Using Barnacle-Specific Primers to Quantify Larva Attachment on Nano-Filled Coatings during the Early Stages of Marine Biofouling

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Abstract. This study tried to set up an approach to quantify the attached barnacle larvae in marine biofilms. Biofilms were collected from nano-filled coatings that previously characterized with different antifouling scores for 3-months. Quantification was achieved by 18S rDNA copy number measurement in quantitative real-time PCR (qPCR) using barnacle-species specific PCR (polymerase chain reaction) primers. Interestingly, the coatings with best anti-fouling scores were not the ones with less barnacle larva attachment in the early stage of marine biofouling, suggesting that some nano-filled coating be able to attract barnacle larvae’s attachment but can prevent their further growth either by directly inhibiting the growth or evacuating the larvae through modulated biofilm dynamics, and the relative amount of barnacle larvae attachment cannot be regarded as a prediction for long-term antifouling efficacy of a coating.

1. Introduction

Barnacle is the No.1 marine fouling species that brings huge economical loss worldwide [1-3]. The endeavor to inhibit barnacle attachment has never ceased [4-7]. In theory, one can inhibit the attachment of barnacle larvae on engineered surfaces, but one can also inhibit the larva’s growth after it attaches.

Many different technologies were tested and being developed [8-10]. Macro- or nano-surface structure is one of the promising approaches. For example, Long Y et al [11] prepared novel nanostructures with porous architecture that exhibit effective biocide performance, and the disinfection rate of the typical biofouling bacteria Bacillus sp. and Pseudoalteromonas sp. could reach 99.9% and 99.8% in 60 min, respectively. Beigbeder A et al [12] found slight addition of multiwall carbon nanotubes in a silicone matrix can dramatically increase the ability to hinder marine alga Ulva and barnacle. Chen TY et al [13] found that addition of silicone oil in a low surface energy coating leads to apparent less attachment of barnacle larvae but little effect on algae’s attachment. Guo ZW et al [14] found that nanosilver particles can differentially affect biofilm formation of two marine bacteria. All these examples suggested that surface nano-structure may induce some specific patterns in biofilm dynamics thus achieving anti-fouling consequences.
In this study, we used different carbon nanotubes (CNTs) as nano-fillers to make polydimethylsiloxane (PDMS) based coatings subjected to field test and biofilm sampling. In order to measure the barnacle larvae attachment during the early stage of biofouling, barnacle-specific primers was designed and quantitative polymerase chain reaction (qPCR) was employed to assess the relative amount of barnacle larvae in the biofilm biomass from different coatings. The results showed interesting relationship between early-stage barnacle larvae attachment amounts and the coating’s three-month field test anti-fouling scores.

2. Materials and Methods

2.1. Nano-coating design
Ship hull steel panels, with the dimension of 10 cm × 10 cm × 0.3 cm, were rubbed using sand papers to obtain relatively uniform surfaces. All the panels were carefully washed with the sterile deionized water, air-dried and then coated with the primer coat (the chlorinated rubber iron-red antirust paint, provided by HaoYing Company (Weihai, China), and can be cured in about 72 h at room temperature) to provide a background before coating the PDMS matrix. The silicone elastomer matrix was the Sylgard 184 kit (Dow Corning, USA) supplied as a two-part kit consisting of a pre-polymer (base, part A) and a cross-linker (curing agent, part B). The CNTs fillers (Table 1) were purchased from the Timesnano Company (Chengdu, China). To prepare the unfilled PDMS, the PDMS pre-polymer (part A) and the curing agent (part B) were thoroughly mixed in a ratio of 10:1 (w/w) to obtain a cross-linked PDMS via hydrosilylation. Both the pre-polymer and the curing agent were well mixed for 15 min, and then degassed to remove air bubbles at room temperature. Nano-filled coatings were prepared as above but with selected CNTs (Table 1). CNTs fillers with highest or lowest anti-fouling scores in the three-month filed test were selected from previous study [15-18]. The final concentration of all the CNTs fillers in the PDMS matrix was 0.1% (w/w). Afterwards, these silicone mixtures were painted on one side of surfaces of the pre-treated panels respectively. After 6 h of curing at 105°C, the silicone-based coatings with a thickness of 300 μm were obtained for the subsequent field studies.

![Figure 1. The nano-filled coating plates used for biomass sampling at four time points.](image)

2.2. Marine field test site
The field assays were carried out at the Xiaoshi Island Harbor waters (N37°31′51″; E121°58′19″) in Weihai, China. A wooden raft bridge allowed the immersion of tested panels at different depths for long periods if necessary. There were rich fouling organisms at the immersion site during the field studies, such as invertebrate larvae (i.e., juvenile barnacles, oysters, mussels and ascidian), algae,
seaweeds as well as sponges. All the tested panels will be faced with heavy fouling pressure in the marine environment. For each coating, its three replicates were immersed at three sites 10-15 meter distance from each other.

Table 1. Parameters for nano-coatings.

| Coatings | CNTs          | Diameter | Antifouling score[15-18] |
|----------|---------------|----------|--------------------------|
| PDMS     | -             | -        | 61                       |
| PDMS(12) | Carboxyl modified Short-SWNT2 | 1~2nm | 91                       |
| PDMS(26) | Hydroxyl modified MWNT2 | 8~15nm | 89                       |
| PDMS(29) | Hydroxyl modified MWNT5 | 30~50nm | 89                       |
| PDMS(32) | Carboxyl modified MWNT2 | 8~15nm | 62                       |
| PDMS(58) | Graphitized MWNT3 | 30~50nm | 63                       |
| PDMS(59) | Graphitized MWNT4 | >50nm | 65                       |

2.3. Biofilm sampling and metagenome extraction

The four-week in situ experiment (Oct.10–Nov.3, 2017) was performed in order to collect the continuous biofilm samples from the silicone-based coating surfaces at different points. For each of the silicone-based coatings, triplicate panels immersed at a depth of 1.5 m for sampling were used throughout, aiming to collect sufficient biofilm samples. Biofilm samples were collected by scraping using the sterile brushes. The steel panels were carefully washed with the sterile deionized water before scraping. All the replicate biofilm samples from the identical silicone-based coating surface at the same point in time were collected into a sterile 2.0 mL Eppendorf tube. The panels for sampling were brought back to the laboratory in one hour. Then all the mixed biofilm samples were re-suspended in 400μL of the sterile deionized water, and centrifuged at 4000 rpm for 5 min in order to obtain a biomass pellet. Afterwards, they were preserved at −80°C for further analysis.

Genomic DNA was extracted with the hot phenol-chloroform method. Briefly, five-fold volumes phenol (pH8.0)-chloroform were well mixed with one volume of biofilm sample, then vigorously vortexed for 1 min, before being heated in a 65°C water bath for 15 min with frequent shaking. After incubation for 3 min at room temperature, the sample was centrifuged at 10,000 rpm for 10 min. The supernatant was extracted again, with an equal volume of phenol (pH8.0)-chloroform. After a second centrifugation at the same speed and for the same length of time, the supernatant was collected mixed with equal volume of chloroform, vortexed for another 1 min and centrifuged at 10,000 rpm for 15 min. The final supernatant was used directly as a PCR template.

![Figure 2](image-url) **Figure 2.** The average biomass accumulation of each coating during 24 days of field test. Each point represented 3 replicates’ average value.
2.4. Barnacle-specific primers design and validation
Species-specific primers were designed using Simple Allele-discrimination PCR (SAP) technology [19-20]. PCR primers specific for the 18S ribosomal genes of barnacle were designed and tested for their specificity. Firstly, several kinds of barnacles, Amphibalanus cirratus, B. improvisus, and Fistulobalanus albicostatus, were collected in Weihai coastline. Genomic DNA was extracted from them and the near full length 18S rDNA was amplified for DNA sequencing. About 300 18S full length sequences were also downloaded from public databases (NCBI) and these 18S rDNA sequences were subjected to multiple alignments using MAFFET in order to identify Weihai barnacle-specific SNPs that are further used to design primers by SAP technology. PCR conditions for each primer pair were optimized using genomic DNA of the above three types of barnacle. Besides, several other types of macrofoulers (Crassostrea gigas, Hydroides, Mytilus edulis, Bugula neritina, Calloporidae, Styelaclava) were also collected to extract genome DNA as control PCR templates to confirm that barnacle-specific primers’ specificity.

Annealing temperature gradients were used to determine the best PCR conditions. PCR product was visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. In each 12μL PCR reaction there were 0.25μL Taq DNA polymerase (5U/μL), 6μL 2×NPK02 buffer, 1.8μL primer, 0.5μL template and 3.45μL ddH2O. The optimized PCR conditions were: initial denaturation for 3min at 94°C, followed by 40 cycles (30s at 94°C, 30s at annealing temperature, 35s at 72°C), followed by 2 min of final extension at 72°C. In qPCR, the above NPK02 was replaced by NPK62 buffer (GREDBIO) [21].

Figure 3. Primer-specificity determination using equal-molar mixture DNA from different macrofoulers’ genome DNA as a PCR template. T: annealing temperature; C: PCR cycle. From 1 to 10 the primer pairs were designed for other macrofoulers (data not shown) except that No.7 and No.8 were designed for barnacle (Balanus improvisus, Amphibalanus poecilotheca, respectively).

2.5. QPCR quantification of the attachment efficiency of barnacle larvae on nano-coatings
With the optimized conditions qPCR was done to analyze the biofilm samples. The amplification efficiency of each primer pair were determined by standard procedure; making dilution series of the standard target DNA (sequencing-confirmed PCR product of barnacle 18S rDNA fragment), calculating a linear regression based on the Ct data points and inferring the efficiency from the slope of the line. Serial dilutions from 10-1 to 10-8 were utilized. Technical triplicates were tested for each dilution point and primer pair. A Non-Template Control (NTC) was included in each assay to confirm that the Ct value generated by the lowest concentrated DNA was not an artifact. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artifacts. The standard DNA concentration of the first dilution point was quantified with a UV–VIS spectrophotometer (ACTGene UVS-99), by which the concentrations of barnacle 18S rDNA copy numbers (representing the relative amount of attached barnacle larvae) from each sample can be calculated with the standard curve built through serial dilutions.
3. Results and Discussion

3.1. Plate sampling and observation
The samplings were undertaken at day 3, day 8, day 15 and day 24 with the average sea water temperature of 17.5°C. During the observation in the past few years at the test site, barnacle growth can still be readily seen around 10-15°C each year during the autumn-winter period. So during the sampling period in this study, barnacle larvae attachment can still be readily measured. The three hanging sites for different plates in the seawater also had effects on biofilm formation and development. Some sites were easier to aggregate sea muds that had hindering effects on biomass accumulation. So every three biofilm samples were equally mixed to represent each coating.

3.2. Biomass determination
Different coatings had apparent differential effects on biofilm formation and development. One of the apparent effects was seen as average biomass amount (Figure 2), in which one coating (PDMS (32)) had less biomass than the control at the fourth time point, while all other coatings had more biomass accumulation than the control. Meanwhile, the speed of biomass accumulation for each coating was also different in the first two weeks. It seemed that PDMS (12) and PDMS (26) had the largest accumulation speed, but three other coatings (PDMS (29), PDMS (32) and PDMS (58), accumulated more biomass in the first three days. These observations suggested that the compositional change and its dynamics in the tested biofilms were modulated by the nano-fillers.

3.3. Primer specificity assessment
Primer specificity was checked using barnacle-specific primers and 10 different macrofoulers’ genome DNA templates (as a mixture) (partial data seen in Figure 3). TH1-F566 (Universal primer) and TH2-F566 were satisfactory pairs of primer with amplicons 410bp (No.7) and 215bp (No.8) respectively. TH1: 5’-ATG CTT TCG CAG TAG TTC GTTG-3’; TH2: 5’-ACC GTA AGGAT TGA CAG ACT GGT-3’. TH1-F566 was further used for qPCR.

Considering that this study only employed limited number of barnacle types and limited number of other macrofouler species, the designed primers may encounter the need to revise for both more types of barnacle and more species of other macrofoulers in the near future for more stringent specificity.

3.4. QPCR results
For qPCR, PCR product of 18S rDNA using TH1-F566 primer pair and B.improvisus genome DNA as template was employed to be the standard DNA (42 ng/ul). By series of dilution from 10-1 to 10-8, the standard curve was made to quantify biofilm samples from different coating surface at different intervals. The optimized annealing temperature for qPCR was 62°C (Figure 3). The expected results were decreased barnacle larva attachment on nano-filled coatings with higher antifouling scores. Even though the antifouling score represents the whole attachment/growing contents of all macrofoulers (not only for barnacles) [Table 1, 15-18], but generally high antifouling score correlates with little attachment and growth of barnacles. In Figure 4, the PDMS(12) coating had highest value for barnacle larva attachment, PDMS (26) coating also had a high attachment value, strongly suggesting that the early attachment of barnacle larvae may have to experience de-attachment on some nano-filled surfaces, and this observation needs further confirmation in the near future.

Also, the coatings with best anti-fouling scores were not the ones with less barnacle larva attachment, suggesting some nano-filled coating be able to attract barnacle larvae’s attachment but can prevent their further growth either by directly inhibiting the growth or evacuating the larvae through modulated biofilm dynamics.
Figure 4. Detection of barnacle larvae attachment on coating surfaces by qPCR. Data were averaged by three replicates.

4. Conclusion
In this study, barnacle-specific primer was employed to detect barnacle larva attachment amount on the biofilm samples from different nano-filled surfaces during the early stage of marine biofouling. Though the short term nature of the above analysis is limited in value for predictions of overall antifouling efficacy, it is useful for observing the biofilm dynamics’ influence on larva attachment of macrofoulers, thus judging the value of a concept that prevention of barnacle larva attachment in the early stage of marine biofouling may not be a good prediction for an efficient antifouling coating.

Species-specific primers can be designed for any kind of macrofouler. So this is an example approach expandable to other macrofoulers in the near future to detect the existence of macrofoulers’ larvae on any surface thus preliminarily assess the surfaces’ antifouling potential. Plus, the approach can be used to investigate the biofilm’s early inner dynamic changes of different species soon.

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