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ORIGINAL PAPER

Bacterial assay for the rapid assessment of antifouling and fouling release properties of coatings and materials

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Abstract An assay has been developed to accurately quantify the growth and release behaviour of bacterial biofilms on several test reference materials and coatings, using the marine bacterium *Cobetia marina* as a model organism. The assay can be used to investigate the inhibition of bacterial growth and release properties of many surfaces when compared to a reference. The method is based upon the staining of attached bacterial cells with the nucleic acid-binding, green fluorescent SYTO 13 stain. A strong linear correlation exists between the fluorescence of the bacterial suspension measured (RFU) using a plate reader and the total bacterial count measured with epi-fluorescence microscopy. This relationship allows the fluorescent technique to be used for the quantification of bacterial cells attached to surfaces. As the bacteria proliferate on the surface over a period of time, the relative fluorescence unit (RFU) measured using the plate reader also shows an increase with time. This was observed on all three test surfaces (glass, Epikote and Silastic T2) over a period of 4 h of bacterial growth, followed by a release assay, which was carried out by the application of hydrodynamic shear forces using a custom-made rotary device. Different fixed rotor speeds were tested, and based on the release analysis, 12 knots was used to provide standard shear force. The assay developed was then applied for assessing three different antifouling coatings of different surface roughness. The novel assay allows the rapid and sensitive enumeration of attached bacteria directly on the coated surface. This is the first plate reader assay technique that allows estimation of irreversibly attached bacterial cells directly on the coated surface without their removal from the surface or extraction of a stain into solution.

Keywords Antifouling · Biofilm · *Cobetia* · Fluorescence · SYTO 13

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Introduction

Marine biofouling is the result of the growth of microorganisms, plants and animals on surfaces immersed in the natural environment. The organisms involved in marine fouling are primarily the attached or sessile forms commonly occurring in shallow waters along the coastline. The biofouling process occurs in the natural environment in sequential steps. Within minutes of immersion, a pristine surface becomes 'conditioned' through the adsorption of organic layers of macromolecules [5, 9, 19, 27]. The conditioned surface is later colonized by microorganisms, including bacteria, algae (especially diatoms), fungi and protozoa. The attachment, colonization and growth of microorganisms on the surface results in the formation of a slimy layer called biofilm. The biofilm layer can aid (or deter) the subsequent succession of 'macrofouling' species, such as barnacles, by facilitating adhesion or through the production of bioactive molecules [15, 21, 42].

Biofouling is an ongoing problem for water-immersed man-made structures such as ship's hulls, oceanographic instrumentation, pipelines, membranes, heat exchangers and aquaculture equipment, resulting in severe economic

consequences [31]. There is therefore a great need for novel coatings and material systems to combat these challenges. It is well known that different surfaces can behave in contrasting ways with regard to fouling. Some manufacturers focus their research on surfaces or coatings that resist fouling formation, whereas others focus on fouling-release properties. These are application specific, and certainly it is important to understand both fouling formation and fouling release mechanisms on such surfaces [13, 23, 41]. For the screening process of these materials, there is a need for rapid test methodologies to effectively assess the antifouling and fouling-release performance.

In this paper, we describe a rapid and reproducible bacterial assay to assess both antifouling and fouling-release performance of coatings and materials. The assay is based on the quantification of bacterial growth and adhesion using biofilms of a single bacterial species.

With regard to adhesion or fouling-release assays, a few of the pieces of ocean immersion testing equipment have been miniaturised and adapted for laboratory evaluations. For example, the automated water jet apparatus was developed for screening of a range of potential fouling-release coatings that were deposited in multiwell plates [17, 34, 35, 37, 39]. A water channel apparatus with fully calibrated turbulent flow was also used to quantify the fouling-release potential of coatings [32, 36]. In our study, the release was achieved by applying hydrodynamic shear forces with a custom-made laboratory-based high-speed rotary device. This rotating drum testing method was originally designed for the determination of erosion behaviour and antifouling performance of marine antifouling coatings in the aquatic environment [2]. The relatively large-sized coated panels normally used on this rotary have now been adapted for laboratory bacterial assays on standard microscope slides.

In any bacterial assay, enumeration of the attached bacterial cells is an important and challenging step. Numerous direct and indirect methods have been employed for quantifying the biofilm developed on surfaces. The recently developed general nucleic acid fluorochromes are becoming more and more common for biofilm quantification [11]. This includes the fluorochromes DAPI [20, 25, 26], SYTO 60 [28] and SYTO 9 [22]. These nucleic acid stains provide greater specificity and higher sensitivity as compared to other methods, such as ATP, DNA, crystal violet staining, viable cell count and AODC [4, 6, 7, 12, 29, 33, 38, 40]. For example, simple quantification by microscopy is difficult due to bacterial aggregation and slime formation [18]. Although the ATP content of microorganisms can be accurately measured, it is influenced by the extraction method used. The widely used crystal violet staining method depends upon the quantity of exopolysaccharides (EPS) present in the biofilm, and it therefore becomes less sensitive when the bacterial biomass is low [12, 24]. Fluorochrome-conjugated lectin is another sensitive method for biofilm quantification, which takes advantage of the selective binding to sugar moieties in the EPS [12]. The absence of specific sugars in the EPS will reduce the specificity and sensitivity of this method. Each of these methods, including the nucleic acid stains, are mostly involved in the extraction of stain or attached bacteria into solution in order to determine the bacterial biomass. This has been taken care of in our assay wherein the bacteria are quantified directly on the surface without extraction of stain into solution. The method is centered on the use of the molecular probe SYTO 13 green fluorescent nucleic acid stain. The advantage of using SYTO 13 is that it is highly cell penetrating, sensitive and gives high fluorescence intensity with a negligible background fluorescent signal. SYTO 13 has been used in this assay to quantify bacterial biomass following the growth and subsequent release step. Finally, the bacterial assay protocol developed has been demonstrated by screening of three different antifouling and reference coatings.

Materials and methods

Chemicals

SYTO 13 (5 mM, 250 µl) was purchased from Molecular Probes, Inc. (Invitrogen). A concentrated solution of 25 µM in dimethyl sulfoxide (DMSO) was prepared from the main stock and stored at −20°C. The working stain solution of desired concentration was freshly prepared in deionised water (filtered through a 0.2-µm filter) on the day of the assay. Sea salt and bacteriological peptone used for the bacterial medium were purchased from Sigma-Aldrich, Germany.

Bacterial strain and growth conditions

The marine bacterium *Cobetia marina* (DSMZ4741) was used in this study. The strain was obtained from the DSMZ culture collections, Germany. The growth medium used was Sea Salt Peptone (SSP) (sea salt 40 g; peptone 18 g; deionised water 1,000 ml). The culture was maintained on Sea Salt Peptone Agar (SSPA) slants (sea salt 40 g; peptone 18 g; agar 30 g; deionised water 1,000 ml).

The culture was grown in the SSP medium at 28 ± 2°C for 18 h. This was then used as a source of inoculum for another flask containing fresh SSP medium. Subsequently, this flask was incubated on a rotary shaker at 150 rpm at 28°C. The culture was grown for 20–22 h, and cells were collected by centrifugation at ~7,000*g* and 4°C using a cold centrifuge. The cells were washed once with

phosphate buffered saline (PBS; 5 mM, pH 7.4) and diluted to a known absorbance at a wavelength of 595 nm using the plate reader (SPECTROFluor multi-well plate reader of TECAN GENios, Magellan software).

Test surfaces

Standard glass microscope slides (76 × 26 × 1 mm; Menzel-Glaser, Germany) and coated Epikote and Silastic T2 slides were used for method development. The glass slides were coated with a commercial epoxy-based coating (Epikote, Euroresins, UK), serving as a reference for bacterial growth (being a relatively inert material) and silicone-based coating (Silastic T2, Dow Corning Ltd., UK), serving as a reference for the adhesion assay. In brief, all the glass slides were first cleaned with 20% HCl solution, deionised water and ethanol, and dried. For Epikote preparation, the glass slides were pre-treated with a silane solution (Silquest A1100 silane, Contivema BV, The Netherlands) for improved adhesion. After drying of the silanised slides for 15–30 min, Epikote was applied with a spray gun (Eminent, E13HTE 1.5 mm). For Silastic T2, slides were initially cleaned in the same way as Epikote, followed by rinsing in acetone. The application of a thin layer of primer was applied (1200 OS Primer, Dow Corning Ltd., UK). After air drying for 16 h, Silastic T2 (diluted 60% with xylene) was applied with the spray gun. These coatings were used as the reference materials for the assay development along with non-coated standard microscope slides.

In addition to the above coatings, a few coatings were used only for illustration of the developed assay. Three different sol-gel-based coatings were deposited on glass microscope slides having different nano- and micro-surface roughness (for application details see [30]). The roughness in sol-gel matrix was obtained with the introduction of organically modified clay nanoparticles [30]. The concentration and distribution of these nanoparticles in the sol-gel alters the roughness of the surface. Based on the roughness level, coatings were classified as nano (up to 400 nm), intermediate (up to 2 μm) and micro (up to 6 μm), and designated as En, Ei and Em, respectively. Additionally, sol–gel coatings are known for their superior hardness and scratch resistance, and therefore are potentially suitable for underwater applications.

To minimise any leaching effects, all coated slides were pre-leached in sterile deionised water for 7 days before the experiments. It also helped to check coating integrity prior to the commencement of the assay (i.e., surface cracks or delamination) as the coating has to remain intact against the intense rotor release test. To avoid biocontamination during the leaching step, the deionised water was continuously circulated through an activated charcoal filter and exposed to UV-C radiation.

Calibration of RFU values and total bacterial count

A calibration was carried out to assess the correlation between the relative fluorescence units (RFU) measured with the plate reader and the total bacterial count (TBC). Additionally, this also gives an indication of the plate reader sensitivity for the detection of the minimum number of bacteria using SYTO 13 fluorescent stain.

Calibration: The bacterial culture was made to 0.1 absorbance in PBS as detailed above. Serially diluted aliquots of the cells (50, 100, 150 and 200 μl of 0.1 absorbance were made to 2 ml with PBS) were pipetted into 96-well round-bottom plates, and SYTO 13 (1.5 μM) stain was added. The stained samples were first allowed to stand for 10 min in the dark, and then fluorescence was measured as RFU (excitation 485 nm, emission 535 nm) using the plate reader. Sub-samples of the diluted cell suspensions were also taken to determine the TBC by epifluorescence microscopy using the same SYTO 13 stain. Briefly, 200 μl of the SYTO 13 stain was added to test tubes (final concentration 1.5 μM) containing 2 ml of the diluted bacterial cell suspension. Staining was performed under dark conditions, and SYTO 13 was allowed to react with the cells for 10 min. The samples were then filtered through a 0.22-μm nucleopore filter (previously stained with amido black). A drop of immersion oil was added onto the top of the filter and then covered with a cover slip. Another drop of oil was applied onto the cover slip, and the slide was viewed. Images were acquired under UV light with a blue filter using an epifluorescence microscope (Olympus BX51) with Soft imaging software (AnalySIS). Under these conditions, the stained cells were revealed by a bright green fluorescence. Multiple images (10–12) of the bacteria were taken, and the bacteria were counted using image analysis software (AnalySIS). The average was then used to calculate the TBC as follows:

$$\mathrm{TBC/ml} = \mathrm{Mean\ count} \times (\mathrm{filter\ area/field\ area}) \times (1/\mathrm{ml\ filter}).$$

A calibration curve was plotted using RFU and TBC values. These calibration plots can be subsequently used to estimate the quantity of bacterial cells adhering to the surface.

Bacterial assay

Bacterial attachment and growth: The slides of Epikote and Silastic T2 were pre-leached in deionised water for 7 days before the start of the assay. These coated materials along with cleaned glass microscope slides were conditioned for 1 h in artificial seawater before being transferred to the bacterial cell suspensions. A freshly grown (log-phase) bacterial suspension was prepared with an

absorbance of 0.2 at 595 nm. The conditioned replicate slides (three for each assay) were placed into individual compartments of plastic quadric plates (quadriPERM plates; Greiner Bio-one Ltd.), and 8 ml of the bacterial suspension was added, fully immersing the slides. The plates were placed in an incubator at 28°C on a rotary shaker (150 rpm). After 1 h of incubation, the slides were soaked by dipping in sterile seawater to remove any suspended cells. Three replicate slides of each were taken out at this stage of the assay to determine the amount of initial irreversibly attached cells. This was carried out using the plate reader and fluorochrome SYTO 13 as depicted in detail below. The remaining slides containing the settled cells were transferred back into new quadriPERM plates containing 8 ml of seawater-enriched growth medium. The plates were incubated again for 4 h at 28°C on a rotary shaker (150 rpm) for the attached cells to proliferate onto the surface. At the end of the incubation, the slides were gently rinsed again to remove any suspended cells. The growth of the bacteria cells were then measured as detailed below.

The slides were placed into the Tecan slide holder (four slides in one holder) and partially air-dried (~10 min). Six spots of 20 µl drops of 1.5 µM SYTO 13 stain were added onto the partially dried biofilm [8]. The drops were covered with a rectangular glass cover slip (24 × 50 mm, Menzel-Glaser, Germany) to evenly distribute the stain across the surface. This is vital in the case of hydrophobic coatings. The slide holder was placed in the dark for 10 min, followed by the measurement of fluorescence in the plate reader. The fluorescence was measured with the top mode using three flashes. The plate reader method was programmed to counterfeit the 384-well flat bottom plates, and this resulted in a maximum number of points across each slide, i.e., a total of 18–25 RFU points across each slide (Fig. 1). The average number of these points was taken to determine the bacterial biomass attached on the surface after subtracting with the negative control. A negative control was a slide of each individual coating that had been exposed to the same conditions, but in the absence of bacteria serving as a coating blank and also to take care of coating autofluorescence.

Adhesion assay: After 4 h of growth, replicate slides were used for the measurement of bacterial adhesion through exposure to hydrodynamic force. The measurement was performed on a custom-made rotary device (Fig. 2). The set-up contains 1,000 l of filtered natural seawater maintained at 20 ± 3°C. The equipment was adapted to fit standard microscope slides onto the rotating drum (diameter 50 cm). The drum is rotated by an electric motor that can be controlled to give various rotation speeds from 5 to 30 knots. This exposes the adhered bacteria to shear forces (turbulent flow), resulting in a complete or partial removal of the biofilm from the slide surfaces. Fixed rotor speeds of 5, 9, 12 and 17 knots were used for 10 min, corresponding to peripheral speed of the drum surface of 100, 228, 325 and 475 m/min, respectively [2]. Based on the analysis of release data at these speeds, subsequent experiments were conducted at 12 knots for 10 min to provide a standard shear force. The bacterial cells that remained attached on the surface were measured using SYTO 13 as described earlier and then compared to the slides that had not been subjected to shear force. The results are expressed as percentage of bacteria removed or detached by shear [(RFU of attached bacteria − RFU of bacteria remained after release step)/RFU of attached bacteria × 100]; 95% confidence limits were calculated.

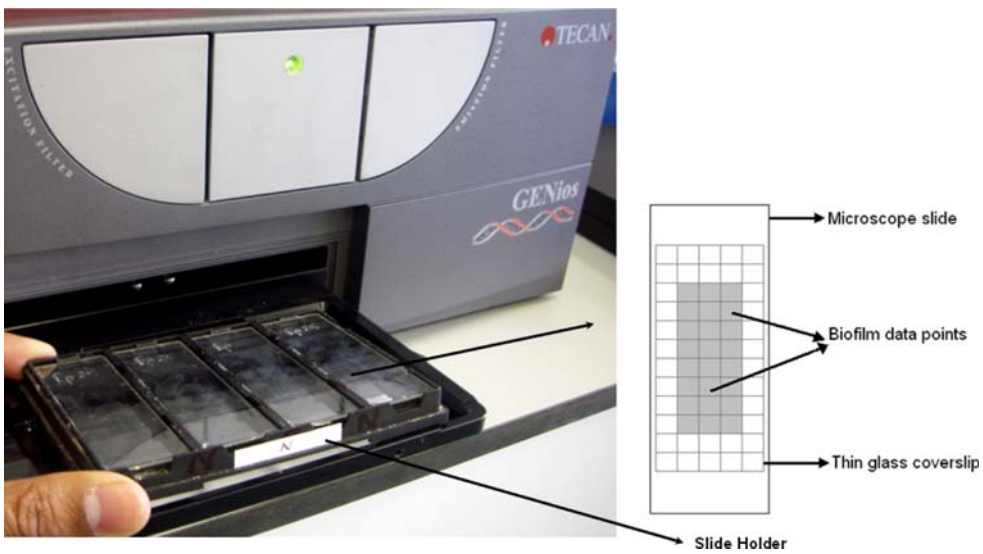

Fig. 1 Slide holder for the measurement of fluorescence of the stained biofilm (as relative fluorescence unit; RFU) directly on the coated surface using the fluorescence plate reader. The measurement gives around 18–24 data points on each slide

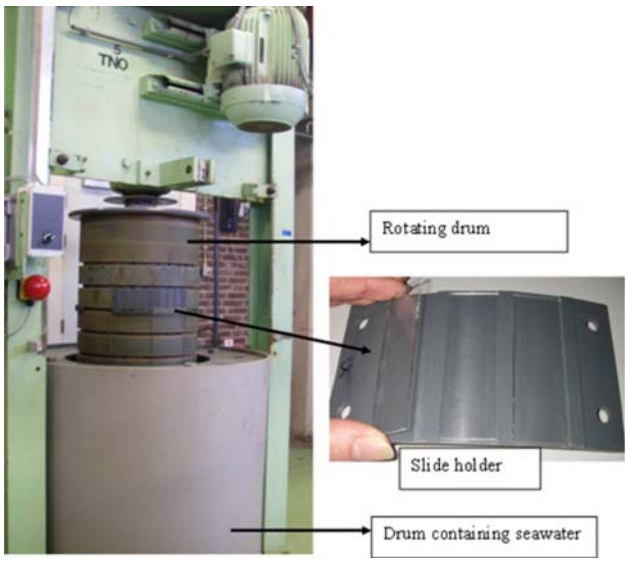

Fig. 2 A custom-made rotary device for the measurement of adhesion strength of the biofilm after subjection to different rotation speeds (*knots*)

Results

Calibration

A clear positive linear correlation ($p < 0.001$) was observed between the fluorescence intensity (RFU) measured using plate reader and total bacterial count as determined by epifluorescence microscopy using *Cobetia marina* bacterium (Fig. 3). SYTO 13 stained *Cobetia* cells bright green against a black background. The calibration factor obtained from the correlation graph can also then be used to directly quantify bacterial cell numbers on the surface.

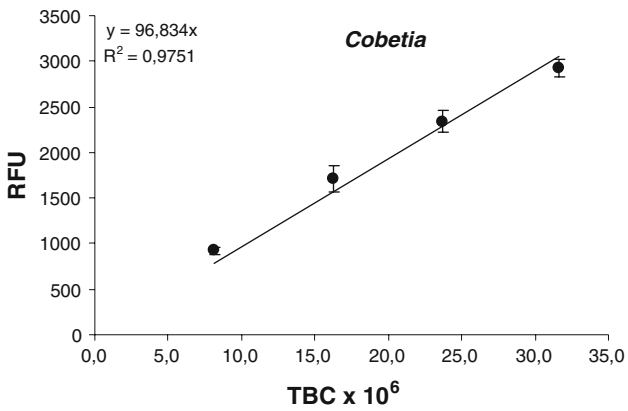

Fig. 3 Relationship between RFU (relative fluorescence unit) and total bacterial count measured using plate reader and epifluorescence microscope, respectively, for *Cobetia marina* bacterium

Bacterial assay and antifouling coatings

Cell suspension of density 0.2 absorbance resulted in an adequate amount of cell attachment with a 1-h settlement period for all three test surfaces (Fig. 4).

The subsequent 4-h growth step resulted in an increased proliferation of bacteria cells on the surface, as shown by the increase in RFU values (Fig. 4). The attached cells (after 4 h) were then subjected to shear forces to determine the fouling-release performance of the coatings.

To ascertain the optimum rotation speed for bacteria release, experiments were carried out with speeds from 5 to 17 knots. The release from the glass was higher than the epoxy and silicon coatings. At the lower speed of 5 knots, there was minimal release of biofilm. As the speed and hence shear stress increased, the detachment of bacteria also increased. The release reached a maximum for the Epikote and Silastic T2 at 17 knots, whereas glass showed complete release (Fig. 5). At 12 knots the release for Silastic T2 and Epikote was ~50%, and this speed was subsequently selected for the release assay.

The assessment of bacterial growth and release of the antifouling sol–gel coatings are shown in Fig. 6. None of the coatings showed bacterial growth inhibition as compared to reference surfaces. The release was high on Ei compared to the reference surfaces and also to the other two sol–gel coatings.

Discussion

In this investigation, a spectrofluorometeric plate reader technique is used to quantify bacteria growth directly on the substrate. This technique is quick, simple and sensitive, and numerous samples can be analysed simultaneously. The fluorochrome SYTO 13 was chosen and preferred over other nucleic acid fluorescent stains because of its high permeability, greater fluorescence yield and low background fluorescence. The concentration range recommended by the manufacturer of SYTO 13 for bacterial application is 50 nM–20 μM and 1–30 min incubation. It was observed that 1.5 μM SYTO 13 stain with a 10-min incubation period results in a good fluorescence intensity (RFU values) [8]. Hence, this combination was used, with the added benefit of being relatively economical.

The fluorescence intensity measured by the plate reader and the total bacterial count measured by epifluorescence microscopy for the same samples showed a significant positive relationship (Fig. 3). This means that fluorescence intensity of the stained cells increased proportionally with the increase in bacterial cell numbers. Thus, this reveals that the method can be applied for the estimation of bacterial numbers. The above staining technique was then used

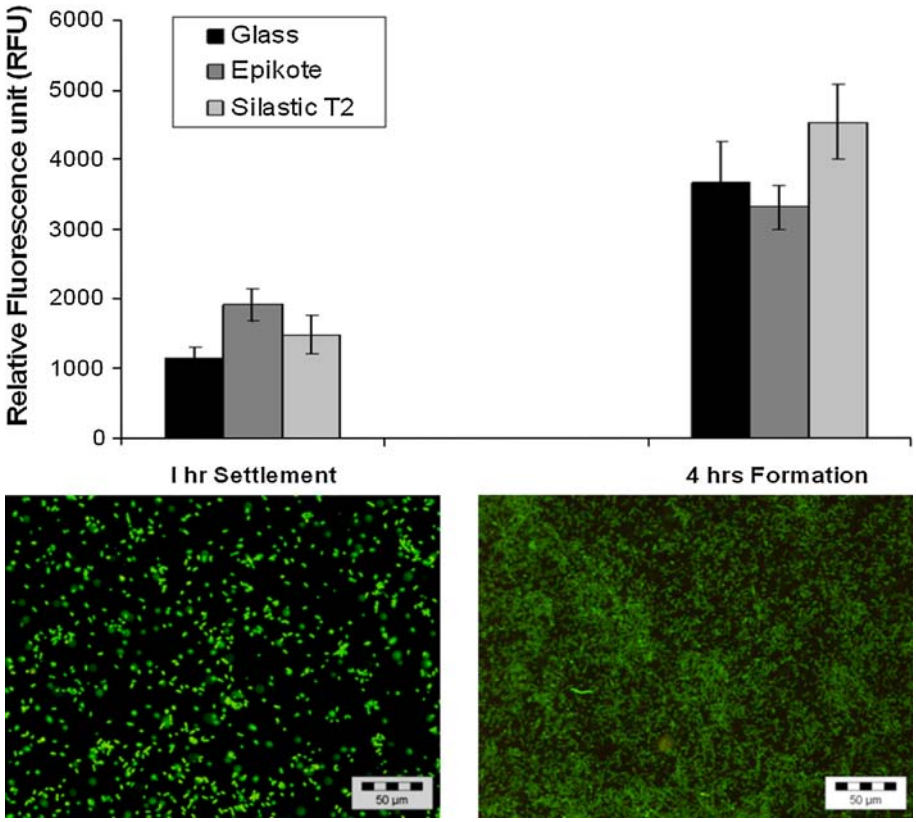

Fig. 4 *Above* are the RFU measured after 1-h settlement and 4-h biofilm formation of *Cobetia marina* using the plate reader; *below* are the corresponding fluorescence images obtained using SYTO 13 staining technique on glass

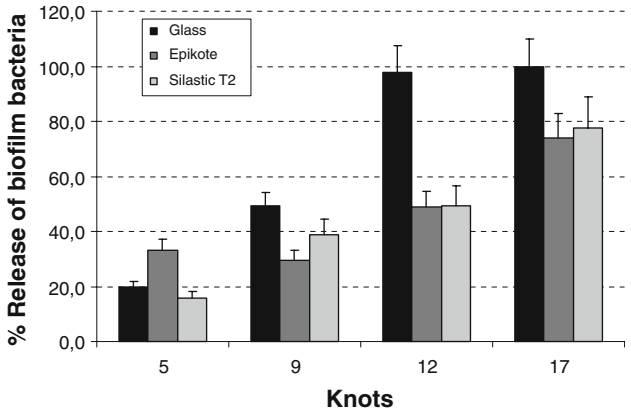

Fig. 5 Percent (%) biofilm release of the *Cobetia* biofilm on glass, Epikote and Silastic T2 coatings at different rotor speed (*knots*). *Error bars* represent plus one standard error of the mean (18–20 data points each slide)

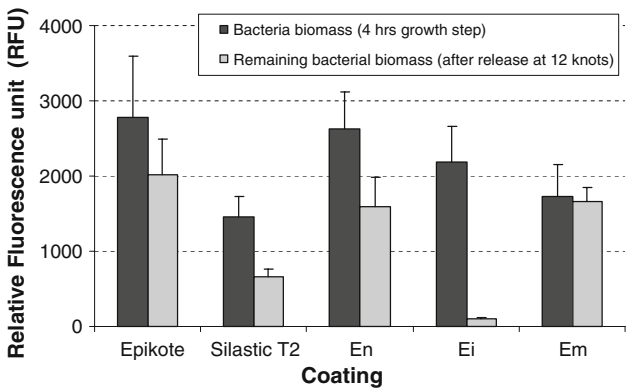

Fig. 6 RFU values representing bacterial biomass after 4-h growth step and the remaining bacterial biomass after the release test on modified sol-gel antifouling coatings and standards (Silastic T2 and Epikote). *Error bars* represent plus one standard error of the mean (18–20 data points each slide)

for the growth (after a 4-h growth period) and adhesion (or release) assay to quantify the bacterial biomass directly on the surface.

As seen from the Fig. 4, the initial settlement and the 4-h growth step resulted in an increase in RFU values measured using the plate reader directly on all three surfaces. The results are comparable with the microscopic observations (Fig. 4). Thus, this technique can be used to quantify bacterial growth and proliferation directly on the surface. The growth study was carried out for a short period of 4 h so as to reduce the effects of crowding and aggregation of bacterial cells or prevent 3D biofilm formation. For instance, the release assay will reveal the true cell-surface adhesion rather than cell–cell interactions when subjected to a shear force. Hence, this 4-h period of incubation was used for the bacterial growth assay and subsequently for the adhesion (release) assay (Fig. 4).

The attached cells after the 4-h growth step were subjected to the release test at different rotor speeds. The smoothness of the glass surface resulted in high release as compared to Epikote and Silastic T2 at all the speeds tested. Based on the difference in release performance observed at 12 knots and 17 knots, the rotation speed of 12 knots was selected for the assessment of biofilm release performance. Both Silastic T2 and Epikote resulted in ~50% bacterial detachment at 12 knots (Fig. 5). This indicates that there is still biofilm remaining attached on the silicone Silastic T2 coating after subjection to a rotation of 12 knots speed. Silicon-based coatings are the most commercially used fouling-release coatings [3, 10], and hence 12 knots would be a suitable speed for further testing of superior release performance coatings. From these results, i.e., a short period of growth step, in order to reduce aggregation of cells and the selected 12 knots speed, a final assay protocol was developed as illustrated in the flow chart (Fig. 7).

The utility and simplicity of the developed assay are illustrated by the assessment of the modified sol-gel antifouling coatings. All the three tested coatings have different surface roughness [30]; however, all showed similar growth, i.e., no inhibition of biofilm formation of *Cobetia* (Fig. 6). On the other hand, the intermediate roughness surface (Ei) showed high bacterial release compared to En (nano) and Em (micro) coatings and the reference surfaces (Fig. 6). From this illustration, it can be concluded that it is relatively easy to assess or screen the performance of coatings for bacterial inhibition or release properties when compared to a reference. In addition, the assay was also briefly referred and illustrated for the assessment of other coating types [1, 14, 16].

In conclusion, the assay developed is relatively simple, fast and can be used to screen several coating surfaces simultaneously for bacterial growth inhibition and release properties. The fact that no solvent extraction of stain is carried out in this assay as well as the use of microscope slides offers an additional advantage to asses and visualise the bacterial interaction on the surface after the assay. Finally, this method is the first plate reader assay for quantification of bacterial biomass directly on coated glass microscope slides.

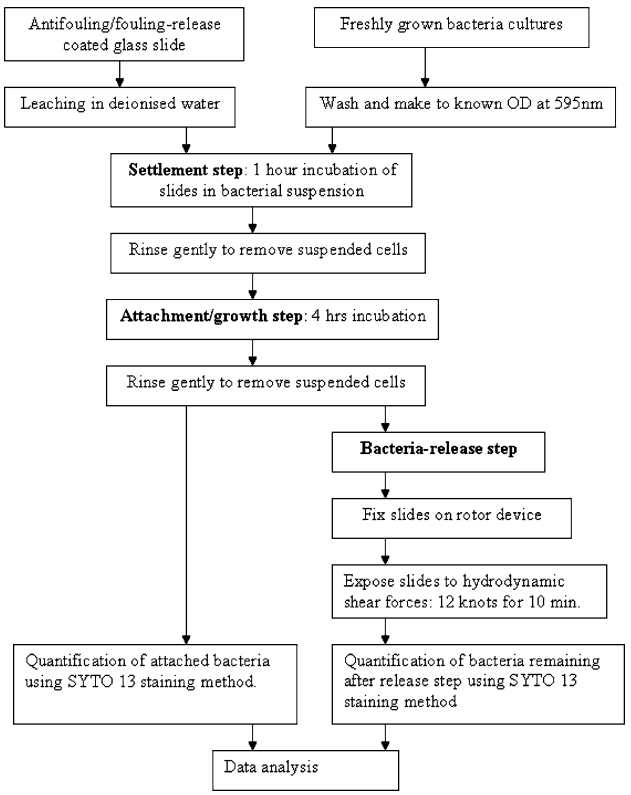

Fig. 7 Flow chart illustrating the assay protocol for screening of the antifouling coatings for the bacterial attachment and release performance

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