

Efficacy of amorphous TiO_x-coated surfaces against micro- and macrofouling through laboratory microcosms and field studies

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Abstract

In this study, Soda Lime Glass (SLG) and Stainless Steel (SS316L) substrata coated with Titanium oxide (TiO_x) were tested for their efficacy in the laboratory microcosms and in field against micro- and macrofouling. Laboratory microcosm studies were conducted for five days using natural biofilms, single-species diatom (*Navicula* sp.), and bacterial biofilms, whereas field observations were conducted for 30 days. The TiO_x-coating induced change in the mean contact angle of the substratum and rendered SS316L more hydrophilic and SLG hydrophobic, which influenced the *Navicula* sp. biofilm, and bacterial community structure of the biofilm. Overall, the TiO_x-coated SS316L showed minimal microfouling, whereas non-coated SLG exhibited greater efficacy in deterring/preventing macrofouling organisms. Moreover, the reduction in macrofouling could be attributed to high abundance of Actinobacteria and unraveling its mechanism of action needs future studies emphasizing biochemical processes and pathways.

Keywords: Biofilm, Macrofouling, TiO_x, Barnacle, Bacteria, Anti-fouling

Introduction

Biofouling, an undesired attachment of micro- and macro-organisms to any man-made structure immersed in seawater, is a highly dynamic and complex process in the aquatic environment which has considerable economic significance (Callow and Callow 2011). This process can be categorized into two steps: (a) the micro-fouling process, which generally begins with the formation of conditioning film followed by the settlement of microorganisms to form a biofilm (Wahl 1989; Bhosle et al. 2005), and (b) the macro-fouling process, where the macro-organisms adhere to the initial microbial biofilm (Wieczorek and Todd, 1998; Dang and Lovell, 2016). Macrofouling organisms, especially barnacles, adhere with a powerful adhesive that prevents them from dislodging from any substratum (Khandeparker and Anil 2007). Moreover, their attachment to ship hulls causes a significant increase in the hydrodynamic drag of the vessel and raises fuel penalties by as much as 40% (Townsin 2003; Yebra et al. 2004; Schultz et al. 2011). Economically, it is a huge problem that the expenditure of the US Navy alone is estimated to be ~ \$180-260M per annum (Schultz et al. 2011). Biofilms or microbial assemblages provide an optimal ecological condition for the settlement of many macrofouling organisms (Unabia and Hadfield 1999; Armstrong et al. 2001; Dobretsov et al. 2006; Khandeparker et al. 2002, 2003, 2006; De Gregoris et al. 2012; Sahoo and Khandeparker, 2018a). The importance of microbes and the associated polymers/metabolites in the settlement of macrofoulers and their implications in biofouling has been well studied (Khandeparker et al. 2002, 2003; Hadfield, 2011; Dobretsov and Rittschof 2020 and references within; Rajitha et al. 2020a, b). It has also been reported that the age of the biofilm, and the species composition of the microbes influence the settlement of the most dominant fouling barnacle, *Amphibalanus amphitrite* cyprids (Khandeparker et al. 2003; Qian et al. 2003; Hung et al. 2008).

Antifouling paints have been used extensively as a solution to inhibit biofouling on substrates in contact with seawater, but highly effective antifouling paints based on metal biocides (mainly tributyltin, copper- and zinc-based systems) had unintended environmental impacts (Gibbs et al. 1988; Champ 2003; Pistone et al. 2021 and references therein). Finding more ecological (“green”) alternatives to the traditional technologies, such as developing a non-toxic, i.e., biocide-free coating and modifying the substrate surface properties, has been a challenge to both industries as well as academia (Callow and Callow 2011). Extensive use of biocide-containing paints to curb this issue also targets the non-target organisms, driving research toward non-biocidal solutions based on altering substrate surface properties, especially the application of coatings (Dobretsov and Thomason 2011). Recently, the interplay between Nitric Oxide and Serotonin signaling playing a vital role in the pelago-benthic transition of barnacle *Amphibalanus amphitrite* was demonstrated, and it was suggested that

such signaling pathways could provide antifouling solution against the recruitment of barnacles (Sahoo and Khandeparker 2018a, b). Conversely, surface coating techniques have shown promising results as they are economical and efficient strategies to protect subsea surfaces (Jin et al. 2022). As the properties of substrate surfaces can be manipulated, antifouling approaches to control biofilm formation primarily focus on modifying the surface properties (Unepetty et al. 2022). Hydrophobicity has been widely used in anti-biofouling surfaces in the marine environment (Genzer and Efimenko 2006; Marmur 2006; Li et al. 2020). In the field of antifouling coating, titanium oxide has been gaining a lot of attention because of its insoluble nature in water. It can be embedded well into the coating matrix, owing to which the leaching of titanium particles is minimal (Szeto et al. 2021). In recent years, numerous articles have been published investigating the antifouling effects of titanium oxide, and it has been demonstrated that titanium oxide (TiO₂) based coatings are a new member of the antifouling coating family (Soethe et al. 2017; Szeto et al. 2021 and references therein). An earlier study demonstrated that TiO₂ could alter the biomass and community composition of biofilms, and subsequently inhibited mussel, *Mytilus coruscus* plantigrade settlement (Yang et al. 2016). A recent study demonstrated the effect of non-stoichiometric amorphous TiO_x-coated surfaces against viral particles (Mittireddi et al. 2021). However, not much is known about the efficacy of such environmentally friendly TiO_x-coated surfaces (with modified surface properties) on micro and macrofouling.

Considering the above, Soda Lime Glass (SLG) and Stainless Steel (SS316L) substrata coated with TiO_x were used in this study. The 316L stainless steel is an engineering alloy in liquid-handling systems and hydraulic machinery with wide-ranging marine applications (Zexu et al. 2021). In comparison, SLG is a transparent material with a wide range of applications in optical, bio-medical, semi-conductor industries, etc. The influence of TiO_x-coated SLG and SS316L substrata was assessed against micro and macro fouling in laboratory microcosm studies and in field (Mandovi Bay - adjoining Arabian Sea) along the west coast of India. It was hypothesized that TiO_x coating would modify the nature of the substrata, thus influencing the microbial community structure in the biofilms and subsequently result in either promotion or inhibition of settlement and recruitment of fouling organisms.

Materials and methods

Coating Synthesis and Characteristics

The TiO_x coatings were applied on SLG and SS316L (ultrasonically pre-cleaned) substrates at room temperature, using RF Magnetron sputtering technique (Excel instruments), at 1.42 Pa working

pressure. A base pressure of 2.5×10^{-4} Pa was achieved prior to the coating process using a series of pumps. Note that, a TiO_x ($X=1.8$) ceramic titanium oxide (Abletarget Limited) target (99.99% pure; 2" dia and 3 mm thick) was used for sputter coating the TiO_x compound onto the respective substrates under argon (99.99% pure) atmosphere at 50 sccm flow rate. Further note that, the sputtering process was carried out at constant RF power (50W), substrate rotation (20 rpm), coat time (180 min), and target to substrate distance (8 cm) to ensure the formation a uniform coating on various substrates.

The coatings were found to be amorphous in structure, transparent (~ 93% in the visible-NIR region with respect to SLG) with an average thickness of 47.1 ± 0.8 nm and nominal composition of 45.4 at. % Ti and 54.6 at. % O (Figure 1; Mittireddi et al. 2021). Static contact angle measurements were carried out before and after coating these samples using deionized (DI) Water (Figure 2). Note that the mean contact angle value of uncoated SLG and SS 316L substrates were $40.6^\circ \pm 11.8^\circ$ and $88.6^\circ \pm 5^\circ$ respectively, implying them to be originally slightly hydrophilic and hydrophobic. However, when coated with TiO_x material, these substrates had opposing effects induced in them, as evident from the change in their mean contact angles ($110.5^\circ \pm 3.8^\circ$ for coated SLG and $58.4^\circ \pm 7.6^\circ$ for coated SS 316L substrates), which altered their hydrophilic and hydrophobic properties.

Laboratory microcosm experiments

The testing of TiO_x coating with respect to substratum type was first performed in the laboratory. For this, the TiO_x -coated and non-coated SLG and SS316L coupons (1 cm x 1 cm) ($n = 4$) were exposed for five days in the laboratory to a) natural seawater (50 μm filtered) containing a gamut of microorganisms, b) a fouling diatom (*Navicula* sp.), which is phytoplankton, and c) three species of biofilm-forming bacteria (isolated earlier from glass substrata) resulting in three types of biofilms as described below.

Biofilm development

Natural Biofilm (NBF)

Surface water samples were collected from the Mandovi estuary ($15^\circ 30' 09.5''\text{N}$, $73^\circ 48' 42.0''\text{E}$) situated in Goa, southwestern coast of India. The biofilm formation on TiO_x -coated SLG, SS316L coupons (1 cm x 1 cm), and non-coated coupons ($n = 4$) was tested by exposing the coupons to 5 ml of freshly collected seawater filtered through 50 μm mesh containing a gamut of microorganisms for a period of 5 days in the laboratory using sterile polystyrene multiwells (six well Corning-430262, Corning, New York, USA). These plates were maintained on a shaking incubator at 25°C (± 1). The biofilm formed on these coupons is referred to as Natural Biofilm (NBF).

Diatom Biofilm (DBF)

The laboratory-maintained pure culture of diatom *Navicula* sp. was sub-cultured in 100 ml (n = 4) of f/2 medium (Guillard and Ryther 1962), and the culture was incubated for 10 days under light: dark (12:12) cycle. Subsequently, 100 ml of culture (1×10^5 Cells ml⁻¹) was used to inoculate 6 well multiwells (Corning-430262, Corning, New York, USA), containing TiOx-coated SLG and SS316L coupons (1 cm x 1 cm) (n = 4), and these coupons were maintained for a period of 5 days in a shaking incubator at 25 °C (± 1) temperature. The observations on the diatom abundance were taken after 2 and 5 days, respectively. The biofilm formed on the coupons is referred to as Diatom Biofilm (DBF).

Artificial Bacterial Biofilm (ABF)

Bacteria were isolated from the 5-d-old biofilms developed in-situ on pre-treated glass slides in estuarine (Mandovi estuary-15°30'09.5"N 73°48'42.0"E, west coast of India), and marine (Open ocean; Arabian Sea, 15° 51.482' N, 072° 43.511' E) environments (Hede and Khandeparker, 2020). *Bacillus indicus* and *Bacillus cereus* from the estuarine biofilms and *Staphylococcus lentus*, marine biofilm-forming bacteria were selected for the present study. The bacterial biofilms were developed following the methods by Maki et al. (1988) and Khandeparker et al. (2006). Briefly, all three bacterial cultures were grown separately in the Zobell Marine Broth (ZMB) for 18-24 hours at 25 °C (± 1). Subsequently, these bacterial cultures were harvested by centrifugation (30,000 X g for 30 min). The cells were washed several times with autoclaved filtered seawater and centrifuged. The supernatant was discarded, and the cells were re-suspended using autoclaved filtered seawater. The final concentration of the bacteria in the suspension was 1×10^6 to 1×10^7 Cells ml⁻¹. These suspensions were introduced in polystyrene six well multiwells (Corning-430262, Corning, New York, USA) containing TiOx-coated SLG and SS316L coupons (1 cm x 1 cm) (n = 4), and the coupons were maintained for 5 days in a shaking incubator at 25 °C (± 1). The bacterial density in natural autoclaved seawater was adequate to sustain for five days. The biofilm formed on the coupons is referred to as Artificial Bacterial Biofilm (ABF).

Subsequently, in order to quantify the bacterial numbers, the biofilm on the coupons was scraped off using filtered autoclaved seawater, and the samples were fixed using formaldehyde at a final concentration of 1–2%; v/v. The quantification of the biofilm bacteria was carried out using flow cytometry, as described below.

Field Experiment

Before deployment, the TiOx-coated and non-coated SLG and SS316L panels (10 cm x 10 cm) were cleaned as described by Hede and Khandeparker (2018). Subsequently, the panels (n = 3) supported

by PVC frames were deployed in the field (Mandovi Bay, (15°30'09.5"N 73°48'42.0"E) for a period of 30 days (Figure 3). The panels were exposed at the subsurface level from an existing jetty located at the tropical monsoon-influenced Mandovi Bay, west coast of India (Figure 3), for a period of 30 days. The panels were retrieved on days 5, 17, and 30. The in-situ natural biofilms (INBF) developed on the panels were scraped and the samples were stored appropriately for different microbiological analyses (Total bacterial count (TBC), total viable count (TVC), protists, bacterial community structure), and macrofouling analysis using a stereo microscope (SZX10 OLYMPUS Tokyo, Japan).

Microbiological analysis

Enumeration of TBC using Flow cytometry

For the analysis of TBC, the biofilm samples were processed and analysed as described by Hede and Khandeparker (2018, 2022). Briefly, the thawed biofilm samples were sonicated (30W for 60s), and the samples were passed through the BD cell strainer cap (pore size, 40 µm) to remove the larger particles. Moreover, the subsamples (1 ml) were stained with SYBR Green I (1:10000 final concentration), incubated in dark incubation for 15 mins, and were analyzed using the flow cytometer BD FACS Aria Fusion. The gating was done against SSC versus green fluorescence, and 1µm fluorescent beads (Polysciences, USA) were used as internal standard. The TBC is expressed as Cells dm⁻² (cells per square decimetre) of the coupon/panel surface area.

Enumeration of protists

The flow cytometry settings for protists were similar to TBC; however, the detector voltage was reduced concerning SSC vs green fluorescence. This was done to avoid the interference of bacteria with protist populations during the analysis (Hede and Khandeparker, 2022). BD FACS Diva version 6.2 software was used to process the data. The protists abundance is expressed as Cells dm⁻² (cells per square decimetre) of the coupon/panel surface area sampled.

Enumeration of TVC and Pathogenic bacteria

For the enumeration of TVC, the subsamples were serially diluted, and spread (100 µl) on Zobell Marine Agar and Nutrient Agar (HiMedia). MacConkey Agar was used for the enumeration of Total coliforms, and Thiosulphate Citrate Bile Salt (TCBS) agar for *Vibrio* spp. primarily, *Vibrio alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*. Identification of these specific bacterial species has been confirmed earlier by both the biochemical tests and MALDI-TOF MS Biotyping (MTB) (Khandeparker et al. 2015, 2017). The TVC is expressed as CFU dm⁻² (colony-forming units per square decimetre) of the coupon /panel surface area sampled.

Diatom Biofilm (DBF)

The *Navicula* sp. biofilm was scraped from the coupons and 10 ml of the sample was fixed using Lugol's iodine solution. Cell abundance was determined ($n = 3$) using a haemocytometer at a magnification of 400 x using an inverted microscope (IX73 OLYMPUS, Tokyo Japan) and expressed as Cells. dm^{-2} (Cells per square decimetre) of the coupon surface area sampled.

Elucidation of bacterial diversity using quantitative PCR (qPCR)

For elucidation of bacterial diversity, the biofilm samples were processed and analysed as described by Hede and Khandeparker (2022). Briefly, the DNA extraction was performed using the MO BIO Power Biofilm™ DNA extraction kit. Next, 1 μl of eluted DNA ($\sim 1.0\text{--}5.0$ ng) from the biofilm samples was subjected to real-time quantitative polymerase chain reaction (qPCR) using specific primer sets for major bacterial phyla (Proteobacteria classes (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria), Chloroflexi, Verrucomicrobia, Firmicutes, Bacteroidetes, and Actinobacteria (Table 1).

Before metagenomic DNA analyses, the qPCR bacterial standards containing amplified 16S rRNA gene PCR products of each target group were prepared and serially diluted, ranging from 10^9 to 10^6 copies (refer to Hede and Khandeparker 2022). The qPCR assays were performed in triplicates using Rotor-Gene Q (Qiagen). The PCR cocktail mixture consisted of 10 μl of the master mix, 2 μM of each primer, 1 μl of DNA template ($\sim 1.0\text{--}5.0$ ng of environmental DNA), and made up with nuclease-free water (Ambion TM), to a total volume of 20 μl . The raw data were processed, and abundance was calculated by comparing it with a standard curve (raw value, gene copies μL^{-1}) using Rotor-Gene software (v.2.3.1, Qiagen). The efficiency of the PCR reaction was between 0.98 and 1.0. The major bacterial phyla were expressed as the relative abundance based on the sum of the absolute abundances of bacterial phyla estimated from gene copy numbers.

Data analysis

Prior to data analysis, the biological data were $\log(x+1)$ transformed. Furthermore, Statistica 6.0 program was used to check the data for the normality and homogeneity of variances (Stat Soft Inc. Tulsa, OK, USA). The data was then analysed using one-way ANOVA analysis followed by Tukey's HSD (Honest Significant Difference) post-hoc test at $\alpha = 0.05$ to compare different treatments with respect to coated and non-coated surfaces using SPSS 16.0 software.

Results

The results of the laboratory microcosm experiment indicated that the total bacterial count (TBC) in natural biofilms (NBF) was significantly low in the 5-d-old biofilms developed on TiOx-coated SLG when compared to non-coated SLG as well as TiOx-coated SS316L (Wilk's lambda, $p < 0.001$; Tukey's HSD, $p < 0.001$) (Table 2). The coverage of *Navicula* sp. also reduced significantly on SLG after coating with TiOx compared to non-coated ones (Wilk's lambda, $p < 0.001$; Tukey's HSD, $p < 0.001$) (Table 2). In the case of TiOx-coated SS316L, the total viable count (TVC) in natural biofilm (NBF) was significantly low in the 5-d-old biofilms when compared to TiOx-coated SLG (Wilk's lambda, $p < 0.001$; Tukey's HSD, $p < 0.001$) (Figure S1). Among the two substrates the diatom abundance was lowest on SS316L, which reduced further after coating with TiOx (Table 2).

The results of the in situ experiment also indicated significantly low viable bacterial count (TVC) in 5-d-old in-situ natural biofilm (INBF) developed on TiOx-coated SS316L panels (Wilk's Lambda, $p < 0.001$) (Figure S2). Similarly, low numbers of TBC ($5.32 \pm 0.617 \times 10^6$ Cells dm^{-2}) and protists ($2.20 \pm 0.14 \times 10^5$ Cells dm^{-2}) were observed on TiOx-coated SS316L panels when compared to TiOx-coated SLG panels ($8.99 \pm 0.889 \times 10^6$ and $3.19 \pm 0.38 \times 10^5$ Cells dm^{-2} , respectively). On the contrary, the TBC and protist numbers were high on the non-coated SLG panels, i.e. $7.54 \pm 0.71 \times 10^6$ Cells dm^{-2} and $8.01 \pm 2.80 \times 10^5$ Cells dm^{-2} respectively. No such trend was observed in the pathogenic bacteria.

The macrofouling was documented on the coated and non-coated surfaces at the Mandovi Bay for 5, 17, and 30 days, respectively. Upon inspection, the frames were raised, and digital photographs of the plates were taken. In the laboratory, surface coverage and composition on the entire panel were estimated using a stereo microscope. Results indicated that till day 5, the surfaces were not colonized by hard fouling or macrofouling organisms. Subsequently, both the surfaces were colonized by barnacle *Amphibalanus amphitrite*, hydroids, oysters, bryozoans, and algal filaments (Figure 4) as observed on day 17. The TiOx-coated SS316L panels favoured the settlement of barnacles and hydroids on day 17 (Figure 5). A considerable decrease in the settlement was observed with time, i.e., on day 30 in the case of barnacles and hydroids. The bryozoans and algal filaments appeared after day 17 (Figure 5). On the other hand, the settlement of barnacles and hydroids was significantly lower on SLG panels, as observed on day 17. Due to episodic rains during the sampling period, the SLG panels were lost and unavailable for observation on day 30 (Figure 4-5).

Variations in the bacterial community composition in terms of abundance in the in-situ natural biofilm (INBF) is depicted in Figure 6. The bacterial community composition was significantly

different with respect to the substratum type, days and the treatment, i.e., whether the substratum was coated/non-coated (Wilk's Lambda, $p < 0.001$). Gammaproteobacteria were abundant, followed by Bacteroidetes in the INBF formed on TiO_x-coated SLG on day 5. However, these groups decreased drastically over a period of time, and on day 17, Actinobacteria followed by Betaproteobacteria were the dominant taxonomic groups. In the case of TiO_x-coated SS316L biofilms also, Gammaproteobacteria and Bacteroidetes were abundant. However, these groups were taken over by Firmicutes and Actinobacteria on day 17.

Subsequently, Alphaproteobacteria also contributed almost equally to Actinobacteria and Betaproteobacteria on day 30 in the case of TiO_x-coated SS316L biofilm. Alphaproteobacteria were also detected in non-coated SS316L biofilms on day 17 (Figure 6). Overall, it seems that macrofouling was influenced by the substrate type, SLG was more effective against macrofouling organisms than SS316L, whereas, bacterial settlement was strongly influenced by TiO_x.

Discussion

The laboratory microcosm experiment showed significantly low microfouling (i.e., bacteria and diatom numbers) on TiO_x-coated SS316L panels. Coating with TiO_x resulted in a significant reduction in the density of diatoms in the *Navicula* sp. biofilm. Yang et al. (2016) also demonstrated that TiO₂ altered the community composition of biofilms and reduced the diatom density in the biofilms. In the in-situ condition, the numbers of bacteria and protists were also low in biofilms developed over SS316L panels. The decrease in their numbers could be attributed to an increase in the oxidative stress within algal cells (reactive oxygen species or ROS), membrane damage of microorganisms by inducing plasmolysis, etc., either due to titanium oxide particles (Szeto et al. 2021 and references therein) or due to changes induced in the substrata after applying the coating. However, it is important to note that titanium oxide-coated materials are categorised as environmentally beneficial (Szeto et al. 2021). Agostini et al. (2019) evaluated the effect of Zn and Cu₂O-based coatings on micro and macrofouling on steel surfaces and pointed out that Cu₂O-based antifouling painted surfaces showed high microfouling inhibition. However, none of these coatings were effective in avoiding the total biofouling establishment. Thus, it seems that the changes in the surface chemistry or hydrophilicity of SS316L surface after TiO_x coating led to lower microfouling.

The in situ experimental results also point to significantly low viable (culturable) bacteria in 5-d-old in-situ natural biofilm (INBF) developed on TiO_x-coated SS316L panels. After 5 days, both the surfaces were colonized by hard fouling or macrofouling organisms dominated by barnacle *A. amphitrite*, hydroids, oysters, bryozoans, and algal filaments. The macrofouling organisms in a

biofouling community can be categorized into soft foulers, which lack a rigid exoskeleton, e.g., soft corals, sponges, tunicates, hydroids, etc. and hard foulers, those possess a hard exoskeleton in the form of a shell, plate or dwelling tube, e.g., barnacles, oysters, mussels, calcareous tubeworms, etc. (Hunsucker et al. 2019). Among the hard foulers, barnacles, particularly *A. amphitrite* is a dominant fouling organism in the tropics and subtropics (Anil et al. 1990; Venugopalan and Wagh, 1990; Khandeparker et al. 2002; Otani et al. 2007; Clare and Hoeg, 2008, Desai and Prakash, 2009). Moreover, *Chthamalus* sp. and *Megabalanus tintinnabulum* are also major biofouling barnacles in tropical environments (Thiyagarajan et al. 1997; Yan et al. 2009; Eswaran and Khandeparker 2014). In the present study the fouling community was dominated by the barnacle, *A. amphitrite* and Hydroids. Among both the substrata, the SLG was more effective against macrofouling. On the contrary, the TiO_x-coated SS316L favoured the settlement of hydroids and barnacles. This could be attributed to the physical property (i.e., contact angle) that seem to change without a corresponding change in the antifouling property. Earlier studies have pointed out that the antifouling effect of TiO₂ is due to the generation of reactive oxygen species from the excited titanium oxide (or photocatalytic activities of titanium oxide) (Szeto et al. 2021 and references therein). However, the toxicity is limited to suspended TiO₂ particles and does not apply to the immobilized TiO₂ in antifouling coatings. Changes in the substratum characteristic induced after applying a coating, and its impact on biofilm formation and microbial diversity, governed by the environmental settings, seem to influence the macrofouling.

The in-situ natural biofilm formed on TiO_x-coated SLG showed a high abundance of Gammaproteobacteria, followed by Bacteroidetes on day 5. The biofilms developed in estuarine environments are dominated by Gammaproteobacteria and Bacteroidetes (Hede and Khandeparker, 2022). The dominance of these heterotrophic bacterial phyla in the biofilms could be attributed to their close association with the diatoms (Amin et al. 2012). However, these groups decreased drastically over a period of time, and on day 17, Actinobacteria followed by Betaproteobacteria were the dominant taxonomic groups. Both Actinobacteria and Betaproteobacteria are known to play a significant role in the recycling of organic matter (Nold and Zwart 1998; Glöckner et al. 2000). Moreover, the marine Actinobacteria have been important in the bioprospecting of bioactive compounds in terms of antifouling and antibiofilm effects (Pereira et al. 2020; Sabido et al. 2020). Many studies have demonstrated the antifouling activity of several species of *Streptomyces* isolated from marine sediments against major macrofoulers such as diatoms, microalgal spores, *Hydroides elegans*, etc. (Stalin et al. 2022 and references therein). Future studies should explore the potent antifouling activity of Actinobacteria from marine biofilms. In the case of TiO_x-coated SS316L biofilms also,

Gammaproteobacteria and Bacteroidetes were abundant. Bacteroidetes are known as efficient colonizers on steel surfaces in the marine environment and are also associated with biological corrosion (Kirchman 2002; Dang and Lovell 2016; Antunes et al. 2018, 2020). However, these groups were taken over by Firmicutes and Actinobacteria on day 17, Firmicutes being more dominant. Firmicutes are well-known colonizers of immersed artificial surfaces known for their spore-forming ability (Dang and Lovell, 2000). The present study reported a significantly higher abundance of this phylum on the SS316L coated with titanium oxide. Subsequently, on day 30, Alphaproteobacteria were additionally detected only in the case of TiO₂-coated SS316L biofilms. This taxon was also seen in non-coated SS316L biofilms on day 17. Alphaproteobacteria are pioneer surface colonizers possibly owing to their adaptation to relatively oligotrophic environments (Dang and Lovell 2000). Overall, in the present study, there was a significant difference in the bacterial community composition when the substratum was coated with TiO₂. When these results were compared with the fouling by different organisms, it seems that a high abundance of Actinobacteria (known as producers of biofouling inhibitors) on SLG panels on day 17 led to a significant reduction in the settlement of barnacles and hydroids. Whereas, a higher abundance of Firmicutes and Betaproteobacteria along with Alphaproteobacteria exclusively present on SS316L panels could have resulted in the higher settlement of macrofouling communities. Liang et al. (2019) also reported altered bacterial community composition on three coated substrata (polyurethane (PU), polydimethylsiloxane (PDMS), and epoxy resin (EP)), which lowered mussel settlement against these biofilms. These results suggest that the heterogeneity within the biofilms provides an ecological opportunity for bacterial populations to diversify and evolve, resulting in a competitive or defensive response against other strains or species (Oliveira et al. 2015). As a result, vacant niches may emerge in biofilms, which can be occupied by novel genotypes (Steenackers et al. 2016 and references within).

Thus, the evolution of bacterial populations within biofilms plays a significant role in promoting diversity and ecological stability in microbial communities (Steenackers et al. 2016 and references within). Earlier studies have indicated that the biofilm age and microbial species composition influence the settlement of barnacle *A. amphitrite* cyprids (Khandeparker et al. 2003; Qian et al. 2003; Hung et al. 2008). The larvae of fouling organisms perceive information about their ultimate habitats via exogenous (chemical) signals released by these microbes either from the water column or via direct contact with the substratum, and these organisms have evolved mechanisms to harbor specific microbes in their biofilms which facilitate conspecific settlement to protect the success of their population in aquatic ecosystems (Khandeparker et al. 2002, 2003, 2006, De Gregoris 2012). In order to have an improved and fundamental understanding of the interactions between the materials

and the colonizing flora, fauna, and microorganisms, the biochemical processes should be given more attention under the local environmental conditions, especially the tropical climate (Zhang et al. 2019). The biochemical degradation pathway of any targeted toxicant provides basic information before the degradability of the selective pollutant by a microorganism can be claimed (Gu 2020).

In conclusion, applying TiO_x coating to SS316L and SLG substrates modified their surface characteristics, thereby influencing the biofilm composition and macrofouling community structure. The mean contact angle of substrates, which changed on coating with TiO_x, rendered SLG more hydrophobic and SS316L more hydrophilic. The photocatalytic properties of TiO_x through the generation of free radicals and hydrophilicity of SS316L surface after TiO_x coating possibly led to lower microfouling. Moreover, a significant difference in the bacterial community composition was observed on SLG, which seems to have exhibited excellent anti-fouling property. Future studies should emphasize on unravelling the biochemical pathways involved in altering the micro/macrofouling communities after applying TiO_x coatings.

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Data availability statement

Data will be made available on request

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Legends to Figures

Figure 1 (a) Cross-sectional HRTEM image of the coated SLG substrate depicting the thickness, phase structure and uniformity of the deposited film; (b) Transmittance spectra of the uncoated and coated SLG substrates.

Figure 2 Measurement of static contact angles on SLG and SS 316L substrates before and after TiO_x coatings.

Figure 3 Deployment of panels at Mandovi Bay.

Figure 4. Fouling (micro and macrofouling) on TiO_x-coated SLG and SS316L panels exposed in the field (Mandovi Bay) on day 5 and day 17. SLG – Soda Lime Glass; SS - Stainless steel.

Figure 5. Abundance of macrofouling organisms on TiO_x-coated SLG and SS316L panels deployed in the field (Mandovi Bay) for 17 and 30 days respectively. Due to episodic rains during the sampling period the soda lime glass panels were lost on day 30. PL - Panel Lost. (A) Barnacles and Oysters (B) Hydroids, Bryozoans and Algae. SLG – Soda Lime Glass; SS - Stainless steel.

Figure 6. Relative abundances of major bacterial phyla demonstrating changes in the bacterial communities in INBF formed on TiO_x-coated SLG and SS316L panels exposed in the field (Mandovi Bay) for 5, 17 and 30 days respectively. Due to episodic rains during the sampling period the SLG panels were lost on day 30. SLG – Soda Lime Glass; SS - Stainless steel.

Legends to Supplementary Figures

Supplementary Figure 1. Total Viable Count (TVC), Total Coliforms (TC), *V. alginolyticus*, and *V. parahaemolyticus* abundance on the TiO_x-coated and non-coated SLG and SS316L panels exposed to natural seawater in the laboratory microcosm experiment. Error bars indicate the standard deviation from the mean based on four replicate samples. SLG – Soda Lime Glass; SS - Stainless steel.

Supplementary Figure 2. Abundance of different pathogenic bacteria in the in-situ 5-day-old natural biofilm (INBF) developed on the TiO_x-coated and non-coated SLG and SS316L panels exposed in the field (Mandovi Bay). Error bars indicate the standard deviation from the mean based on three replicate samples. SLG – Soda Lime Glass; SS - Stainless steel.

Legends to Tables

Table 1 Group-specific primers for real time quantitative PCR

Table 2 The influence of substratum type (SLG and SS316L) and TiO_x coating on the total bacterial count (TBC) in 5-day-old Artificial Bacterial Biofilms (ABF) of *B. cereus*, *B. indicus* and *S. lentus*, Natural Biofilms (NBF), and Diatom (*Navicula* sp.) biofilms in the laboratory microcosm experiment. ± indicates the standard deviation from the mean based on four replicate samples.

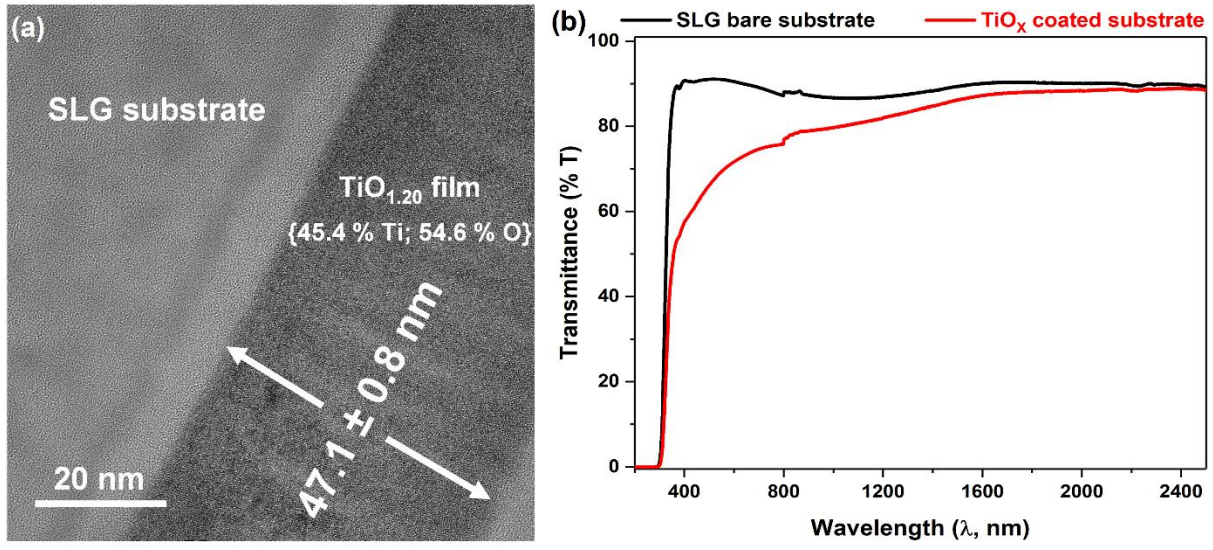


Figure 1

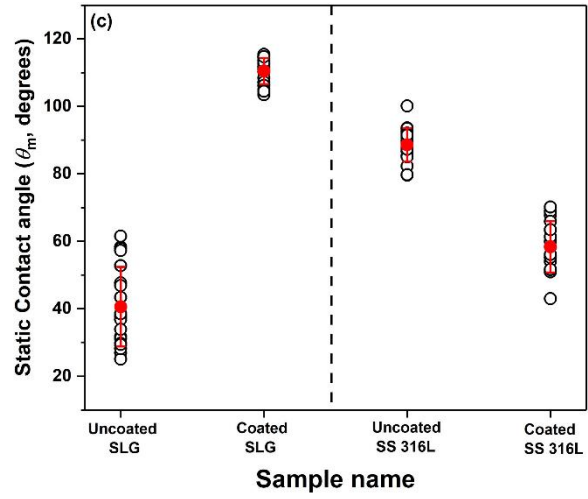
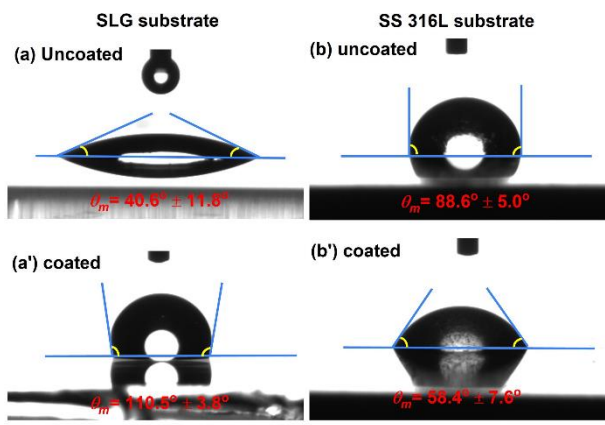


Figure 2



Figure 3

TiOx coated soda lime glass (SLG)



Day 5

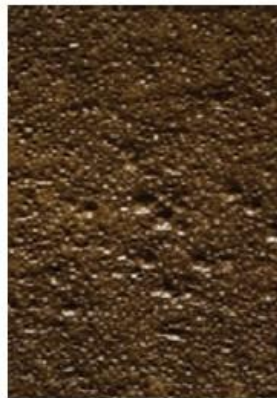


Day 17

TiOx coated SS316L



Day 5



Day 17

Figure 4

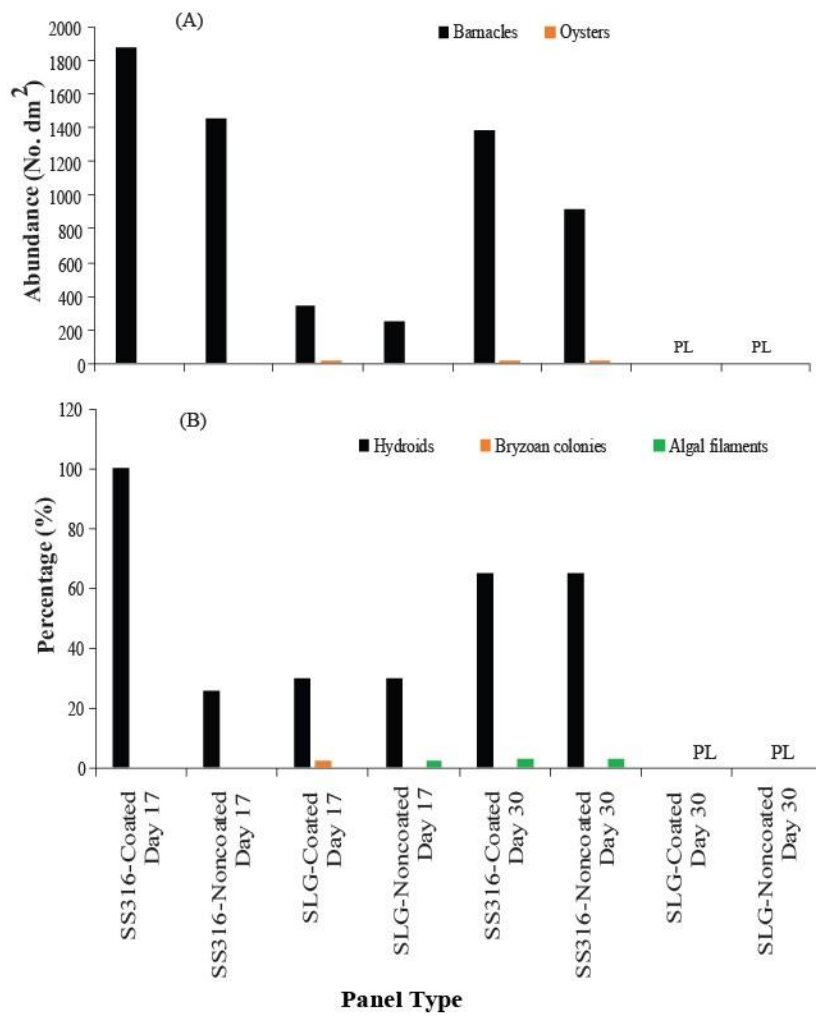


Figure 5

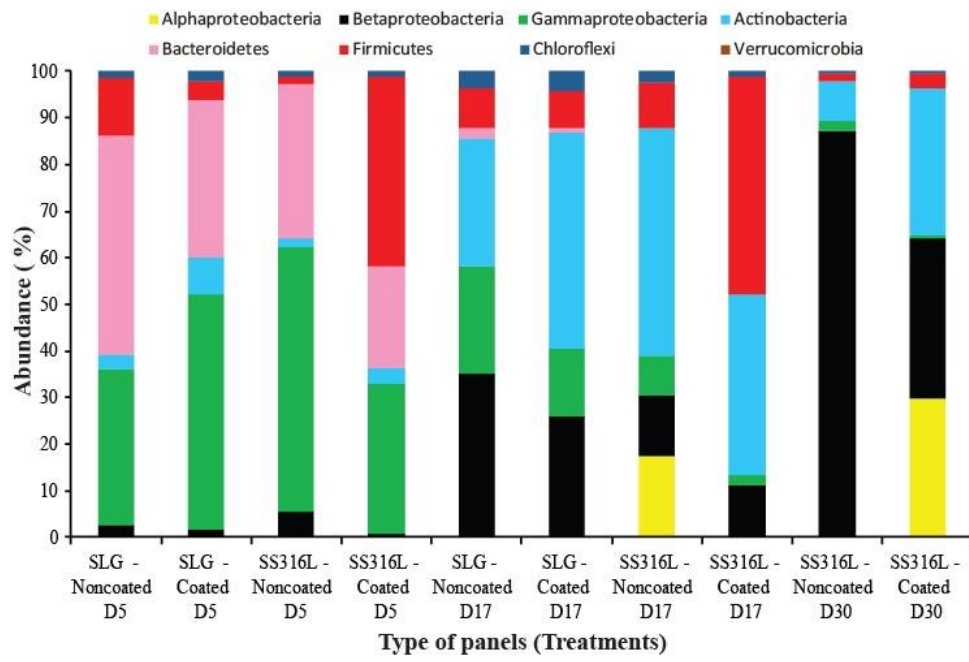
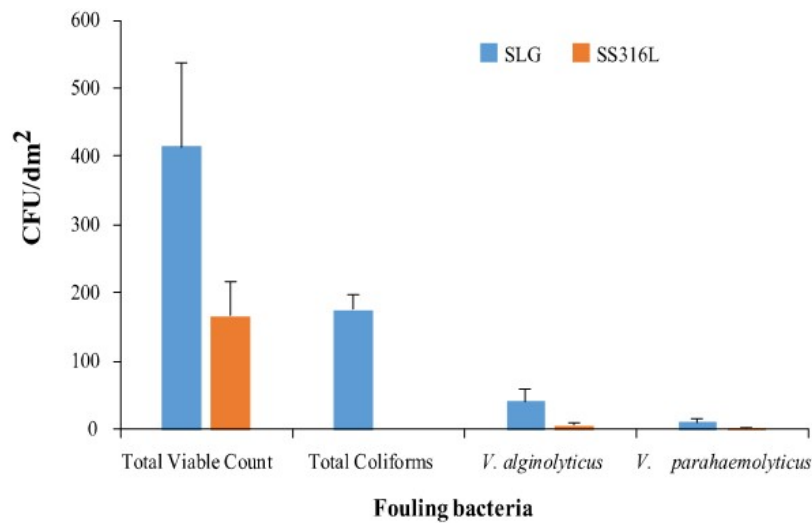
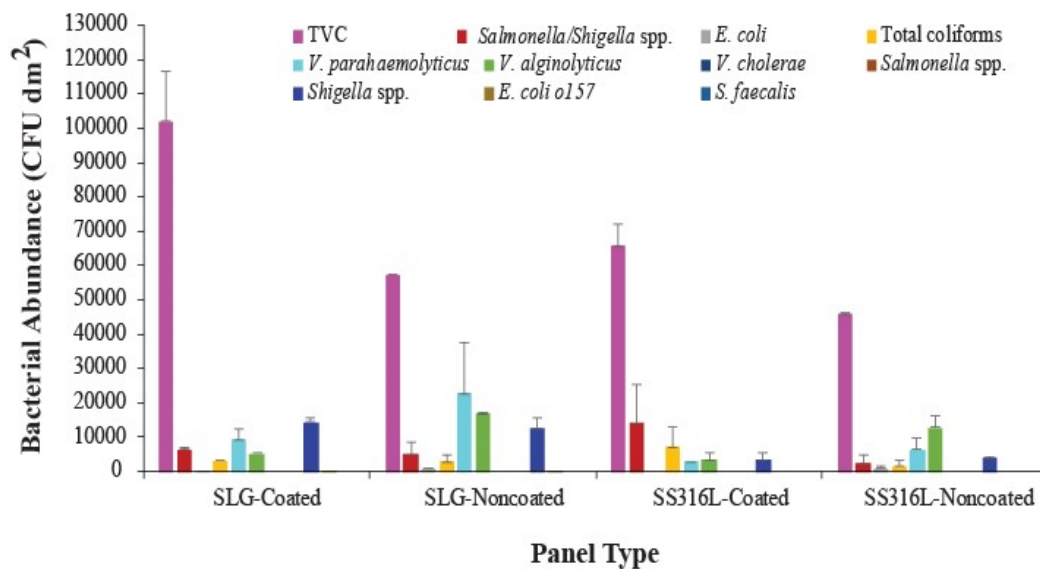


Figure 6



Supplementary Fig. 1. Total Viable Count (TVC), Total Coliforms (TC), *V. alginolyticus*, and *V. parahaemolyticus* abundance on the TiO_x-coated and non-coated SLG and SS316L panels exposed to natural seawater in the laboratory microcosm experiment. Error bars indicate the standard deviation of the mean based on four replicate samples. SLG – Soda Lime Glass; SS - Stainless steel.



Supplementary Fig. 2 Abundance of different pathogenic bacteria in the in-situ 5-day-old natural biofilm (INBF) developed on the TiO_x-coated and non-coated SLG and SS316L panels exposed in the field (Mandovi Bay). Error bars indicate the standard deviation of the mean based on three replicate samples. SLG – Soda Lime Glass; SS - Stainless steel.

Table 1

Target group name	Product size (base pairs)	Primer name and sequence (5'-3' direction)	Reference
Alphaproteobacteria	250	α-682F – CIAGTGTAGAGGTGAAATT α-908R – CCCCGTCAATTCCTTTGAGTT	De Gregoris et al. 2011
Betaproteobacteria	340	β-359f – GGGGAATTTTGGACAATGGG β-682r – ACGCATTTCACTGCTACACG	Ashelford et al. 2002
Gammaproteobacteria	170	γ-1080F – TCGTCAGCTCGTGTGTGA γ-1202R – CGTAAGGGCCATGATG	De Gregoris et al. 2011
Firmicutes	200	928FirmF – TGAAACTYAAAGGAATTGACG 1040FirmR – ACCATGCACCACCTGTC	De Gregoris et al. 2011
Bacteroidetes	200	798cfbF – CRAACAGGATTAGATACCCT cfb967R – GGTAAGGTTCCCTCGCGTAT	De Gregoris et al. 2011
Actinobacteria	300	Act920F3 – TACGGCCGCAAGGCTA Act1200R – TCRTCCCCACCTTCCTCCG	De Gregoris et al. 2011 Gich et al. 2001 and
Chloroflexi	280	941F – AGCGGAGCGTGTGGTTT 1223r – CCATTGTAGCGTGTGTMG	Björnsson et al. 2002
Verrucomicrobia	97	Ver1165F – TCAKGTCAGTATGGCCCTTAT Ver1263R – CAGTTTTYAGGATTCCTCCGCC	Yang et al. 2015

Table 2

Type of substrata	Type of biofilm	Abundance Average Cells dm ⁻²	
		Coated	Noncoated
SLG	<i>B. cereus</i>	$2.39 \times 10^8 \pm 2.80 \times 10^6$	$2.42 \times 10^8 \pm 1.66 \times 10^6$
	<i>B. indicus</i>	$5.71 \times 10^7 \pm 8.13 \times 10^6$	$5.27 \times 10^7 \pm 2.86 \times 10^6$
	<i>S. lentus</i>	$1.04 \times 10^8 \pm 1.05 \times 10^7$	$1.00 \times 10^8 \pm 2.12 \times 10^6$
	Natural Biofilm	$8.78 \times 10^7 \pm 0 \times 10^7$	$1.18 \times 10^8 \pm 2.46 \times 10^6$
	Diatom Biofilm (<i>Navicula</i> sp.)	$3.10 \times 10^4 \pm 1.44 \times 10^4$	$5.21 \times 10^4 \pm 0 \times 10^4$
SS316L	<i>B. cereus</i>	$1.90 \times 10^8 \pm 2.80 \times 10^7$	$1.67 \times 10^8 \pm 1.41 \times 10^7$
	<i>B. indicus</i>	$1.32 \times 10^8 \pm 1.67 \times 10^7$	$1.30 \times 10^8 \pm 7.32 \times 10^6$
	<i>S. lentus</i>	$1.24 \times 10^8 \pm 1.41 \times 10^7$	$1.38 \times 10^8 \pm 8.30 \times 10^6$
	Natural Biofilm	$1.35 \times 10^8 \pm 0 \times 10^8$	$1.36 \times 10^8 \pm 1.81 \times 10^7$
	Diatom Biofilm (<i>Navicula</i> sp.)	$1.74 \times 10^3 \pm 1.32 \times 10^2$	$2.65 \times 10^3 \pm 6.68 \times 10^2$