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Effects of natural biofilms on settlement of plantigrades of the mussel *Mytilus coruscus*

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ABSTRACT

The effect of natural biofilms on settlement of plantigrades of *Mytilus coruscus* was investigated in the laboratory. Plantigrades settled in response to natural biofilms, and the percentages of plantigrade settlement increased with biofilm age. The settlement-inducing activity of biofilms was positively correlated with age-related characteristics of the biofilm, such as dry weight, thickness, chlorophyll *a* concentration and densities of bacteria and diatoms. Cluster analysis of denaturing gradient gel electrophoresis revealed high similarity between bacterial communities in biofilms according to biofilm age, indicating that bacterial community structure may not play an important role in settlement of plantigrades in this species. Therefore, natural biofilms may be used to enhance settlement of plantigrades for the Chinese aquaculture industry.

1. Introduction

In the marine environment, biofilms exist on all surfaces and play a key role in mediating biotic interactions and biogeochemical activities (Qian and Dahms, 2009). Additionally, they mediate larval settlement and metamorphosis of many marine invertebrates (Hadfield, 2011; Hadfield and Paul, 2001; Qian et al., 2007), provide a suitable food source to newly metamorphosed juveniles, and serve as biofilters to absorb and biodegrade excessive nutrients (Qian et al., 2007). Yu et al. (2010), for example, demonstrated that natural biofilms could promote larval settlement of the pearl oyster *Pinctada fucata*, and Campbell et al. (2011) showed that biofilms on hard surfaces enhanced larval settlement of the native Eastern oyster *Crassostrea virginica*.

Mussels possess a planktonic larval phase preceding a benthic adult phase. Larvae will survive to the stage of competence for settlement if suitable environmental conditions are available, but they can delay settlement until they found a suitable substrate (Crisp, 1974), such as biofilms (Alfaro et al., 2011; Bao et al., 2007a,b; Dobretsov, 1999; Ganesan et al., 2010; Satuito et al., 1995, 1997). However, mussel settlement is not permanent and the metamorphosed individuals, termed plantigrades, post-larvae, spat or juveniles could detach from primary settlement sites and reattach on alternative habitats (Bayne, 1964; Buchanan and Babcock, 1997; Kavouras and Maki, 2003). The secondary settlement behavior of mussels is important for the distribution of populations and could contribute to local recruitment dynamics (Corre et al., 2013). Despite the long-recognized role of biofilms as potential inducers of mussel larval settlement and metamorphosis, knowledge of the interactions between marine biofilms and resettlement of metamorphosed plantigrades of mussels is limited.

The mussel, *Mytilus coruscus* Gould, 1860, which inhabits the temperate zone along the coastal waters of East Asia (Chang, 2007), is an important aquaculture species in China (Chang and Wu, 2007). Recently, due to the overexploitation of wild resources of this species, it is difficult to meet the market demands (Chang and Wu, 2007). Thus, development of hatchery techniques has become of economic interest in this species. Efforts have been made to understand the role of biofilms on larval settlement and metamorphosis. However, there has been no effort to clarify the effects of biofilms on settlement of plantigrades beyond the larval settlement, and the patterns that mediate the mechanism of settlement of plantigrades remain unknown for this species. In the present study, we investigated the effects of natural biofilms on settlement of plantigrades of the mussel *M. coruscus*. The characteristics of biofilms of different ages were investigated according to their dry weight, thickness, chlorophyll *a* (chl *a*) concentration, bacterial and diatom densities and bacterial community structure. The purpose of this study was to gain insights into the settlement

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mechanism of plantigrades of *M. coruscus*, and to provide valuable information for artificial culture of plantigrades of this species.

2. Materials and methods

Details of the bioassays used in this study are provided in Table 1 to facilitate understanding of the experimental design. Briefly, the settlement-inducing activities of natural biofilms with different ages (7 days, 14 days, 21 days and 28 days) on plantigrades were investigated using 9 replicates. The characteristics of those biofilms including dry weight, thickness, chl *a* concentration, bacterial and diatom densities and bacterial community structure were analyzed.

2.1. Preparation of natural biofilms

Biofilm slips were prepared by immersing clean glass slips (half portions of microscope glass slides; 38 mm × 26 mm) in coastal seawater at Gouqi Island (122°46′E; 30°43′N), Zhejiang, China. The glass slips were placed on PVC holders and immersed at a depth of 0.5–1.0 m below the water surface for 7–28 days. Slips were brought back to the laboratory on the same day of the bioassay and were thoroughly washed with autoclaved 1.2 μm-filtered sea water (AFSW) before the assays.

2.2. Measurement of biofilm dry weight

Dry weight was measured by the method of Bao et al. (2007a). The biofilms on each glass slip were scraped off using a sterile glass slide and separately suspended in AFSW. Each suspension was collected on a pre-weighed GF/C filter (Whatman glass fiber 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 weighing. The dry weight of the biofilm was determined after subtracting the weight of the filter.

2.3. Quantifications of densities of bacteria and diatoms

Biofilms were fixed in 5% formalin for a maximum of 3 weeks. Samples were washed with AFSW and stained with acridine orange (AO, 0.1%) for 5 min. Bacterial densities of stained samples were counted under the microscope at 1000× magnification using an Olympus BX51 epifluorescence microscope. 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 quantified from ten random fields of view.

Table 1
Schematic representation of experimental design.

General	Experimental setup
Biofilm work	Preparation of natural biofilms
	The characteristics of biofilms of different ages
	Measurement of biofilm dry weight
	Quantifications of densities of bacteria and diatoms
	Determination of biofilm thickness
	Chl *a* concentration in biofilms
	Bacterial community analysis
	DNA extraction
	PCR amplification of 16S rDNA
	Bacterial community profiling by denaturing gradient gel electrophoresis
Mussel work	Culture of plantigrades
	Settlement bioassays

2.4. Determination of biofilm thickness

Biofilm thickness was measured by the method of Yang et al. (2013). The biofilms with four ages were fixed in 5% formalin solution for 24 h. Biofilms were stained with propidium iodide (5 μg ml^{-1}) and incubated for 15 min in the dark. Slides were washed three times and then observed at 400× magnification under an Olympus FluoView™ FV1000 Laser-Scanning Confocal microscope. Three replicate biofilms were examined for each age of biofilms. 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.

2.5. Chl a concentration in biofilms

Chl *a* concentration was measured by the method of Wang et al. (2012). Biofilms were scraped from 3 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 chlorophyll, samples were vortexed for 1 h at the end of the extraction period and 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* concentration was calculated using the following equation (Ma et al., 2011):

$$\text{chl}\,a = \frac{[12.12 \times (D664-D750) - 1.58 \times (D647-D750) - 0.08 \times (D630-D750)] \times Ve \times d}{A}$$

where, chl *a* is chl *a* concentration (μg cm^{-2}) in the biofilm; D630, D647, D664 and D750 are the absorptions at 630, 647, 664 and 750 nm; Ve, the extraction volume; A, the area of substratum surface; d, the optical length of the cuvette.

2.6. DNA extraction

Biofilms were scraped from 3 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).

2.7. 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), 2.5 μl of 10 × PCR buffer, 2 μl of $MgCl_2$ (25 mM), 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 15 additional 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.