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Influence of darkness and ageing on marine and freshwater biofilm microbial communities using microcosm experiments

Niyati Hede and Lidita Khandeparker*

Abstract:

Ballast tank biofilms pose an additional risk of microbial invasion if sloughed off during ballasting operations, yet their significance and invasion biology is poorly understood. In this study, biofilms developed in marine and freshwater locations were exposed to prolonged darkness and ageing by mimicking ballast water conditions in the laboratory. Upon prolonged darkness, the decay of phytoplankton, as indicated by the decrease in chlorophyll *a* in marine biofilms led to remineralization and enhanced bacterial and protist populations. However, the same trend was not observed in the case of freshwater biofilms wherein the microbial parameters (i.e., bacteria, protists) and chlorophyll *a*

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decreased drastically. The bacterial community structure in such conditions was evaluated by real-time quantitative PCR (qPCR), and results showed that the biofilm bacterial communities changed significantly over a period of time. α -proteobacteria was the most stable taxonomic group in the marine biofilms under dark conditions. However, β -proteobacteria dominated the freshwater biofilms and seemed to play an important role in organic matter remineralization. γ -proteobacteria, which includes most of the pathogenic genera, were affected significantly and decreased in both the types of biofilms. This study revealed that marine biofilm communities were able to adapt better to the dark conditions while freshwater biofilm communities collapsed. Adaptation of tolerant bacterial communities, regeneration of nutrients via cell lysis, and presence of grazers appeared to be key factors for survival upon prolonged darkness. However, the fate of biofilm communities upon discharge in the new environment and their invasion potential is an important topic for future investigation.

Keywords: Biofilms. Bacterial diversity. Microcosms. Marine. Freshwater. qPCR. Bioinvasion. Ageing. Protists

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Introduction

Shipping carries over 90% of the world's cargo and transfers approximately 10 billion tons of ballast water (BW) around the world every year, mainly used for vessel stability [1]. The water loaded in the ballast tanks contains a gamut of nonindigenous organisms such as vertebrates, invertebrates, plants, microscopic algae, bacteria including pathogenic types, and their propagules, etc. [2-7]. The physical and chemical environment inside a ballast tank is different from outside, and the vast majority of species carried in the BW do not survive the journey [8]. Even though all the organisms

taken on board into ballast tanks may not survive, studies have shown that bacteria, microalgae, dinoflagellates, and zooplankton are well capable of surviving prolonged periods of harsh conditions by forming spores, cysts or other resting stages [9-14].

Microorganisms are found in several locations within a ship that includes ballast water, residual sediments, and biofilms formed on interior tank surfaces [4, 6]. These act as vectors leading to the introduction of new species and pathogens that can influence human health and economy, for example, by affecting the fisheries. Several areas of concern in BW management programs that are very well addressed, include introduction of new species and pathogens [5- 7, 15- 17], and the role of plankton and their associated microorganisms [3, 7, 18]. However, the transport of microorganisms through biofilms as vector in the ship's ballast, is not well addressed. Progress has been made towards removing or killing the suspended species in the ballast water, but the risk of retained tank wall biofilms has not been well studied [19]. Biofilms on submerged surfaces are known to harbour pathogenic bacteria [20, 21]. They act as seed banks, releasing microorganisms and signify an additional risk of microbial invasion, if released into the water or sloughed off during ballasting operations [4].

Previous studies have documented the fate and survival of variety of ballast water organisms such as phytoplankton assemblages, dinoflagellates, zooplankton, and microbial communities during transit [10, 12, 14, 22-29]. However, little is known about how the ballast tank conditions would influence biofilm microbial communities, and their fate when released into a novel environment. The stressors found in the ballast tanks such as darkness, ageing, predation, and nutrient availability would eliminate the less tolerant communities, leaving open niches for the most adaptable communities [30]. BW taken on board can be either fresh, brackish or marine, depending on the locality of the source port. Thus, the bacterial composition in the biofilms differs in different environments. The factors affecting the survivability of organisms in ballast water include, for example, temperature, salinity, oxygen, light, and availability of food [25, 31, 32]. Ballast tanks are unfavourable environments with no light to support photoautotrophs, and the ability of organisms to survive during transport also depends on the length of the voyage which would determine the survivorship of transported organisms [14, 30]. Earlier it was hypothesised that the decline in phytoplankton and zooplankton populations due to unfavourable conditions in the ballast tanks, would influence heterotrophic mode of nutrition, and thus act as incubators for microorganisms [33, 34]. However, the study by Drake et al. [33], presented results that did not support 'incubator

hypothesis', wherein, the ballast water did not serve as an incubator for microorganisms; instead, the microbial abundance decreased during the voyage.

Taking the above points into consideration, experiments were conducted on biofilms developed in the marine (open-ocean) and freshwater environments to understand how darkness and ageing would influence the biofilm communities by mimicking ballast water tank conditions. Since the darkness would prevent the photosynthesis, we hypothesized that these conditions would contribute to the heterotrophic mode of nutrition that might fuel heterotrophic protists. Therefore, such environments could either act as the incubator or may present unfavourable conditions for biofilm communities. The survival of bacteria may depend on the type of bacterial species, the presence of grazers, competition for resources available, regeneration of nutrients via cell death, and lysis of darkness intolerant species.

Materials and methods

Experiment I: Marine biofilms

Before deployment, the glass slides (25.4 mm x 76.2 mm) were cleaned as described by Bhosle et al. [35]. Briefly, the slides were treated with the chromic acid solution for five hours, then washed with UV treated Milli-Q water, followed by methanol, and dried in an oven. They were then covered in aluminium foil and ashed at 450 °C for five hours in the muffle furnace.

The location of the marine station for the development of biofilms is depicted in Fig. 1. The study was carried in an open ocean environment (15°51.482' N, 072°43.511' E) during cruise CRV *Sindhu Sankalp 072* (SSK 072) in November 2014. The ship drifted and was not stationary at one location, but moved from 15°51.482' N, 072°43.511' E up to 15°31.99220' N, 072°19.90430' E during the period of deployment. The temperature in the surface waters varied from 28.82 - 29.1 °C and salinity ranged from 35.1 - 35.4 during the period of deployment. The depth of the water column was 200 m.

Tank and in situ marine biofilms

A schematic representation of the experimental design is depicted in Fig. 2. Briefly, a deck experiment was conducted on board wherein biofilms were developed for a period of five days on clean pre-treated glass slides, which were suspended in two different dark tanks (~ 80 L capacity) with enriched and unenriched conditions. This initial period of biofilm development was used to achieve a homogenous biofilm community with sufficient biomass to carry out the microbiological analysis. For enriched conditions, a CTD cast of surface water with minimal nutrients (nitrate - 0.3 μM) and bottom (150 m) nitrate-rich water (nitrate - 25 μM) was sampled for the experiment. The first tank was filled with 1:1 ratio of surface water with minimal nutrients (nitrate - 0.3 μM) and bottom nitrate rich waters (nitrate - 25 μM), the final concentration of nitrate was $\sim 7 \mu\text{M}$, in which the slides were suspended. These biofilms are termed as enriched (EN) marine biofilms. In the case of unenriched conditions, the second tank was filled with only surface water with minimal nutrients wherein another set of slides were suspended. These biofilms are termed as unenriched (UN) marine biofilms. The water within the tanks was not changed until the end of the experiment. These five-day old marine tank biofilms were harvested for different microbiological analysis as described below.

A set of forty-nine slides were collected for microbiological analysis from each tank. Four slides were sampled and each one was scraped separately into 10 mL of 0.22 μm filtered autoclaved seawater for microbial counts ($n = 1 \text{ slide} \times 4 \text{ replicates}$). 1 mL of subsample was used for the analysis of viable and pathogenic bacterial abundance while 9 mL of subsample was fixed with paraformaldehyde (0.2 % final concentration), quick frozen in liquid nitrogen, for enumeration of total bacterial counts and protists. For bacterial diversity, thirty slides were sampled ($n = 10 \text{ slides} \times 3 \text{ replicates}$), and ten slides were scraped and pooled into 20 mL of 0.22 μm filtered autoclaved seawater. Lastly, fifteen slides were sampled ($n = 5 \text{ slides} \times 3 \text{ replicates}$) for chlorophyll *a* content wherein five slides were scraped and pooled using 20 mL of 0.22 μm filtered autoclaved seawater.

The in situ marine biofilms were also developed by suspending the clean pre-treated glass slides into the sea (subsurface water) of the station and anchored outside to the ship for a period of five days. These five-day old biofilms were evaluated for different microbiological analysis as described above.

Translocation experiment

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After five days of incubation in the dark, these tank biofilms were translocated to four replicate microcosms and exposed to prolonged darkness for a period of 30 days. The microcosm experiments were carried out to assess, whether, the conditions in the ballast tanks would support the incubator hypothesis for the marine biofilm communities or present unfavourable environment. The glass slides were then placed in each of the sterile polycarbonate square jars (75 x 74 x 138 mm, 450 mL, Himedia) containing the same tank water. All the jars were closed and static during the incubations. Replicate biofilm samples for microbial counts (1 slide per microcosm), bacterial diversity (10 slides per microcosm), and chlorophyll *a* content (5 slides per microcosm) were collected on day 30. The ageing experiments with in situ samples could not be achieved as slides were lost due to harsh sea conditions.

Experiment II: Freshwater biofilms

The location of the freshwater station for the development of biofilms is depicted in Fig. 1. It was located in the Kidhirpur Dock area (22°32.45994' N, 088°18.95370' E) across the Hooghly estuary (Kolkata). The surface water temperature was low (21 - 21.5 °C) in the surrounding environment, and salinity ranged from 0.19 - 0.23 during the period of deployment.

Tank and in situ freshwater biofilms

A schematic representation of the experimental design is depicted in Fig. 2. Two different experiments (in situ and laboratory) were performed simultaneously during February 2015 in the Kidhirpur Dock area at Kolkata port. The glass slides were deployed in the freshwater station for the development of biofilms upto five days. These biofilms are termed as in situ freshwater biofilms. Nutrient concentrations were higher in the freshwater, especially, nitrate with 27.35 µmol. A laboratory experiment was also carried out wherein, a tank (~ 80 L capacity) was filled with the same station water and slides were suspended for a period of 5 days. These biofilms are termed as freshwater tank biofilms. This initial period of biofilm development was used to achieve a homogenous biofilm community with sufficient biomass to carry out the microbiological analysis. The tank was concealed to avoid entry of light. The water within the tank was not changed until the

end of the experiment. The slides with five day old freshwater biofilms were sampled from both (tank and in situ) as described above but the biofilms were scraped using 0.85 % autoclaved saline water instead of seawater.

Translocation experiment

After five days incubation, these mature freshwater biofilms were translocated to four replicate microcosms and exposed to prolonged darkness for a period of 30 days. The microcosm experiments were carried out to assess, whether, the conditions in the ballast tanks would support the incubator hypothesis for the freshwater biofilm communities or present unfavourable environment. All the jars were closed and static during the incubations. For each treatment, the slides were sampled from the microcosms as described above, except for the scraped freshwater biofilms, which were suspended in 0.85 % autoclaved saline water instead of seawater.

Environmental physicochemical parameters

Vertical profiles of temperature and salinity were collected using portable Seabird CTD (SBE 19 plus) for Experiment I in the marine environment, whereas a Multiparameter Sonde S5X (Hydro lab) was used for Experiment II in the freshwater environment. Nutrients (nitrate, phosphate, and nitrite) were analyzed by SKALAR SAN^{plus} ANALYSER.

Chlorophyll *a* concentration in biofilms

For chlorophyll *a* estimation, the scraped and pooled biofilm samples were filtered on GF/F 25 mm Whatman filters in dark condition and kept at -20 °C until further analysis. The samples were analyzed within 15 days of sampling date. The filters were extracted using 90 % acetone, and extracts were filtered through 0.22 µm PTFE sterile syringe filter (Millipore). Chlorophyll *a* (Chl *a*) was measured following standard methods [36] and expressed as µg/cm² of the slide surface area sampled.

Total Viable Counts and pathogenic bacteria in biofilms

Total viable counts (TVC) and pathogenic bacteria were carried out in the field laboratory by serially diluting 1 mL of subsample, which was spread plated (0.1 mL) on five different media, namely Zobell Marine Agar (ZMA) 2216 (marine bacteria), Nutrient Agar (NA) (freshwater bacteria), MacConkey Agar (Total coliforms, TC), XLD Agar (*Salmonella/Shigella* spp.), and TCBS Agar (*Vibrio* spp., especially, *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae*). *Vibrio* spp. were distinguished as described by Pfeffer and Oliver [37]. Briefly, the yellowish colonies with > 2mm diameter were counted as *V. alginolyticus*, those with < 2mm diameter were counted as *V. cholerae* and greenish colonies as *V. parahaemolyticus*. Identification of these bacterial species has been confirmed previously by appropriate biochemical tests and verified by protein profiling using MALDI-TOF MS Biotyping (MTB) [38, 39]. ZMA and NA plates were incubated at room temperature, whereas; plates of other three media were kept at 37 °C. The viable abundance is expressed as colony forming units per square centimetre (CFU/cm²) of the slide surface area sampled.

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Total Bacterial Counts in biofilms

For the total bacterial counts, the frozen samples were kept at - 80 °C until further analysis. The samples were analyzed within 15 days of sampling date. Before analysis, the frozen samples were thawed, and 1 mL of subsample was sonicated (30 W for 60 s) following which samples were passed through the BD cell strainer cap (pore size, 40 µm) to remove larger particles. The samples were then stained with SYBR Green I (1:10000 final concentration, Molecular Probes, USA) and incubated in the dark for 15 mins before measurement. After incubation, samples were analyzed by FACS Aria II equipped with the 488 nm blue laser. The emitted light was passed through the following filter sets 488/10 band pass (BP) for right angle light scatter (SSC) and 530/30 band pass (BP) for green fluorescence. The calibration of the above parameters was done by using fluorescent beads (1 µm, Polysciences, USA). Gating was done against SSC versus green fluorescence. Flow cytometry data were processed using BD FACS Diva software. The total bacterial counts (TBC) is expressed as cells/cm² of the slide surface area sampled.

Protist abundance enumeration

Protists were analyzed using a modified protocol by Christaki et al. [40]. Before analysis, the frozen samples were thawed, and stained with SYBR Green I (1:10000 final concentration, Molecular Probes, USA), and incubated in the dark for 15 mins before measurement. After incubation, samples were analyzed by a flow cytometer (FACS Aria II) equipped with the blue (488 nm) laser. The emitted light was passed through the following filter sets: 488/10 band pass (BP) for right angle light scatter (SSC), 530/30 band pass (BP) for green fluorescence and 695/40 for red fluorescence. The fluorescence voltage of the detector was reduced with reference to SSC vs. green fluorescence so that bacteria are below the detection threshold. Gating was done against green versus red fluorescence. Data obtained were processed with the BD FACS Diva software. The protist abundance is expressed as cells/cm² of the slide surface area sampled.

Standardization of protocol for DNA extraction from biofilms

The scraped and pooled biofilm samples for bacterial diversity were filtered using autoclaved 0.22 µm filter papers (Millipore) and transferred to 70 % ethanol and further stored at - 20 °C until analysis. The samples were extracted and analyzed within 15 days of sampling date. DNA was extracted using a MO BIO PowerBiofilm™ DNA extraction kit according to the manufacturer's instructions. A modified manual method by Miller et al. [41]; was also used for comparison. The latter method combines a bead-beating methodology with chloroform-isoamyl alcohol extraction, followed by precipitation of the extracted DNA with isopropanol. Both the methods showed comparable results (data not shown). Thus, the kit method was adopted.

Quantification of group specific bacteria in biofilms using quantitative PCR

The extracted DNA was subjected to an absolute quantitative polymerase chain reaction (qPCR) method used to characterize the bacterial communities from the biofilms. The major bacterial groups, including α -proteobacteria, β -proteobacteria, γ -proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes in different samples were quantified by real-time PCR. The chosen primers are shown in Table 1 according to previously established real-time PCR protocols [42], and corresponding

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annealing temperatures were standardized in the laboratory. The qPCR assays were designed to measure group specific bacteria using Rotor-Gene Q (Qiagen). Standard curves were obtained by using bacterial standards containing the 16S rRNA gene PCR products of each target group. These PCR products were serially diluted 10-fold, ranging from 10^6 to 10^9 copies.

One μL of DNA from the biofilm samples ($\sim 1.0 - 5.0$ ng of environmental DNA) was amplified via qPCR with group-specific primer pairs as described by De Gregoris et al. [42]. The Power SYBR® Green PCR Master Mix (Applied Biosystems) was used for different qPCR reactions with group-specific PCR primers. All the qPCR reactions were performed in triplicates contained in the total volume of $20 \mu\text{L}$, $10 \mu\text{L}$ of master mix, $0.25 \mu\text{M}$ of each primer, $1 \mu\text{L}$ of DNA template and made up with nuclease free water (Ambion™). For group-specific primers, an initial enzyme activation step at 95°C for 5 min was followed by 45 cycles of denaturation at 95°C for 15s, primer annealing at 53°C for 20s and primer extension at 72°C for 20s. The melting curve analysis of the products was carried out for each qPCR reaction, to determine the specificity of amplification and confirm that the fluorescence signals are originated from specific PCR products and not from primer dimers or other artifacts. Data were further processed using Rotor-Gene software. Resulting values (Raw value, DNA copies/ μL) were \log_{10} transformed. PCR efficiency (E) and the Threshold cycle (C_T) values were determined using the software. Data were expressed as the gene copy numbers/ cm^2 of the slide surface area sampled.

Statistical analysis

The experimental data were $\log(x+1)$ transformed. Statistical analyses were preceded by checking for normality and homogeneity of variances using the Shapiro-Wilk's W test. This analysis was carried out using the Statistica 6 program (Stat Soft Inc., Tulsa, OK, USA). The datasets fulfilling the assumptions for parametric analysis were analyzed using two-way analysis of variance (ANOVA) with the sampling days (5 and 30) and enrichment (enriched and unenriched) as independent factors, to determine the variations in the communities of marine biofilms (microbial cell counts, viable and pathogenic bacteria, bacterial diversity, and chlorophyll *a* concentration), followed by post hoc Tukey's HSD test (Bonferroni corrected). Whereas, in the case of freshwater biofilms, the sampling days (5 and 30) and the type of biofilm (in situ and tank) were taken as independent factors. This statistical analysis was performed by using the SPSS statistical software program (Version 16). The relationship between the environmental variables, nutrients, and

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pathogenic bacteria as well as bacterial diversity in the in situ and laboratory experiments were determined by using CANOCO version 4.5 [43]. The Detrended Correspondence Analysis (DCA) was performed to determine variability in the data set. The length of the first gradient axis was < 2.0 indicating the linear variation in the data [44]. Due to the linear character of the data, RDA (Redundancy Analysis) was conducted to estimate how environmental parameters influenced the biological parameters. A forward selection was achieved on the set of environmental variables, and statistical significance of each variable was tested using Monte Carlo permutation test under the reduced model (999 permutations). The length of the arrows (environmental variables) and their orientation indicates their relative importance and approximate correlations to the axes [45]. Further, correlation analysis was performed between abiotic (temperature, salinity, and dissolved nutrients) and bacterial components using Statistica 6.0 statistical package at a significance level of ≤ 0.05 (Stat Soft, OK, USA).

Results

Experiment I

Marine tank biofilms

Chl *a* was comparatively low in the 5-day old EN ($0.00254 \pm 0.0001 \mu\text{g}/\text{cm}^2$) and UN marine tank biofilms ($0.00213 \pm 0.0001 \mu\text{g}/\text{cm}^2$). After prolonged darkness, Chl *a* decreased drastically on day 30 to a minimum of 0.0015 ± 0.0005 (UN, $p < 0.001$, Tukey's HSD) - 0.0024 ± 0.0002 (EN) $\mu\text{g}/\text{cm}^2$.

The TBC (2.81×10^6 cells/ cm^2) and protists (1.25×10^5 cells/ cm^2) increased significantly in the EN biofilms on day 30 ($p < 0.001$, Tukey's HSD, Fig. 3a-b) during which nitrate levels decreased drastically (data not shown). The sampling days and enrichment factor had a significant effect on the viable and pathogenic bacterial abundance (two-way ANOVA, $p < 0.05$). The TVC abundance was significantly more in the EN (1.04×10^4 CFU/ cm^2) and least in the UN biofilms (1.68×10^3 CFU/ cm^2) ($p < 0.001$, Tukey's HSD, Fig. 3c). Upon prolonged darkness, the viability dropped significantly upto 10 fold in both the marine tank biofilms (EN - 1.23×10^3 CFU/ cm^2 and UN - 5.05×10^2 CFU/ cm^2) ($p < 0.001$, Tukey's HSD). In RDA plot, Axis 1 separated freshwater from the marine biofilms (Fig. 4). According to RDA analysis, the first two axis accounted for 75.6% of the variability. TVC was

influenced by higher temperature (27.1 - 27.9 °C) and significantly correlated with salinity in the marine biofilms, while TBC was significantly correlated with nitrite ($p < 0.05$).

Vibrio alginolyticus were abundant in EN biofilms (2.13×10^2 CFU/cm²) followed by *V. parahaemolyticus* (1.22×10^2 CFU/cm²) which is indicated in the RDA plot (Fig. 4). However, *V. cholerae* were not detected in the marine biofilms on day 5 and 30 as well. Both the *V. alginolyticus* and *V. parahaemolyticus* abundance decreased significantly in the EN biofilms when exposed to darkness ($p < 0.001$, Tukey's HSD, Fig. 3e).

Differences in the bacterial community composition in terms of gene copy numbers per square centimeter in the marine tank biofilms are depicted in the Fig. 5. The gene copy numbers of α -proteobacteria were high in both the EN and UN biofilms. This group was stable over a period of time upon the prolonged darkness (Fig. 5). The sampling days and enrichment had a significant effect on bacterial community composition (two-way ANOVA, $p < 0.001$). β -proteobacteria and Actinobacteria were significantly different among the sampling days (two-way ANOVA, $p < 0.05$), while enrichment had a significant effect on α -proteobacteria, Actinobacteria, and Firmicutes (two-way ANOVA, $p < 0.05$).

In situ marine biofilms

Chl *a* concentration was 0.0101 ± 0.009 $\mu\text{g}/\text{cm}^2$ in the in situ marine biofilms. The viable and pathogenic bacterial abundance (i.e., TVC, TC, *Vibrio* spp.) was low except for TBC and protists, which were higher in the in situ marine biofilms. The bacterial community was dominated by γ -proteobacteria followed by α -proteobacteria, while Bacteroidetes, Firmicutes, and Actinobacteria contributed the least (Fig. 5).

Experiment II

In situ freshwater biofilms

Chl *a* was 0.351 ± 0.001 $\mu\text{g}/\text{cm}^2$ in the in situ freshwater biofilms. It was significantly influenced by phosphate ($p < 0.05$) and nitrite as shown in the RDA plot (Fig. 4). However, upon prolonged darkness, the Chl *a* concentration decreased significantly with a minimum value of 0.033 ± 0.005 $\mu\text{g}/\text{cm}^2$ ($p < 0.001$, Tukey's HSD), even though nutrients such as nitrate (27.35 μM) and phosphate (18.76 μM) were higher in the water column.

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The TBC and protists declined significantly upon exposure to dark conditions on day 30 in the in situ biofilms ($p < 0.05$, Tukey's HSD, Fig. 6a-b). Similarly, the TVC abundance which was more in the in situ biofilms on day 5 (2.69×10^3 CFU/cm²), also decreased significantly on exposure to prolonged darkness ($p < 0.001$, Tukey's HSD, Fig. 6c). This was evident from an increase in the nitrate levels (64.03 μ M) observed at the end of the dark experimental period.

Freshwater biofilms were mainly dominated by TC (Fig. 6d). They were more in the in situ biofilms on day 5 (2.05×10^3 CFU/cm²) and reduced drastically upon prolonged darkness ($p < 0.001$, Tukey's HSD, Fig. 6d). TC were strongly influenced by phosphate ($p < 0.05$) and nitrite (Fig. 4). *Vibrio* spp., especially *V. alginolyticus* were dominant (2.21×10^2 CFU/cm²) followed by *V. cholerae* (7.6×10^1 CFU/cm²) and *V. parahaemolyticus* (6.0 CFU/cm²) in the freshwater biofilms on day 5 (Fig. 6e). It was observed that these pathogenic bacteria were not able to persist upon prolonged darkness on day 30. The *V. alginolyticus* and *V. parahaemolyticus* abundance did not show any significant correlation with any of the parameters, whereas *V. cholerae* were significantly influenced by phosphate ($p < 0.05$) and nitrite (Fig. 4) in the in situ biofilms.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the freshwater biofilms are depicted in Fig. 7. The bacterial community composition was significantly different with respect to the sampling days and type of biofilms (two-way ANOVA, $p < 0.001$). γ -proteobacteria and Bacteroidetes were significantly influenced by the sampling days (two-way ANOVA, $p < 0.05$). γ -proteobacteria were abundant in the in situ freshwater biofilms with high gene copy numbers on day 5 and were significantly correlated with nitrite ($p < 0.05$). However, this group decreased drastically on day 30 upon prolonged darkness ($p < 0.001$, Tukey's HSD, Fig. 7). A similar trend was shown by Bacteroidetes, wherein, it decreased significantly upon prolonged darkness on day 30 ($p < 0.001$, Tukey's HSD, Fig. 7). Bacteroidetes were influenced by phosphate and nitrite. β -proteobacteria was the dominant taxonomic group in the in situ freshwater biofilms and significantly correlated with nitrate concentration ($p < 0.05$) (Fig. 4).

Freshwater tank biofilms

The microbial parameters (TBC, TVC, and TC) and the chlorophyll *a* was low in the freshwater tank biofilms (Fig. 6). *Vibrio* spp. were not detected in the tank biofilms. Upon prolonged dark conditions, all the microbial parameters except for protists and TC resulted in 10 fold reduction in their abundance ($p < 0.05$, Tukey's HSD, Fig. 6). An increase in the nitrate levels (49.86 μ M) was observed

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at the end of the dark experimental period. Tank biofilms showed a different pattern of bacterial communities in comparison to the in situ freshwater biofilms (Fig. 7). However, no significant differences in bacterial biofilm community composition were observed in the case of freshwater tank biofilms upon prolonged darkness.

Discussion

Ballast water is known as a major vector for the invasion of alien marine and freshwater species and has found recognition through the International Maritime Organization (IMO) [12]. Ballast-tank biofilms signify an additional risk of microbial invasion, yet their fate and invasion biology is poorly understood. The present study was carried out at pilot scale simulating ship's ballast tank conditions in the laboratory. The most scrutinized bacteria in the invasive studies of ballast water have been pathogens like *V. cholerae* and indicator organisms like *Escherichia coli*, but one of the specific aims of this study was to look, not only at pathogenic bacteria, but to examine the changes in the biofilm bacterial community composition when exposed to prolonged darkness and ageing in order to mimic ballast water conditions in the laboratory.

A significant decrease in the chlorophyll *a* was observed in the case of unenriched marine biofilms, which could be attributed to the darkness that inhibited photosynthesis, leaving only heterotrophic mode of nutrition. This was evident from the increased TBC and protist abundance upon prolonged dark conditions. Thus, the results on marine biofilms supported the incubator hypothesis, which states that the ballast water tanks act as incubators for microorganisms [33]. The dark conditions result in the death of the phytoplankton thus influencing zooplankton, which feed on them leading to their mortality. Subsequently, they are colonized and decomposed by ambient bacteria, which further fuel bacterial production [33, 46]. It is also reported earlier that a large number of bacteria emerge from the decomposing zooplankton contributing to an increased bacterial production [18]. A study by Tomaru et al. [17] suggested that this detritus also fuels heterotrophic protists. Since the results are in agreement with the proposed incubation hypothesis, it is expected that bacterial community in the biofilms would change over a period of time.

In the present study, bacterial diversity in the biofilms was characterized by quantitative polymerase chain reaction (qPCR), increasingly used in microbial ecology to determine the abundance of target genes by absolute standard curve [42]. This technology merges the PCR chemistry with the use of fluorescent molecules (e.g., SYBR Green) to monitor the amplified

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products during each cycle of the PCR reaction [47]. In the current study, the composition of the biofilm bacterial community changed significantly over a period of time. Tomaru et al. [17] used DGGE for bacterial community profiling and findings indicated a shift in the bacterial community composition. Bacterial community distribution differs in different environmental conditions that exist in the fresh, marine, pelagic, and benthic waters [48, 49].

A clear difference in marine biofilm bacterial community composition was observed between in situ and tank biofilms during the study period. The marine biofilms were represented by abundant γ -proteobacteria, followed by α -proteobacteria, while Bacteroidetes, Firmicutes, and Actinobacteria contributed the least. The α -proteobacteria and γ -proteobacteria are known to be ubiquitous groups in both freshwater and marine ecosystems [49-51]. In the present study, α -proteobacteria was the most stable taxonomic group in the marine biofilms over a period of time under the dark conditions. They are a cosmopolitan group of gram-negative bacteria found in both freshwater and marine ecosystems [49, 50]. This group possesses different metabolic strategies such as photosynthesis, nitrogen fixation, ammonia oxidation, etc. [52, 53]. An earlier study reported that the family Rhodospirillaceae, belonging to α -proteobacteria, were able to survive in the ballast tank, making this a common component of ballast water bacteria, resulting in multiple introductions and potential bioinvasions [51].

In the case of freshwater biofilms, chlorophyll *a* decreased significantly upon prolonged dark conditions. An increase in the levels of nitrate was observed at the end of the dark experimental period, which could be attributed to cell lysis and disintegration. The released cellular matter can be broken down further into dissolved organic matter (DOM), which can support heterotrophic bacteria in the dark period [14]. However, the number of TBC and protists decreased drastically after exposure to darkness when compared to marine biofilms. Our investigation did not support the incubator hypothesis for freshwater biofilms but was in agreement with the general rule that the abundance and species diversity decreases with the ageing upon prolonged darkness conditions. The decline in the numbers could be attributed to the presence of grazers (personal observation), that could have played an important role in controlling the bacterial and protist abundance in the freshwater biofilms. Major predators of protozoa, namely amoeba, flagellates, and ciliates, are reported to be present in freshwater biofilms [54].

A distinct variability in biofilm bacterial community composition was observed between in situ and tank freshwater biofilms. β -proteobacteria and Firmicutes were dominant taxa in the in situ

freshwater biofilms. Earlier studies reported that β -proteobacteria is the most diverse group within biofilms and attach more easily to surfaces during the initial stages of biofilm development when compared to members of other bacterial groups [55, 56]. They are dominant in freshwater ecosystems, known for their versatile, degrading capabilities, whereas they are relatively low in the ocean [49]. This group is also found in the marine biofilms, but occur at lower levels compared to other Proteobacteria [57, 58]. In the present study, β -proteobacteria abundance correlated with high nitrate concentration in the in situ freshwater biofilms when exposed to prolonged darkness. They are known to play an important role in nitrogen fixation [59] and derive nutrients from the decomposition of organic matter. An interaction between this bacterial class and nitrate, showing a positive relationship has been reported earlier [60, 61]. Studies have also indicated that this class showed rapid growth rates in the presence of increased nitrogen and phosphate concentrations as well as low predation [62].

γ -proteobacteria, which includes most of the pathogenic genera, were dominant in both the marine and freshwater biofilms but decreased under dark conditions. They are known to be predominant in most of the ecosystems mainly saltwater environments, such as saline lakes or oceans [63-65] than freshwater. Among *Vibrio* spp., primarily, *V. alginolyticus* and *V. parahaemolyticus*, belonging to γ -proteobacteria, were more prevalent in the enriched marine biofilms but decreased significantly when exposed to dark conditions. Moreover, it is said that microbes can survive hostile environments in an inactive state until they reach a suitable habitat with favourable conditions for growth [66].

V. cholerae and total coliforms were more abundant in the freshwater biofilms. Earlier studies have reported that river biofilms acts as a reservoir for total coliforms and can represent the health risk when these are resuspended from biofilms to water [67]. Total coliforms and *V. cholerae* decreased drastically upon prolonged darkness. The possible reason could be that under stress conditions, many species of bacteria move into a starvation mode of physiologically viable but non-culturable (VBNC) state. It is a unique survival strategy acquired by many bacteria especially *V. cholerae*, in response to harsh environmental conditions [68]. Previous studies have reported that several human pathogenic bacteria enter into the VBNC state and cannot be recovered using culture media, although they retain their viability and virulence [68-70].

Our investigation revealed the prevalence of different bacterial groups in the biofilms (marine/freshwater) that were able to thrive in the dark environments. Marine biofilm communities

were able to adapt better to the dark conditions and were mainly dominated by α -proteobacteria. However, freshwater biofilm communities collapsed, except for β -proteobacteria, which seemed to play an important role in organic matter remineralization. The resulting prevalence of bacterial taxa in the respective biofilms could also be attributed to the smaller influence of grazers on them compared to others. Although grazers are known to feed on bacteria, the experimental studies have shown species-specific prey preferences by different protistan grazers [71-74]. Even though the selective feeding of grazers is an important mechanism for the shaping of bacterial community composition and diversity, the bacterial cell size is also considered as a major feature that influences susceptibility towards different grazers [72, 75]. Thus, increasing the survival of several bacterial communities under grazing pressure. This study revealed that prolonged dark conditions serve as an incubator for marine biofilm communities, whereas the dark conditions did not act the same for freshwater biofilm communities. Therefore, the adaptation of tolerant bacterial communities, regeneration of nutrients via cell lysis, and the presence of grazers appeared to be key factors for survival upon prolonged darkness.

It was interesting to note that the freshwater and marine biofilms seemed to behave very differently when exposed to ageing and darkness, but this may not be true for all the freshwater and marine biofilms. The biofilm bacterial community composition is niche specific and influenced by interactions with the surrounding environment [76, 77]. The bacterial community composition of marine and freshwater biofilms were distinctly different. These results were in agreement with the Baas-Becking hypothesis for microbial taxa which states that ‘everything is everywhere, but the environment selects.’ The composition of naturally occurring bacterial communities exhibits a marked biogeography [78, 79], with distinct freshwater, coastal, and oceanic populations, which may further alter the community composition of the recipient community. Since the marine biofilms were able to survive better in the dark conditions, it can be proposed that the introduced populations will either succeed or fail to survive depending on the characteristics of the recipient bacterial community. In contrast, the collapsed freshwater biofilms would fail to establish. The ecological impact of ballast biofilm bacteria on the recipient communities may also depend on the number of bacteria introduced, the composition of the bacterial assemblages, and recipient environmental conditions.

Microbial invasions occur worldwide and are much harder to notice than invasions by macroorganisms [80]. All species transported in ballast tank biofilms may not be invasive nor will all survive the journey. Earlier it was reported that non-pathogenic microbes could cause shifts in the community composition and alter various aspects of ecosystem functioning [80]. The effective risk

assessment relies on identifying important factors, which drive their successful invasion or proliferation. The factors such as the number of organisms discharged (propagule pressure), recipient environmental conditions or resident biota may play a major role as they can govern their ability to colonize and invade in the new environment. Further studies should focus on the fate of biofilm communities upon discharge in the new environment, and their invasion potential is an important topic for future investigations.

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Legends to Figures

Fig. 1 Sampling site

Fig. 2 Schematic representation of experiment I and II. TBC= Total bacterial counts; TVC= Total viable counts; TC= Total coliforms

Fig. 3 Abundance of (a) total bacteria counts, (b) protists, (c) total viable counts, (d) total coliforms, and (e) *Vibrio* spp. (*V. alginolyticus* and *V. parahaemolyticus*) in the in situ and tank (enriched and unenriched) marine biofilms during the sampling days (day 5 and day 30). The solid bars represent averaged data ($n = 4$) of the samples. Error bars indicate the standard deviation of the mean based on four replicate samples. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the enriched and unenriched marine biofilms (two-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). Bars labelled by the same letter on the graph are not significantly different from each other. The asterisk (*) above bars indicate significant differences between enriched and unenriched marine biofilms

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Fig. 4 RDA analysis of marine (●) and freshwater (▲) biofilms during the study period. MD5 In situ= 5-day old in situ marine biofilms; MD5 UN= 5-day old unenriched marine biofilms; MD5 EN= 5-day old enriched marine biofilms; MD30 UN= 30-day old unenriched marine biofilms; MD30 EN= 30-day old enriched marine biofilms; FD5 In situ= 5-day old in situ freshwater biofilms; FD5 T= 5-day old freshwater tank biofilms; FD30 In situ= 30-day old in situ freshwater biofilms; FD30 T= 30-day old freshwater tank biofilms; Chl *a*= Chlorophyll *a*; TBC= Total bacterial counts; TVC= Total viable counts; TC= Total coliforms; VA= *Vibrio alginolyticus*; VP= *V. parahaemolyticus*; VC= *V. cholerae*; S= *Shigella* spp., Alpha= α -proteobacteria; Beta= β -proteobacteria; Gamma= γ -proteobacteria. All data points are averages of several replicates

Fig. 5 Gene copy numbers of α -proteobacteria (dark blue), β -proteobacteria (dark red), γ -proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank (enriched and unenriched) marine biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples \pm SE. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the enriched marine biofilms (two-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). Bars labelled by the same letter on the graph are not significantly different from each other

Fig. 6 Abundance of (a) total bacterial counts, (b) protists, (c) total viable counts, (d) total coliforms, and (e) *Vibrio* spp. (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*) in the in situ and tank freshwater biofilms during the sampling days (day 5 and day 30). The solid bars represent averaged data ($n = 4$) of the samples. Error bars indicate the standard deviation of the mean based on four replicate samples. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the in situ and tank freshwater biofilms (two-way ANOVA, $p <$

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0.05, followed by Tukey's HSD post hoc test). The asterisk (*) above bars indicate significant differences between in situ and tank freshwater biofilms

Fig. 7 Gene copy numbers of α -proteobacteria (dark blue), β -proteobacteria (dark red), γ -proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank freshwater biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples \pm SE. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the in situ freshwater biofilms (two-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). Bars labelled by the same letter on the graph are not significantly different from each other

Legend to Table

Table 1 Primers for real-time PCR



Fig. 1

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Fig.2

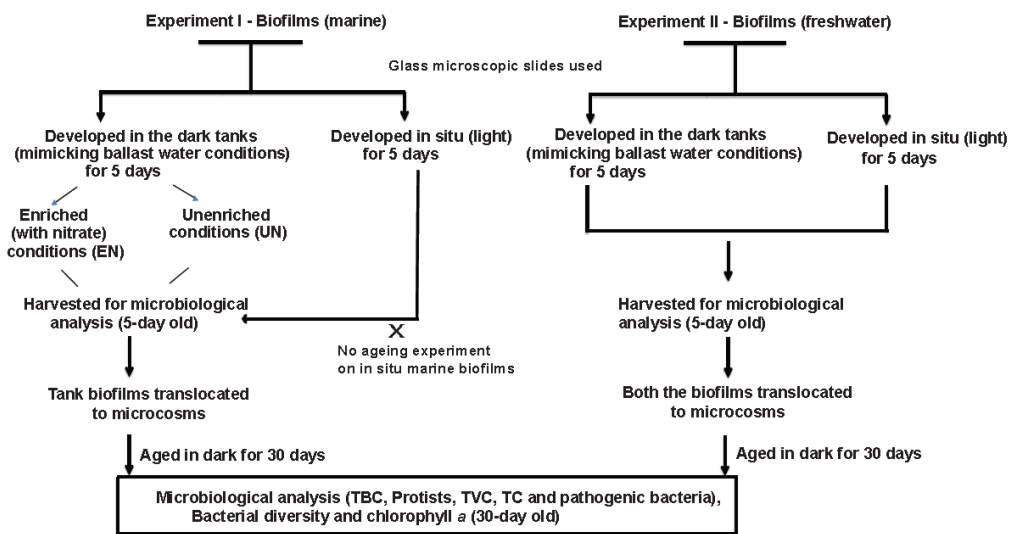


Fig. 2

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Marine biofilms

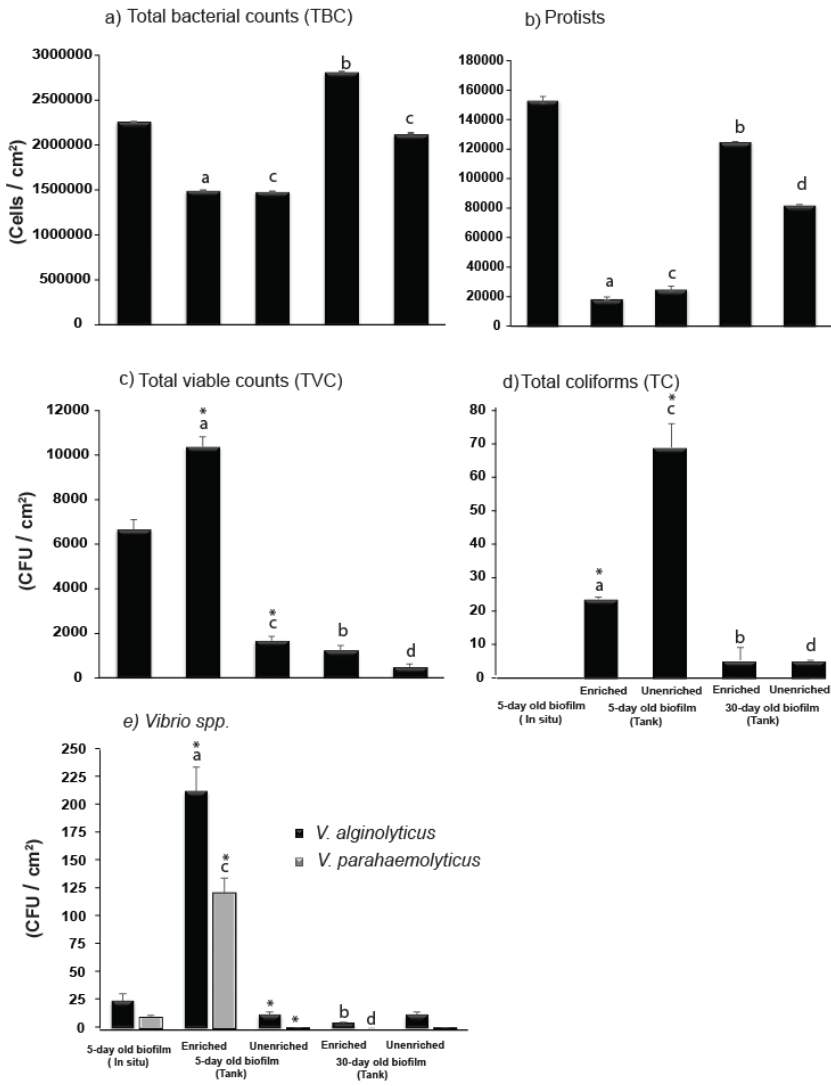


Fig. 3

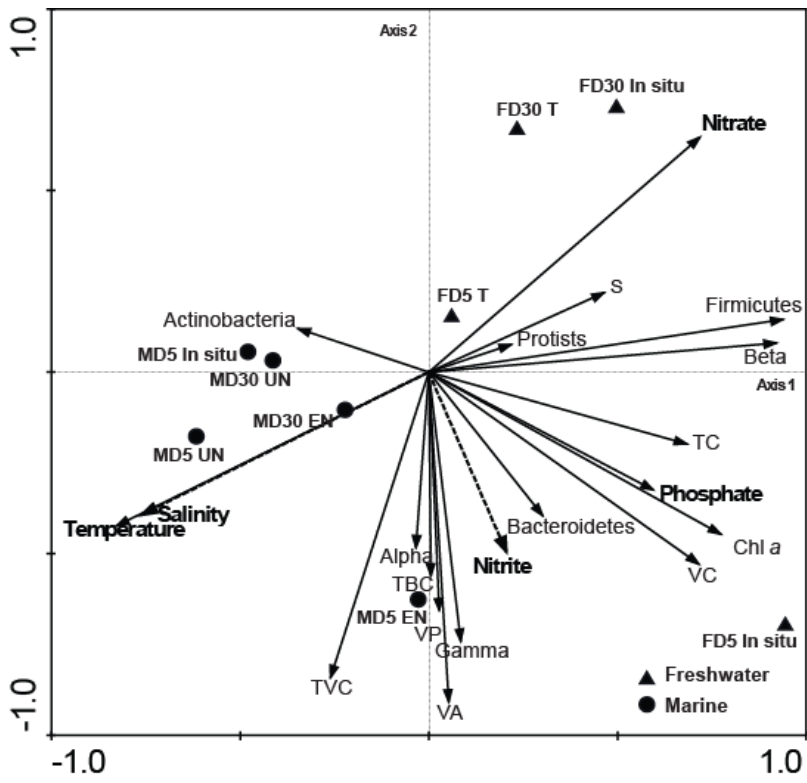


Fig. 4

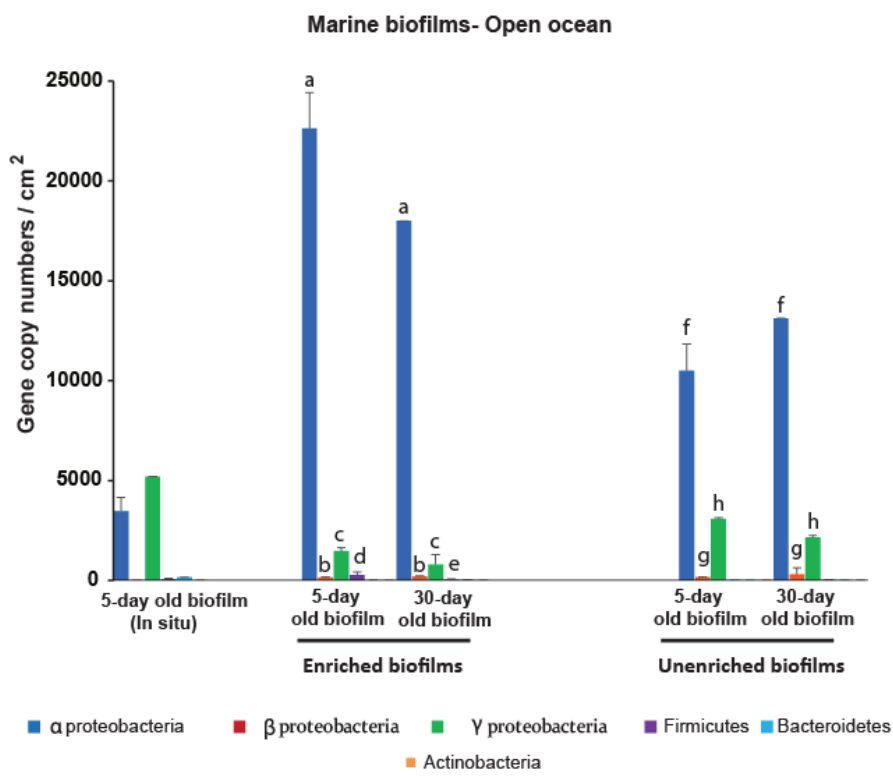


Fig. 5

Freshwater biofilms

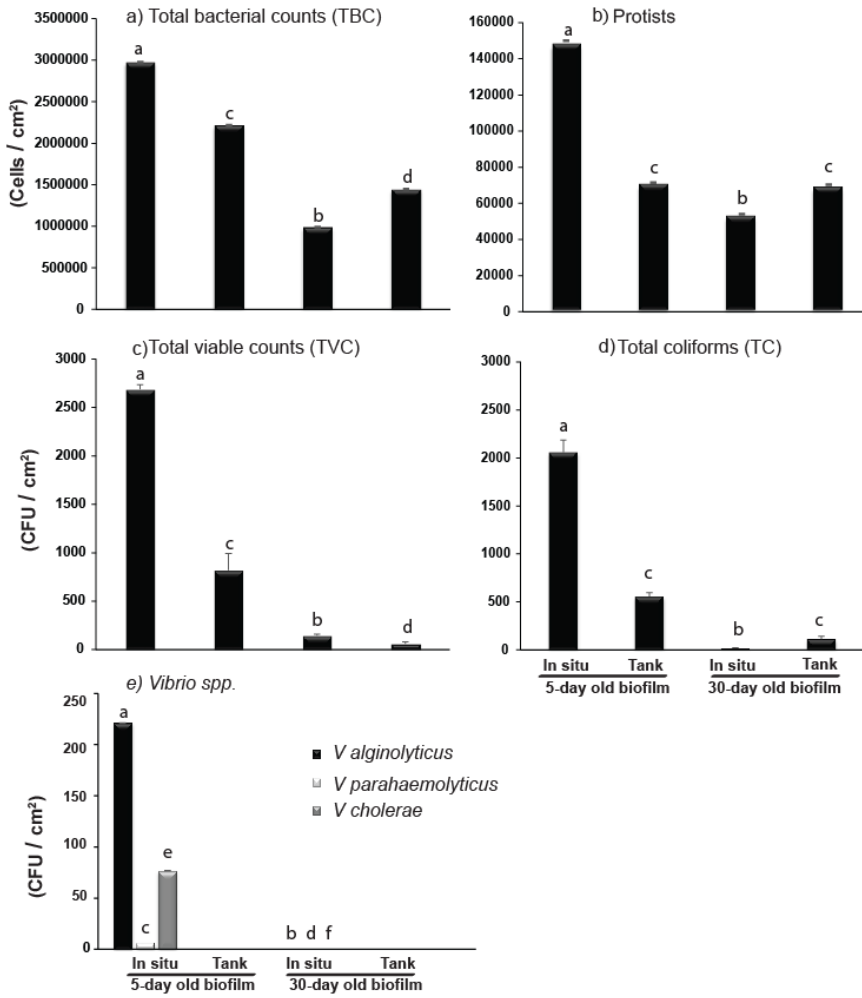


Fig. 6

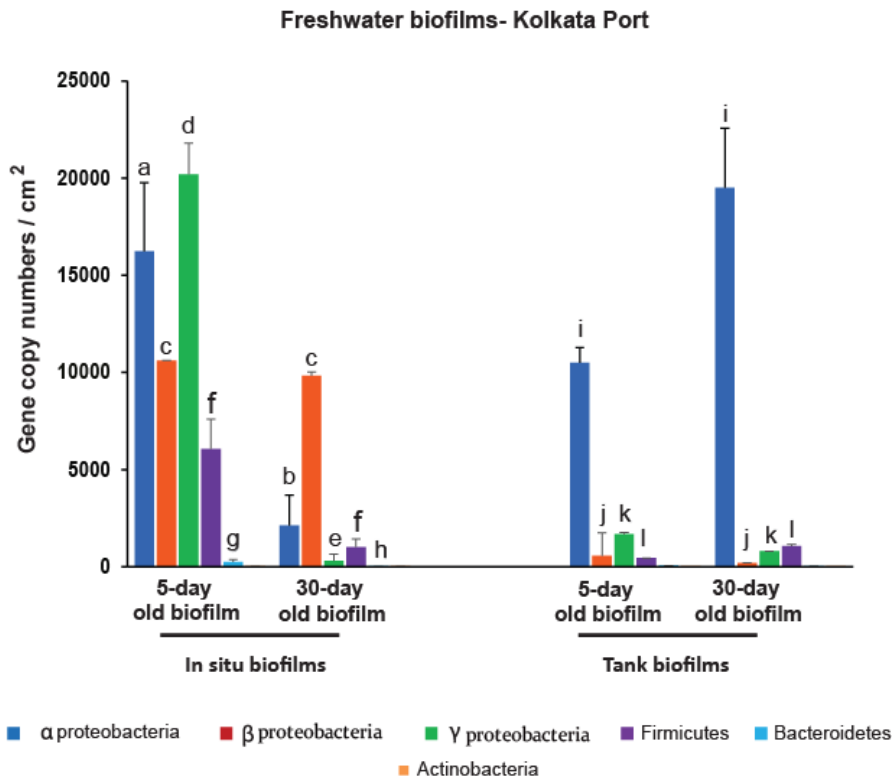


Fig. 7

Table 1 Primers for real time PCR

Target group	Annealing Temp.(°C)	Primer name and sequence (5'-3' direction)	Reference
α -proteobacteria	53	682F: CIAGTGTAGAGGTGAAATT 908R: CCCCGTCAATTCCTTTGAGTT	(39)
β -proteobacteria	53	Beta359f: GGGGAATTTGGACAATGGG Beta682r: ACGCATTCTACTGCTACACG	(79)
γ -proteobacteria	53	1080F: TCGTCAGCTCGTGYGTGA 1202R: CGTAAGGGCCATGATG	(39)
Bacteroidetes	53	798cfbF: CRAACAGGATTAGATACCCT cfb967R: GGTAAGGTTCTCGCGTAT	(39)
Firmicutes	53	928F: TGAAACTYAAAGGAATTGACG 1040FirmR: ACCATGCACCACCTGTC	(39)
Actinobacteria	53	Act920F3: TACGGCCGCAAGGCTA Act1200R: TCRTCCCCACCTTCCTCCG	(39)

