, 38 and 37% of the sequences in biofilm samples on black, red, orange, blue, white, yellow and green substrata, respectively (Figure 3, Table S2). These two dominant phyla, *Bacteroidetes* and *Proteobacteria* accounted for >95% of the bacterial reads from the seven biofilm samples (Figure 3, Table S2).

The bacterial communities at lower taxonomic were examined. *Flavobacteria* (51–56%) was the dominating group in seven biofilm samples, and no significant difference was observed in seven samples (Tukey–Kramer HSD test, $P > 0.05$; Table S3). The *Alphaproteobacteria* and *Gammaproteobacteria* were respectively the second and the third highly enriched in all biofilm samples, while no significant difference was observed in the same class (Kruskal–Wallis test, $P > 0.05$; Table S3).

The author used Illumina sequence data, based on the bacterial community, to construct a heatmap analysis comparing the seven biofilm samples of the top 100 genera (Figure 4). The *Flavobacteriaceae*_unclassified was highly enriched in black, red and white samples (Table S4). The relative abundance of *Rhodobacteraceae*_unclassified was less abundant in blue, white, yellow and green samples (Table S4). Genera *Tenacibaculum* gained a higher proportion in orange (28%) and yellow (25%) samples (Table S4). The relative abundance of *Vibrio* was less abundant in black (2.2%) samples (Table S4).

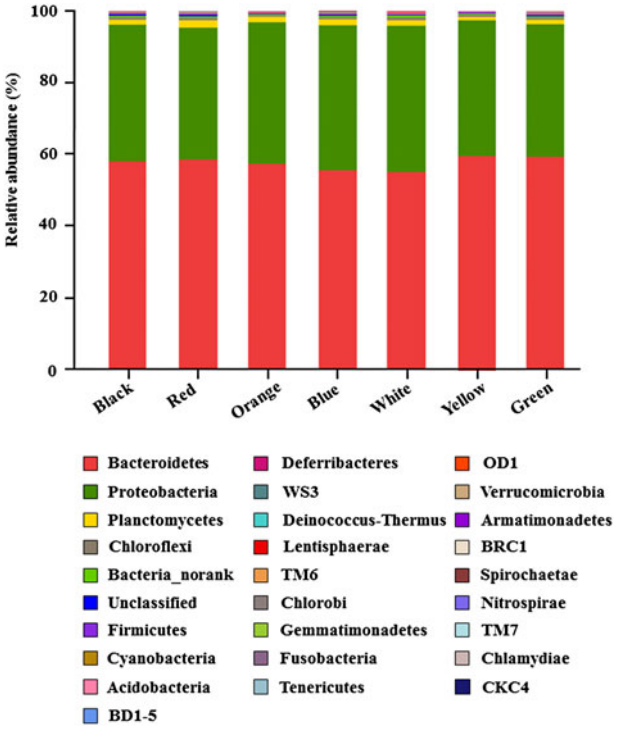

Fig. 3. Relative abundance of bacterial reads classified at the phylum level. Sequences that could not be classified into any known group were assigned as 'Unclassified'.

Similarity analysis among bacterial communities

Bacterial communities in biofilms developed in green and red substrata were clustered together (Figure 5), indicating that a similar community structure existed between the two biofilm samples. The lowest similarity was observed in white and blue samples with other five biofilm samples. The principal component analysis (PCA) indicated that red, green and black samples were largely separated from the other four samples by PC2, which accounted for 11.44% of variance (Fig. S2). The white and blue samples were separated from the other five samples by PC1, which accounted for 77.64% of variance. The bacterial species in the libraries of biofilms developed on the seven substrata were further investigated to evaluate presence of a core microbiota. Overall, only 309 OTUs were shared among seven libraries (Table S5).

ANOSIM test was conducted to determine the similarity between seven biofilm samples. R-values were interpreted as follows: $R > 0.75$ as well separated, $0.50 < R \leq 0.75$ as separated but overlapping groups, $0.25 < R \leq 0.50$ as separated but strongly overlapping groups, and $R \leq 0.25$ as barely separable at all, in accordance with the PRIMER-manual (Clarke & Warwick, 2001). For seven biofilms, significant difference between bacterial communities on the biofilms was observed, which had an R value of 0.255 ($P < 0.05$).

DISCUSSION

Surface colour, one physical characteristic of substrata, influenced larval settlement of some macrofouling marine organisms including barnacles, polychaetes and pearl oysters (Yule & Walker, 1984; James & Underwood, 1994; Kon-ya & Miki, 1994; Saucedo *et al.*, 2005; Robson *et al.*, 2009). Thus far, studies on relationship between recruitment of marine invertebrates, biofilm communities and substratum colour are very limited. Dobretsov *et al.* (2013) initially demonstrated that black and white substrata affected both the formation of biofilm community and settlement of macroorganisms. However, whether more colours such as red, green, blue, orange and yellow affect the formation of biofilm community and subsequent recruitment of marine invertebrates remains unknown. In the present study, we demonstrated that *M. coruscus* plantigrades settled differently on biofilms developed on seven coloured substrata.

In the aquatic environment, bacteria can quickly adhere on a surface, over a few seconds to a few minutes and the interaction between cell attachment and surface has attracted more attention from researchers (Whitehead & Verran, 2009). Previous studies have shown that substratum characteristics have an important impact on the process of the adhesion of microorganisms (Fletcher & Loeb, 1979; Whitehead & Verran, 2009; Hsu *et al.*, 2013). Dobretsov *et al.* (2013) demonstrated that a microfouling community could be

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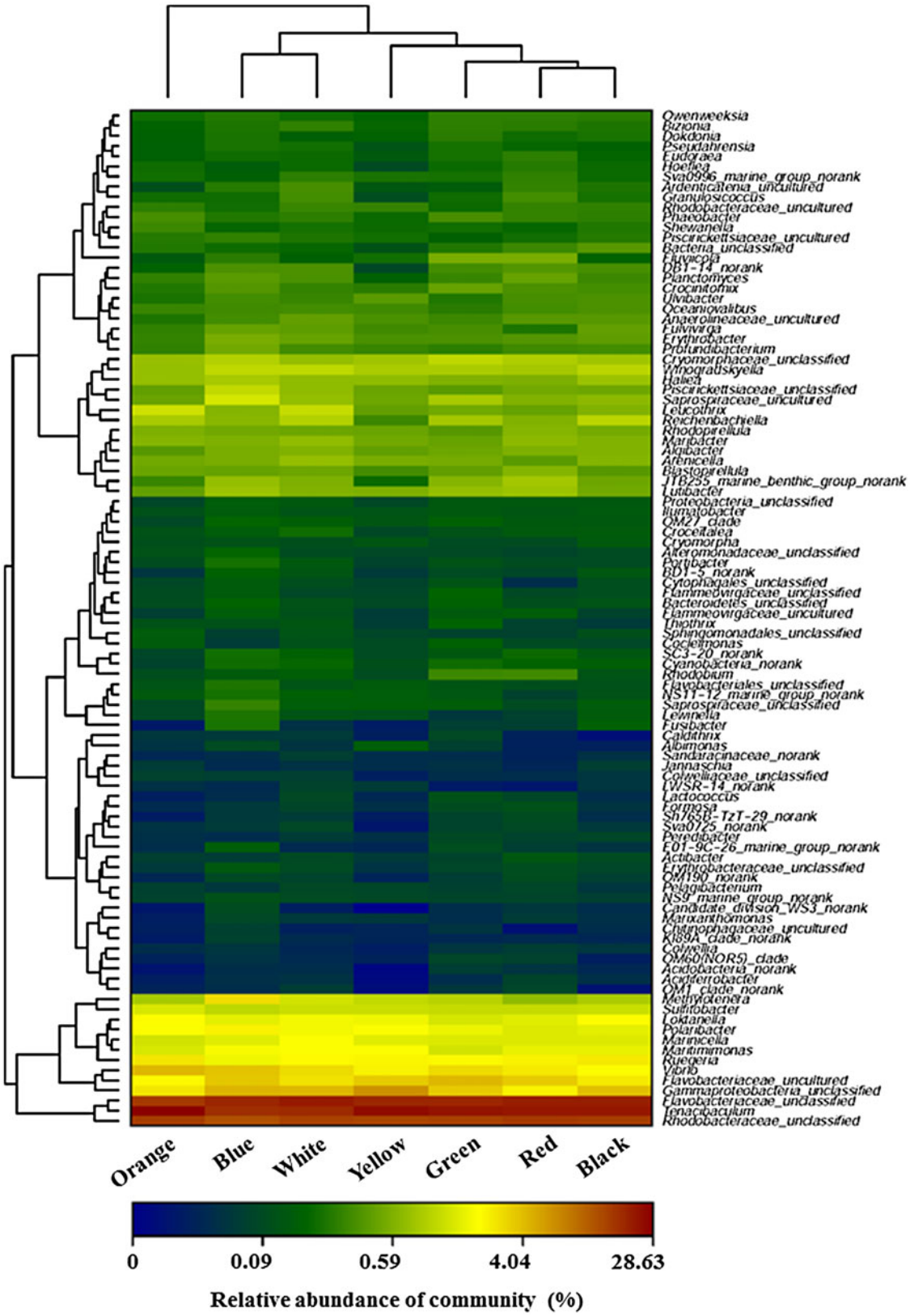

Fig. 4. The relative abundance and distribution among seven biofilm samples. The colour intensity indicates relative values of bacterial family.

influenced by black and white surfaces. The results of the present investigation showed that bacterial density and bacterial community varied among biofilms developed on seven coloured substrata, indicating that the formation and communities of biofilm in a dynamic environment is correlated to substratum colour. This is consistent with our hypothesis that substratum colours could affect bacterial attachment and formation of bacterial community.

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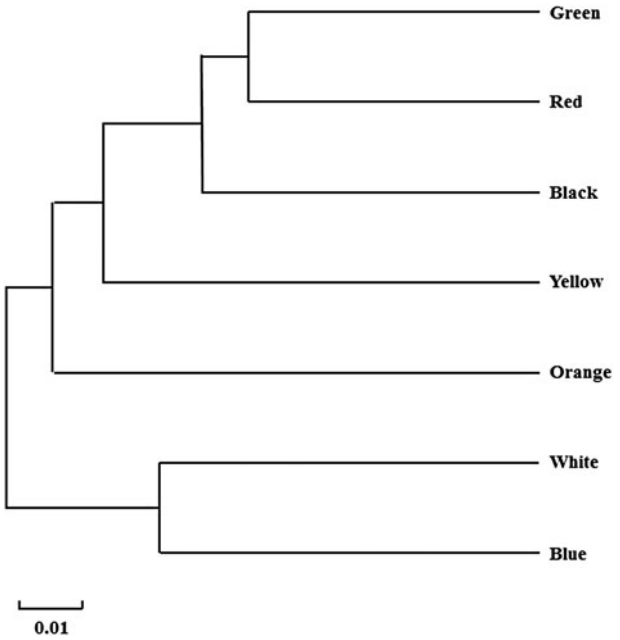

Fig. 5. Cluster analysis of biofilm community based on the Bray–Curtis distance of seven samples.

Previous studies have shown that bacterial communities varied among the substratum characteristics, substratum type and environment conditions (Huggett *et al.*, 2009; Chung *et al.*, 2010; Burke *et al.*, 2011; Lee *et al.*, 2011; Qian *et al.*, 2011; Sharp *et al.*, 2012). Recently, Dobretsov *et al.* (2013) showed that *Alphaproteobacteria* (>35%) and *Firmicutes* (>20%) were the major groups in 21-day biofilms formed on black and white substrata. In contrast, *Bacteroidetes* was the most abundant phylum (>55%) in 28-day biofilms developed on seven coloured substrata in this study. This discrepancy may be due to differences in the formation period of biofilms or sea areas. Within *Bacteroidetes*, bacterial communities formed on seven coloured surfaces were dominated by *Flavobacteria* (>50%). Previous studies also have shown that *Bacteroidetes* was one of the most abundant groups in marine bacterioplankton in the coastal areas (Alonso *et al.*, 2007; Fernández-Gómez *et al.*, 2013). *Bacteroidetes* had the tendency to form biofilms and then constituted the highest abundance among biofilm samples (Patel *et al.*, 2003). *Proteobacteria* was the second most abundant phylum (>35%) in 28-day biofilms developed on seven coloured surfaces. Earlier studies also showed that the *Proteobacteria* dominated the biofilm communities (Jones *et al.*, 2007; Chung *et al.*, 2010). Within *Proteobacteria*, bacterial communities developed on coloured surfaces were dominated by *Alphaproteobacteria* (20–25%) and *Gammaproteobacteria* (12–16%). In addition, *Alphaproteobacteria* are dominant in bacterial communities developed on black, red and orange surfaces, and *Gammaproteobacteria* are dominant in bacterial communities developed on blue, white, yellow and green surfaces. The ANOSIM analysis in the present study showed that the *R* value between the various biofilm samples was 0.255, indicating that difference exists in the bacterial community of biofilms formed on coloured substrata. This variation may reflect that substratum colours had different influences on the diversity and composition of bacterial community. The explanation of the reaction of bacterial communities to surface colour could be that coloured surfaces may cause variation in quantities of radiant energy and subsequently result in variation in bacterial communities in biofilms. Dobretsov *et al.* (2013) also suggested that the amount of radiant energy absorbed or reflected may explain the effect of substratum colour. Further research is needed.

Bacterial attachment is very important for subsequent biofilm formation and recruitment of macrofouling organisms (Thomas *et al.*, 2008). Previous investigations suggested bacteria isolated could produce cues and impact larval recruitment of many marine invertebrate species (Zobell & Allen, 1935; Kirchman *et al.*, 1982; Unabia & Hadfield, 1999; Hadfield, 2011; Huang *et al.*, 2012; Yang *et al.*, 2013b), whereas the inducing activity of natural biofilms might be not only due to the presence of inducing bacterial strains (Chung *et al.*, 2010). Bacterial community structure might be one of the most important factors determining whether a biofilm inhibits or facilitates larval recruitment of marine invertebrates (Wieczorek & Todd, 1998; Qian *et al.*, 2003; Lau *et al.*, 2005; Thiyagarajan *et al.*, 2006; Wang *et al.*, 2012). The data presented here showed that bacterial community changed with the surface colour during the biofilm formation, and their corresponding inductive activities of these biofilms on *M. coruscus* plantigrade settlement also varied. Within *Proteobacteria, Alphaproteobacteria* were found in higher fraction in black, red and orange surfaces, whereas *Gammaproteobacteria* were found in lower fraction in these three coloured surfaces. This finding may explain the differences in settlement inducing activities of these biofilms developed on various coloured substrata. In addition, our results showed that the proportions of *Vibrio* were lower in the black surface than those in the others. Previous studies also suggest that some members belonging to the genus *Vibrio* can promote the settlement process of the mussel *M. coruscus* (Yang *et al.*, 2013b) and the coral *Pocillopora damicornis* (Tran & Hadfield, 2011) larvae. Thus, existence of a low fraction of inductive bacterial species may explain the discrepancy of their inducing activity. However, information on the interaction between the many groups of bacteria and settlement of larvae/juveniles of marine invertebrates is limited, and further research should be conducted to understand and determine the relationship between bacteria and settlement of larvae/juveniles of marine invertebrates. Another explanation of the discrepancy on inducing activity of biofilms on seven coloured surfaces might be variation of the cell density and community structure of photosynthetic organisms. Further research on interaction between surface colours and photosynthetic organisms in biofilms needs to be conducted.

In summary, our findings suggest that differences in plantigrade settlement response may be correlated to the variation in bacterial community, and cell attachment of bacteria and bacterial community in biofilms was influenced by the substratum colours. Substratum colours, especially black and red, should be taken into account when investigating the interaction among substratum characteristics, biofilm community and macrofouling. The present study is a starting point for future ecological research on the interaction between the substrata and microbial community in affecting the recruitment of marine invertebrates.

SUPPLEMENTARY MATERIAL

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