

Ecological impacts of aged freshwater biofilms on estuarine microbial communities elucidated through microcosm experiments: a microbial invasion perspective

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Abstract

Inadvertent introductions of alien species via biofilms as a vector released through ballast water are of environmental importance, yet their consequences are not much known. In the present study, biofilm communities developed in an inland freshwater port under in situ and dark conditions were subjected to long-term dark incubations. Subsequently, the impact of these aged biofilms as vectors on estuarine water column communities were evaluated using microcosm experiments in the laboratory. Variations in biofilm and planktonic microbial communities were quantified using quantitative PCR.

Upon prolonged dark incubation, a shift in bacterial diversity with an increase in tolerant bacterial communities better adapted to stress was observed. Actinobacteria were the dominant taxa in both aged biofilms upon dark incubations. The laboratory studies indicated that on exposure of these biofilms to estuarine water, resuscitation of *Vibrio alginolyticus*, *V parahaemolyticus*, and *V cholerae* from a dormant state existing in these biofilms to culturable form was observed. Moreover, the results revealed that both the biofilm types can pose a threat to the environment, but the degree of risk can be attributed to the imbalance caused by significant changes in the surrounding estuarine microbial communities. Consequently, this may result in either proliferation or decline of some genera with different metabolic potential and resuscitation of pathogenic forms not present earlier, thereby influencing the ecology of the environment. Quantifying these effects in the field using biofilm metagenomes with an emphasis on virulent species and understanding traits that enable them to adapt to changing environments is a way forward.

Keywords Freshwater biofilms; Bacterial diversity; Estuaries; Environmental impact; Microbial invasion; Microcosms .

Introduction

One of the major consequences of globalization is the transportation of microorganisms through shipping. The successful introduction and establishment of novel microorganisms into ecosystems is termed as microbial invasion [1-2]. Over the past many decades, a diverse range of freshwater, estuarine, and marine organisms have been translocated from their native environments through ballast water (BW) discharge [3]. This ballast water which contains a gamut of microorganisms, is used to weigh down empty ships and then discharged while reloading the cargo. BW is a major mechanism known for introducing new aquatic organisms (including pathogenic ones), thereby causing a significant threat to aquatic biodiversity and the ecology of the environment worldwide [2-4].

Other than ballast water, microorganisms can also be transferred in either an attached biofilm state or as detached clusters of bacteria [5-7]. These biofilms act as seed banks comprising rich microbial communities harbouring pathogenic bacteria and coliforms [8-10]. The biofilm communities on the interior walls of ballast tanks have been implicated in the transport of microorganisms and pathogens [11]. The age and composition of these biofilm microbial communities in the interior ballast tanks will differ depending on the voyage duration, which can range from a few weeks to about 100 days or more. However, the consequences of these biofilms when released in the new environment are little explored. Their release through the ship's ballast water can cause or is likely to cause harm to the ecology of the environment, economy (e.g., fouling of water supply systems) and human health indirectly by affecting the fisheries, which is an additional concern.

The present investigation is a case study aimed at elucidating the microbial dynamics of freshwater biofilm microbial communities developed under in situ/dark conditions, which were further subjected to long-term dark incubations. These aged biofilms were then translocated to the microcosms containing estuarine water in the laboratory. Subsequently, their impact on the estuarine water column microbial communities was assessed. The rationale for considering the estuarine environment for translocation studies is based on reports that many of the shipping ports are located in river mouths and estuaries characterized by broad temporal salinity changes [12-13]. These estuaries are susceptible to anthropogenic disturbances by the introduction of alien species via major vectors and can pose a significant threat [14]. The introduced biofilm species can thus potentially influence the ecology and composition of estuarine microbial communities, but to our knowledge, no previous research has specifically addressed this topic. It was anticipated that the biofilm bacterial community pattern in freshwater biofilms developed under in situ/dark conditions would differ with ageing, eliminating less tolerant communities and leaving behind the most adaptable ones. Subsequently, we hypothesized that translocating these aged biofilm communities into estuarine water would cause modulation of estuarine

water column characteristics, resulting in alterations in the community structure and introduction of new taxa that could be beneficial or harmful to the environment.

Materials and Methods

Experimental design and sampling

The whole investigation was divided into two parts, as given below:

Freshwater biofilm communities subjected to long-term dark incubations

Two different experiments (in situ and laboratory) were conducted on freshwater biofilms developed in the freshwater station located in the Kidderpore Dock area (22°32.45994' N, 088°18.95370' E) across the Hooghly River in West Bengal, India. A schematic illustration of the experimental workflow is depicted in Fig. 1. Biofilms were initially developed on standard glass slides (25.4 mm x 76.2 mm) by deploying them in the station (subsurface layer) for five days during February 2015. 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 dark freshwater biofilms. This initial period of biofilm development was used to attain a heterogeneous biofilm community with sufficient biomass [15].

Since the voyage duration differs and lasts for 100 days or more, the 5-day old freshwater biofilms were exposed to 100 days' dark incubation to evaluate the influence of long-term dark incubation on these biofilm microbial communities. For this, the 5-day old freshwater biofilms were transferred to polycarbonate rectangular cage microcosms (43 x 27 x 18 cm, Tarsons, 15L) containing the same field and tank water, which were incubated and aged in the dark for 100 days. These microcosms were static during incubations and covered with a thick black cover to avoid the entry of light. After incubation, the 100-day old in situ and dark freshwater biofilms were evaluated for different microbiological parameters as described below.

A set of seventeen slides in duplicates ($n = 17 \times 2$) were randomly chosen and collected for microbiological analyses. Out of seventeen slides, two slides ($n = 2 \times 2$) were sampled for microbial counts, and each slide was scraped separately using a sterile cell scraper (BD Biosciences) into 10 ml of 0.22 μm autoclaved filtered seawater. Subsequently, 1 ml of the subsample was used for the analysis of total viable count (TVC) and pathogenic bacterial abundance, while the remaining 9 ml was fixed with paraformaldehyde (0.2 % final concentration), quick-frozen in liquid nitrogen, for enumeration of total bacterial count (TBC) and protist abundance. For biofilm bacterial diversity, a set of ten individual slides ($n = 10 \times 2$) were scraped and subsequently pooled using 20 ml of 0.22 μm filtered

autoclaved seawater. Lastly, five slides ($n = 5 \times 2$) were scraped using 20 ml of 0.22 μm filtered autoclaved seawater for chlorophyll *a* content analysis.

Laboratory-based microcosm experiments

The microcosm experiments were carried out in the laboratory under controlled conditions. These experiments aimed to evaluate the microbial dynamics of the aged biofilms (100-day old) and their impact when released into the estuarine water. For this, the 100-day old aged in situ and dark freshwater biofilms were subjected to microcosms containing the freshly collected estuarine water (EW) from the Dona Paula Bay situated at the mouth of the Zuari estuary, Goa, India (salinity ~ 35) in May 2015. The microbiological analyses were also conducted for the freshly collected estuarine water to determine their initial microbial community, and these results are presented in Table 1.

For each experiment, two microcosms were established, and the individual microcosm was considered a replicate containing sixty-eight slides each. The glass slides were placed on slide holders and deployed in each sterile polycarbonate rectangular cage microcosms (43 x 27 x 18 cm, Tarsons, 15L) filled with the estuarine water (12L). All the microcosms were static during the incubations for 15 days under 12:12 light/dark conditions at room temperature. The slides with biofilms and the water samples following immersion were retrieved on the 1st, 5th, 10th, and 15th day, respectively. For each treatment, the slides were sampled from the microcosms as described above. In addition, the water samples (1000 mL per microcosm) for bacterial diversity were also collected in duplicates on each day (0, 1st, 5th, 10th and 15th) during the study period.

Physico-chemical parameters

The estuarine water samples for physico-chemical parameters (salinity, nutrients, and chlorophyll *a*) were collected from the mouth of the bay on Day 0 for both experiments. Salinity was measured using a Salinometer. The measurements of nutrient concentrations (mainly nitrate, phosphate, nitrite, ammonia) in the estuarine water and microcosm samples were carried out by SKALAR SANplus ANALYSER, using skalar methods and expressed as micromole per litre (μM).

Chlorophyll *a* concentration in biofilms

The scraped and pooled biofilm samples were filtered through GF/F Whatman filters, then extracted with 90% acetone overnight and measured following the standard methods [16]. Chl *a* is expressed as $\mu\text{g cm}^{-2}$ of the slide surface area sampled.

Microbiological analysis

For enumeration of viable and pathogenic bacteria in the biofilms, 1 mL of subsample was serially diluted and spread plated (0.1 mL) using four different media: Nutrient Agar (NA), Thiosulphate Citrate Bile Salt (TCBS) Agar (*Vibrio* spp. primarily, *Vibrio alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*), and MacConkey Agar (Total coliforms). *Vibrio* spp. grown on the TCBS agar were differentiated as described by Pfeffer and Oliver [17]. In brief, the sucrose fermenting yellow, < 2 mm diameter colonies were counted as *Vibrio cholerae*, followed by > 2 mm diameter colonies as *Vibrio alginolyticus*, while greenish colonies were counted as *Vibrio parahaemolyticus*. Identification of these bacterial species has been confirmed previously by using appropriate biochemical tests (HiMedia, India) and MALDI-TOF MS Biotyping (MTB) method [18-19]. In addition, the Zobell Marine Agar (ZMA) 2216 was used to count marine bacteria from the biofilms subjected to microcosm experiments. TVC is expressed as colony forming units per square centimeter (CFU cm⁻²) of the slide surface area sampled. Also, the total viable counts in the estuarine water were enumerated and expressed as colony forming units per millilitre (CFU mL⁻¹)

The standard protocol for total bacterial count enumeration in biofilms was used [15]. Briefly, the thawed samples were sonicated at 30W for 60 s in a water bath sonicator (Ultrasonic cleaner, Equitron), then passed through the BD cell strainer cap (pore size, 40 µm) to remove larger particles. Further, 1 mL subsamples were stained using SYBR Green I (1:10000 final concentration, Molecular Probes, USA) and subjected to dark incubation for 15 mins. Subsequently, the samples were analyzed using the flow cytometer BD FACS Aria II equipped with the blue laser (wavelength: 488nm) and calibrated using 1µm fluorescent beads (Polysciences, USA) as an internal standard. The emitted light signals were passed through the 488/10 bandpass filter for right-angle light scatter/side scatter (RALSS/SSC) and 530/30 bandpass filter for green fluorescence (FL-1). The samples were run at low flow rates to keep the number of events below 500 per second for better precision, or else the samples were diluted. Gating was done against SSC versus green fluorescence.

For enumeration of protists, the only difference was that the detector voltage was reduced with reference to SSC vs green fluorescence to avoid the interference of bacterial populations with protists, as described by [15]. The emitted light signals were recorded through the 488/10 bandpass filter for SSC, 530/30 bandpass filter for green fluorescence (FL-1), and 695/40 for red fluorescence (FL-2). Gating was done against green versus red fluorescence. The data were processed using BD FACS Diva version 6.2 software. TBC and protists are expressed as cells per square centimeter (Cells cm⁻²) of the slide surface area sampled. Also, the TBC and protists in the estuarine water were enumerated and expressed as cells per millilitre (Cells mL⁻¹).

DNA extraction from biofilm samples

The scraped and pooled biofilm samples were filtered through autoclaved 0.22µm filter papers (Millipore), preserved in 70% ethanol and stored at - 20°C until analysis (analyzed within a month). DNA's were extracted using the MO BIO PowerBiofilm™ DNA extraction kit as stated in the manufacturer's protocol.

DNA extraction from water samples

The water samples (n = 2) were filtered (1000 mL) through autoclaved 0.22 µm filter papers (Millipore) and stored at -20°C until analysis (analyzed within a month). DNA's were extracted using the MO BIO Water DNA extraction kit as per the manufacturer's protocol.

Bacterial diversity of water and biofilm samples by quantitative PCR (qPCR)

The major bacterial phyla, namely Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, were quantified in different samples by real-time quantitative polymerase chain reaction (qPCR) using group-specific primers as listed in the electronic supplementary table [ESM_1] [20-21]. The corresponding annealing temperatures for the group-specific primers were standardized in the laboratory. Before metagenomic DNA analyses, the qPCR standards were prepared by using genomic DNA extracted from different bacterial pure cultures categorized under different taxa (For example, *Alcaligenes faecalis* used for Betaproteobacteria, *Vibrio alginolyticus* used for Gammaproteobacteria, *Micrococcus* spp. used for Actinobacteria, and *Bacillus cereus* used for Firmicutes primer set). Furthermore, the standards were amplified via qPCR using group-specific primers. These amplified PCR fragments were purified using the pure link PCR purification kit (Invitrogen, USA) and pooled to specific concentrations (checked using a Bio Spectrometer, Eppendorf). The standard curves were then obtained via qPCR by serially diluting these bacterial standards containing 16S rRNA gene PCR products of each target group, ranging from 10⁹ to 10⁶ copies.

The Fast SYBR® Green PCR Master Mix (Applied Biosystems) and the appropriate PCR primers were used to run qPCR reactions using Rotor-Gene Q (Qiagen). All the qPCR mixtures were run in triplicates, contained a total volume of 20 µL, 10 µL of the master mix, 2 µM of each primer, 1 µL of DNA template (~1.0 - 5.0 ng of environmental DNA) and made up with nuclease-free water (Ambion™). The protocol for qPCR amplification using group-specific primers was as follows: an initial enzyme activation step at 95°C for 20 secs, followed by 45 cycles of denaturation at 95°C for 3s, the 30s of annealing at 53°C (except for Alphaproteobacteria, annealing at 60°C) and the final extension at 72°C for 30s. Amplification of specific targets observed as the sigmoidal curve was further

confirmed by analyses of melt curves. Melt curve analysis (T_m) was done to determine the specificity of amplification for each qPCR reaction. A single peak indicated the presence of a site-specific PCR amplicon, as shown in the electronic supplementary figure [ESM_2]. The data were further processed using Rotor-Gene software (v.2.3.1, Qiagen). The PCR amplification efficiency (E), the threshold cycle (CT), and gene copy numbers achieved by comparing with a standard curve (raw value, gene copies μL^{-1}) were calculated using the software. The PCR efficiency was always between 0.98 and 1.0. The major bacterial phyla from water and biofilm were expressed as the relative abundance (estimated from the gene copy number) based on the sum of the absolute abundances of major bacterial phyla.

Statistical analysis

The biotic data were $\log(x+1)$ transformed, while the abiotic data were normalized. To meet the parametric test criteria, the data were checked for the normality and homogeneity of variances using the Statistica 6.0 program (Stat Soft Inc. Tulsa, OK, USA). The data sets fulfilling the assumptions for the parametric analyses were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's HSD test (Bonferroni corrected) to determine the significant variations in the bacterial parameters (microbial cell counts, viable and pathogenic bacteria) between the aged biofilms and the experimental data (IBM statistics, SPSS version 16.0). The significant changes in the bacterial communities (major bacterial phyla data) between the treatments were evaluated with non-metric multidimensional scaling (NMDS) based on the Bray-Curtis similarities and confirmed with an analysis of similarity (ANOSIM) test using PRIMER version 6.0 software [22]. Also, Simpson's and Shannon diversity indices used to measure diversity were calculated using PRIMER software. The relationship between the biotic and abiotic variables was determined by correlation analysis using Statistica 6.0 at a significance level of $p \leq 0.05$ (Stat Soft, OK, USA).

Results

Aged biofilm microbial communities before microcosm experiments

When the 5-day old freshwater biofilms developed under in situ and dark conditions with distinct communities were subjected to prolonged dark incubation (100 days), the TBC, protists, TVC, *Vibrio* spp., and total coliforms declined significantly, irrespective of the type of biofilms (Table 2, Tukey's HSD, $p < 0.001$). However, the diversity of bacteria in biofilms developed under dark conditions was higher than in situ conditions after 100 days of incubation (Table 2). Moreover, the Simpsons and Shannon diversity indices also pointed to a higher diversity (0.41; 0.70) in the aged dark freshwater

biofilms, compared to aged in situ freshwater biofilms (0.087; 0.225). Actinobacteria dominated both these aged biofilms.

Dynamics of aged biofilms when exposed to the estuarine seawater

When the 100-day old in situ and dark biofilms with distinct communities were exposed to EW in the laboratory, all the microbial parameters, i.e. TBC, protists, TVC, total coliforms, increased significantly in both these biofilms, except on Day 15, where the counts decreased ($p < 0.001$, Tukey's HSD, Fig. 2a-e). Moreover, *Vibrio* spp., i.e., *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Vibrio cholerae* appeared and proliferated on Days 1 and 5 in both the aged biofilms when subjected to EW (Fig. 2f-h). The bacterial communities in both the aged biofilms were significantly different when exposed to EW (Fig 3a-b), as visualized using NMDS ordination (Fig. 4a). Analysis of Similarity (ANOSIM) confirmed these differences ($R = 0.428$, $p < 0.05$). When the aged in situ biofilms were subjected to EW, the biofilm bacterial communities, initially dominated by Actinobacteria, changed significantly to Gammaproteobacteria and Bacteroidetes (Fig 3a). Moreover, Gammaproteobacteria abundance showed a significant correlation with Chl *a* and phosphate ($p < 0.05$). On the other hand, when the aged dark biofilms were subjected to the EW, Gammaproteobacteria peaked on Day 1, followed by Actinobacteria, which was the dominant taxa at the end of the experimental period (Fig. 3b).

Influence of aged freshwater biofilms on the estuarine water in the tank microcosms

Significant changes were observed in the estuarine water column communities, regardless of the type of biofilms exposed (Fig. 3c-d), as visualized using NMDS ordination (Fig. 4b). Analysis of Similarity (ANOSIM) also confirmed these differences ($R = 0.33$, $p < 0.05$). When the estuarine water dominated by Alphaproteobacteria was exposed to aged in situ biofilms, the bacterial communities of the EW changed significantly to Gammaproteobacteria, which peaked on Day 1 followed by Betaproteobacteria that increased drastically at the end of the experimental period (Fig. 3c). The Betaproteobacteria abundance was influenced by ammonia ($p < 0.05$). Whereas, Alphaproteobacteria and Firmicutes abundance decreased significantly in the EW exposed to aged in situ biofilms ($p < 0.001$, Tukey's HSD).

On the other hand, when EW was subjected to aged dark freshwater biofilms, the bacterial communities initially dominated by Alphaproteobacteria changed significantly to Gammaproteobacteria, which dominated during the initial days of incubation, followed by Actinobacteria (Fig. 3d). The abundance of Actinobacteria showed a significant positive correlation with nitrate and ammonia ($p < 0.05$). Whereas Alphaproteobacteria, Betaproteobacteria, Bacteroidetes,

and Firmicutes decreased significantly in the EW exposed to aged dark biofilms ($p < 0.001$, Tukey's HSD).

Discussion

The present study revealed that both the in situ and dark freshwater biofilms initially dominated with distinct communities changed to Actinobacteria as dominant taxa upon the prolonged dark incubations. Actinobacteria are one of the largest bacterial phyla with high G+C content containing Gram-positive bacteria [23]. The lifestyles and ecology of these bacteria are diverse. They can thus survive in most aquatic habitats, including open-ocean, coastal, estuarine, and within a ballast tank [24]. Actinobacteria taxon plays a significant role in the carbon cycling, recycling of organic matter, and nutrient regeneration [25-28]. Moreover, they feed on dead decaying organisms, thus acting as scavengers. Alternatively, the viable and pathogenic bacteria were not detected in the freshwater biofilms on exposure to prolonged dark incubations, which possibly entered into an inactive or viable but non-culturable state (VBNC) (a survival strategy) in response to harsh dark conditions. Moreover, it is known that several human pathogenic bacteria enter into the VBNC state by reducing their cell size and cannot be recovered using culture media, although they retain their viability and virulence [29-31]. Furthermore, these aged biofilms were subjected to microcosm experiments, and their response on exposure to estuarine water is discussed below. A pictorial representation of the response of aged freshwater biofilms exposed to estuarine water is depicted in Fig. 5.

When aged in situ freshwater biofilms were exposed to estuarine water, these biofilms were significantly colonized by the microbial communities present in the estuarine water, depending on the receptivity of these biofilms. This was evident from an increase in all the microbial parameters in these biofilms, i.e., TBC, protist abundance, TVC (marine bacteria), total coliforms, pathogenic bacterial abundance, i.e., *Vibrio* spp. and chlorophyll *a* concentration suggesting establishment and proliferation of the water column communities. A similar trend was observed in the aged freshwater biofilms developed in the dark. These results point out the lack of competition between the communities in the biofilm niche and the estuarine communities, which resulted in the successful establishment of water column microbial communities in these biofilms. The aged in situ freshwater biofilms, dominated by Actinobacteria, significantly reduced when exposed to estuarine water. These biofilms were colonized by the surrounding estuarine communities dominated by Gammaproteobacteria and Bacteroidetes. The dominance of Gammaproteobacteria and Bacteroidetes in the biofilms could be attributed to their close association with the diatoms. This was evident from the strong correlation between Gammaproteobacteria and Chl *a* concentration. Several studies have demonstrated that the Proteobacteria and Bacteroidetes are the main heterotrophic bacterial phyla associated with the

diatoms [32-34]. Specific genera within these phyla (e.g. *Vibrio*, *Alteromonas*, and *Flavobacterium*) appear to be strongly associated with the diatoms [35-38]. When the aged dark freshwater biofilms were exposed to estuarine water, a similar trend was observed, i.e., colonization of biofilms by surrounding estuarine communities. Gammaproteobacteria dominated these biofilms during the initial days of incubation, but Actinobacteria were dominant towards the end of the experiment. Thus, it seems that the aged freshwater biofilms had plenty of empty spaces that resulted in the colonization of the microbial communities from the surrounding water.

The surrounding estuarine water, initially dominated with distinct communities, showed a clear shift in bacterial communities subsequent to their colonization in both the aged freshwater biofilms. The resulting shifts influenced other bacterial genera of the estuarine water column communities, as evident from a significant increase in Betaproteobacteria when exposed to aged in situ freshwater biofilms. Moreover, the Betaproteobacteria abundance showed a significant positive correlation with ammonia concentration during the study period. This could be attributed to the proliferation and dominance of ammonia-oxidizing Betaproteobacteria (AOB), which play a significant role in the biogeochemical cycling of nitrogen by converting ammonium to nitrite [39-41]. Many microcosm studies suggest that the bacterial nitrifiers strongly increase in the presence of different groups of protozoa due to the increased availability of ammonium released by protozoan grazing [42-44]. A similar trend has been observed in the present study. An increase in ammonia concentration and protist numbers was evident in the water column at the end of the experimental period. These high levels of ammonia are believed to favour the growth of *Nitrosomonas* spp. [45]. As pointed out earlier ammonia-oxidizing Betaproteobacteria, are known for their crucial role in the biogeochemical cycling of nitrogen. Such a hypothetical condition, wherein the waters are enriched with ammonia-oxidizing Betaproteobacteria, could be beneficial if translocated to the new environment. However, this requires further validation using in situ studies as the volume of water used in a microcosm is not comparable with the volume of water in an estuary. Moreover, the possible translocation of ammonia-oxidizing Betaproteobacteria from ballast waters may or may not have a real impact on the biogeochemical cycling of nitrogen in the recipient environment.

Among the culturable *Vibrio* spp., *V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae* belonging to Gammaproteobacteria appeared in both the aged freshwater biofilms exposed to estuarine water. It is possible that these culturable *Vibrio* spp. existed in a viable but nonculturable (VBNC) state during the prolonged dark conditions along with the plankton, as they were not detected in the aged biofilms. Their existence in these aged biofilms is anticipated from gene copy numbers of Gammaproteobacteria as detected by qPCR. It is a survival strategy attained by many bacteria,

especially *V. cholerae*, in response to harsh environmental conditions [29-31]. This phenomenon has been widely referred to as resuscitation, and there are only a few reports on the potential of *Vibrio* spp. to revert from the VBNC state to culturable form [46-48]. The significance of salinity in the distribution of *V. cholerae* in the aquatic environment was reported earlier [49-51]. Other than salinity and nutrients, temperature and plankton are also significant for the ecology of *Vibrio* spp. [52-53]. As biofilms mature, dispersal becomes a choice. Hence, bacteria can sense whether it is beneficial to reside within the biofilms or whether it is time to resume the planktonic lifestyle [54]. Thus, it is suggested that the possible contamination of ambient water bodies with *Vibrio* spp. and the harmful effects of their pathogenic forms, causing cholera, will depend upon the environmental factors that influence the expression of virulence genes [51].

Ballast tanks are generally made up of metals with protective coatings (hydrophobic), and the surfaces used to develop biofilms in the present study are glass slides (hydrophilic), which have been extensively used for aquatic biofilm studies [15, 52, 55-56]. Hence, it can be expected that biofilm community composition would differ based on the surface properties (hydrophobic versus hydrophilic) to be colonized [57-58]. Moreover, the experimental conditions outlined for this study were conducted under a controlled environment compared to the dynamic tidally influenced estuarine environment. Hence, differential community response is expected and must be considered while extrapolating these results to natural conditions.

Conclusions

This research is anticipated as an initial step towards understanding the possible discharge effects of aged biofilms on the aquatic environment, which has not been addressed earlier. The aged freshwater biofilms (100 days) on exposure to estuarine water resulted in the modification of bacterial community structure, proliferation or reduction in some bacterial genera, and the emergence of microbes not present earlier. Interestingly, the dormant *Vibrio* spp. i.e. *V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae* belonging to Gammaproteobacteria existing in aged freshwater biofilms could resuscitate back to culturable form when subjected to estuarine water. However, their degree of pathogenicity or virulence will depend on environmental conditions. Thus, it seems that the interaction and exchange between biofilm communities and surrounding inoculum characteristics can influence the characteristics of native or newly introduced species. This may result in (1) alterations in the water column community composition, which can contribute to the reduction or proliferation of some genera with different metabolic potential, thus influencing the ecology of that environment, and (2) changes in the water quality, which could have a cascading effect on the environment if the species that emerge turns out to be pathogenic. Thus, the risks emerge from the fact that pathogenic bacteria that travel by

“hiding out” within the biofilms can be non-virulent in the dormant VBNC state but can regain virulence after resuscitation into culturable forms under suitable conditions. Future studies should focus on quantifying these effects in the field using advanced molecular tools, with an emphasis on virulent species, which will help in understanding traits that enable them to adapt to the changing environment and is an important topic for future investigation.

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Author Contributions

NH: Conceptualization, methodology, formal analysis, investigation, writing - original draft, review & editing. **LK:** Conceptualization, methodology, validation, formal analysis, investigation, resources, writing - original draft, review & editing, supervision. All the authors have read and approved the final manuscript.

Data Availability

The data sets supporting the results of this article are included within the article.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The authors state that the experiments were carried out under controlled conditions in the laboratory. All the aspects of the study were conducted in compliance with relevant Institutional biosafety and biosecurity protocols, and the manuscript has been approved by the Patents and Publication Committee of the Institute.

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Legends to Tables

Table 1 Different biotic variables in the estuarine water. All the data points are averages of several replicates.

Table 2 Different biotic variables in the in situ, dark and aged freshwater biofilms. All the data points are averages of several replicates.

Legends to Figures

Fig 1 Schematic diagram illustrating the experimental workflow. TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms.

Fig 2 Mean values of (a) total bacterial count, (b) protists, (c) total viable count (ZMA), (d) total viable count (NA), (e) total coliforms, (f) *V. alginolyticus*, (g) *V. parahaemolyticus*, and (h) *V. cholerae*, in the aged in situ (■) and dark (■) freshwater biofilms exposed to estuarine water (EW) during the sampling days. ZMA = Zobell Marine Agar, NA = Nutrient Agar, FBF = freshwater biofilm. 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 alphabets above the bars represent significant differences between the aged biofilms and the experimental data (one-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 3 Relative abundances of major bacterial phyla demonstrating changes in the (a) aged in situ freshwater biofilms and (b) aged dark freshwater biofilms exposed to the (c) and (d) estuarine water, respectively. Alphaproteobacteria (dark blue), Betaproteobacteria (dark red), Gammaproteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange). FBF = Freshwater biofilm, EW = Estuarine water.

Fig 4 Non-metric multidimensional scaling (NMDS) analyses of (a) bacterial communities in the aged in situ and dark freshwater biofilms subjected to microcosm experiments, and b) water column bacterial communities in the tank microcosms when exposed to in situ and dark freshwater biofilms. Symbols indicate the treatments (Green up-triangle (▲) = In situ freshwater biofilms exposed to EW, Purple down-triangle (▼) = Dark freshwater biofilms exposed to EW, Blue square (■) = EW (Control, Day 0), whereas Day 0, Day 1, Day 5, Day 10, and Day 15 indicate the days of sampling in the EW. Day 100 FBF = 100-day old freshwater biofilms, EW = Estuarine water.

Fig 5 Pictorial representation of the response of the aged freshwater biofilms exposed to estuarine water. VA = *Vibrio alginolyticus*, VP = *V. parahaemolyticus*, VC = *V. cholerae*.

Electronic Supplementary Material

ESM_1 Group-specific primers for real time quantitative PCR

ESM_2 Melt curve analysis

Table 1 Different biotic variables in the estuarine water. All the data points are averages of several replicates.

S. No		Estuarine water
1	Chl <i>a</i> ($\mu\text{g L}^{-1}$)	0.18 ± 0.002
2	TVC (marine)	$4.5 \pm 0.01 \times 10^4$
(CFU mL ⁻¹)	<i>Vibrio alginolyticus</i>	$2.2 \pm 0.3 \times 10^3$
	<i>V. parahaemolyticus</i>	$2.1 \pm 0.5 \times 10^2$
	<i>V. cholerae</i>	ND
	Total coliforms	$7.5 \pm 3.3 \times 10^1$
3	TBC	$1.3 \pm 0.004 \times 10^6$
(Cells mL ⁻¹)	Protists	$4.1 \pm 0.9 \times 10^4$
4	Alphaproteobacteria	$1.4 \pm 0.3 \times 10^5$
(Gene copy numbers μL^{-1})	Betaproteobacteria	$1.3 \pm 0.2 \times 10^4$
	Gammaproteobacteria	$3.2 \pm 1.6 \times 10^4$
	Bacteroidetes	$3.5 \pm 1.6 \times 10^4$
	Firmicutes	$9.4 \pm 4.5 \times 10^3$
	Actinobacteria	$5.7 \pm 1.1 \times 10^3$
	Simpson's Diversity of Index	0.45
	Shannon's Diversity of Index	0.91

S. No = Serial number, ND = Not detected, TVC =Total Viable Count, TBC = Total Bacterial Count, Chl *a* = Chlorophyll *a*, \pm values = Standard deviation.

Table 2 Different biotic variables in the in situ, dark and aged freshwater biofilms. All the data points are averages of several replicates.

S. No		5-day old in situ biofilm	100-day old in situ aged biofilm	5-day old dark biofilm	100-day old dark aged biofilm
1	Chl <i>a</i> ($\mu\text{g cm}^{-2}$)	0.351 ± 0.001	ND	0.046 ± 0.001	ND
2	TVC (freshwater)	$2.7 \pm 0.05 \times 10^3$	$1.2 \pm 0.1 \times 10^2$	$8.2 \pm 1.7 \times 10^2$	$2.0 \pm 0.2 \times 10^1$
(CFU cm^{-2})	TVC (marine)*	NS	$1.7 \pm 0.2 \times 10^1$	NS	$3.9 \pm 0.3 \times 10^1$
	<i>Vibrio alginolyticus</i>	$2.2 \pm 0.1 \times 10^2$	ND	ND	ND
	<i>V parahaemolyticus</i>	$6.0 \pm 0.9 \times 10^0$	ND	ND	ND
	<i>V cholerae</i>	$7.6 \pm 2.4 \times 10^1$	ND	ND	ND
	Total coliforms*	$2.1 \pm 0.1 \times 10^3$	ND	$5.6 \pm 0.4 \times 10^2$	ND
3	*TBC	$3.0 \pm 0.003 \times 10^6$	$3.4 \pm 0.2 \times 10^5$	$2.2 \pm 0.01 \times 10^6$	$2.5 \pm 0.1 \times 10^5$
(Cells cm^{-2})	*Protists	$1.5 \pm 0.3 \times 10^5$	$9.5 \pm 8.1 \times 10^2$	$6.9 \pm 1.0 \times 10^4$	$1.9 \pm 0.2 \times 10^3$
4	Alphaproteobacteria*	$1.6 \pm 0.1 \times 10^4$	$4.0 \pm 2.7 \times 10^2$	$1.1 \pm 0.1 \times 10^4$	$5.0 \pm 0.1 \times 10^4$
(Gene copy numbe rs cm^{-2})	Betaproteobacteria*	$1.1 \pm 0.01 \times 10^4$	$3.0 \pm 0.7 \times 10^1$	$5.7 \pm 3.7 \times 10^2$	$2.3 \pm 0.1 \times 10^3$
	Gammaproteobacteria*	$2.0 \pm 0.3 \times 10^4$	$1.2 \pm 0.9 \times 10^3$	$1.6 \pm 0.6 \times 10^3$	$3.4 \pm 0.1 \times 10^2$
	Bacteroidetes*	$2.4 \pm 0.03 \times 10^2$	$1.9 \pm 0.4 \times 10^2$	$6.4 \pm 0.8 \times 10^1$	$4.6 \pm 0.3 \times 10^0$
	Firmicutes*	$6.0 \pm 7.8 \times 10^3$	$3.2 \pm 0.1 \times 10^1$	$4.5 \pm 7.3 \times 10^2$	$3.9 \pm 0.1 \times 10^3$
	Actinobacteria*	ND	$3.9 \pm 0.1 \times 10^4$	ND	$1.6 \pm 0.002 \times 10^5$
	Simpson's Diversity of Index	0.71	0.087	0.27	0.41
	Shannon's Diversity of Index	1.32	0.225	0.72	0.70

S. No = Serial number, ND = Not detected, TVC = Total Viable Count, TBC = Total Bacterial Count, Chl *a* = Chlorophyll *a*, NS = Not sampled, \pm values = Standard deviation. The asterisk (*) indicates significant differences observed between the aged freshwater biofilms (Tukey's HSD, $p \leq 0.05$).

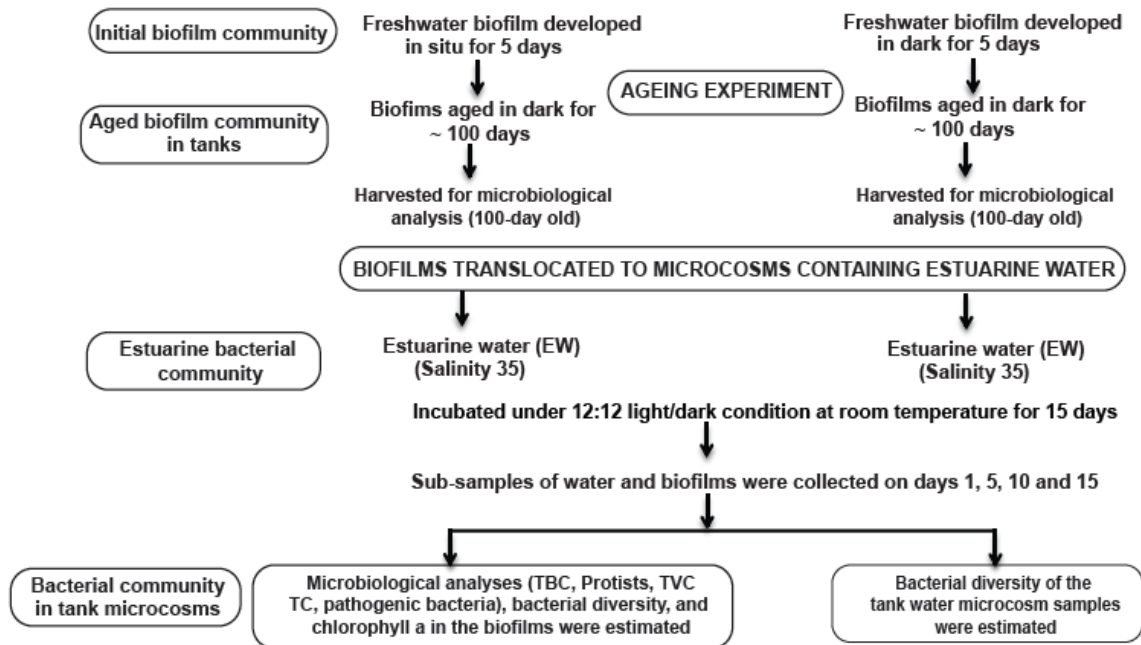


Fig. 1

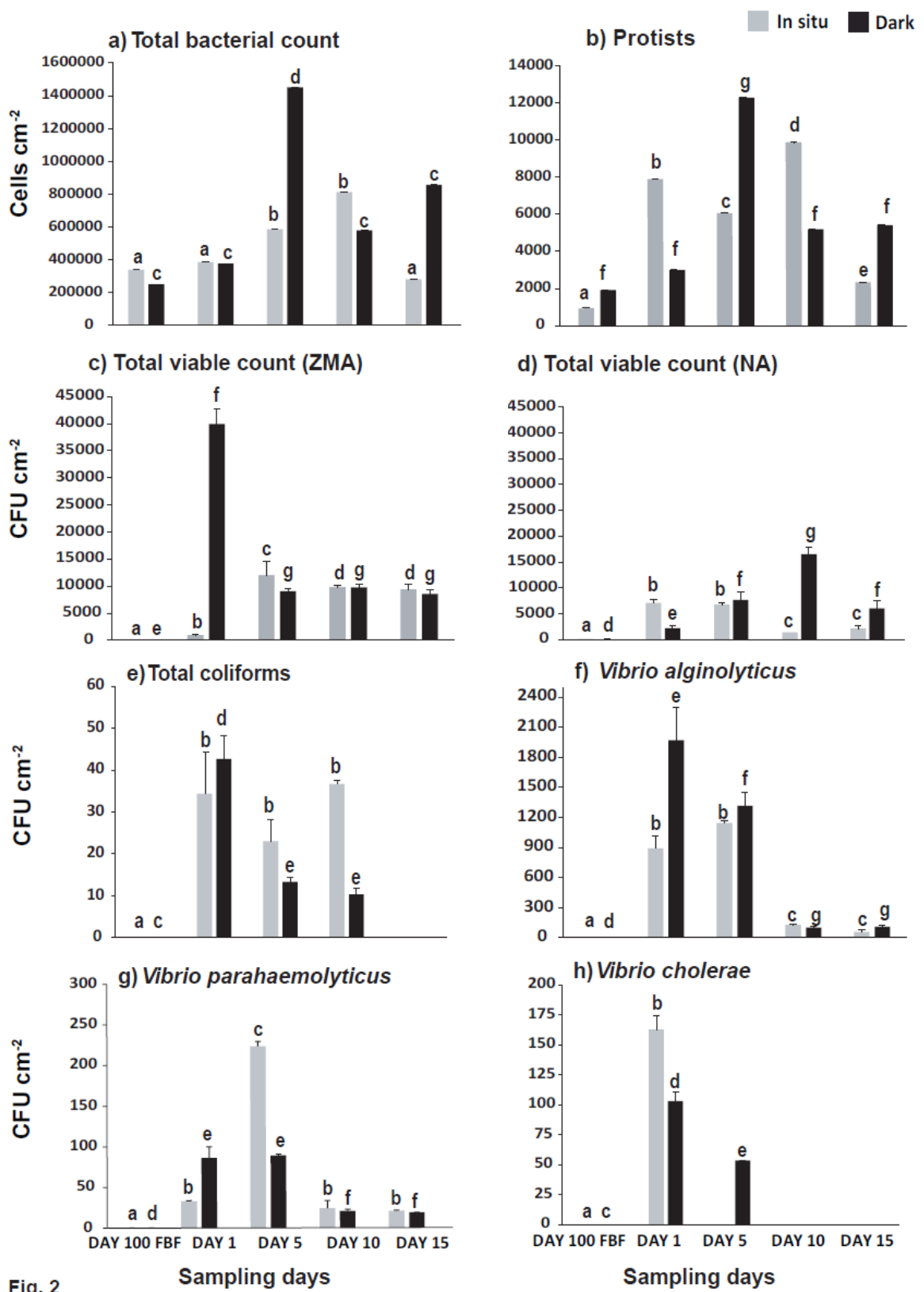


Fig. 2

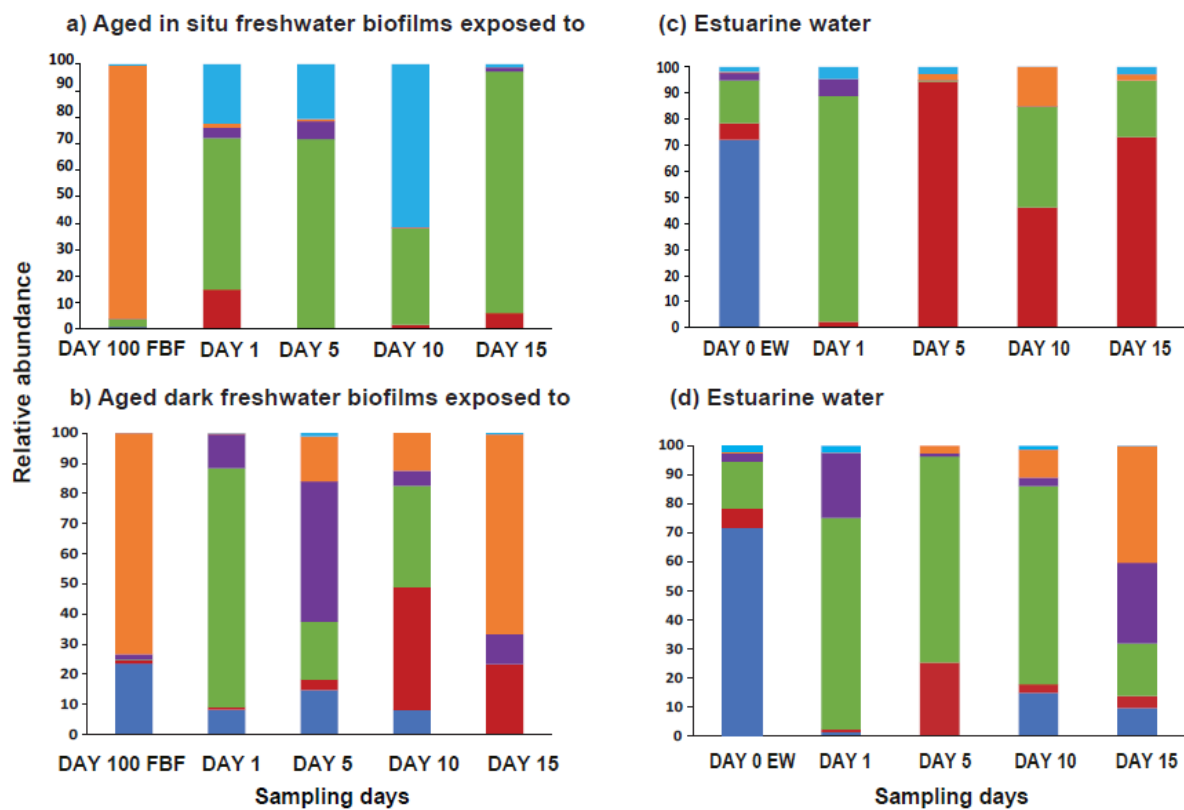


Fig. 3 ■ Alphaproteobacteria ■ Betaproteobacteria ■ Gammaproteobacteria ■ Firmicutes ■ Bacteroidetes ■ Actinobacteria

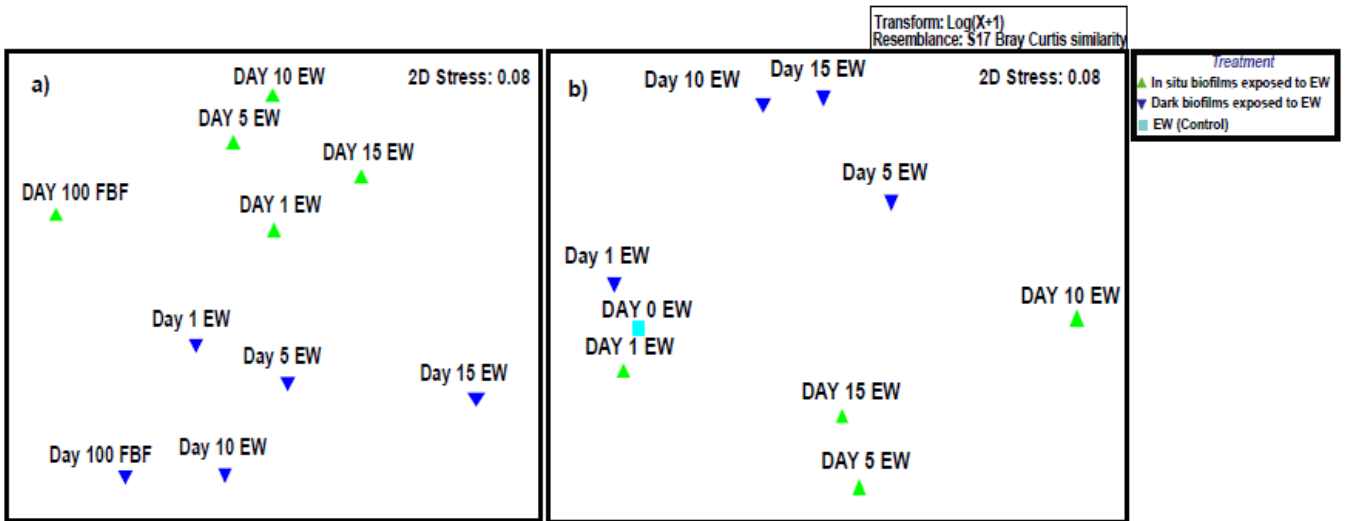


Fig. 4

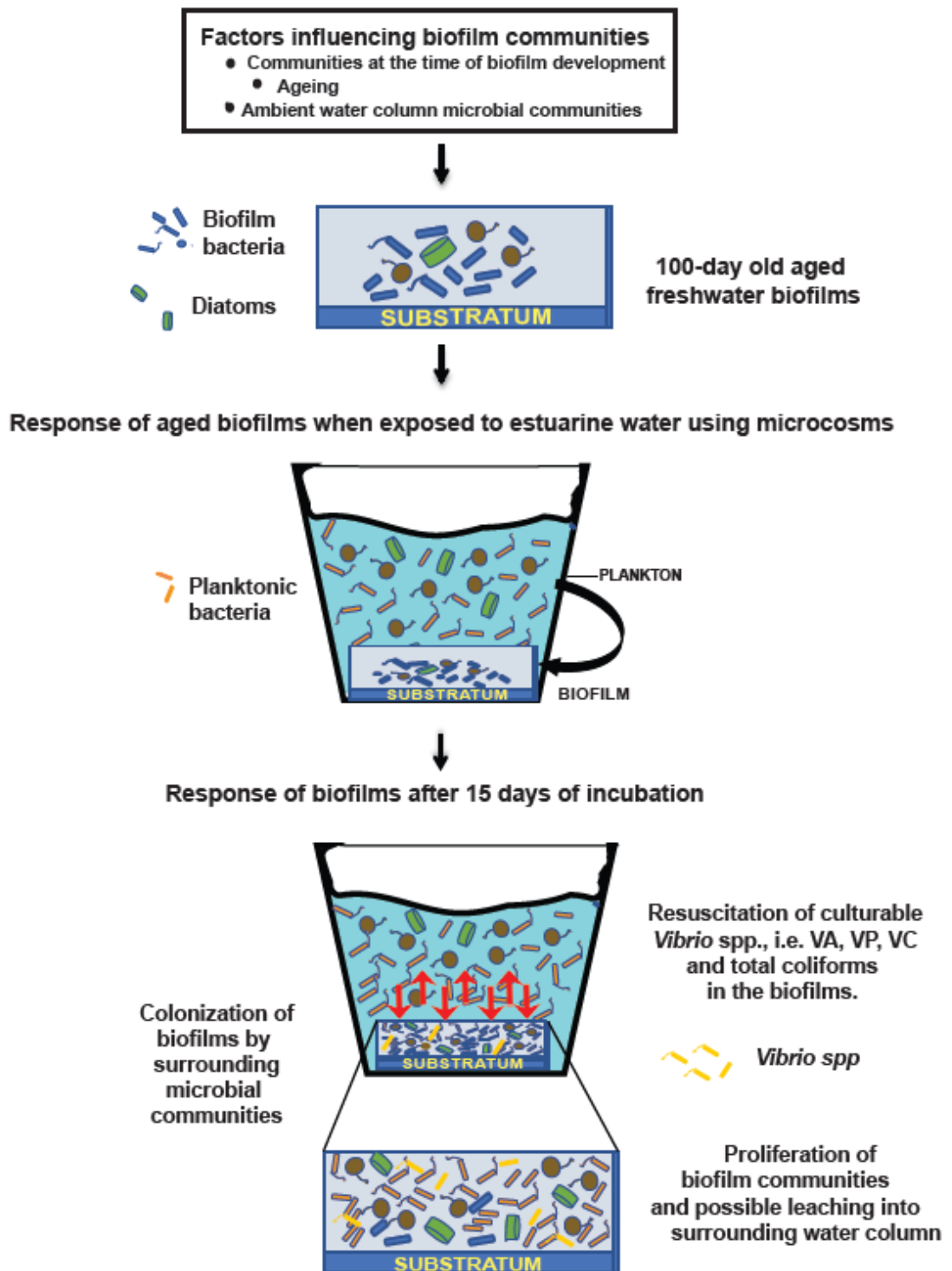


Fig. 5

ESM_1 Group-specific primers for real time quantitative PCR

Target group name	Product size (base pairs)	Primer name and sequence (5'-3' direction)	Reference
Alphaproteobacteria	250	α - 682F - CIAGTGTAGAGGTGAAATT α - 908R - CCCCGTCAATTCCTTGAGTT	[20]
Betaproteobacteria	340	β - 359f - GGGGAATTTGGACAATGGG β - 682r - ACGCATTTCCTGCTACACG	[21]
Gammaproteobacteria	170	γ - 1080F - TCGTCAGCTCGTGYGTGA γ - 1202R - CGTAAGGCCATGATG	[20]
Firmicutes	200	928FirmF - TGAAACTYAAAGGAATTGACG 1040FirmR - ACCATGCACCACCTGTC	[20]
Bacteroidetes	200	798cfbF - CRAACAGGATTAGATACCCT cfb967R - GGTAAGGTTCTCGCGTAT	[20]
Actinobacteria	300	Act920F3 - TACGGCCGCAAGGCTA Act1200R - TCRTCCACCTTCTCCG	[20]