Studies on response of biofilms to changing environmental conditions

A Thesis submitted to Goa University for the degree of

DOCTOR OF PHILOSOPHY

in

Marine Sciences

By

Niyati Pandurang Hede

Goa University, Goa – 403 206, India

July, 2021

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July, 2021

Dedicated to my parents

STATEMENT OF THE CANDIDATE

As required under the University Ordinance OB-9A.5, I hereby state that the present thesis entitled "**Studies on response of biofilms to changing environmental conditions**" is my original research work carried out in the CSIR-National Institute of Oceanography, Dona Paula, Goa and the same has not been submitted in part or in full elsewhere for any other degree or diploma.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

9/7/2021

Niyati Pandurang Hede

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on response of biofilms to changing environmental conditions**" submitted by Miss Niyati Pandurang Hede, for the award of the degree of Doctor of Philosophy in Marine Sciences is based on original studies carried out by her under my supervision. This thesis or any part thereof has not been previously submitted for any other degree or diploma in any Universities or Institutions.

9/7/2021

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Niyati Pandurang Hede

Abbreviations and symbols

°C	Degrees Celsius
°E	Degrees East
°N	Degrees North
%	Percentage
1-λ’	Simpson’s diversity index
cm	Centimeter
cm^{-1}	Reciprocal wavelength
H’	Shannon diversity index
Hr(s)	Hour(s)
× g	Number of times the gravitational force
NO_2	Nitrite
PO_4	Phosphate
nm	Nanometer
m	Meters
mg	Milligrams
mL	Milliliters
mm	Millimeters
mM	Millimolar
qPCR	Quantitative polymerase chain reaction
µm	Micrometers
µM	Micromoles per litre
$µg/cm^2$	Microgram per centimetre square
µg/L	Microgram per litre
µl	Microliters
s	Seconds
L	Litres
W	Watts
α-proteobacteria	Alphaproteobacteria
β-proteobacteria	Betaproteobacteria
γ-proteobacteria	Gammaproteobacteria
ANOVA	Analysis of variance
ANOSIM	Analysis of similarities

Arab	Arabinose
AS	Arabian Sea
ASW	Aged Seawater
bp	Base pairs
BP	Band pass
BF	Biofilms
BW	Ballast water
BD	Becton, Dickinson (company)
BLAST	Basic Local Alignment Search Tool
CANOCO	Canonical correspondence analysis
CB-EPS	Cell-bound extracellular polymeric substances
CFU	Colony forming units
CSIR	Council of Scientific and Industrial Research
CT	Threshold cycle
CTD	Conductivity Temperature Depth
Chl *a*	Chlorophyll *a*
Con A	Concanavalin A
DAPI	4′,6-diamidino-2-phenylindole
DCA	Detrended Correspondence Analysis
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EN	Enriched
EPS	Extracellular polymeric substances
EW	Estuarine water
Expt.	Experiment
FACS	Fluorescence-activated cell sorting
FALS	Forward angle light scatter
FITC	Fluorescein isothiocyanate
FT-IR	Fourier transformed Infra-red
FBF	Freshwater biofilms
FBF In situ-D5	5-day old freshwater biofilms
FBF In situ-D30	30-day old freshwater biofilms
FBF Tank-D5	5-day old freshwater tank biofilms
FBF Tank-D30	5-day old freshwater tank biofilms

Fruc	Fructose
Gal	Galactose
GF/F	Glass Fiber Filter
HPLC	High-pressure liquid chromatography
HSD	Honestly significant difference
IR	Infrared
KPD	Kidderpore Dock
Man	Mannose
MALDI-TOF	Matrix-Assisted Laser Desorption-Ionisation-Time of Flight
MBF	Marine biofilms
MBF In situ-D5	5-day old in situ marine biofilms
MBF UN-D5	5-day old unenriched marine biofilms
MBF UN-D30	30-day old unenriched marine biofilms
MBF EN-D5	5-day old enriched marine biofilms
MBF-EN-D30	5-day old enriched marine biofilms
M-EBF	Mandovi estuarine biofilms
M-EBF In situ-D5	5-day old in situ Mandovi biofilms
M-EBF In situ-D30	30-day old in situ Mandovi biofilms
M-EBF Tank-D5	5-day old Mandovi tank biofilms
M-EBF Tank-D30	30-day old Mandovi tank biofilms
MS	Mass Spectrometry
Min	Minutes
n	Number of slides
N	Total number of individuals in the sample
NA	Nutrient Agar
GlcNAc	N-acetyl-D-glucosamine
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NMDS	Non-metric Multidimensional Scaling
p	Probability value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
nRIU	nano Refractive Index Units

Rib	Ribose
RID	Refractive Index Detector
Rpm	Revolutions per minute
rDNA	Ribosomal Deoxyribonucleic acid
RALS	Right angle light scatter
RDA	Redundancy Analysis
Rha	Rhamnose
S	Total number of species in the community
SEM	Scanning Electron Microscopy
SBA	Soybean Agglutinin
SIMPER	Similarity percentages
Spp.	Species
SSC	Side scatter
SSK	Sindhu Sankalp
T	Tank
TBC	Total Bacterial Count
TM	Trademark
TVC	Total Viable Count
TC	Total Coliforms
TRITC	Tetramethylrhodamine
TCBS	Thiosulphate Citrate Bile Salt
USA	United States of America
UN	Unenriched
VA	*Vibrio alginolyticus*
VP	*Vibrio parahaemolyticus*
VC	*Vibrio cholerae*
WGA	Wheat Germ Agglutinin
XLD	Xylose lysine deoxycholate
ZD5	Five-day old Zuari
ZMA	Zobell Marine Agar
ZMB	Zobell Marine Broth
Z-EBF	Zuari estuarine biofilms
Z-EBF In situ-D5	5-day old in situ Zuari biofilms
Z-EBF In situ-D30	30-day old in situ Zuari biofilms

Z-EBF Tank-D5	5-day old Zuari tank biofilms
Z-EBF In situ-D30	30-day old Zuari tank biofilms

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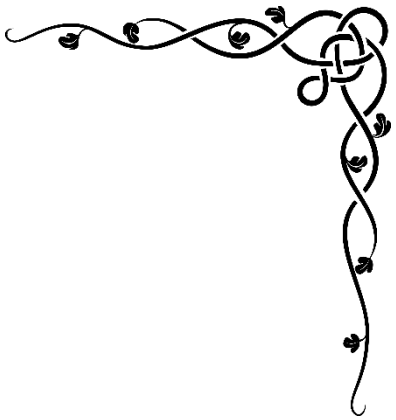


Chapter 1

Introduction

1. Introduction

1.1 Biofilms: a preferred mode of existence

Bacteria live in a wide range of habitats in the aquatic environments. They are known to exhibit two modes of existence, either a free-living (planktonic) or a surface-associated (biofilms) lifestyle (Salta et al., 2013; Dang and Lovell, 2016 and references within). In nature, biofilms are the most preferred mode of existence of microbes, wherein more than 90 % of bacteria reside within them (Costerton et al., 1995). Biofilms were first discovered and documented by Zobell in 1943, wherein he reported that bacteria in seawater are mostly sessile and preferentially attach to a solid surface by producing a mucilaginous holdfast. The first recorded microscopic observation of the microbial assemblages' dates back to the 17th century when Antonie van Leeuwenhoek experimented with plaque samples taken from human teeth (Leeuwenhoek, 1684). However, it was only in 1978, when the term biofilm was coined by Bill Costerton (Costerton et al., 1978), who is considered as the "father of the field of biofilms" (Lappin-Scott et al., 2014). These biofilms are microecosystems, wherein attached microbial communities comprising of autotrophic (diatoms, green algae, cyanobacteria) and heterotrophic (bacteria, fungi, protozoa) components are embedded in the glue-like extracellular polymeric substances (EPS) (Lock, 1993; Denkhaus et al., 2007).

There has been a growing interest in biofilms, owing to their significance in environmental, industrial, and medical areas (Donlan, 2002). Biofilms are ubiquitous and are commonly found in the natural environments such as streams, lakes, river beds, oceans, seas, and on hulls of vessels, boats, ships, and interior ballast tanks; industrial settings (e.g., paper mills, nuclear power plants, aquaculture ponds, wastewater treatment plants, nuclear reactors, and water pipelines), and medically related (e.g., dental plaque, implants (contact lenses, catheters) (Venugopalan and Nair, 1990; Rajagopal et al., 1991; Marsh and Bradshaw, 1995; Stickler, 1996; Callow and Callow, 2002; Zegans et al., 2002; Coetser and Cloete, 2005; Drake et al., 2005, 2007; Rao et al., 2009; Balamurugan et al., 2011; Schultz et al., 2011; Flemming et al., 2016; Venugopalan et al., 2012; Trepos et al., 2014; Salta et al., 2013;

Sharma et al., 2015; Battin et al., 2016; Dang and Lovell, 2016; Di Pippo et al., 2018; Colombo and Tanner, 2019; Procópio, 2019).

Biofilm formation is the most favoured survival mechanism opted by the microorganisms which is advantageous to them as compared to the planktonic existence (Costerton et al., 1987; Costerton and Lappin-Scott, 1989; Jefferson, 2004; Dang and Lovell, 2016; Charles et al., 2017; de Carvalho, 2018 and references within). Some of the advantages are as follows, a) enhances tolerance to harsh environmental conditions, b) avoids being carried away by fluid-driven or shear forces, c) resistance to predation by grazers, d) enhanced access to nutritional resources (via accumulated biogenic particles such as phytoplankton detritus, zooplankton faecal pellets, and marine snow which are rich in organic matter), due to closer proximity between cells facilitating mutualistic or synergistic associations and protection, e) production of abundant amount of extracellular polymers (EPS), which protects them from pH shifts, osmotic shock, desiccation, and UV radiation, etc., thus providing environmental stability. The formation of biofilms, either via cell-surface or cell-cell interactions, involves different molecules (e.g., lipids, polysaccharides, proteins, nucleic acids, and some other biomolecules), and processes (e.g., coaggregation and quorum sensing) which contribute to the development of biofilms (Rickard et al., 2000; Watnick and Kotler, 2000; Stoodley et al., 2001; Sauer et al., 2002; Purevdorj-Gage et al., 2005; Jain and Bhosle, 2009; Dang and Lovell, 2016). Organisms within these biofilms play an important role in the bottom-up supply of energy and organic matter to the food web as well as contribute to energy flow and nutrient cycling (Battin et al., 2003).

The processes governing biofilm formation involves a sequence of chemical and biological events (Figure 1.1). Once the surface is immersed in an aquatic environment, irrespective of its physicochemical properties (Baier, 1973; Loeb and Neihof, 1975; Fletcher and Pringle, 1986; Taylor et al., 1997; Donlan, 2002; Ista et al., 2004), the adsorption of macromolecules takes place. This layer is termed as conditioning layer, which results in the accumulation of proteins, polysaccharides, and other molecules that provide a metabolically favourable environment for bacterial cells and serve as nutritional cues to trigger biofilm formation (Bhosle et al., 1989;

Venugopalan et al., 1994; Bhosle et al., 2005; Garg et al., 2008; Jain and Bhosle, 2009). The composition of the conditioning film differs depending on the kind of environment the surface is exposed to and results in alterations in its surface properties, such as hydrophobicity, surface charge, surface roughness, and wettability, which influences microbial attachment (Bakker et al., 2003; Jain and Bhosle, 2009; Lorite et al., 2011; Dang and Lovell, 2016).

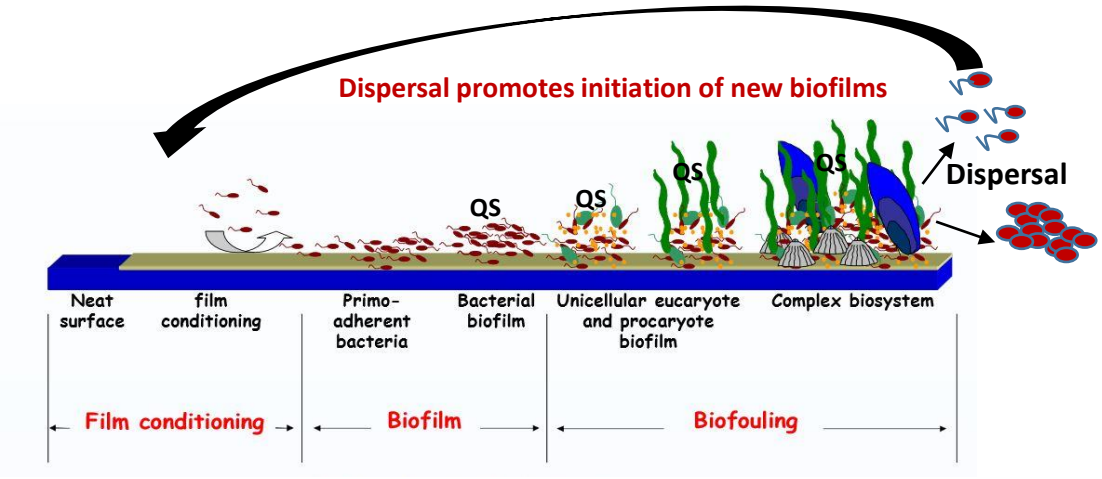


Figure 1.1 Processes governing biofilm formation (QS – Quorum sensing, Source: Ritter et al., 2010).

The transport of microorganisms towards these pre-conditioned surfaces, which is the second step in biofilm formation, occurs by different mechanisms, such as physical forces, which may include, Brownian motion, gravitation, diffusion, convection, or the intrinsic motility of a microorganism (Gottenbos et al., 2002). The bacterial adhesion to the surfaces is also influenced by cell surface characteristics such as cell surface hydrophobicity, surface charge, and cell appendages (flagella, pili, curli, lipopolysaccharides), that play a significant role in the bacterial adhesion (Kimkes and Heinemann, 2020 and references within). Moreover, the bacterial movement towards the surfaces is also influenced by the concentration gradients via chemotaxis (diffusible) or haptotaxis (surface-bound) using chemoattractants which are sensed by binding to the receptors (Sourjik and Wingreen, 2012; Ricoult et al., 2015; Dang and Lovell, 2016). Other than substratum and bacterial cell surface properties, the ambient bulk

fluid which serves as the source of temperature, pH, nutrients, ions, flow velocity (laminar or turbulent flow) strongly influences the cell attachment, and biofilm formation (Characklis and Cooksey, 1983; Sonak, 1998; Donlan, 2002).

Furthermore, the mechanisms involved in the bacterial adhesion to the cell surfaces comprise of the biochemical and physicochemical processes. The biochemical processes include specific interactions via ligand-receptor or ligand-sugar interactions between the bacterial cell surface and the substratum (Dalton and March, 1998). Whereas, the physicochemical processes, include non-specific interactions that are explained by the thermodynamic model (based on surface free energies of the interacting surfaces), DLVO (Derjaguin, Landau, Verwey, Overbeek) and extended DLVO theories (Hermansson, 1999; Bos et al., 1999 and references within). Alternatively, some species of bacteria tend to recognize and attract potential partners forming clumps, thereby contributing to coaggregation (Figure 1.2), a type of cell-cell (lectin-sugar) interaction, which also forms an integral part in the development and formation of multispecies biofilms (Rickard et al., 2003). Coaggregation was first reported by Gibbons and Nygaard, between different species of human dental plaque bacteria in the 1970s. It is not a random process, but highly specific recognition and adherence of the bacterial cells that employ specific molecules (protein adhesins or carbohydrate moieties) present on their cell surface to recognize partner bacteria (Rickard et al., 2002; Kolenbrander et al., 2006). This process is a key mechanism and phenomenon in the biofilm formation, which also facilitates interaction among different bacterial species in the biofilms (Rickard et al., 2003). These interactions are known to occur between members of the same genus (intra-generic), same species (intra-species), or with different bacteria (inter-species) (Rickard et al., 2002; Katharios-Lanwermeyer et al., 2014). This process differs from coadhesion, wherein the free-floating species adhere to species that are already attached to the surface as a part of a biofilm (Figure 1.2).

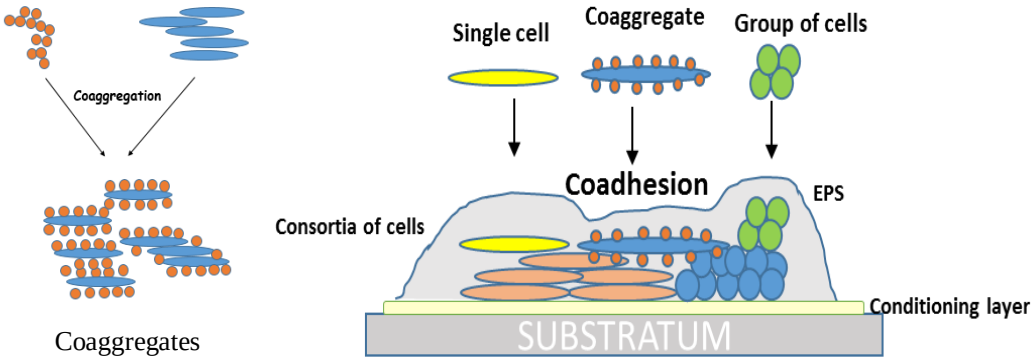


Figure 1.2 Coaggregation and coadhesion processes involved in the biofilm formation (Source: reproduced from Rickard et al., 2003).

Earlier it was known that biofilms could only be formed when free-living bacteria come in contact with the surface, as depicted in Figure 1.1. However, recent studies have demonstrated that the individual biofilm-forming bacteria tend to aggregate in their planktonic phase, thereby possibly resulting in natural biofilms, originating from these preformed cell aggregates as depicted in Figure 1.3 (Kragh et al., 2016; Melaugh et al., 2016).

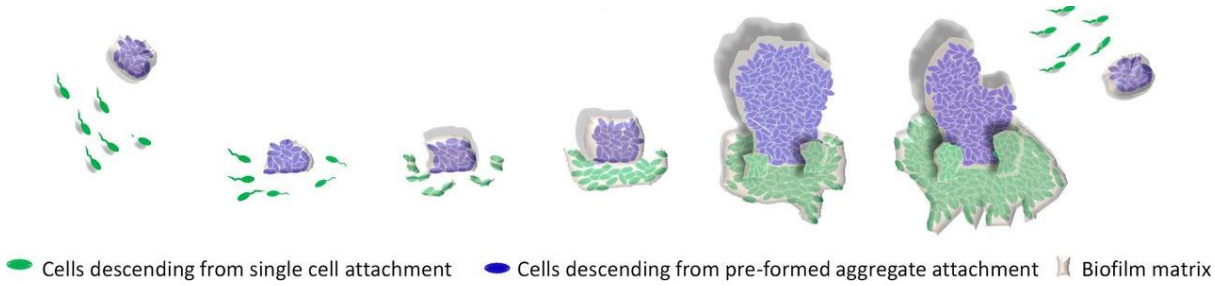


Figure 1.3 Biofilm formation originating from preformed aggregates (Source: Kragh et al., 2016).

The microbial adhesion (either of single organisms or (co)aggregates), an important process in the biofilm formation occurs, which is often initially reversible and turns irreversible with time (Hinsa et al., 2003; Caiazza and O'Toole, 2004). Once bacteria adhere to the surface, they often proliferate to form clusters of microcolonies that spread over the surface (Lappin-Scott and Costerton, 1995). As the density of bacteria increases, they regulate the expression of specific genes and release chemical signalling molecules when the population reaches a critical threshold. This process is called quorum

sensing (Annous et al., 2009; Deep et al., 2011; Hmelo, 2017). These signalling molecules vary among the gram-positive and gram-negative bacteria. There are three types of molecules, (1) Acyl-homoserine lactones (AHLs) which mediate intracellular communication in gram-negative bacteria, (2) Autoinducer peptides (AIPs) expressed in the case of gram-positive bacteria, and Autoinducer-2 (AI-2) mediates interspecies communication in both the gram-positive and gram-negative bacteria (van Bodman et al., 2008; Li and Tian, 2012). The formation of biofilms is a quorum-sensing controlled process (involving cell-cell communication and gene regulation), which involves various bacterial physiological activities, including EPS production (Dobretsov et al., 2009; Hmelo, 2017; Jemielita et al., 2018).

After attachment and adhesion stages, the bacterial cells produce glue-like sticky materials, which are termed as extracellular polymeric substances (EPS), establishing the formation of a biofilm (Steinberg and Kolodkin-Gal, 2015; Flemming et al., 2016). EPS have been classified based on their proximity to cells. When they are tightly attached to the cell surface are termed as cell-bound EPS (capsular EPS), and when loosely attached to the cell surface are referred to as free-EPS or slime, which may be released into the surrounding media as depicted in Figure 1.4 (Eboigbodin and Biggs, 2008).

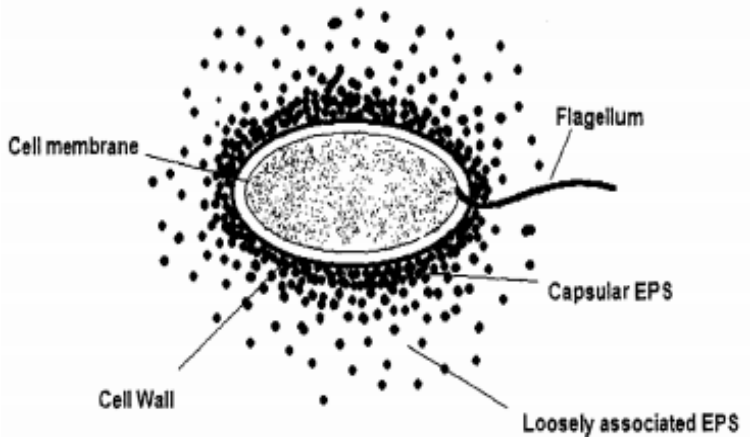


Figure 1.4 Bacteria cell with capsular and loosely associated extracellular polymeric substances (EPS) (Source: Jain A, 2008).

The EPS are mainly composed of polysaccharides, and the current studies have shown that along with proteins, considerable amounts of other

macromolecules such as lipids, DNA and humic acids are also found (Davey and O'Toole, 2000; Flemming and Wingender, 2001; Flemming et al., 2007; Flemming and Wingender, 2010; Flemming et al., 2016; Neu and Lawrence, 2016; Sutherland, 2016). EPS surrounding the cell membranes, protect the cells from the external environmental stress, and the ionic nature of the capsules helps nutrients to accumulate near bacteria (Weiner et al., 1995; Flemming et al. 2016; Neu and Lawrence, 2016). Moreover, EPS also plays an important role in the formation and maintenance of microbial aggregates (Zeng et al., 2016). Most of the studies have elaborated on characterizing the cell-free EPS and its importance in biofilm formation from different bacteria and habitats (Khandeparker et al., 2002, 2003; D'Souza et al., 2005; Saravanan and Jayachandran, 2008; Kavita et al., 2013, 2014). Furthermore, the physicochemical properties of the environment can influence the biofilms or change the biological properties of the cells within the biofilms, leading to the final stage of biofilm cycle which is the detachment process (Moore et al., 2000; Kaplan, 2010).

In the final stages of the biofilm cycle, the escape of clumps of cells, or the release of individual cells from biofilms occurs via desorption, detachment, and dispersion (Figure 1.5). Desorption involves the transfer of individual bacteria to the surrounding medium. Detachment includes external forces, such as erosion, sloughing, and abrasion or else larger organisms feeding on the biofilms can indirectly cause detachment of aggregates (Moore et al., 2000; Stoodley et al., 2001; Petrova and Sauer, 2016). The dispersion, which is an active process, involves physiological changes that facilitate the release of cells from the biofilms to the surrounding (Petrova and Sauer, 2016). Moreover, the dispersal events are regulated by several inter- or intracellular signalling mechanisms, including quorum-sensing systems. An increase in the levels of the intracellular messenger, cyclic-di-GMP [(Bis-(3'-5')-cyclic-dimeric guanosine monophosphate)], determines the transition of a microbial cell from planktonic phase to biofilm growth, while a reduction causes biofilm dispersal (Römling et al., 2013; Sisti et al., 2013, Chua et al., 2014, Ha and O'Toole, 2015; Toyofuku et al., 2016; Valentini and Filloux, 2016; Jenal et al., 2017; Armbruster and Parsek, 2018). The single or clusters of escaped cells are then able to attach on to a new

surface (abiotic or biotic) and create new colonies, thus initiating new biofilm formation or merge with existing colonies (Annous et al., 2009; Kragh et al., 2016).

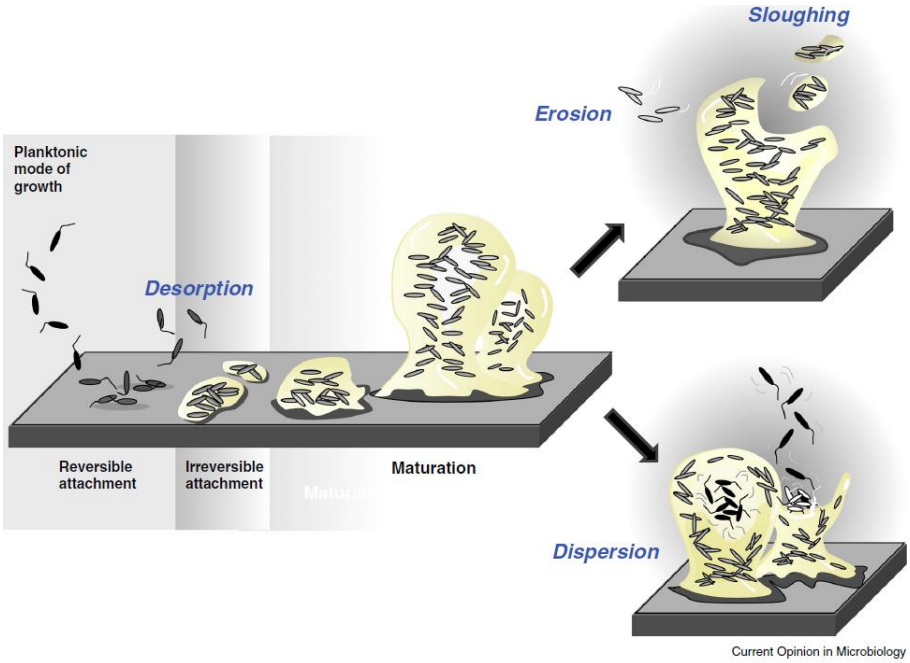


Figure 1.5 Modes of escape from biofilms (Source: Petrova and Sauer, 2016).

Cumulative knowledge and evidence suggest that it is the specific cell-surface, cell-cell, and interspecies interactions (cooperative or competition) that shape up the composition, structure, and functions of biofilm microbial communities (Dang and Lovell, 2016). The enhanced and unique physiological activities of these biofilm-associated microbial communities lay the foundations for biogeochemical functions that can sharply differ from those of free-living (i.e., planktonic) microbial communities. Hence, it can be expected that the microbial communities that exist within the aggregates or biofilms may thrive better in extreme or hostile environments where individual planktonic microorganisms would find the survival, challenging (Karunakaran et al., 2011; Dang and Lovell, 2016).

1.2 Implications of biofilms in aquatic environments

Different kinds of surfaces, whether natural or artificial, are rapidly colonized by microorganisms when exposed to aquatic environments (Salta et al., 2013; Dang and Lovell, 2016). Biofilms are present everywhere in nature and known to have many implications in different environments

(Wilson and Devine, 2003; de Carvalho, 2018; Caruso, 2020). Bacteria along with the diatoms and other microorganisms are responsible for the formation of microfouling, thus serving as cues for the adhesion of larger organisms such as algae, mussels, and barnacles, leading to macrofouling (Khandeparker et al., 2002, 2003; Cao et al., 2011; Hadfield, 2011; Dobretsov and Rittschof, 2020; Rajitha et al., 2020 and references within). As a result, they contribute to detrimental effects, such as biofouling (Salta et al., 2013), biocorrosion (Dang et al., 2011) leading to the deterioration of the surfaces, including ship hulls, interior ballast tanks, etc., and other aquatic establishments (pipelines, cooling towers and heat exchangers of nuclear power plants), and economic loss. Some of the examples include, added fuel consumption incurred through excessive frictional drag on the vessel caused by fouled ship hulls, the impaired heat transfer efficiency of the heat condensers due to fouling, increased fluid resistance due to clogging of cooling water intakes (nuclear power plants), and biodeterioration of submerged materials (e.g., corrosion of metals), etc. (Characklis, 1973; Nair and Venugopalan, 1996; Melo and Bott, 1997; Walker, 1998; Rao, 1999; Hall-Stoodley et al., 2004; Rao, 2012; Di Pippo et al., 2018).

Many studies have been carried out in the area of biofilm research pertaining to initial adhesion, and biochemical-molecular characterization (Bhosle et al., 1989; Venugopalan et al., 1994; Bhosle and Wagh, 1997; D'Souza and Bhosle, 2003; D'Souza et al., 2005; Bhosle et al., 2005; Jain and Bhosle, 2009). Earlier studies have also reported the role of substrate type (Sharma et al., 1990; Devi et al., 1995; D'Souza and Bhosle, 2003; Sekar et al., 2004; Dobretsov et al., 2013; Masó et al., 2016; Briand et al., 2017; Balqadi et al., 2018, etc.), fouling diatoms on submerged surfaces (Mitbavkar and Anil, 2000; Patil and Anil, 2005; Kanavillil et al., 2012), and bacteria-diatom interactions with respect to *V. cholerae* (Rehnstam-Holm et al., 2010; Khandeparker et al., 2014) in the biofilm formation. Studies have also reported the role of protozoans and grazing on biofilms (Chavez-Dozal et al., 2013; Kanavillil and Kurissery, 2013; Scherwass et al., 2016; Raghupathi et al., 2018), and cues responsible for settlement of biofouling organisms (Kirchman and Mitchell, 1983; Khandeparker et al., 2002, 2003, 2006; Qian et al., 2007; Huggett et al., 2009; Hadfield et al., 2011; Khandeparker and

Kumar, 2011; De Gregoris et al., 2012; Rosenhahn and Sendra, 2012; Murthy et al., 2013; Thiyagarajan et al., 2013; Sahoo and Khandeparker, 2018; Antunes et al., 2019; Khandeparker et al., 2019, Siddik and Satheesh, 2019; Dobretsov and Rittschof, 2020; Peng et al., 2020; Rajitha et al., 2020; Vijayan and Hadfield, 2020).

Like any other submerged surfaces, these biofilms are also formed on the interior walls of the ship's ballast water tanks (Figure 1.6). Other than ballast water, these ballast tank biofilms have also been implicated in the transport of microorganisms and pathogens, i.e., microbial invasions (Drake et al., 2005; 2007). Moreover, these microbial biofilms are also responsible for the transmission of harmful or pathogenic microorganisms and their genetic determinants (Cottingham et al., 2003; Huq et al., 2008). However, little is known about the ecological consequences of these biofilms on the environment and the risks involved, as their release through the ship's ballast water can cause or is likely to cause, harm to the environment (ecology), which is an additional concern.

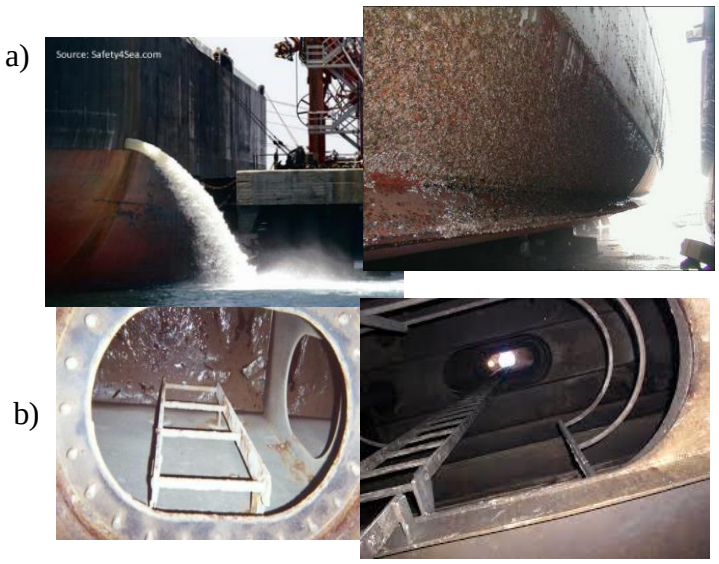


Figure 1.6 Images showing (a) clean and fouled surface of a vessel, (b) interior of an ocean-going ship's ballast tank (Sources: https://www.environmental-expert.com/products/harsonic-for-the-hull-of-vessels-674499, https://www.usgs.gov/media/images/ballast-tank-ocean-going-ship).

Transportation of aquatic microorganisms can occur either naturally through currents, tides, storms, or climatic changes or else through human-mediated dispersal. Over past decades, many species have been transported

around the world at speeds which would not be possible without any anthropogenic influence. Shipping known to be the most cost-effective and convenient mode of transportation serves as an important vector for the unintentional spread of aquatic organisms. The movement of ships requires the intake of ballast, mainly used for the stability of the vessel. Ballast is any material that can be either solid or liquid, including sediment, placed in a ship to regulate the stability or to maintain stress loads within acceptable limits (National Research Council, 1996). The concept of ballast is not novel and has been followed since ancient times. In the olden days, the sea-going vessels used solid ballast such as sandbags, rocks, iron blocks, etc. which were loaded/unloaded by hand once the cargo loading or discharge operation was done (Carlton, 1985; Minchin et al., 2009) (Figure 1.7).

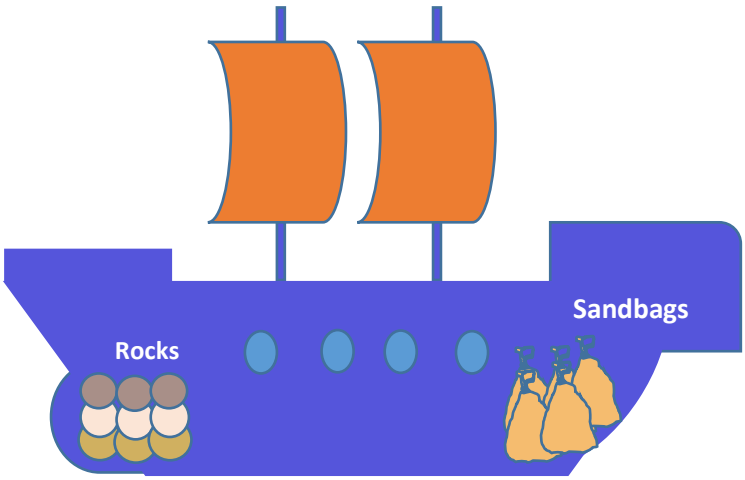


Figure 1.7 Ancient method of ballasting using solid ballast (Source: reproduced from www.marineinsight.com).

The shipping industry started using ballast water back in the 1880s (Gollasch et al., 2000a; Minchin et al., 2009). Since the need is quite variable and can be weather dependent, ships started using water as ballast material, to avoid the dangers and difficulties of solid ballast (Minchin et al., 2009). Water has a good weight-to-volume ratio and is carried in the ballast, or in empty cargo tanks to keep the propellers submerged when the ship is not fully loaded (Gollasch et al. 2000b). Using seawater was a logical evolution since it is free, always present in abundant quantities, and easily handled by means of pumps (De Baere, 2011).

Ballast tanks are filled with ambient water when the cargo is off-loaded from a ship to compensate for the lack of weight. This water contains a gamut of non-indigenous aquatic organisms such as vertebrates, invertebrates, plants, microscopic algae, bacteria including pathogenic types, and their propagules (Drake et al., 2005, 2007; Mimura et al., 2005; Rivera et al., 2013). They serve as onboard aquaria, wherein; many organisms are able to survive in the ballast tanks (Gollasch et al., 2000a; Carney et al., 2011; Desai et al., 2018). The conditions within these tanks are harsh compared to the natural environment and can vary throughout the journey. Moreover, there is complete darkness within the tanks (Carlton, 1985), and since darkness would prevent photosynthesis, it has been suggested that phytoplankton abundance declines within days of uptake. This would then leave only mixotrophic or heterotrophic feeding options (Gollasch et al. 2000a). Thus, 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 ones (Villac et al., 2013). Once the ship reaches the next destination, this ballast water is emptied as the new cargo is loaded on board (Figure 1.8).

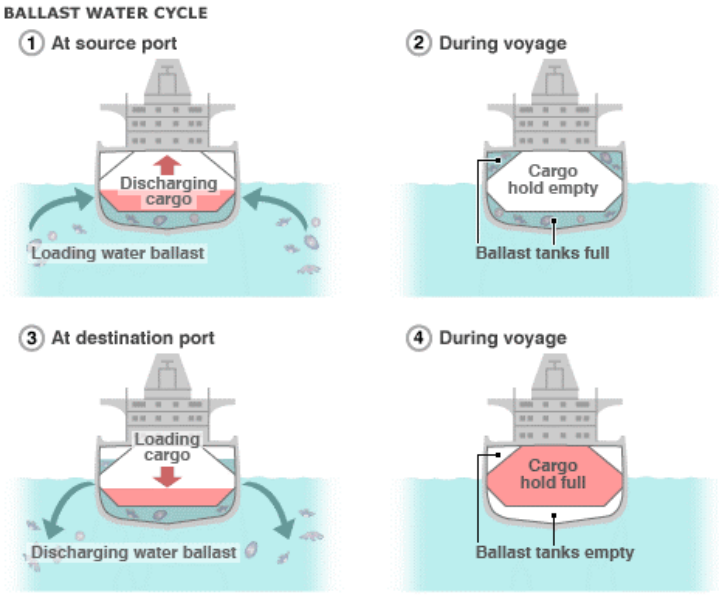


Figure 1.8 Ballast water uptake and discharge via cargo ships (Source: www.globallast.imo.org).

Ballast water has received tremendous attention in recent years and has been recognized as a vector for transfer and introduction of alien aquatic

organisms (i.e., bioinvasion) from one location into new environments, and approximately 12 billion tons of ballast water is annually transported world-wide, thereby threatening the aquatic biodiversity (Anil et al., 2002; Carlton et al., 2011; Khandeparker and Anil, 2017; Hess-Erga et al., 2019). Although the ballast water is discharged every time, the tanks still harbour biofilms, which are formed on the interior walls, and little is known about the response of the biofilm organisms to ballast tank conditions, risks involved in translocation of these biofilm microorganisms and their impact on the environment. These ballast tank biofilms comprise of rich microbial communities harbouring pathogenic bacteria, wherein microbial concentrations are ten times greater than the ballast water (Drake et al., 2005, 2007). They act as seed banks, releasing microorganisms (including pathogenic bacteria), leading to an additional risk of microbial invasion if released into the water or sloughed off during ballasting operations (Drake et al., 2005). This can potentially lead to the introduction of new species and pathogens, causing harm to the environment (ecology), including biodiversity by altering community structure, food webs, ecological processes, as well as causing damage to economic sectors (such as fisheries, aquaculture), and affecting human health.

From the ecological perspective, biofilms can reflect the real conditions of the ambient environment and respond rapidly to environmental changes (Kropfl et al., 2006; Xuemei et al., 2010). They are recognized as hot spots where different bacteria, including pathogens, interact with each other and are influenced by various environmental and biological interactions. Biofilms respond more rapidly to environmental changes because of their short life cycle than higher-level organisms. Moreover, biofilms are easy to manipulate under controlled conditions, thus being an ideal system for assessing the impact of changing environmental conditions, which is not feasible in the field. As attached communities are microecosystems where complex interactions conflict, responses to multiple stressors can be different depending on target organisms, and thus, both direct and indirect effects can be inferred. Therefore, it is of utmost importance to understand the response and mechanism(s) of biofilms under different environmental conditions, which may affect the behaviour of microorganisms within that environment.

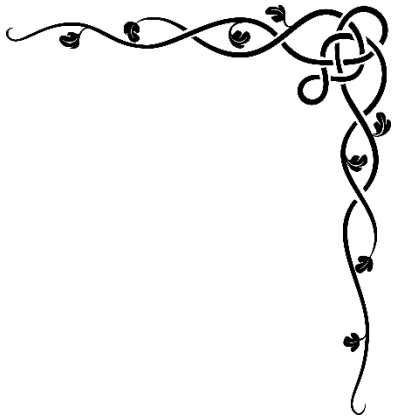
Experimenting with biofilms gives an opportunity to understand the influence of changing environmental characteristics on the biofilm populations in the field conditions through translocation studies.

In view of this, the present study involved many case studies wherein the implications of ballast tank biofilms as a vector of microbial invasion, i.e., translocation of biofilm microorganisms, including pathogens, the potential risk involved and their impact on the environment was investigated. This information is of great interest from the perspective of ballast tank biofilm management issues.

1.3 Objectives of the study

Keeping the above into consideration, the following objectives were proposed,

- ***Evaluation of fouling bacterial diversity in biofilms developed in different environmental conditions.***
- ***Response of biofilm communities to environmental changes.***
- ***Characterize specific compounds produced by chosen marine fouling strains and to identify their role in the biofilm formation.***



Chapter 2

Objective 1: Evaluation of fouling bacterial diversity in biofilms developed in different environmental conditions

2. Evaluation of fouling bacterial diversity in biofilms developed in different environmental conditions

2.1 Introduction

Biofilms are ubiquitous assemblages of surface-associated microorganisms (bacteria, protozoa, archaea, algae, fungi, etc.) enclosed in the extracellular polymeric matrix and can develop on almost all surfaces (natural/artificial) submerged in the aquatic environments (Donlan, 2002; Salta et al., 2013; Dang and Lovell, 2016). Just like any other submerged surfaces, biofilms are also formed on the internal walls of the ship's ballast tanks (Drake et al., 2005). The formation of these ballast tank biofilms depends on the type of fresh inoculum loaded in the ballast tanks which can be either of freshwater, estuarine, or marine origin, depending on the locality of the source port (Carlton, 1985, 2010).

Differing patterns of bacterial distribution exist between fresh and marine, pelagic and benthic waters (Nold and Zwart, 1998; Methé et al., 1998). The marine pelagic zone is dominated by α-proteobacteria and γ-proteobacteria but carries a relatively low abundance of β-proteobacteria, which are predominant in freshwater, and to some extent, estuarine systems where freshwater wedges exist (Bouvier and del Giorgio, 2002; Cottrell and Kirchman, 2003, 2004). In estuaries, Bacteroidetes and α-proteobacteria fractions are dominant, and trends have been observed that both α-proteobacteria and γ-proteobacteria increase as salinity increases, with a decrease in β-proteobacteria (Bouvier and del Giorgio, 2002; Castle and Kirchman, 2004; Eswaran and Khandeparker, 2017). Unlike coastal and estuarine waters that are rich in nutrients and planktonic forms, open ocean waters contain relatively fewer organisms and low nutrient levels (Rigby et al., 1995). However, an earlier study has reported that the nutrient dynamics in the open ocean are mostly influenced by the upwelling of nutrients from oceanic deep water masses, thereby temporarily increasing the concentration of nutrients and facilitating enrichment in the oligotrophic waters (Viner and Wilkinson, 1987). Besides inorganic nutrients, the upwelling processes are also known to bring in the seeding populations of plankton species from deeper waters (Pitcher, 1990). Such enrichment of sea surface waters due to upwelling of nutrient-rich water is a common phenomenon in the Arabian

Sea. The northeastern Arabian Sea experiences high production during the winter monsoon due to such convective mixing process (Banse and McClain, 1986; Madhupratap et al., 1996).

Among different aquatic environments, the biofilms formed in the estuaries are the most dynamic, as estuaries are influenced by tides, anthropogenic activities, and experience a continuous influx of freshwater, resulting in the changes in salinity (Smyth and Elliott, 2016). Our recent study on the biofilm microbial dynamics in the tropical monsoon-influenced estuary indicated that biofilm community characteristics differ with the changing environmental conditions and it also depends on the inoculum characteristics and period of exposure (Khandeparker et al., 2017a). The differences in the biofilm bacterial community structure in aquatic environments from freshwater to marine are often linked with environmental factors such as light, salinity, nutrients etc. (Lee et al., 2016; Mora-Gómez et al., 2016). Other than environmental variables, algal-derived exudates and protozoan grazing also influence the biofilm population dynamics (Huws et al., 2005; Morán et al., 2013). Moreover, the dissolved organic matter released by the algae has long been recognized as high-quality substrates for bacteria (Cole et al., 1982; Morán et al., 2013). On the other hand, protozoans are prey-selective, exhibit species-specific responses, and can significantly alter the biofilm community composition (Huws et al., 2005; Pernthaler, 2005; Dopheide et al., 2011; Yang et al., 2013). Thus, it is expected that the biofilm composition will differ in different environmental conditions.

The conditions within the ballast tanks are different from the natural environment and thus can influence the survival of transported organisms. Ballast tanks are hostile, closed dark environments with no light to support the growth of phototrophs, which subsequently affects the grazers. Unlike some phototrophs, the heterotrophic bacteria can withstand prolonged dark conditions and hence the dark tanks act as incubators for them (Drake et al., 2002). In order to understand the role of biofilms as a vector, it is very important to look into the patterns of biofilm bacterial communities across environmental gradients, such as those encountered during transport in the dark ballast tanks. In fact, there are fewer reports on the

biofilms developed in the dark conditions, such as those present in the condensers and heat exchangers of the nuclear power plants, in pipes or benthic biofilms. These studies have focused on culturable and unculturable microbial community composition of biofilms via DGGE profiles and biochemical characterization (Rao, 1999; Choi et al., 2010; Romaní et al., 2014; Tsagkari and Sloan, 2018). With the development of culture-independent methods such as quantitative polymerase chain reaction (qPCR), a rapid quantitative community-level assessment of microbes has become much easier (Bilodeau, 2011; Kim et al., 2013) and was used in the present study.

The present investigation addressed three case studies for unravelling the patterns of biofilm bacterial communities across the environmental gradients. Three separate experiments were carried out for assessing the bacterial communities in the biofilms developed in different aquatic environments (marine, freshwater and estuarine) under darkness, reflecting conditions which occur in the ballast tanks (closed dark environments) and compared with in situ conditions. In Expt. I, an attempt has been made for the first time to develop biofilms in the open ocean environment, off the coast of western India. The marine biofilms were developed in the enriched (mixture of surface and bottom water, simulating upwelled nutrient-rich deeper water) and unenriched (nutrient-poor surface water) dark condition in an onboard deck experiment. In addition, the marine biofilms were also developed in the in situ condition. In Expt. II, the in situ and laboratory experiments were performed simultaneously on freshwater biofilms developed in a navigable port of Kolkata (eastern India). Whereas, in Expt. 3, the in situ and laboratory experiments were performed on the estuarine biofilms developed in the two most important estuaries of Goa, i.e. Mandovi and Zuari estuary. It was hypothesized that the patterns of biofilm bacterial communities would differ in the different aquatic environments, developed under aphotic/dark conditions, leading to the elimination of less tolerant communities, leaving behind the most adaptable ones, in comparison to the photic conditions.

2.2 Materials and methods

2.2.1 Description of the study area and sampling

2.2.1.1 Experiment I: Marine biofilms

The study was carried in an open ocean environment (15° 51.482' N, 072° 43.511' E) off the coast of western India in the Arabian Sea during cruise CRV *Sindhu Sankalp* 072 (SSK 072) in November 2014. The location of the marine station for the development of biofilms is depicted in Figure 2.1. 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 depth of the water column was 200 m. Before deployment, the glass slides (25.4 mm x 76.2 mm) were cleaned as described by Bhosle et al. (2005). 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.

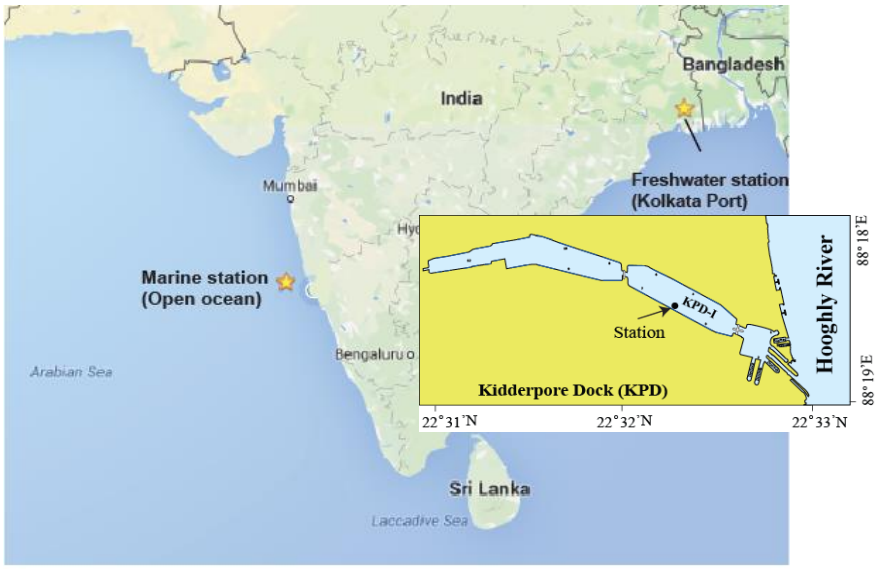


Figure 2.1 Map showing the location of the sampling sites. Inset figure showing freshwater sampling station located at Kidderpore dock (KPD-I) across the Hooghly estuary.

A schematic representation of the experimental design is depicted in Figure 2.2. In the Experiment Ia, the biofilms were developed in the in situ marine open-ocean environment by suspending the clean pre-treated glass slides (n = ~100 slides) into the sea (subsurface water) of the station and anchored outside to the ship for 5 days (natural light/dark cycle). These

biofilms are termed as in situ marine biofilms. This initial period of biofilm development (5 days) was used to achieve a heterogeneous biofilm community with sufficient biomass to carry out the microbiological analyses. For the experiment, a set of forty-nine slides could be retrieved on day 5, while the remaining slides were lost due to the harsh open ocean environment. For the subsequent experiments, the same number of slides (n = 49) were used for the microbiological analyses, as described below.

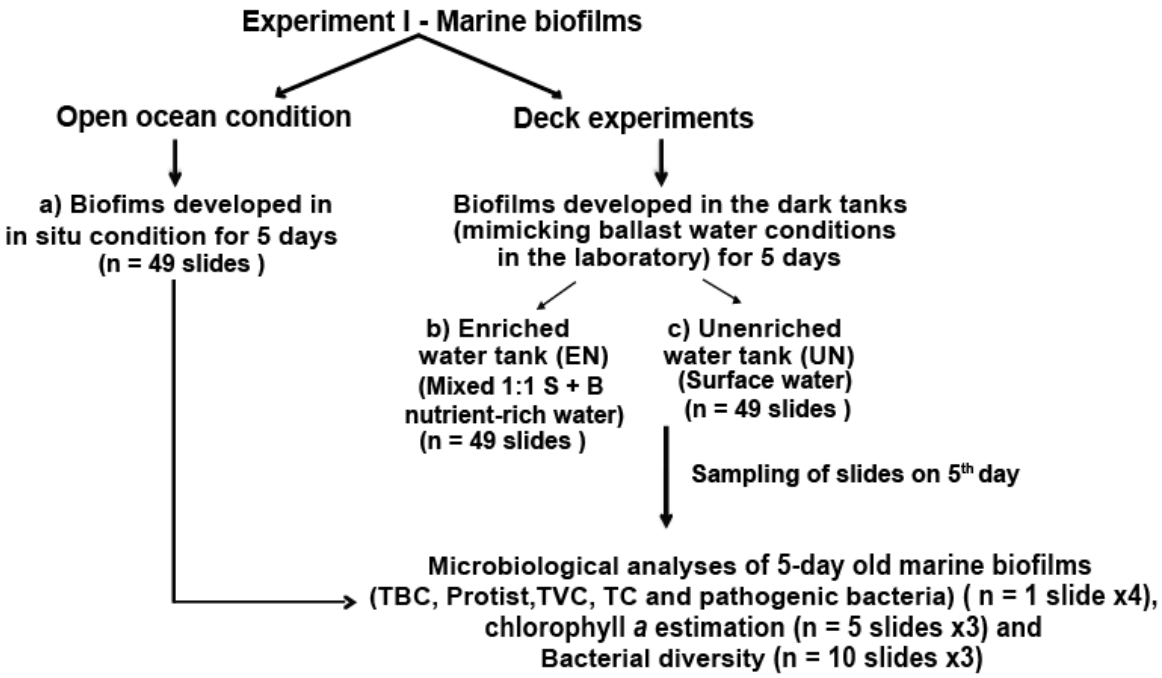


Figure 2.2 A schematic representation of the experimental design of Experiment I. a) In situ marine biofilms, b) Enriched marine biofilms, c) Unenriched marine biofilms, n = Number of slides, S = Surface, B = Bottom, TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms.

For microbiological analyses, four slides were sampled, and each one was scraped separately into 10 mL of 0.22µm filtered autoclaved seawater for microbial counts (n = 1 slide x 4 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 the total bacterial count and protists. For bacterial diversity, thirty slides were sampled (n = 10 slides x 3 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

slides x 3 replicates) for chlorophyll *a* content wherein five slides were scraped and pooled using 20 mL of 0.22 µm filtered autoclaved seawater.

The deck experiments were conducted onboard (Experiment Ib-c), wherein biofilms were developed for 5 days on clean pre-treated glass slides, which were suspended in two different dark tanks (~ 80 L capacity) with enriched (a mixture of surface and bottom water, simulating upwelled nutrient-rich deeper water) and unenriched (nutrient-poor surface water) dark conditions to evaluate the effect of nutrients and darkness on the marine biofilm communities. This initial period of biofilm development (5 days) was used to achieve heterogeneous biofilm community with sufficient biomass to carry out the microbiological analysis. For enriched conditions (Experiment Ib), a CTD cast of surface water with minimal nutrients (nitrate - 0.3 µM) and bottom (150 m) nitrate-rich water (nitrate - 25 µM) was mixed (1:1 ratio), simulating upwelled nutrient-rich deeper water, and the final concentration of nitrate was ~7 µM, in which the slides were suspended. The biofilms developed in these tanks are termed as enriched (EN) marine biofilms. In the case of unenriched conditions (Experiment Ic), the second tank was filled with only the 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, and the five-day old marine tank biofilms (n = 49 slides) were harvested from each tank for different microbiological analysis as described above.

2.2.1.2 Experiment II: Freshwater biofilms

The location of the freshwater station for the development of biofilms is depicted in Figure 2.1. It is located in the Kidderpore Dock area (22° 32.45994' N, 088° 18.95370' E) at Kolkata port across the Hooghly estuary in West Bengal, India. This station is an inland port environment which is exclusively freshwater in origin; wherein all the commercial activities (crude oil, chemicals, automobiles, petroleum products, and several other types of general cargo, such as timber, pulses, coal, and containers) are undertaken (http://www.kolkataporttrust.gov.in).

A schematic representation of the experimental design is depicted in Figure 2.3. Two different experiments (in situ and laboratory) were performed simultaneously during February 2015 in the Kidderpore Dock area at Kolkata port.

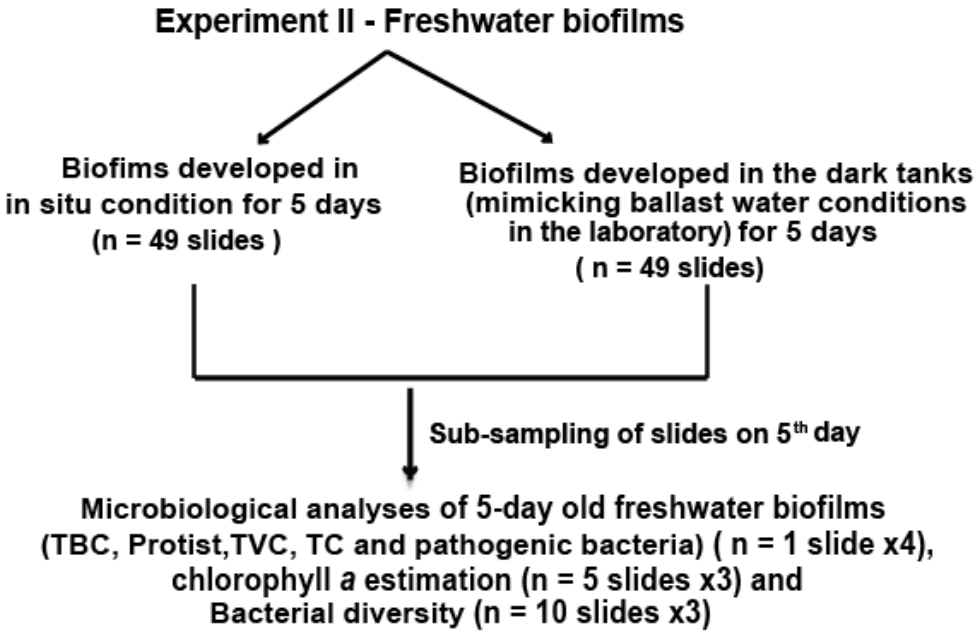


Figure 2.3 A schematic representation of the experimental design of Experiment II. n = Number of slides, TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms.

The pre-treated glass slides were deployed in the freshwater station for the development of biofilms upto 5 days (natural light/dark cycle). These biofilms are termed as in situ freshwater biofilms. Nutrient concentrations were higher in the freshwater; especially, nitrate was 27.35 µM. In the case of the laboratory experiment, a tank (~ 80 L capacity) was filled with the same station water, and the pre-treated glass 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 heterogeneous 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 (in situ and tank) as described above in section 2.2.1.1 but were scraped using 0.85 % autoclaved saline water instead of seawater.

2.2.1.3 Experiment III: Estuarine biofilms

The two most important estuaries of Goa are Mandovi and Zuari, situated along the west coast of India, which are tide driven, and monsoon-influenced tropical estuaries, located along the central west coast of India (Shetye, 1999). The Zuari estuary has a wider mouth region with extended flushing period when compared to the Mandovi estuary (Shetye et al., 2007). The anthropogenic activities have increased in the coastal waters, causing pollution, which has been a global issue (Malham et al., 2014).

The location of the Mandovi and Zuari stations for the development of biofilms is depicted in Figure 2.4. Both these estuarine stations receive inputs from different anthropogenic activities, such as boat traffic (cruising), sewage discharge, agricultural runoff, in addition, the large number of fishing trawlers are close to the Mandovi station. While, the construction of jetties, ship-building, mining, and shipping activities carried out along the Mormugao port situated at Mormugao bay, and waste discharge from the Mormugao sewage treatment plant (STP) are major sources of pollution in the case of Zuari station.

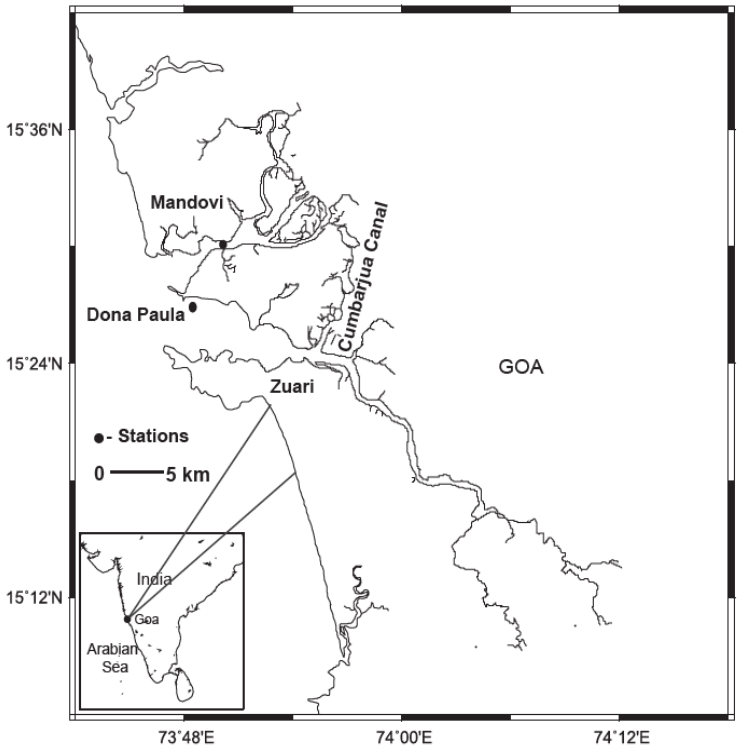


Figure 2.4 Map of study area illustrating sampling locations in the Mandovi and Zuari estuary.

A schematic representation of the experimental design is depicted in Figure 2.5. Two different experiments (in situ and laboratory) were conducted on estuarine biofilms by using two different water inocula for biofilm establishment, one from the Mandovi estuary (15° 30' 17.442" N, 73° 49' 56.2392" E) along the west coast of India, during March 2015 (Experiment IIIa), and the other from the Dona Paula bay (15°27.5′ N, 73°48′ E) which is located at the mouth of Zuari estuary during May 2019 (Experiment IIIb).

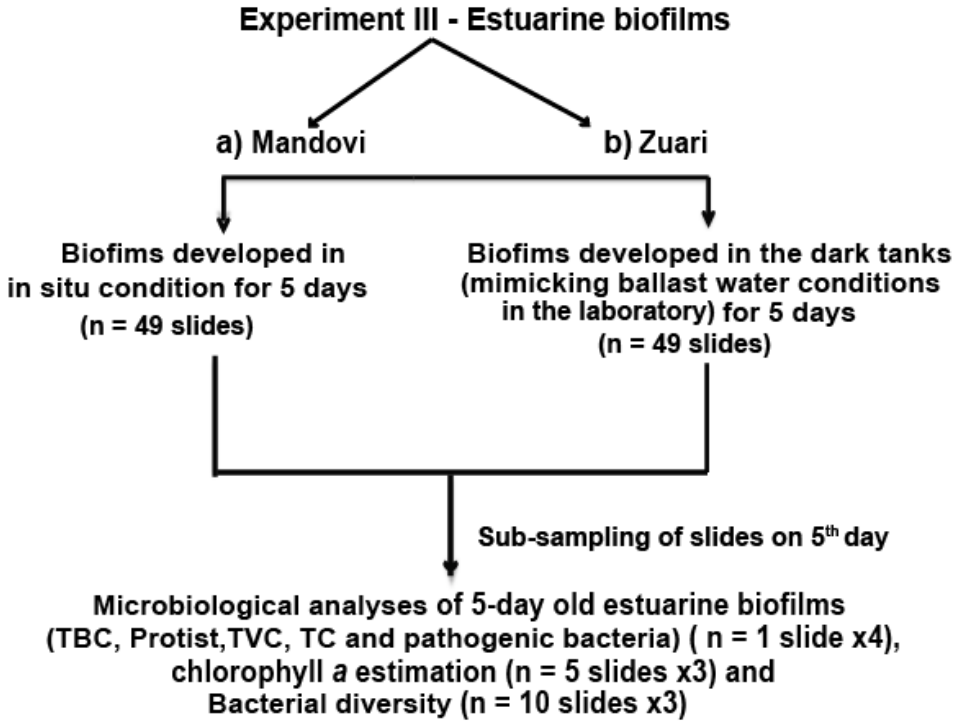


Figure 2.5 A schematic representation of the experimental design of Experiment III. a) Mandovi estuarine biofilms, b) Zuari estuarine biofilms, n = Number of slides, TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms.

In both the experiments, the pre-treated glass slides (25.4 mm x 76.2 mm, Himedia) were deployed in the Mandovi and Zuari stations for the development of biofilms for 5 days (natural light/dark cycle). These biofilms are termed as in situ estuarine biofilms. A laboratory experiment was also carried out wherein, the tanks were filled with the respective station waters, and the pre-treated slides were suspended for a period of 5 days. These biofilms are termed as estuarine tank biofilms. This initial period of biofilm development was used to achieve a heterogeneous 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 until the end of the experiment. The slides with five-day old Mandovi and Zuari estuarine biofilms were sampled from both in situ and tank as described above in section 2.2.1.1.

2.2.2 Physico-chemical 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. For the estuarine water samples, the temperature was measured using portable bucket thermometer, while salinity was measured using Salinometer on Day 0 for both the estuarine stations during the period of deployment. Nutrients (nitrate, phosphate, and nitrite) were analyzed by SKALAR SAN^{plus} ANALYSER and expressed as micromole per litre (µM).

2.2.3 Estimation of Chlorophyll a concentration

Phytoplankton biomass in the water column, in terms of total chlorophyll *a* concentration was assessed by filtering 750 mL of water samples through the GF/F Whatman filters, which were then extracted with 90% acetone overnight and measured following standard methods (Parsons et al., 1984) and expressed as µg/L.

For chlorophyll *a* estimation in the biofilms, 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 the 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 the standard method (Parsons et al., 1984) and expressed as µg/cm^2 of the slide surface area sampled.

2.2.4 Total Viable Count (TVC) and pathogenic bacteria in biofilms

The TVC and pathogenic bacterial analyses were carried out by serially diluting 1 mL of subsamples, which were spread plated (0.1 mL) on four different media, namely Zobell Marine Agar (ZMA) 2216 (marine bacteria), Nutrient Agar (NA) (freshwater bacteria), MacConkey Agar (Total coliforms, TC), and Thiosulphate Citrate Bile Salt (TCBS) Agar (*Vibrio* spp., especially, *V. alginolyticus, V. parahaemolyticus,* and *V. cholerae*). *Vibrio* spp. were distinguished as described by Pfeffer and Oliver, (2003). 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) (Khandeparker et al., 2015, 2017b). ZMA and NA plates were incubated at room temperature, whereas, plates of the other three media were kept at 37 °C. The viable abundance is expressed as colony forming units per square centimetre (CFU/cm^2) of the slide surface area sampled.

2.2.5 Total Bacterial Count (TBC) in biofilms

For the TBC analyses, the samples were kept at - 80 °C until further analysis. These samples were analyzed within 15 days of the sampling date. Before analyses, the frozen samples were thawed, and 1 mL of subsamples were 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). Sample acquisition was set for 10000 events. 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. The flow

rates were calibrated using the equation, R= (Wi- Wf)/ (T x d) where Wi = initial weight of the sample (mg), Wf = final weight of the sample (mg), T = time (minutes), and d = density of the sample (seawater = 1.03, freshwater = 1.00) (Marie et al., 2005). Gating was done against SSC versus green fluorescence. Flow cytometry data were processed using BD FACS Diva software (v.6.2). The TBC is expressed as cells per centimeter square (Cells/cm^2) of the slide surface area sampled.

2.2.6 Protist abundance enumeration in biofilms

Protists were analyzed using a modified protocol by Christaki et al. (2011). For the protists enumeration, the samples were kept at - 80 °C until further analysis. These samples were analyzed within 15 days of the sampling date. Before analyses, 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 (BD 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 per centimeter square (Cells/cm^2) of the slide surface area sampled.

2.2.7 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, USA) and transferred to 70 % ethanol and further stored at - 20 °C until analyses. The samples were extracted and analyzed within 15 days of the 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. (1999); 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 DNA with isopropanol. Both methods showed comparable results. Thus, the kit method was adopted. DNA's of the samples were stored at -20 ºC until further down-stream processing.

2.2.8 Quantification of metagenomic bacterial diversity in biofilms using quantitative PCR

The extracted DNA's from the samples were 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 quantitative PCR. The chosen group-specific primers are shown in Table 2.1, according to previously established real-time PCR protocols (De Gregoris et al., 2011; Ashelford et al., 2002), and corresponding annealing temperatures were standardized in the laboratory. The qPCR assays were designed to measure group-specific bacteria using Rotor-Gene Q (Qiagen). The qPCR standards were prepared by using genomic DNA extracted from different bacterial pure cultures categorized under different taxa (For example, *Alcaligenes feacalis* used for β-proteobacteria, *Vibrio alginolyticus* used for γ-proteobacteria, *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 pure link PCR purification kit (Invitrogen, USA) and pooled to specific concentrations using Bio Spectrometer (Eppendorf). Standard curves were obtained by using these 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.

Environmental DNA's (1 µL) from the biofilm samples (~1.0 - 5.0 ng) were amplified via qPCR with group-specific primer pairs as described by De Gregoris et al. (2011). 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 µL, 10 µL of the master mix, 0.25 µM of each primer, 1 µL of DNA template and made up with nuclease-free water (Ambion^{TM}). 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. Amplification of specific targets observed as the sigmoidal curve was further confirmed by analyses of melt curve. The melting curve analysis of the products (in steps of 0.5 °C for 5s, with temperatures ranging from 75 to 95 °C) was carried out for each qPCR reaction to determine the specificity of amplification and confirm that the fluorescence signals were originated from specific PCR products and not primer-dimers or other artifacts. Data were further processed using Rotor-Gene software (version 2.3.1). The qPCR amplification efficiency (*E*), the threshold cycle (C_T) values and gene copy numbers were calculated using the software. The PCR efficiency was always between 0.98 and 1.0. Resulting values (Raw value, DNA copies/µL) were then $\log_{10}$ transformed. Data were expressed as the gene copy numbers per square centimeter (gene copy numbers/cm^2) of the slide surface area sampled.

Table 2.1 Primers for real-time PCR (De Gregoris et al., 2011, Ashelford et al., 2002*)

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

Temp. = Temperature, α-proteobacteria = Alphaproteobacteria, β-proteobacteria = Betaproteobacteria, γ-proteobacteria = Gammaproteobacteria.

2.2.9 Statistical analysis

The abiotic data were normalized, and the biotic 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 one-way analysis of variance (ANOVA), to determine the significant variations in the bacterial communities between the biofilm types, followed by post hoc Tukey's HSD test (Bonferroni corrected) (IBM statistics, SPSS version 16.0). In addition, Shannon and Simpson's diversity index, which is commonly used as a measure of diversity, was determined using PRIMER software.

The relationship between the environmental variables and biotic factors in the in situ and laboratory experiments was determined by using CANOCO version 4.5 (ter Braak and Verdonschot, 1995). 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 (ter Braak and Smilauer, 2002). Due to the linear character of the data, RDA (Redundancy Analysis) was conducted to assess how environmental parameters influenced the biological parameters. A forward selection was achieved on the set of environmental variables, and the 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 (Leps and Smilauer, 2003). 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 $p \leq 0.05$ (Stat Soft, OK, USA).

2.3 Results

2.3.1 Physico-chemical parameters

The variation in the physico-chemical parameters at the time of slide deployment in the field for different experiments I, II, and III, i.e. marine, freshwater, and estuarine waters indicated that the surface water temperature and salinity were higher in the estuarine waters (Mandovi = 31.2º C and 35; Zuari = 31.9º C and 35) when compared to marine (29.1º C and 35.4), and lower in the freshwater (21.5º C and 0.22). Chlorophyll *a* in the water column varied from 0.046-1.80 µg/L, the concentration was higher in the freshwater (1.80 µg/L) followed by estuarine (Zuari = 0.97 µg/L; Mandovi = 0.42 µg/L) and least in the marine water (0.046 µg/L). Among the studied sites, the phosphate, nitrate and nitrite concentrations were higher at the freshwater site (18.76, 27.35, and 1.53 µM), when compared to the estuarine (Zuari = 3.14, 2.06, and 0.15 µM; Mandovi = 1.09, 1.225, and 0.925 µM), and the marine site (0.658, 0.3, and 0.68 µM), respectively.

2.3.2 Bacterial diversity in biofilms from different environmental conditions

The results showed significant variations in bacterial diversity among the biofilms developed in different aquatic environmental conditions under in situ and dark (aphotic) conditions (Wilk's Lambda, < 0.001). This was also evident from the diversity indices, which were high in the in situ biofilms compared to the tank (dark) biofilms, except for estuarine biofilms as shown in Table 2.2.

Table 2.2 Diversity indices of biofilm types developed in different aquatic environments under in situ and dark conditions.

	S	N	H'	1-λ'
FBF (In situ)	**6**	**53360**	**1.32**	**0.71**
FBF (Dark)	**6**	**13271**	**0.72**	**0.36**
MBF (In situ)	**6**	**8853**	**0.79**	**0.51**
MBF (Dark) EN	**6**	**24532**	**0.33**	**0.15**
MBF (Dark) UN	**6**	**13747**	**0.60**	**0.37**
Z-EBF (In situ)	**6**	**15415**	**1.10**	**0.52**
Z-EBF (Dark)	**6**	**16586**	**1.10**	**0.60**
M-EBF (In situ)	**6**	**33779**	**0.95**	**0.49**
M-EBF (Dark)	**6**	**39632**	**1.09**	**0.58**

FBF = Freshwater biofilms, MBF = Marine biofilms, Z-EBF = Zuari estuarine biofilms, M-EBF = Mandovi estuarine biofilms, EN = Enriched, UN = Unenriched, S = Total number of species in the community, N = Total number of individuals in the sample, H' = Shannon diversity index, 1-λ' = Simpson's diversity index.

2.3.2.1 Experiment I: Marine biofilms

Chl *a* concentration was 0.0101 ± 0.009 μg/cm^2 in the in situ marine biofilms. The viable and pathogenic bacterial abundance (i.e., TVC, TC, *Vibrio* spp.) was low in the in situ biofilms. Whereas, TBC (2.25 × 10^6 Cells/cm^2) and protists (1.53 × 10^5 Cells/cm^2) numbers were high (Figure 2.6). The bacterial community composition in the in situ biofilms was dominated by α-proteobacteria and γ-proteobacteria, while Bacteroidetes, Firmicutes, and Actinobacteria contributed the least (Figure 2.7).

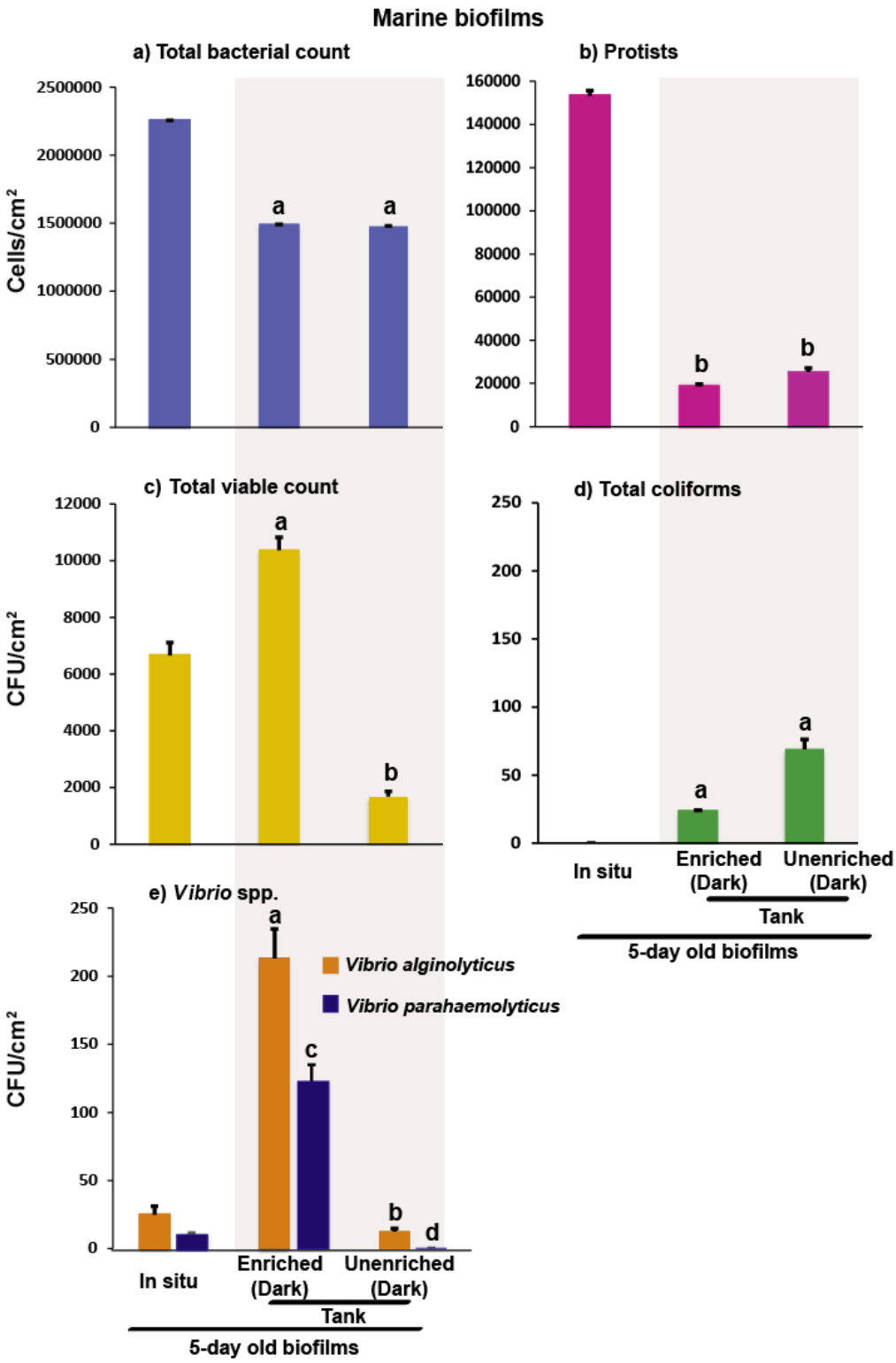


Figure 2.6 Mean values of a) total bacterial count, b) protists, c) total viable count, d) total coliforms, and e) *Vibrio* spp. (*V. alginolyticus* and *V. parahaemolyticus*) in the marine biofilms developed under the in situ and tank (enriched and unenriched dark) conditions for five days. The solid 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 tank (enriched and unenriched) marine biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The shaded area represents dark conditions.

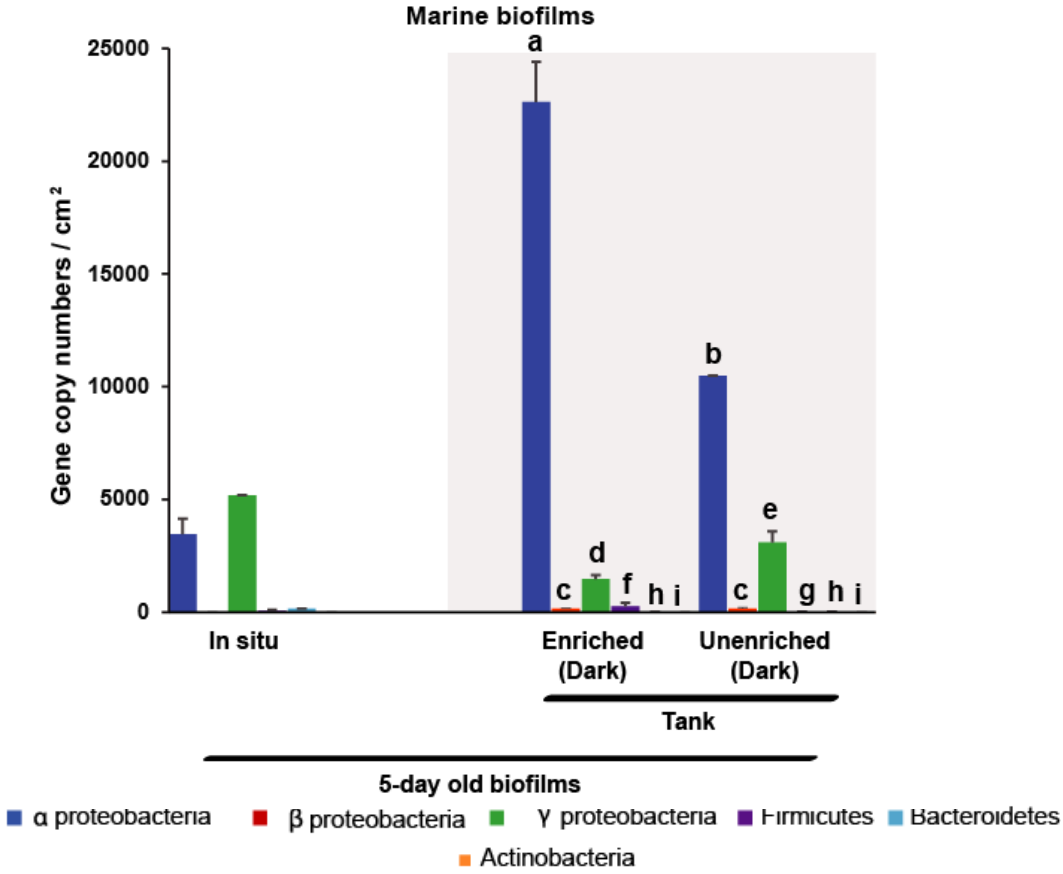


Figure 2.7 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the 5-day old in situ and tank (enriched and unenriched dark) marine biofilms. Values are means of triplicate samples ± SE. Different letters above the bars denote significant differences between the tank (enriched and unenriched) marine biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The shaded area represents dark conditions.

In the case of marine tank biofilms, Chl *a* was comparatively low in the 5-day-old EN (0.00254 ± 0.0001 μg/cm^2) and UN marine tank biofilms (0.00213 ± 0.0001 μg/cm^2). On the other hand, the enrichment factor had a significant effect on the viable and pathogenic bacterial abundance in the tank biofilms (one-way ANOVA, $p < 0.05$). 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, Figure 2.6). A similar trend was observed in the case of *Vibrio* spp., wherein *Vibrio alginolyticus* were abundant in EN biofilms (2.13×10^2 CFU/cm^2) followed by *V. parahaemolyticus* (1.22×10^2 CFU/cm^2) as indicated in the RDA plot (Figure 2.8). On the other hand, the total coliforms were significantly abundant in the UN biofilms ($p < 0.001$, Tukey's HSD).

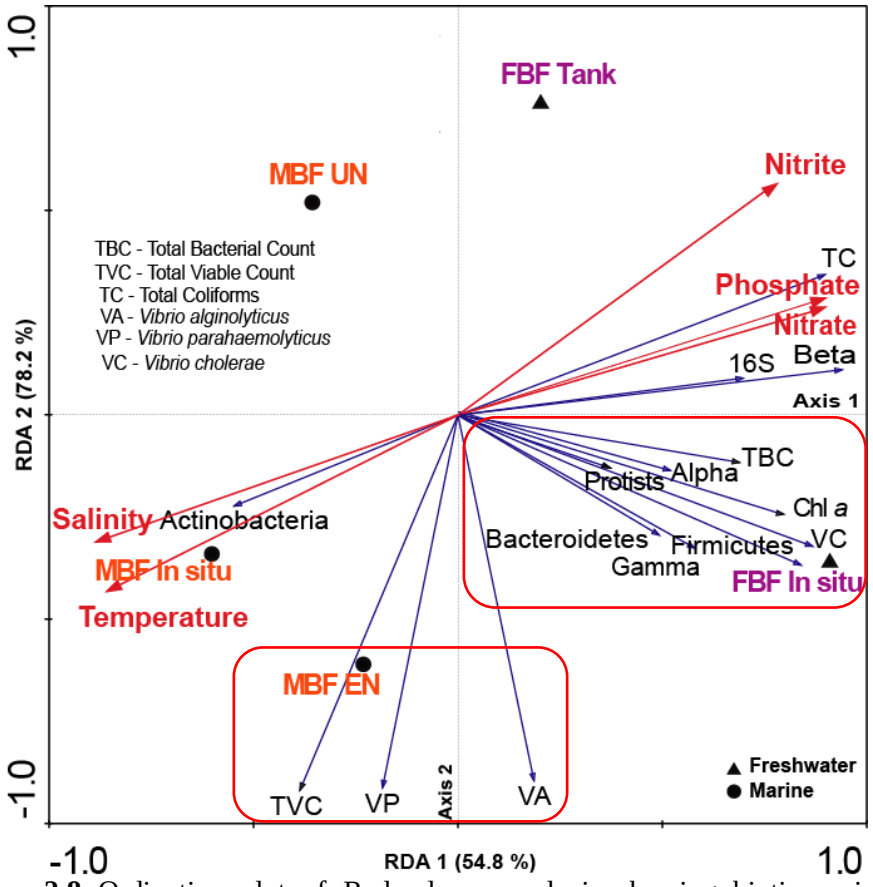


Figure 2.8 Ordination plot of Redundancy analysis showing biotic variables (bacterial species, major bacterial taxa, protists) and their relationship with environmental variables in the marine (●) and freshwater (▲) biofilms developed under in situ and tank (dark) conditions for five days. MBF = Marine biofilms, MBF EN = Enriched marine biofilms, MBF UN = Unenriched marine biofilms, FBF = Freshwater biofilms, Chl *a* = Chlorophyll *a*, Alpha = α-proteobacteria, Beta = β-proteobacteria, Gamma = γ-proteobacteria. Blue and red lines in RDA triplot indicate biotic and abiotic variables, respectively. All data points are averages of several replicates. Red squares highlight significant results ($p < 0.05$).

The enrichment factor had a significant effect on α-proteobacteria, γ-proteobacteria, Bacteroidetes, and Firmicutes (one-way ANOVA, $p < 0.05$, Figure 2.7). The gene copy numbers of α-proteobacteria were dominant in both the EN and UN biofilms (Figure 2.7).

2.3.2.2 Experiment II: Freshwater biofilms

Chl *a* concentration was 0.351 ± 0.001 µg/cm^2 in the in situ freshwater biofilms. TBC (2.98×10^6 Cells/cm^2) and protist numbers (1.47×10^5 Cells/cm^2) were higher in these biofilms (Figure 2.9). Moreover, these in situ biofilms were mainly dominated by total coliforms on day 5 (2.05×10^3 CFU/cm^2). *Vibrio* spp., especially *V. alginolyticus* were abundant (2.21×10^2

CFU/cm^2) followed by *V. cholerae* (7.6 × 10^1 CFU/cm^2) and *V. parahaemolyticus* (6.0 CFU/cm^2) (Figure 2.9). *V. cholerae* showed a significant correlation with chlorophyll *a* in the in situ freshwater biofilms ($p < 0.05$), and this is also clear in the RDA plot (Figure 2.8). On the other hand, the Chl *a* concentration (0.046 ± 0.0003 µg/cm^2) and other microbial parameters (TBC, TVC, and TC) were low in the freshwater tank biofilms, and *Vibrio* spp. were not detected in these biofilms (Figure 2.9).

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter observed in the freshwater biofilms are depicted in Figure 2.10. The bacterial community composition was significantly different with respect to the type of biofilms (ANOVA, $p < 0.05$). γ-proteobacteria were abundant in the in situ freshwater biofilms with high gene copy numbers on day 5 (Figure 2.10). Their abundance was significantly correlated with chlorophyll a ($p < 0.05$) (Figure 2.8). On the other hand, Bacteroidetes showed significant correlation with TBC and protists ($p < 0.05$, Figure 2.8). Whereas, in the case of the tank biofilms, the gene copy numbers of most of the bacterial taxa were low, except for α-proteobacteria (Figure 2.10).

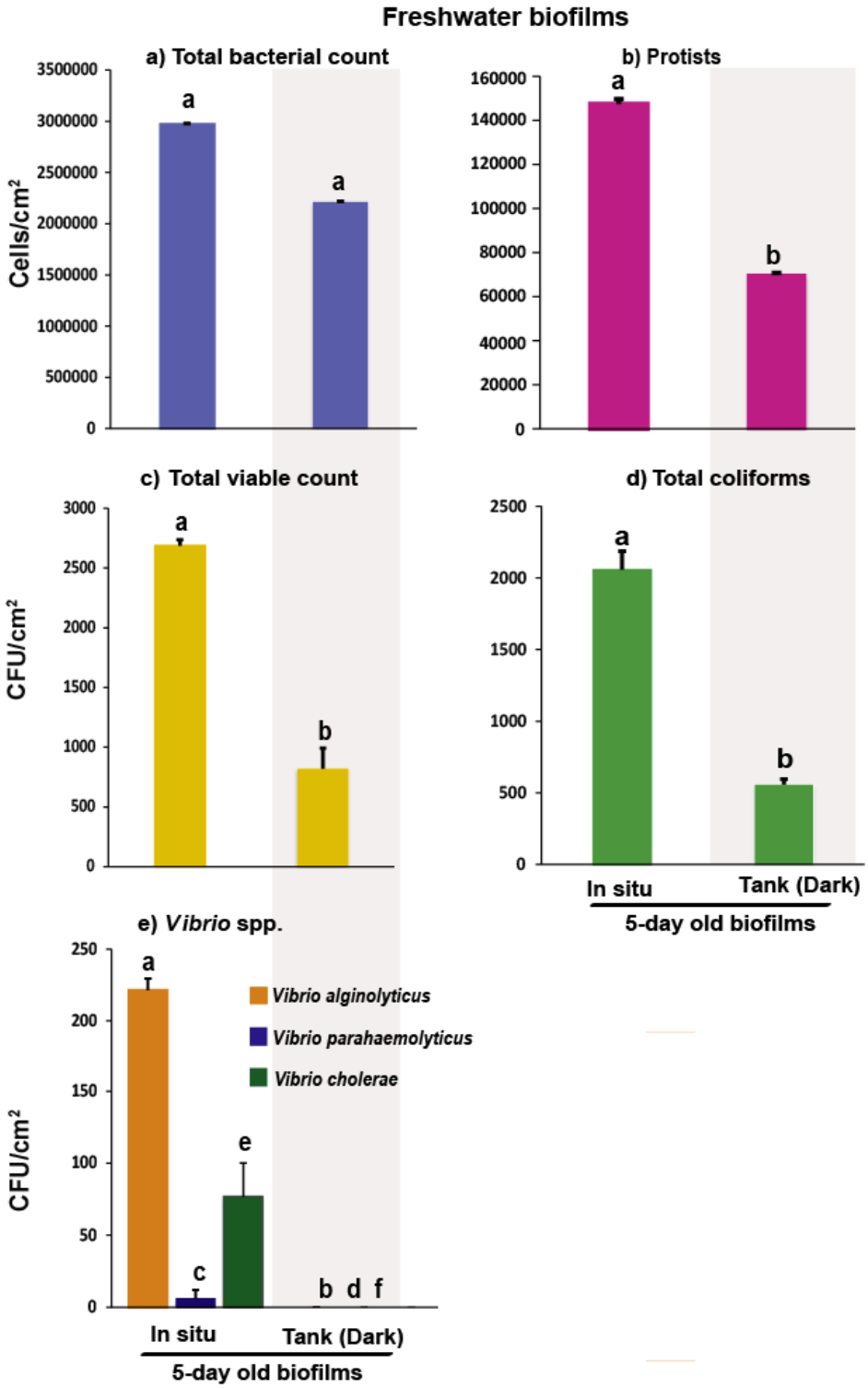


Figure 2.9 Mean values of a) total bacterial count, b) protists, c) total viable count, d) total coliforms, and e) *Vibrio* spp. (*V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae*) in the freshwater biofilms developed under the in situ and tank (dark) conditions for five days. The solid bars represent 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 in situ and tank freshwater biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The shaded area represents dark conditions.

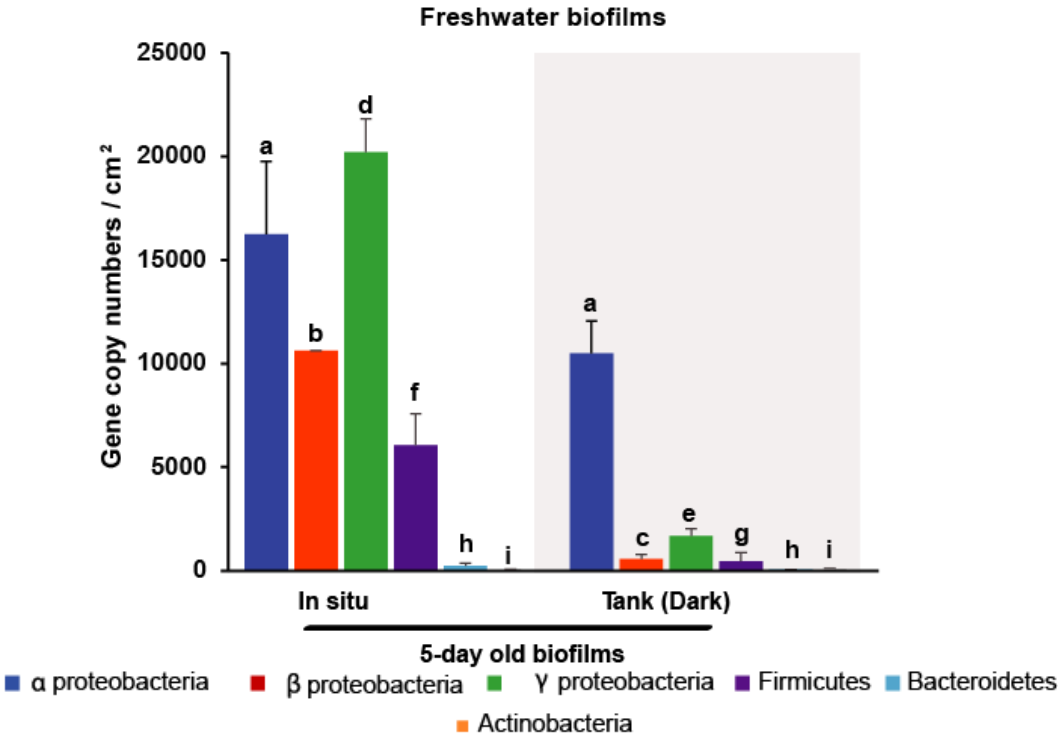


Figure 2.10 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the 5-day old in situ and tank (dark) freshwater biofilms. Values are means of triplicate samples ± SE. Different letters above the bars denote significant differences between the in situ and tank freshwater biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The shaded area represents dark conditions.

2.3.2.3 Experiment III: Estuarine biofilms

(a) Mandovi estuarine biofilms

Chl *a* concentration was 1.2 ± 0.07 µg/cm^2 in the in situ estuarine biofilms and low in the tank biofilms (0.0096 ± 0.0001 µg/cm^2) on day 5. The TBC (3.56 × 10^6 Cells/cm^2), and TVC (9.84 × 10^4 CFU/cm^2) were abundant in the in situ Mandovi estuarine biofilms (Figure 2.11a-b). Moreover, a similar trend was observed in the case of protist abundance (3.88 × 10^5 Cells/cm^2) as well (Figure 2.11c). These biofilms were dominated by *Vibrio* spp., mainly *V. parahaemolyticus* were abundant (1.04 × 10^4 CFU/cm^2) followed by *V. alginolyticus* (8.83 × 10^3 CFU/cm^2) and *V. cholerae* (1.02 × 10^2 CFU/cm^2) on day 5 (Figure 2.11e-f). *V. cholerae* abundance showed a significant positive correlation with nitrite ($p < 0.05$), and this was also evident in the RDA plot (Figure 2.12). In contrast, the microbial parameters (TBC, protist, TVC, and *Vibrio* spp.) were low in the estuarine tank biofilms, except for total coliforms, which did not show any significant change in the abundance (Figure 2.11a-f).

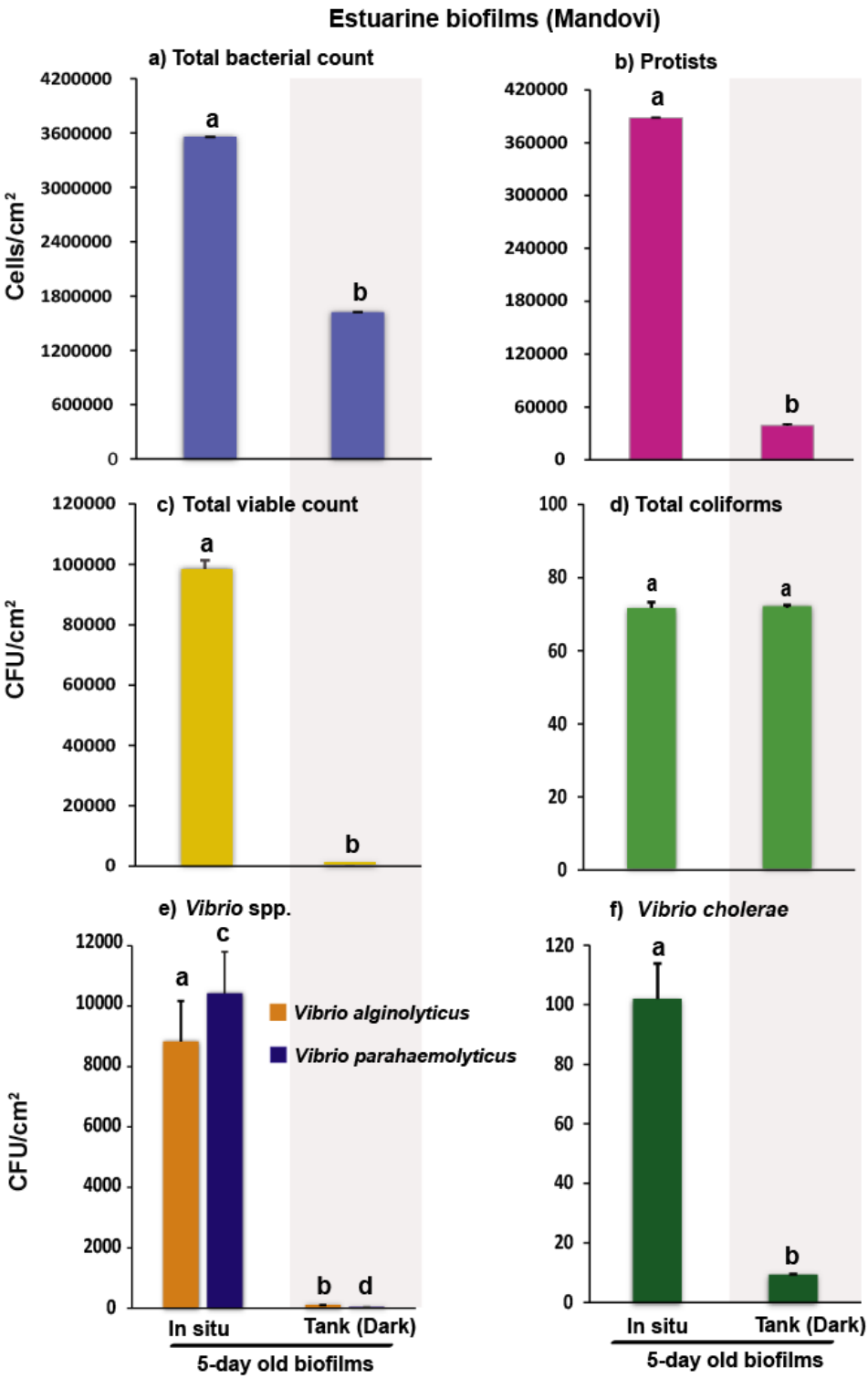


Figure 2.11 Mean values of a) total bacterial count, b) protists, c) total viable count, d) total coliforms, and *Vibrio* spp. e) *V. alginolyticus*, *V. parahaemolyticus*, and f) *V. cholerae* in the Mandovi estuarine biofilms developed under the in situ and tank (dark) conditions for five days. The 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 in situ and tank Mandovi estuarine biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The shaded area represents dark conditions.

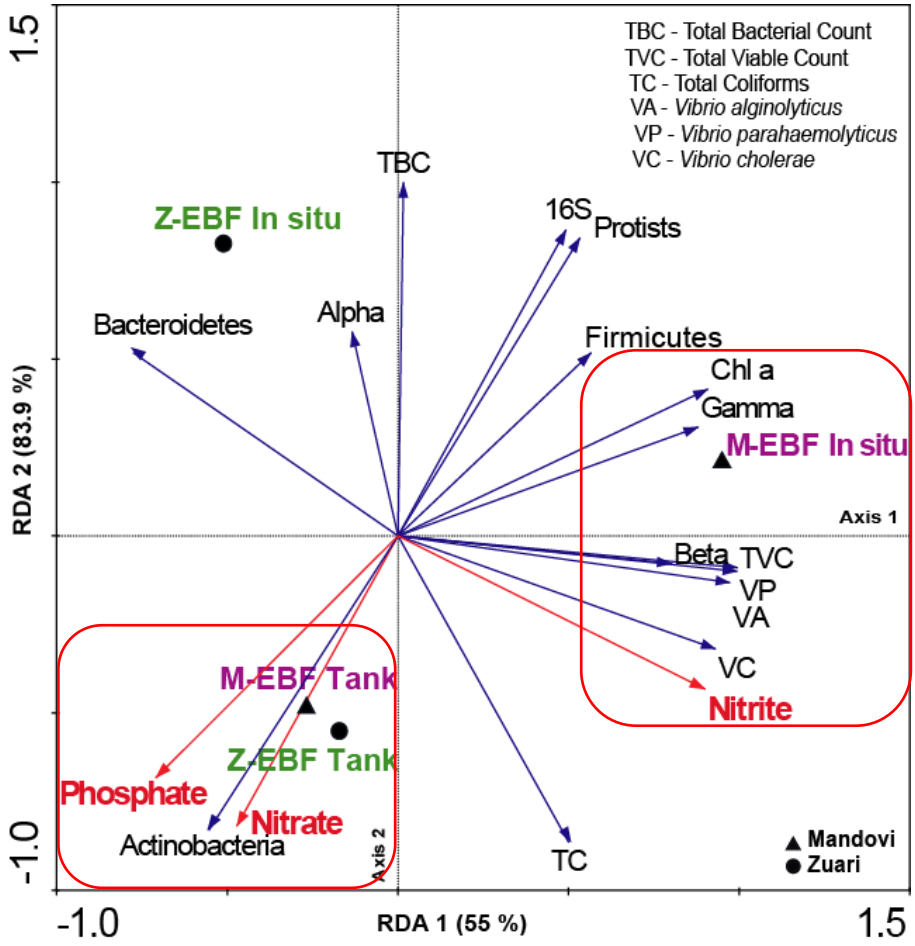


Figure 2.12 Ordination plot of Redundancy analysis showing biotic variables (bacterial species, major bacterial taxa, protists) and their relationship with environmental variables in the Mandovi (▲) and Zuari (●) estuarine biofilms developed under in situ and tank (dark) conditions for five days. M-EBF = Mandovi estuarine biofilms, Z-EBF = Zuari estuarine biofilms, Alpha = α-proteobacteria, Beta = β-proteobacteria, Gamma = γ-proteobacteria, Chl *a* = Chlorophyll *a*. Blue and red lines in RDA triplot indicate biotic and abiotic variables, respectively. All data points are averages of several replicates. Red squares highlight significant results ($p < 0.05$).

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the estuarine biofilms are depicted in Figure 2.13. The bacterial community composition was significantly different with respect to the type of biofilms (one-way ANOVA, $p < 0.05$). γ-proteobacteria were abundant in the in situ Mandovi estuarine biofilms with high gene copy numbers (2.33×10^4 gene copy numbers/cm^2) on day 5 and their abundance showed significant correlation with chlorophyll *a* ($p < 0.05$) (Figure 2.12). On the other hand, high gene copy numbers of Actinobacteria were observed in the estuarine tank biofilms on day 5, and their abundance showed a significant positive correlation with phosphate and

nitrate ($p < 0.05$). This result was evident in the RDA plot as well (Figure 2.12).

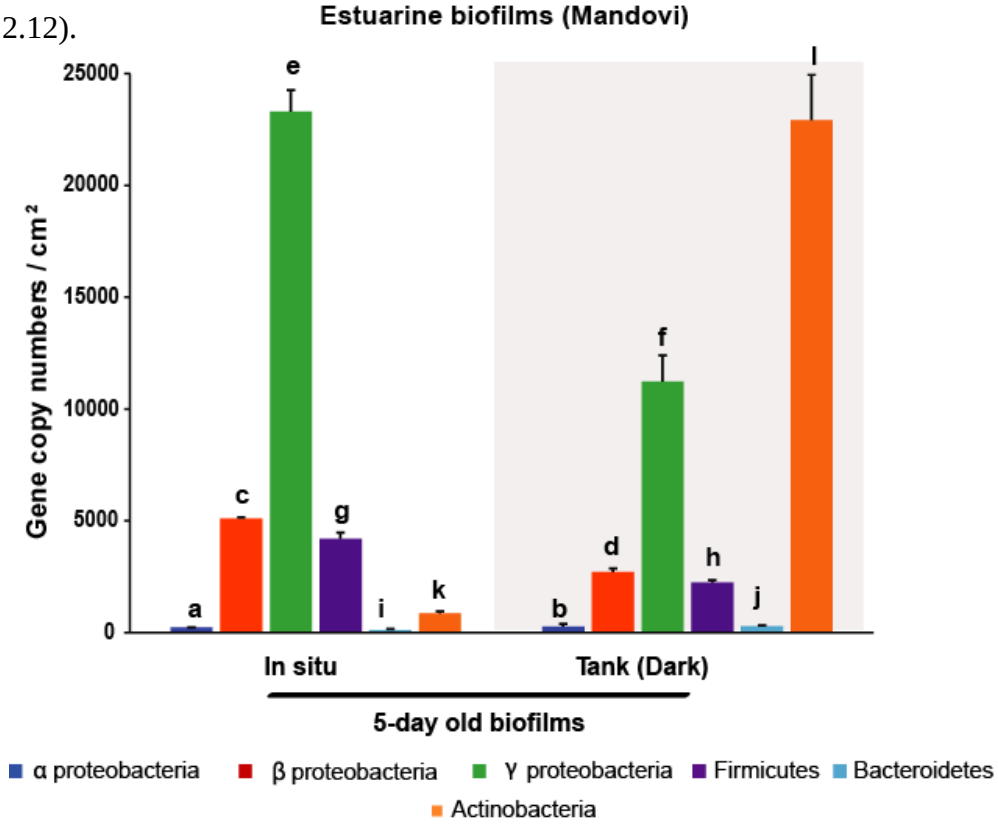


Figure 2.13 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the 5-day old in situ and tank (dark) Mandovi estuarine biofilms. Values are means of triplicate samples ± SE. Different letters above the bars denote significant differences between the in situ and tank Mandovi estuarine biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The shaded area represents dark conditions.

b) Zuari estuarine biofilms

Chl *a* concentration was 0.197± 0.007 µg/cm^2 in the in situ Zuari estuarine biofilms and low in the tank biofilms (0.018 ± 0.0007 µg/cm^2) on day 5. The TBC (6.79 × 10^6 Cells/cm^2) and protists (2.33 × 10^5 Cells/cm^2) were higher in the in situ Zuari estuarine biofilms (Figure 2.14a-b). Although the microbial parameters, i.e. viable and pathogenic bacterial abundance were high in the in situ marine, freshwater, and Mandovi estuarine biofilms, it was interesting to note that the same trend was not observed in the in situ Zuari estuarine biofilms. Unlike in situ biofilms, TVC, TC, and *Vibrio* spp. were abundant in the Zuari tank biofilms on day 5 (Figure 2.14c-f). However, the culturable pathogenic bacteria did not show any significant correlation with any of the abiotic parameters.

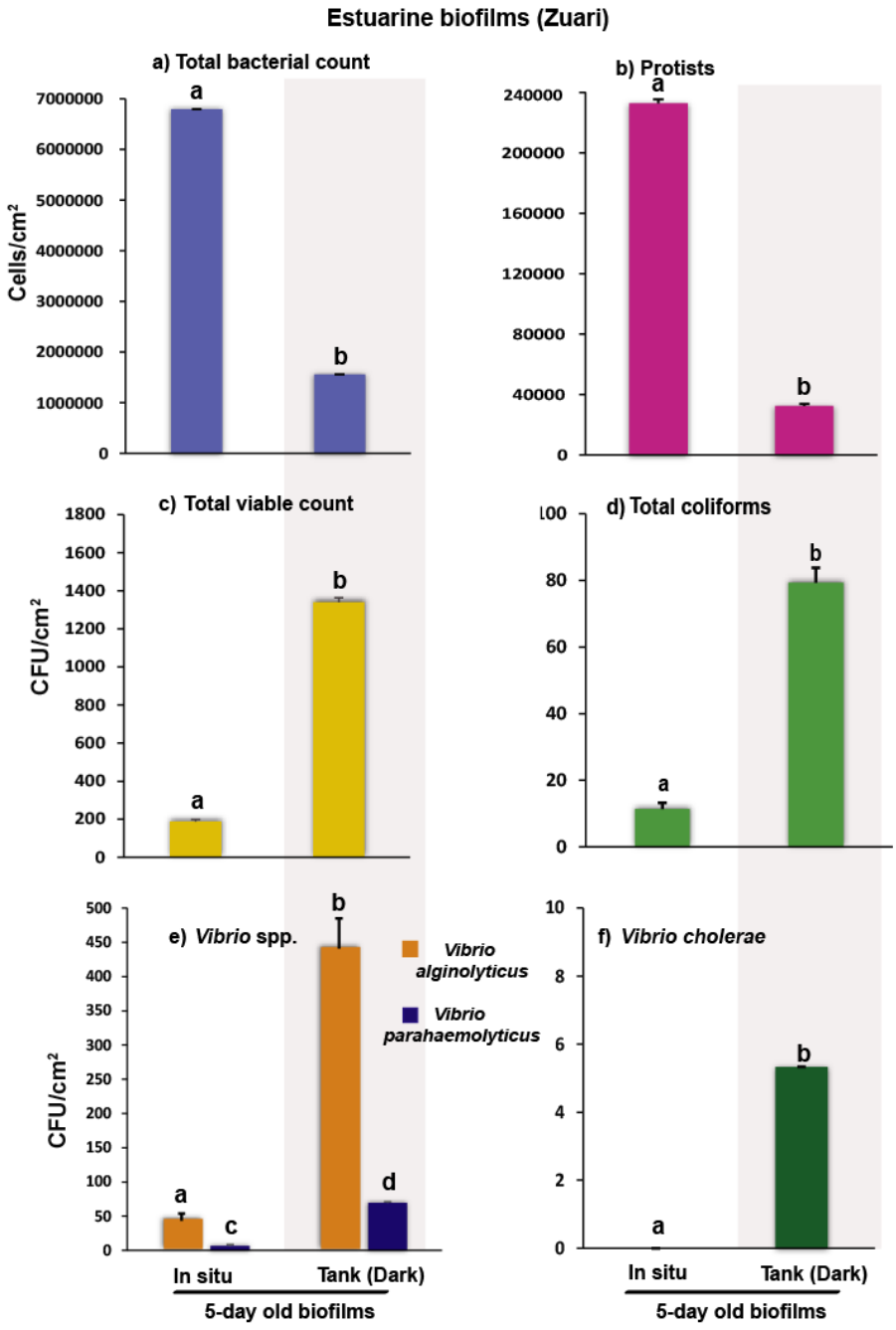


Figure 2.14 Mean values of a) total bacterial count, b) protists, c) total viable count, d) total coliforms, and *Vibrio* spp. e) *V. alginolyticus*, *V. parahaemolyticus*, and f) *V. cholerae* in the Zuari estuarine biofilms developed under the in situ and tank (dark) conditions for five days. The solid 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 Zuari estuarine biofilms (one-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The different letters above the bars indicate significant differences between in situ and tank Zuari estuarine biofilms. The shaded area represents dark conditions.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the estuarine biofilms are depicted in

Figure 2.15. The bacterial community composition was significantly different with respect to the type of biofilms (ANOVA, $p < 0.05$). γ-proteobacteria were abundant in the in situ Zuari estuarine biofilms with 1.03×10^4 gene copy numbers/cm^2 on day 5. On the other hand, γ-proteobacteria and Actinobacteria were dominant microbial taxa in the Zuari estuarine tank biofilms on day 5 ($p < 0.001$, Tukey's HSD, Figure 2.15). Moreover, the gene copy of numbers of Actinobacteria peaked in the Zuari estuarine tank biofilms, and their abundance was influenced by phosphate and nitrate ($p < 0.05$). This result was evident in the RDA plot as well (Figure 2.12).

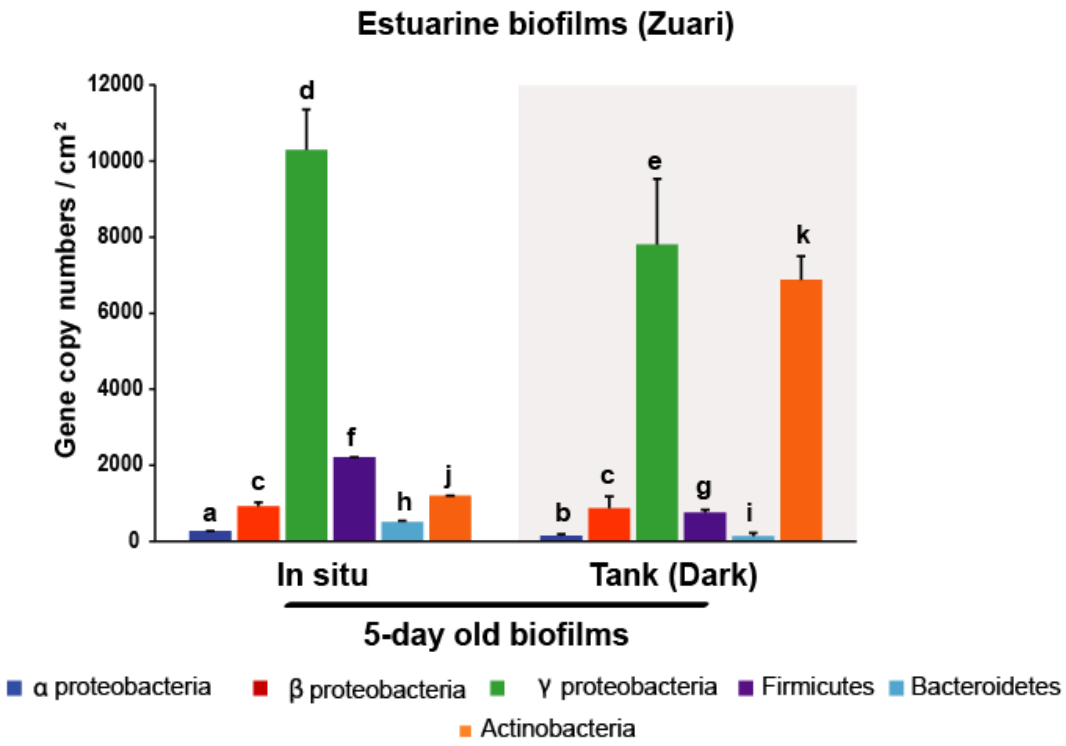


Figure 2.15 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank (dark) Zuari estuarine biofilms during the sampling days (day 5). Values are means of triplicate samples ± SE. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the Zuari estuarine 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.

2.4 Discussion

The present study showed significant variations in the abundance, viability and the bacterial diversity in the biofilms developed in different aquatic environments under in situ and dark (aphotic) conditions. As

expected, the biofilms developed under in situ conditions predominantly consisted of algae (as chlorophyll *a* biomass), and bacteria. These results are in agreement with earlier studies, which also observed an increase in algal biomass (as chlorophyll *a*) and bacterial communities in the light-grown biofilms (Rao et al., 1997; Roman and Sabater, 1999; Sekar et al., 2002). Whereas, dark conditions were mainly dominated by heterotrophic bacteria (Figure 2.16).

In the case of marine biofilms developed under in situ condition, the viable and pathogenic bacterial abundance was low. However, in the case of the biofilms that were developed in the dark marine enriched conditions, the viable bacterial abundance, and *Vibrio* spp. especially *V. alginolyticus*, and *V. parahaemolyticus* were high. Whereas, the total coliforms were high in the case of the unenriched marine condition. An increase in the abundance of *Vibrio* spp., in the enriched marine biofilms could be attributed to the deeper waters which contained *Vibrio* spp., (unpublished result), and also emphasizes on the influence of enhanced nutrient concentrations on this bacterial group. This result is in accordance with previous studies as well (Rehnstam-Holm et al., 2010; Sudhanandh et al., 2010; Lara et al., 2011; Kopprio et al., 2017; Froelich et al., 2019). The *genus Vibrio* spp., belonging to class γ-proteobacteria, comprises of a metabolically diverse group of heterotrophic bacteria that are found in all ocean environments, ranging from coastal to open ocean, and from the surface to the deep waters (Thompson and Polz, 2006 and references within). Moreover, recent studies have also reported that *Vibrio* spp., with broad metabolic and genomic potential, tend to show rapid growth response to nutrient influx (Takemura et al., 2014; Westrich et al., 2016).

Another probable reason for high *Vibrio* spp. numbers could be attributed to the release of these bacteria associated with dead and decayed plankton carcasses due to unfavourable dark conditions. It is well known that *V. cholerae, V. parahaemolyticus*, and even total coliforms are associated with the planktonic copepods and also closely linked with diatoms as well (Huq et al., 1983, 1984; Rehnstam-Holm et al., 2010, Fernandes and Bogati, 2019). An earlier study by Khandeparker and Anil, (2013), also reported that

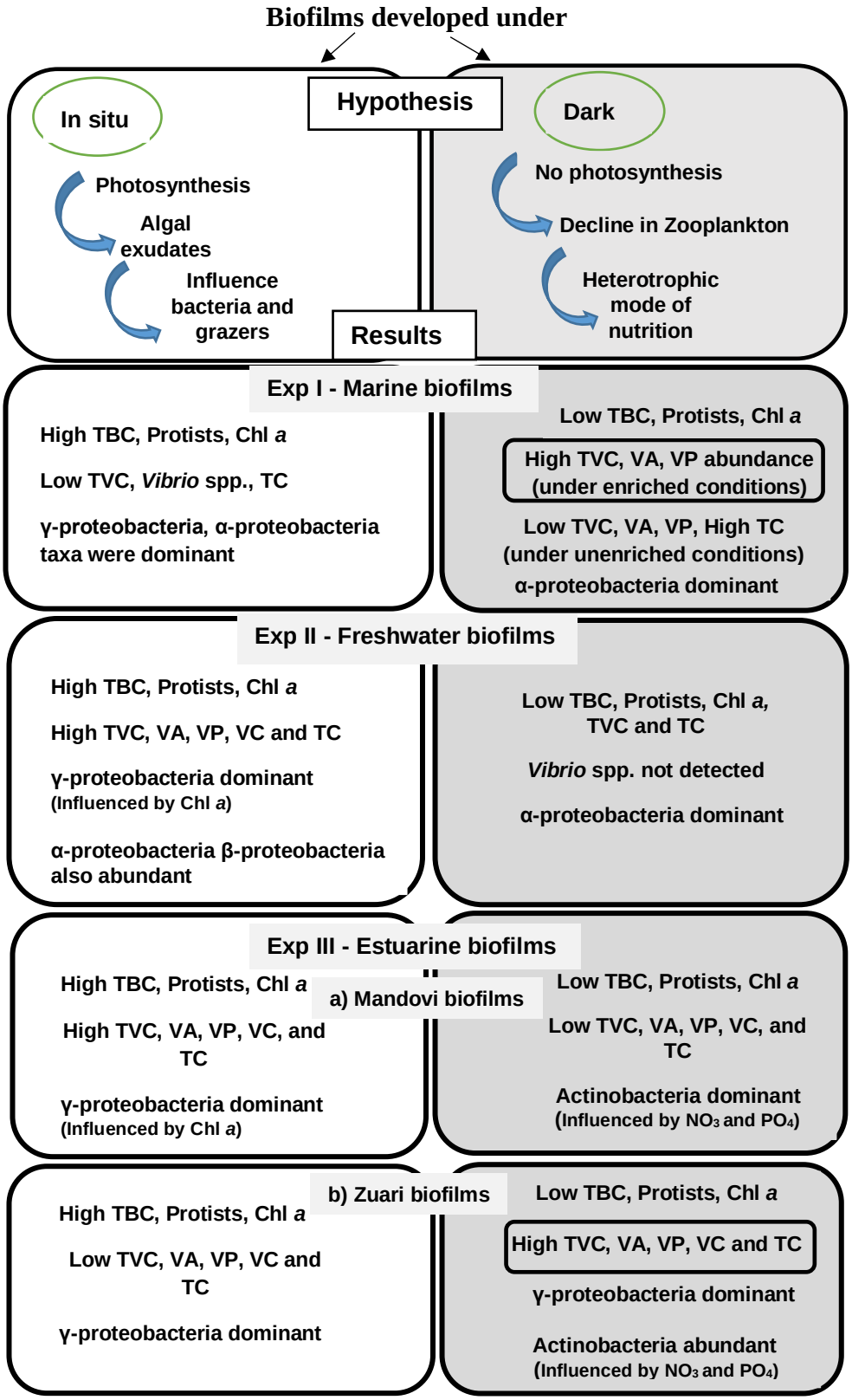


Figure 2.16 A schematic illustration depicting patterns in the response of biofilm microbial communities under in situ and dark conditions (five days) in all the three experiments. Chl *a* = Chlorophyll *a*, TVC = Total Viable Count, TBC = Total Bacterial Count, TC = Total Coliforms, VA = *Vibrio alginolyticus*, VP = *V. parahaemolyticus*, VC = *V. cholerae*, NO_2 = Nitrite, PO_4 = Phosphate. Grey shaded area represents dark conditions.

zooplankton contains a high number of pathogenic bacteria such as *V. cholerae*, *E. coli*, and *S. feacalis*, which emerge from the decomposing plankton contributing to nutrient regeneration and increased bacterial production (Lee and Fisher, 1992). This could also be the probable reason for the presence of total coliforms in the unenriched marine biofilms. Hence, the emergence of virulent pathogenic *Vibrio* species in the marine biofilms under dark conditions and subsequent contamination of ambient water with *Vibrio* spp., as well as their pathogenic forms can be expected, which is a cause of concern. Earlier studies have revealed that the alterations in environmental conditions, especially the nutrient levels or temperature can possibly trigger the expression of virulence genes in the pathogens (Singleton et al., 1982; Miller et al., 1984; Lara et al., 2009). However, their degree of pathogenicity or virulence will depend on environmental conditions. Therefore, future studies should validate the same by addressing virulence gene expression in relation to changes in environmental characteristics under dark conditions.

Another probable reason for high *Vibrio* spp. numbers could be attributed to the release of these bacteria associated with dead and decayed plankton carcasses due to unfavourable dark conditions. It is well known that *V. cholerae*, *V. parahaemolyticus,* and even total coliforms are associated with the planktonic copepods and also closely linked with diatoms as well (Huq et al., 1983, 1984; Rehnstam-Holm et al., 2010, Fernandes and Bogati, 2019). An earlier study by Khandeparker and Anil, (2013), also reported that zooplankton contains a high number of pathogenic bacteria such as *V. cholerae*, *E. coli,* and *S. feacalis*, which emerge from the decomposing plankton contributing to nutrient regeneration and increased bacterial production (Lee and Fisher, 1992). This could also be the probable reason for the presence of total coliforms in the unenriched marine biofilms. Hence, the emergence of virulent pathogenic *Vibrio* species in the marine biofilms under dark conditions and subsequent contamination of ambient water with *Vibrio* spp., as well as their pathogenic forms can be expected, which is a cause of concern. Earlier studies have revealed that the alterations in environmental conditions, especially the nutrient levels or temperature can possibly trigger the expression of virulence genes in the pathogens (Singleton et al., 1982; Miller et al., 1984; Lara et al., 2009). However, their degree of pathogenicity

or virulence will depend on environmental conditions. Therefore, future studies should validate the same by addressing virulence gene expression in relation to changes in environmental characteristics under dark conditions.

The 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 (De Gregoris et al., 2011). The qPCR results indicated that in the case of enriched marine biofilms, α-proteobacteria was an abundant taxon in the dark condition, which was rich in nitrate. While, the relative abundance of other genera was low. A similar trend was observed in the freshwater biofilms developed in the dark; wherein α-proteobacteria was a dominant taxon. These results are in accordance with the copiotrophic hypothesis, which suggests that copiotrophic groups such as Proteobacteria, especially representatives of the *Roseobacter* clade in the α-proteobacteria, are known to exhibit faster growth rates and are more likely to increase in the nutrient-rich conditions in comparison to others having slower growth rates (Harvey et al., 2006; Fierer et al., 2007; Nogales et al., 2011; Buchan et al., 2014; Coutinho et al., 2015; Liu et al., 2017; Wang et al., 2018). Moreover, α-proteobacteria are copiotrophs that are metabolically versatile and capable of rapid growth, indicating they can outcompete other bacteria in utilizing dissolved organic matter (Azam and Malfatti, 2007; Newton et al., 2011; Brown et al., 2014; Yilmaz et al., 2016). These results suggest that the degradation of autotrophic organisms, enhancement of organic matter and enrichment played a significant role in determining the structure of the marine biofilm community composition in dark condition.

In the case of freshwater biofilms, the culturable total coliforms were abundant in the in situ biofilms. The coliforms are known to proliferate in low saline conditions, and are allochthonous organisms, i.e. terrestrial-driven (Karbasdehi et al., 2017; Leight et al., 2018). Moreover, the river biofilms are known to act as a reservoir for total coliforms and can represent the health risk (Balzer et al., 2010). The bacterial diversity was higher in the in situ freshwater biofilms (Proteobacteria (γ-proteobacteria, α-proteobacteria and β-proteobacteria) along with Firmicutes), and was dominated by γ-proteobacteria. Moreover, γ-proteobacteria showed a strong positive

correlation with chlorophyll *a* during the study period. This result points out towards a mutualistic interaction between biofilm-bound microalgal biomass and heterotrophic bacteria within the in situ freshwater biofilms, wherein the biofilm heterotrophs are dependent on algal-derived extracellular organic molecules. Moreover, this algal organic matter is easily consumable, unlike dissolved organic matter in the ambient water, which is mostly high molecular weight and requires extracellular hydrolysis prior to uptake (Roman and Sabater, 1999; Espeland et al., 2001; Ylla et al., 2009; Natrah et al., 2014; Wagner et al., 2015; Dang and Lovell, 2016). Overall, the result suggests that the in situ freshwater bacterial biofilm communities were influenced by the inputs from the phototrophic biomass of the biofilms.

Apart from this, a significant positive correlation between protists and Bacteroidetes taxa suggests protists to play an important role as consumers of this bacterial taxa. The genera belonging to this bacterial group are more susceptible to predation as they are characterized by having higher growth rates than other groups (Kirchman, 2002). Moreover, these results also point out that the protozoans are prey selective and exhibit species-specific responses, and thus are significant in altering the biofilm population dynamics (Huws et al., 2005; Pernthaler, 2005; Dopheide et al., 2011; Yang et al., 2013; Weitere et al., 2018). Alternatively, the dark exposure led to lower microbial parameters (i.e. TBC, viable, and pathogenic bacteria) in the freshwater biofilms suggesting that the microbes might have entered an inactive or viable but non-culturable state (VBNC) (a survival strategy). Moreover, it is known that microbes can survive hostile environments in an inactive state until they reach a suitable habitat with favourable conditions for growth (Ramette and Tiedje, 2007). Also, the dark-grown freshwater biofilms had lower bacterial diversity, dominated by α-proteobacteria and the bacterial communities were compositionally distinct from the in situ freshwater biofilms.

In the case of in situ Mandovi estuarine biofilms, high numbers of culturable *Vibrio* spp., i.e. *V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*, belonging to γ-proteobacteria, were observed, when compared to biofilms developed under dark conditions. This could be attributed to a load of pathogenic bacteria, faecal coliforms, and nutrients, which have

tremendously increased in rivers, estuaries and coastal areas due to anthropogenic activities via sewage discharges, and agricultural runoff (Nagvenkar and Ramaiah, 2009; Pradhan and Shirodkar, 2009; Maya et al., 2011; Shirodkar et al., 2012; Malham et al., 2014). Among *Vibrio* spp., *V. cholerae,* which were abundant in the in situ Mandovi estuarine biofilms, showed a strong correlation with nitrite, which could be due to nitrate reduction. *Vibrio* spp., are known to play a significant role in nitrate reduction in marine environments (Philippot, 2005; Lara et al., 2011), resulting in the reduction of nitrate to nitrite. These species are ubiquitous in the brackish or limnic waters and are autochthonous to estuarine ecosystems, which act as a reservoir for human infections (Tamplin et al., 1990; Colwell, 2000; Yildiz and Visick, 2009; Islam et al., 2020 and references within).

The qPCR analyses also showed the dominance of γ-proteobacteria (includes most of the pathogenic bacteria) in the in situ Mandovi estuarine biofilms, and a strong positive correlation was evident with chlorophyll *a*, which could be attributed to their close association with the diatoms. Several studies have demonstrated that the Proteobacteria and Bacteroidetes (aided by their ability to degrade complex organic matter) are the main heterotrophic bacterial phyla associated with diatoms (Grossart et al., 2005; Sapp et al., 2007; Guannel et al., 2011; Amin et al., 2012). Within these phyla, specific genera (e.g., *Vibrio, Alteromonas,* and *Flavobacterium*) appear to be strongly associated with the diatoms (Rehnstam-Holm et al., 2010; Buchan et al., 2014; Khandeparker et al., 2014; Dang and Lovell, 2016). This result suggests that these Mandovi estuarine biofilm communities developed in the in situ conditions were dependent on the inputs from the phototrophic biomass of the biofilms.

When these Mandovi estuarine biofilms were developed in the dark conditions, the culturable *Vibrio* spp., i.e. *V. alginolyticus, V. parahaemolyticus,* and *V. cholerae,* belonging to γ-proteobacteria, reduced in numbers. It is possible that these culturable *Vibrio* spp. existed in a viable but non-culturable (VBNC) state during the dark conditions along with the plankton, as they were either not detected or low in numbers in the biofilms. It is a survival strategy attained by many bacteria, especially *V. cholerae,* in response to harsh environmental conditions until they reach a suitable habitat

with favourable conditions for growth (Ramette and Tiedje, 2007). Moreover, several human pathogenic bacteria enter into the viable but non-culturable state (VBNC) by reducing their cell size and cannot be recovered using culture media, although they retain their viability and virulence (Colwell, 1985; Colwell et al., 2000; Oliver, 2010; Ramamurthy et al., 2014). However, vice-versa was observed in the case of Zuari estuarine biofilms.

The viable and all culturable pathogenic bacterial numbers were low in the in situ biofilms. However, an increase in the abundance of culturable *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae,* as well as total coliforms, was observed when these biofilms were developed under dark condition. This could be attributed to two possible reasons, (1) these biofilm communities were adaptive to unfavourable dark conditions, and were stable in these conditions, as indicated by diversity indices (Table 2.2), suggesting that these estuarine biofilm communities were less sensitive to dark condition, and (2) it is well known that *V. cholerae, V. parahaemolyticus*, and even total coliforms are associated with the planktonic copepods and also closely linked with diatoms as well (Huq et al., 1983, 1984; Rehnstam-Holm et al., 2010, Fernandes and Bogati, 2019). Moreover, their growth could have been influenced by the decline in the phytoplankton and zooplankton populations due to unfavourable dark condition. An earlier study by Khandeparker and Anil, (2013), has reported that zooplankton contains a high number of pathogenic bacteria such as *V. cholerae, E. coli* and *S. feacalis*. *Vibrio* spp. are ubiquitous across aquatic environments and are biogeochemically important members of the estuarine environments (Froelich et al., 2019). Besides, they are important pathogens for humans (*V. cholerae* and *V. parahaemolyticus*) and fish (*V. alginolyticus*). Thus, it is possible that these bacteria associated with decayed plankton carcasses were released in response to the unfavourable dark condition. Overall, these results suggest the unique ecology of *Vibrio* spp. with different response to dark conditions in both these estuaries, in spite of being geographically closely located but needs further validation.

However, the total bacterial diversity evaluated through qPCR indicated that both the estuarine biofilms, dominated by γ-proteobacteria, changed to Actinobacteria under the dark conditions. Actinobacteria are one of the

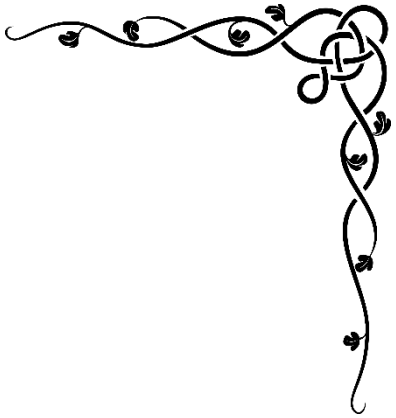
largest bacterial phyla with high G+C content containing Gram-positive bacteria that are ubiquitously distributed in both aquatic and terrestrial ecosystems (Barka et al., 2016). The lifestyles and ecology of these bacteria are diverse, and thus can survive in most of the aquatic habitats, including open-ocean, coastal, estuarine, as well as within a ballast tank (Neyland, 2009). This taxon consists of large phylum and plays a major role in the carbon cycling, recycling of organic matter and nutrient regeneration (Lacey, 1978; Glöckner et al., 2000; Ventura et al., 2007; Hill et al., 2011). Moreover, they wait for other organisms to die and eat up dead organisms, thus acting as scavengers. This was evident from the significant positive correlations observed between Actinobacteria and nitrate as well as phosphate concentrations, irrespective of estuarine biofilm types, during the study period. Another probable reason for their dominance in these estuarine biofilms could be attributed to the lesser preference of grazers on them. Previous studies have reported that Gram-positive bacteria are known to be consumed at significantly lower rates by protists (Iriberri et al., 1994; Pernthaler, 2005). Moreover, the consumption of naturally occurring Gram-positive Actinobacteria is selectively avoided by heterotrophic nanoflagellates (HNFs) as deduced from the analysis of protistan food vacuoles (Jezbera et al., 2004, 2006; Ballen-Segura et al., 2017).

Apart from this, it was interesting to note that α-proteobacteria was a minor component in both the in situ and dark estuarine biofilms, even though it is one of the most abundant, comprising the majority of free-living bacteria and well-studied clades of bacteria in the marine environment (DeLong et al., 1993). Moreover, this group, despite being known to share many metabolic and ecological traits, was apparently not competitive in the estuarine biofilms.

2.5 Conclusions

In conclusion, the estuarine biofilms were mainly dominated by culturable. *Vibrio* spp., whereas total coliforms were more prevalent in the freshwater biofilms. The estuarine and marine biofilm communities were less sensitive and could sustain under dark conditions when compared to freshwater biofilm communities. γ-proteobacteria, which includes most of

the pathogenic bacteria, were dominant in all the three biofilm types but showed major contribution in the estuarine biofilms. Among estuarine biofilms, high numbers of *Vibrio* spp., (*V. alginolyticus, V. parahaemolyticus, V. cholerae*), and total coliforms were detected in the Zuari biofilms under dark conditions. A similar trend was observed in the enriched marine biofilms, except for total coliforms and *V. cholerae*. Moreover, the bacteria that can survive in the dark, and travel by "hiding out" in biofilms would persist within the tank environment and proliferate. When such biofilms loaded with pathogenic forms if present, e.g. in the interior ballast tanks or sloughed off as vector, can possibly contribute to their shedding into the new environment, which is a cause of concern. Thus, in addition to unravelling patterns of biofilm bacterial communities across the environmental gradients; it is also important to understand their response to changing environmental conditions by simulating conditions within ballast tanks (i.e. prolonged darkness and ageing) during a voyage and elucidating their impact on the environment, and the same has been addressed in the subsequent chapters.



Chapter 3

Objective 2: Response of biofilm communities to environmental changes

3. Response of biofilm communities to environmental changes

3.1 Introduction

Shipping carries over 90% of the world's cargo and transfers approximately 12 billion tons of ballast water (BW) around the world every year, mainly used for vessel stability (Tamelander et al., 2010). At the beginning of a voyage, the water loaded in the ballast tanks, either marine, freshwater, or estuarine origin, contains a gamut of nonindigenous organisms such as vertebrates, invertebrates, plants, microscopic algae, bacteria including pathogenic types, and their propagules, etc. (e.g. Williams et al., 1988; Cohen and Carlton, 1998; Ruiz et al., 2000; Drake et al., 2005; Mimura et al., 2005; Drake et al., 2007; Rivera et al., 2013). 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 (To and Jiamin, 2002). 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 (e.g. Roszak et al., 1983; Hallegraeff and Bolch, 1992; Dickman and Zhang, 1999; Gollasch et al., 2000a; Anil et al., 2002; Carney et al., 2011; Wu et al., 2019).

Microorganisms are found in several locations within a ship that includes ballast water, residual sediment, and biofilms formed on interior tank surfaces. 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 (Dobbs and Rogerson, 2005; Mimura et al., 2005; Drake et al., 2007; Emami et al., 2012; Rivera et al., 2013; Tomaru et al., 2014), and the role of plankton and their associated microorganisms (Ruiz et al., 2000, Rivera et al., 2013, Khandeparker and Anil, 2013). However, the transport of microorganisms through biofilms as a 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 (Baier et al., 2014).

Biofilms on submerged surfaces are known to harbour pathogenic bacteria (Hall-Stoodley and Stoodley, 2005, Shikuma and Hadfield, 2010). 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 (Drake et al., 2005).

Previous studies have documented the fate and survival of a variety of ballast water organisms such as phytoplankton assemblages, dinoflagellates, zooplankton, and microbial communities during transit (Hallegraeff and Bolch, 1991, 1992, Carlton and Geller, 1993, Rigby and Hallegraeff, 1993, 1994; Subba Rao et al., 1994; Fukuyo et al., 1995; Macdonald, 1998; Gollasch et al., 2000a, b; Klein et al., 2010; Carney et al., 2011; Tomaru et al., 2014; Steichen and Quigg, 2015; Lymperopoulou and Dobbs, 2017; Desai et al., 2018; Thoha and Rachman, 2018; Wu et al., 2019; Wang et al., 2020; Khandeparker et al., 2020). 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 (Villac et al., 2013). BW taken on board can be either freshwater, brackish, estuarine, or marine origin, depending on the locality of the source port. Thus, the bacterial composition in the biofilm 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 (Dagg, 1977; Carlton, 1985; Rigby and Hallegraeff, 1993). 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 (Carney et al., 2011; Villac et al., 2013). Earlier it was hypothesized that the decline in phytoplankton and zooplankton populations due to unfavourable conditions in the ballast tanks, would influence the heterotrophic mode of nutrition, and thus act as incubators for microorganisms (Drake et al., 2002; Saccà, 2015). However,

the study by Drake et al. (2002), 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.

For a successful translocation of organisms through biofilms as a vector of microbial invasion, the biofilm communities have to pass through a series of stages such as their survival in dark ballast tanks during voyages followed by release and their potential to thrive in a new environment. Taking the above points into consideration, experiments were conducted on biofilms developed in the marine (open-ocean), freshwater (Chapter 3A) and estuarine (Chapter 3B) 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.

Chapter 3 is subdivided into three subchapters (3A, 3B, and 3C). In Chapter 3A-3B, the influence of prolonged darkness and ageing on the biofilm microbial communities developed in the marine, freshwater, and estuarine conditions using microcosm experiments have been discussed. Furthermore, Chapter 3C discusses the ecological impact of freshwater biofilms (which were more fragile to darkness and ageing) as a vector of microbial invasion on the estuarine water using microcosm experiments.

3A. Influence of prolonged darkness and ageing on marine and freshwater biofilm microbial communities

3A.1 Experiment I: Marine biofilms

3A.1.1 Sampling site

Details on the study area are explained earlier in Chapter 2, subsection 2.2.1.1.

3A.1.2 In situ and tank biofilms

A schematic representation of the experimental design is depicted in Figure 3A.1.

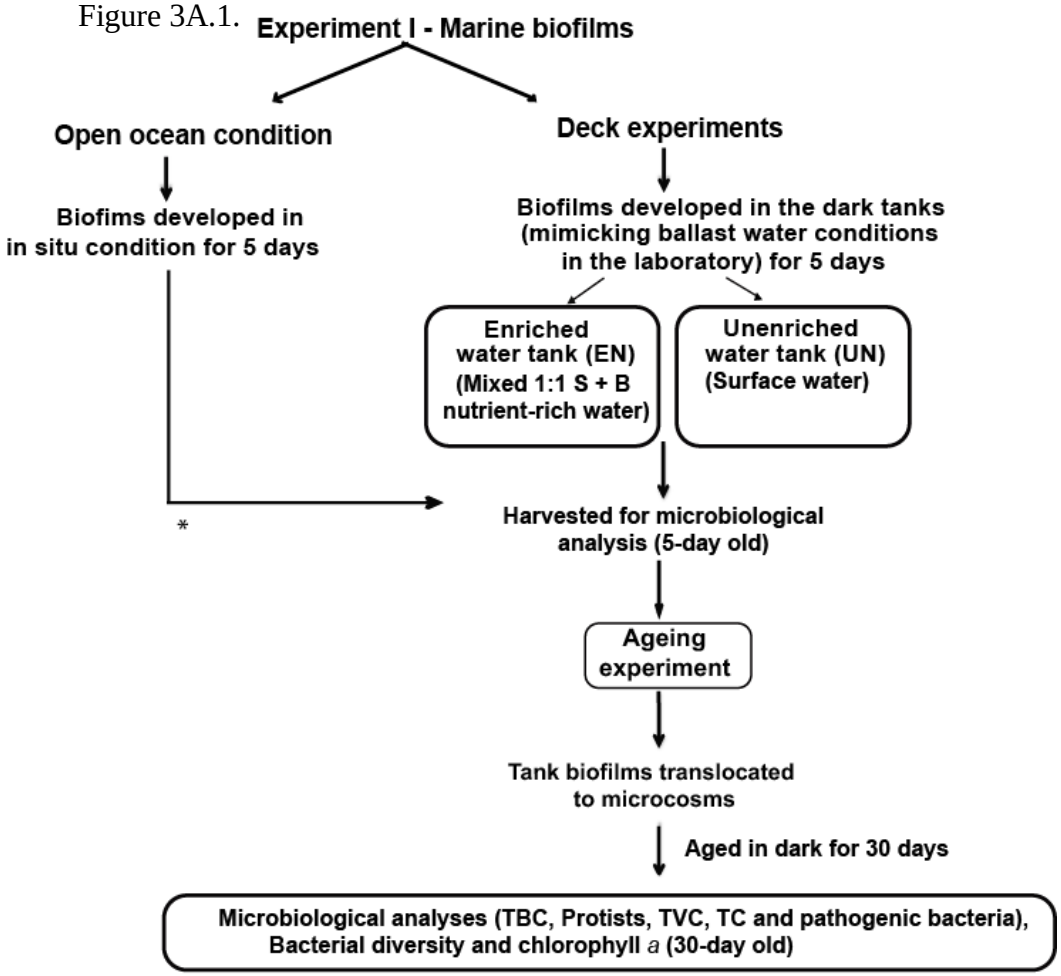


Figure 3A.1 Schematic representation of the experiment I. TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms, S = Surface, B = Bottom. The asterisk (*) indicates that ageing experiment with in situ marine biofilms could not be achieved as slides were lost due to harsh conditions.

The biofilms were developed in the in situ marine open-ocean environment by suspending the clean pre-treated glass slides (n = ~ 100 slides) into the sea (subsurface water) of the station and anchored outside to the ship for 5 days (natural light/dark cycle). These biofilms are termed as in situ marine biofilms. This initial period of biofilm development (5 days) was used to achieve a heterogeneous biofilm community with sufficient biomass to carry out the microbiological analyses.

A set of forty-nine slides could be retrieved on day 5, while the remaining slides were lost due to the harsh open ocean environment. These five-day old biofilms were used for different microbiological analyses. Four slides were sampled, and each one was scraped separately into 10 mL of 0.22µm filtered autoclaved seawater for microbial counts (n = 1 slide x 4 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 the total bacterial count and protists. For bacterial diversity, thirty slides were sampled (n = 10 slides x 3 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 slides x 3 replicates) for chlorophyll *a* content wherein five slides were scraped and pooled using 20 mL of 0.22 µm filtered autoclaved seawater.

For the deck experiments, biofilms were developed for five days on clean pre-treated glass slides, which were suspended in two different dark tanks with enriched (a mixture of surface (S) and bottom water (B), simulating upwelled nutrient-rich deeper water) and unenriched (nutrient-poor surface water) dark conditions. 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 mixed for the experiment (1:1 ratio), and the final concentration of nitrate was ~7 µM, in which the pre-treated slides were suspended. The biofilms developed under these conditions 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 pre-treated slides were suspended. The biofilms are termed as unenriched (UN) marine biofilms. The tanks were concealed to avoid entry of light. The water within the tank was not changed until the end of the experiment. After five days of incubation in the dark, these 5-day old marine tank biofilms were harvested (n = 49 slides) from each tank for different microbiological analyses as described above. While, a separate set of slides (n = 64), with 5-day old biofilms from each of the tank, were used in further experiments, which were conducted to evaluate the influence of prolonged ageing on biofilm microbial communities as described below.

3A.1.3 Translocation experiment

A set of sixteen slides with 5-day old biofilm were retrieved from each tank (enriched and unenriched), and were translocated to four replicate microcosms in the laboratory (n = 64 slides), and further exposed to prolonged darkness for 30 days. These microcosm experiments were carried out to assess the influence of ageing on the enriched and unenriched biofilm communities. The dark conditions would support the incubator hypothesis for the marine biofilm communities or present unfavourable environment. The glass slides with 30-day old biofilms 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. The number of slides sampled for the microbiological analyses were kept constant (n = 49 slides) and analyzed as described above. The ageing experiments with in situ samples could not be achieved as the slides were lost due to harsh sea conditions.

3A.2 Experiment II: Freshwater biofilms

3A.2.1 Sampling site

Details on the study area are explained earlier in Chapter 2, subsection 2.2.1.2.

3A.2.2 In situ and tank biofilms

A schematic representation of the experimental design is depicted in Figure 3A.2.

Two different experiments (in situ and laboratory) were performed simultaneously during February 2015 in the Kidderpore Dock area at Kolkata port. The pre-treated glass slides were deployed in the freshwater station for the development of biofilms for five days. These biofilms are termed as in situ freshwater biofilms. A laboratory experiment was also carried out wherein, a tank (~ 80 L capacity) was filled with the same station water, and the pre-treated slides were suspended for a period of 5 days. These biofilms are termed as freshwater tank biofilms. 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 (in situ and tank) as described above in section 3A.1.2 but were scraped using 0.85 % autoclaved saline water instead of seawater.

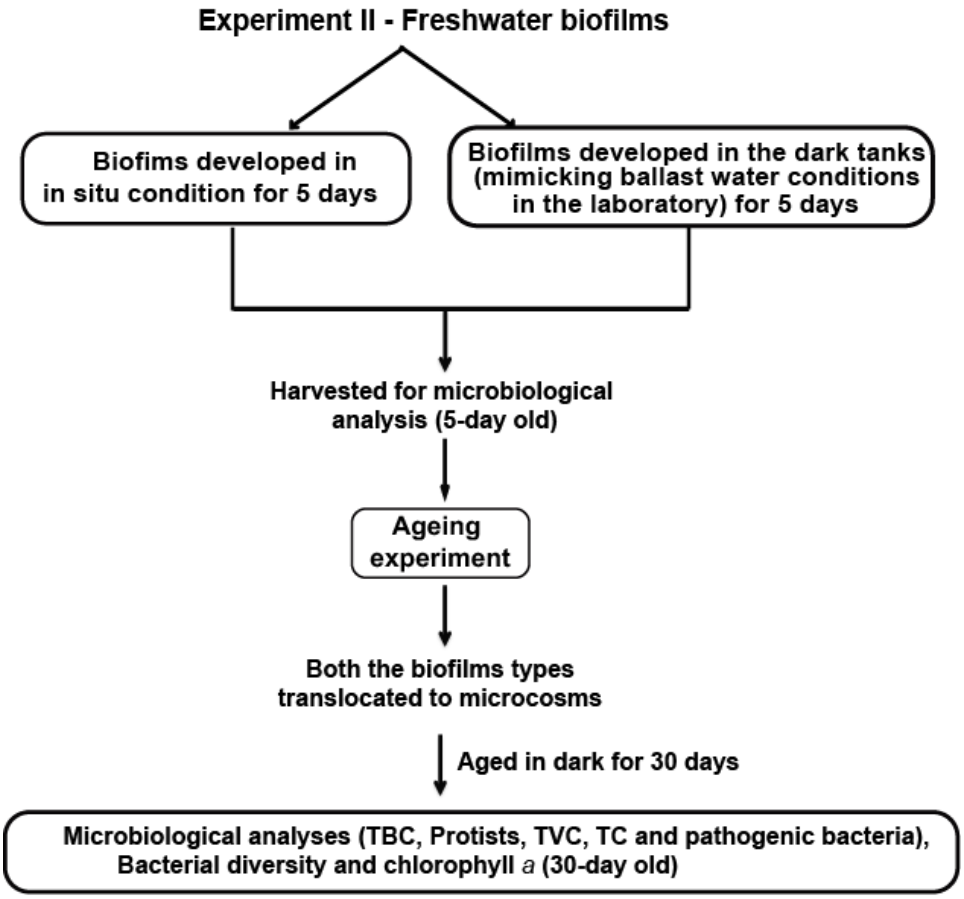


Figure 3A.2 Schematic representation of the experiment II. TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms

3A.2.3 Translocation experiment

After five days of incubation, these 5-day old freshwater biofilms were translocated to four replicate microcosms and exposed to prolonged darkness for a period of 30 days. All the jars were closed and static during the incubations. For each treatment, the slides with 30-day old freshwater biofilms were sampled from the microcosms as described above in subsection 3A.1.3, except for the usage of 0.85 % autoclaved saline water instead of seawater for scrapping freshwater biofilms.

3B. Influence of darkness and ageing on estuarine biofilm microbial communities

3B.1 Experiment III: Estuarine biofilms

3B.1.1 Sampling site

Details on the study area are explained earlier in Chapter 2, subsection 2.2.1.3.

3B.1.2 In situ and tank biofilms

Two different experiments (in situ and laboratory) were conducted on estuarine biofilms by using two different water inocula for biofilm establishment, one from the Mandovi estuary (15° 30' 17.442" N, 73° 49' 56.2392" E) along the west coast of India, during November 2014 (Experiment IIIa), and other from the Dona Paula bay (15°27.5′ N, 73°48′ E) which is located at the mouth of Zuari estuary during May 2019 (Experiment IIIb). A schematic representation of the experimental design is depicted in Figure 3B.1.

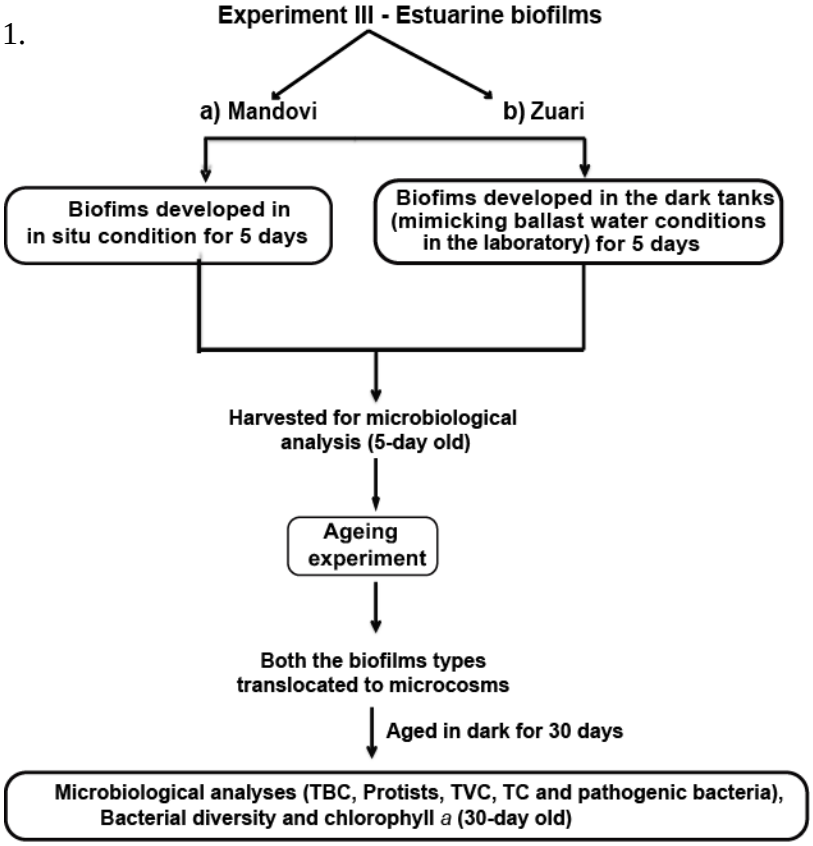


Figure 3B.1 Schematic representation of the experiment III. a) Mandovi estuarine biofilms, and b) Zuari estuarine biofilms. TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms.

In both the experiments, the pre-treated glass slides (25.4 mm x 76.2 mm, Himedia) were deployed in the Mandovi and Zuari station for the development of in situ biofilms for five days. These biofilms are termed as in situ estuarine biofilms. A laboratory experiment was also carried out wherein, tanks were filled with the respective station waters, and the pre-treated slides were suspended for a period of 5 days. These biofilms are termed as estuarine tank biofilms.

3B.1.3 Translocation experiment

The 5-day old estuarine biofilms were translocated to four replicate microcosms and exposed to prolonged darkness for 30 days. All the jars were closed and static during the incubations. For each treatment, the slides with 30-day old estuarine biofilms were sampled from the microcosms as described above in subsection 3A.1.3

3.2 Materials and Methods

3.2.1 Physico-chemical parameters

The details of environmental physicochemical parameters are described previously in Chapter 2 in subsection 2.2.2.

3.2.2 Chlorophyll a concentration in biofilms

The protocol details are described previously in Chapter 2 in subsection 2.2.3.

3.2.3 Total Viable Count (TVC) and pathogenic bacteria in biofilms

The protocol details are described previously in Chapter 2 in subsection 2.2.4.

3.2.4 Total Bacterial Count (TBC) in biofilms

The protocol details are described previously in Chapter 2 in subsection 2.2.5.

3.2.5 Protist abundance enumeration in biofilms

The protocol details are described previously in Chapter 2 in subsection 2.2.6.

3.2.6 Standardization of protocol for DNA extraction from biofilms

The protocol details are described previously in Chapter 2 in subsection 2.2.7.

3.2.7 Quantification of group-specific bacteria in biofilms using quantitative PCR

The protocol details and group-specific primers used are described previously in Chapter 2 in subsection 2.2.8.

3.2.8 Statistical analysis

The 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 bacteria, pathogenic bacteria, bacterial diversity, and chlorophyll *a* concentration), followed by post hoc Tukey's HSD test (Bonferroni corrected). In the case of freshwater and estuarine 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 using the SPSS statistical software program (Version 16). The relationship between the environmental variables, and biotic variables in the in situ and laboratory experiments was determined by using CANOCO version 4.5 (ter Braak and Verdonschot, 1995), as described in Chapter 2, subsection 2.2.9. Further, correlation analyses were performed between abiotic and bacterial components using Statistica 6.0 statistical package at a significance level of ≤ 0.05 (Stat Soft, OK, USA).

3A.3 Results

3A.3.1 Experiment I

3A.3.1.1 In situ marine biofilms

Detailed results on 5-day old in situ marine biofilms are explained in Chapter 2, subsection 2.3.2.1. The ageing experiments with in situ marine biofilms could not be achieved as slides were lost due to harsh sea conditions.

3A.3.1.2 Marine tank biofilms

Chl *a* was comparatively low in the 5-day old EN (0.00254 ± 0.0001 µg/cm^2) and UN marine tank biofilms (0.00213 ± 0.0001 µg/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) µg/cm^2. The TBC and protists increased significantly in the EN biofilms from day 5 (1.49×10^6 and 1.87×10^4 Cells/cm^2) to day 30 (2.81×10^6 and 1.25×10^5 Cells/cm^2) ($p < 0.001$, Tukey's HSD, Figure 3A.3a-b) during which nitrate (0.53 µM) and phosphate (0.98 µM) levels decreased drastically. The sampling days and enrichment factor had a significant effect on the viable and pathogenic bacterial abundance (two-way ANOVA, $p < 0.001$). 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, Figure 3A.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).

Vibrio alginolyticus were abundant in EN biofilms (2.13×10^2 CFU/cm^2) followed by *V. parahaemolyticus* (1.22×10^2 CFU/cm^2) on day 5 which is indicated in the RDA plot (Figure 3A.4). Both the *V. alginolyticus* and *V. parahaemolyticus* abundance decreased significantly in the EN biofilms to 4.67 CFU/cm^2 and below detection, respectively on day 30 when exposed to prolonged darkness ($p < 0.001$, Tukey's HSD, Figure 3A.3e).

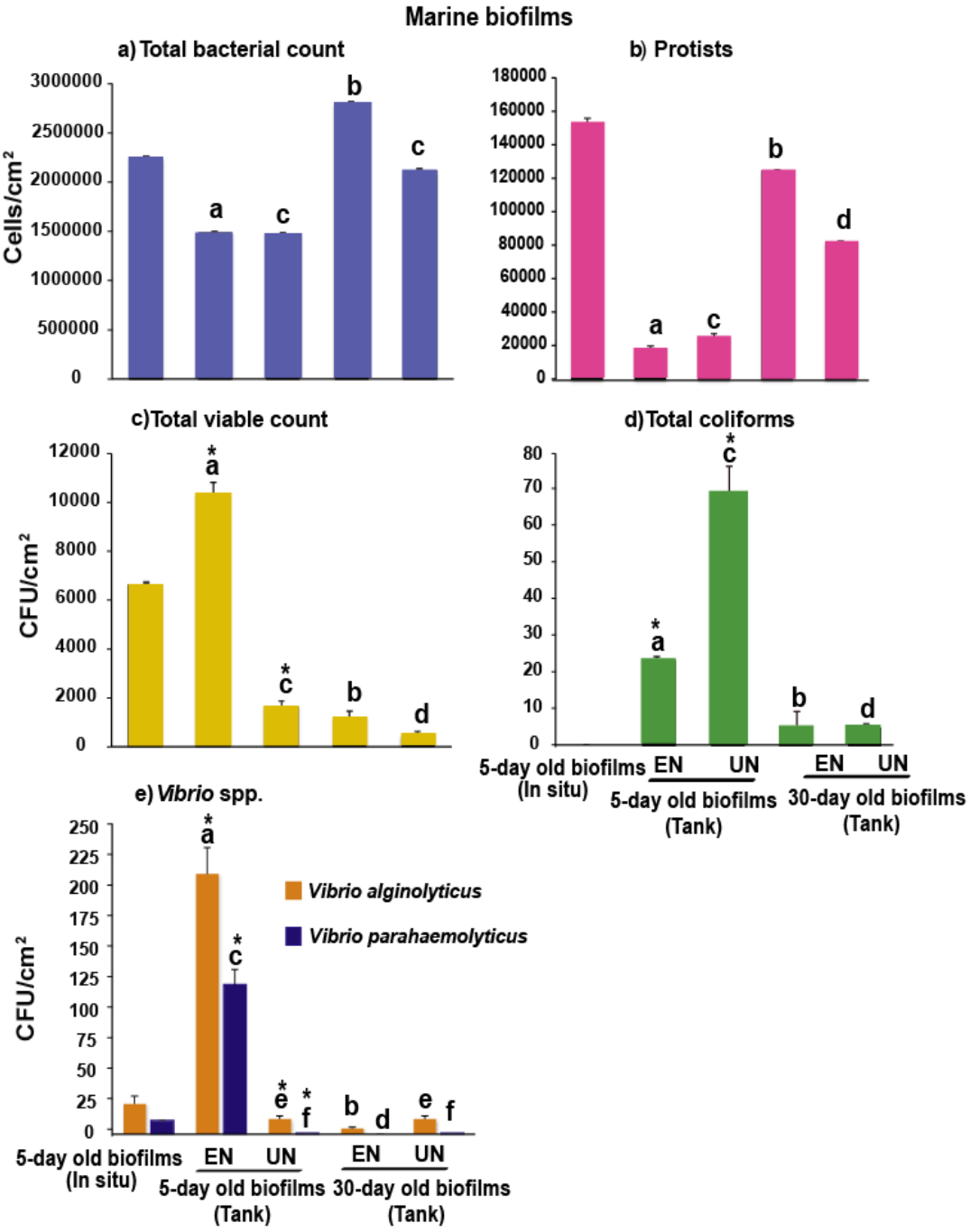


Figure 3A.3 Mean values of (a) total bacterial count, (b) protists, (c) total viable count, (d) total coliforms, and (e) *Vibrio* spp. (*V. alginolyticus* and *V. parahaemolyticus*) in the in situ and tank (enriched and unenriched) marine biofilms during the sampling days (day 5 and day 30). The solid 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 letters on the graph are not significantly different from each other. The asterisk (*) above bars indicate significant differences between enriched and unenriched marine biofilms.

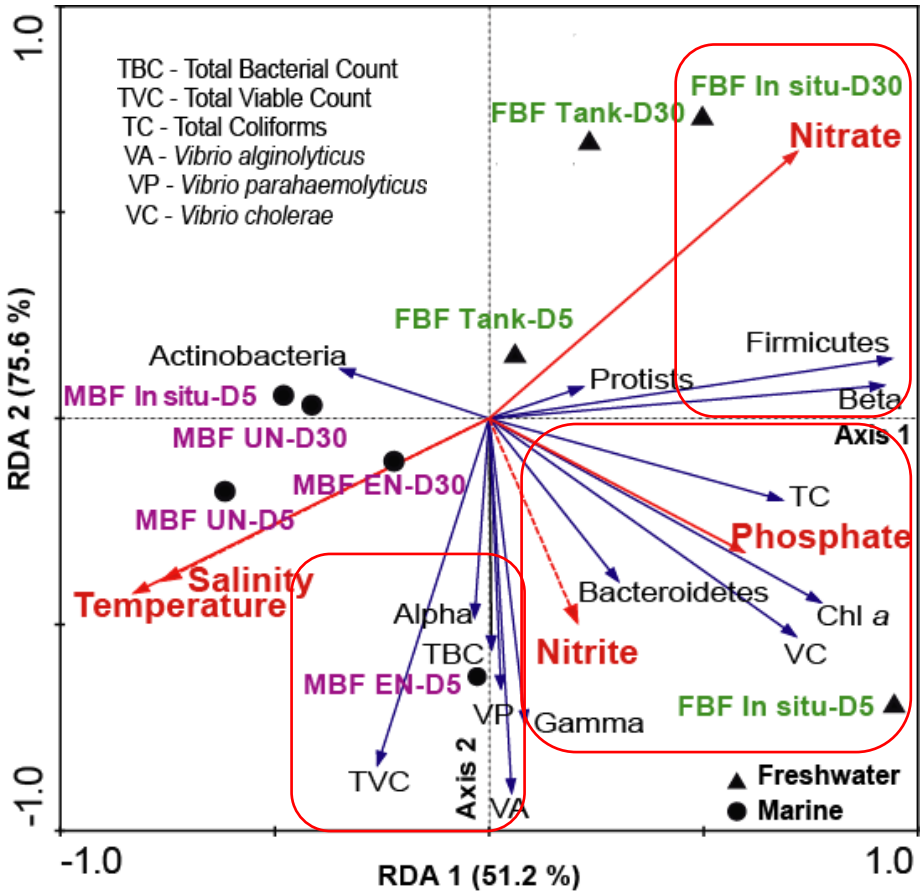


Figure 3A.4 Ordination plot of Redundancy analysis showing biotic variables (bacterial species, major bacterial taxa, protists) and their relationship with environmental variables in the in situ and tank marine (●) and freshwater (▲) biofilms during the sampling days (day 5 and day 30). MBF In situ-D5 = 5-day old in situ marine biofilms, MBF UN-D5 = 5-day old unenriched marine biofilms, MBF EN-D5 = 5-day old enriched marine biofilms, MBF UN-D30 = 30-day old unenriched marine biofilms, MBF EN-D30 = 30-day old enriched marine biofilms, FBF In situ-D5 = 5-day old in situ freshwater biofilms, FBF Tank-D5 = 5-day old freshwater tank biofilms, FBF In situ-D30 = 30-day old in situ freshwater biofilms, FBF Tank-D30 = 30-day old freshwater tank biofilms, Chl *a* = Chlorophyll *a*, Alpha = α-proteobacteria, Beta = β-proteobacteria, Gamma = γ-proteobacteria. All data points are averages of several replicates. Red squares highlight significant results.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the marine tank biofilms are depicted in Figure 3A.5. The sampling days and enrichment had a significant effect on bacterial community composition (two-way ANOVA, $p < 0.001$).The gene copy numbers of α-proteobacteria were dominant in both the EN and UN biofilms. This group was stable over a period of time upon the prolonged darkness (Figure 3A.5).

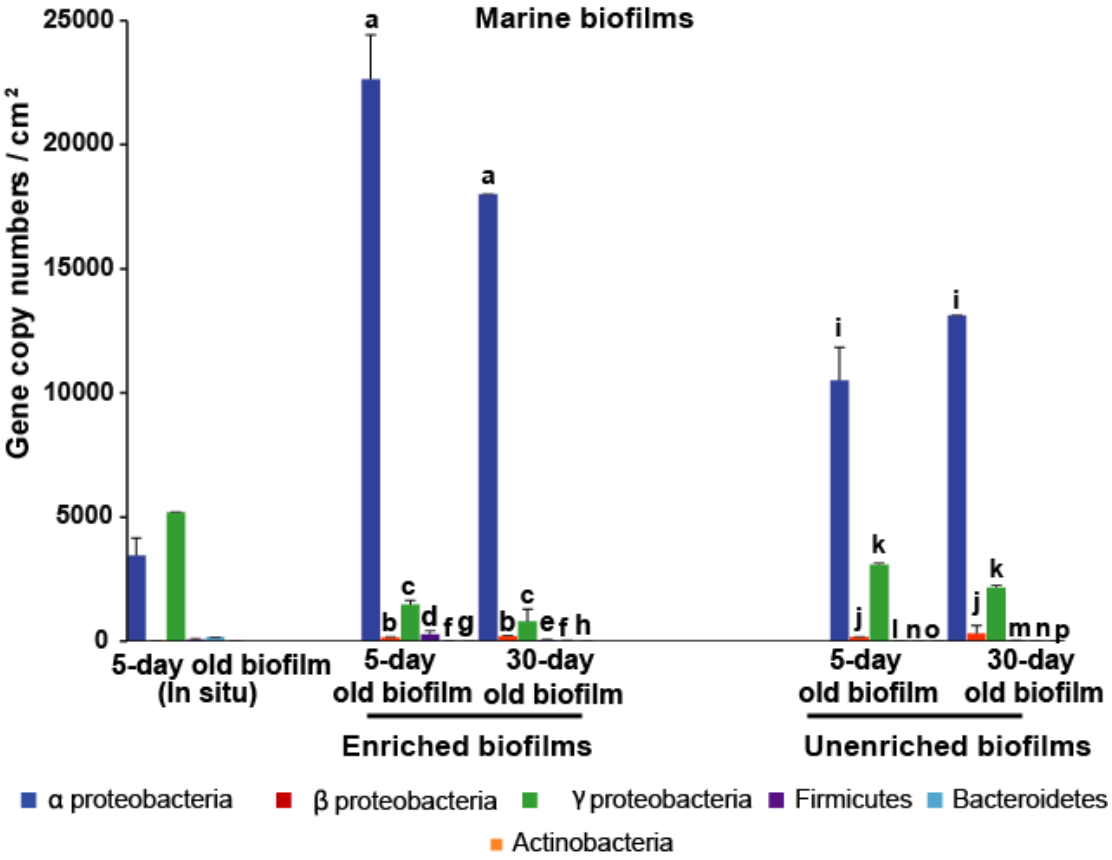


Figure 3A.5 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank (enriched and unenriched) marine biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples ± SE. Bars labelled by the same letter on the graph are not significantly different from each other. 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).

3A.3.2 Experiment II

3A.3.2.1 In situ freshwater biofilms

Chl *a* concentration was 0.351 ± 0.001 µg/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 (Figure 3A.4). However, upon prolonged darkness, the Chl *a* concentration decreased significantly with a minimum value of 0.033 ± 0.005 µg/cm^2 ($p < 0.001$, Tukey's HSD).

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, Figure 3A.6a-b). Similarly, the TVC abundance which was more in the in situ

biofilms on day 5 (2.69×10^3 CFU/cm^2), also decreased significantly on exposure to prolonged darkness ($p < 0.001$, Tukey's HSD, Figure 3A.6c). An increase in the nitrate levels (64.03 μM) was observed at the end of the dark experimental period. Freshwater biofilms were mainly dominated by TC. Their abundance was more in the in situ biofilms on day 5 (2.05×10^3 CFU/cm^2) and reduced drastically (1.87×10^3 CFU/cm^2) upon prolonged darkness ($p < 0.001$, Tukey's HSD, Figure 3A.6d). Their abundance was strongly influenced by phosphate ($p < 0.05$) and nitrite (Figure 3A.4). *Vibrio* spp., especially *V. alginolyticus* were dominant (2.21×10^2 CFU/cm^2) followed by *V. cholerae* (7.6×10^1 CFU/cm^2) and *V. parahaemolyticus* (6.0 CFU/cm^2) in the freshwater biofilms on day 5 (Figure 3A.6e). It was observed that these pathogenic bacteria were not able to persist upon prolonged darkness on day 30. *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 (Figure 3A.4) in the in situ freshwater biofilms.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the freshwater biofilms are depicted in Figure 3A.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 ($p < 0.05$) (Figure 3A.4). However, this group decreased drastically on day 30 upon prolonged darkness ($p < 0.001$, Tukey's HSD, Figure 3A.7). A similar trend was shown by Bacteroidetes; wherein, it decreased significantly upon prolonged darkness on day 30 ($p < 0.001$, Tukey's HSD, Figure 3A.7). β-proteobacteria was the dominant and stable taxonomic group in the in situ freshwater biofilms on day 30 and significantly correlated with nitrate concentration ($p < 0.05$) (Figure 3A.4).

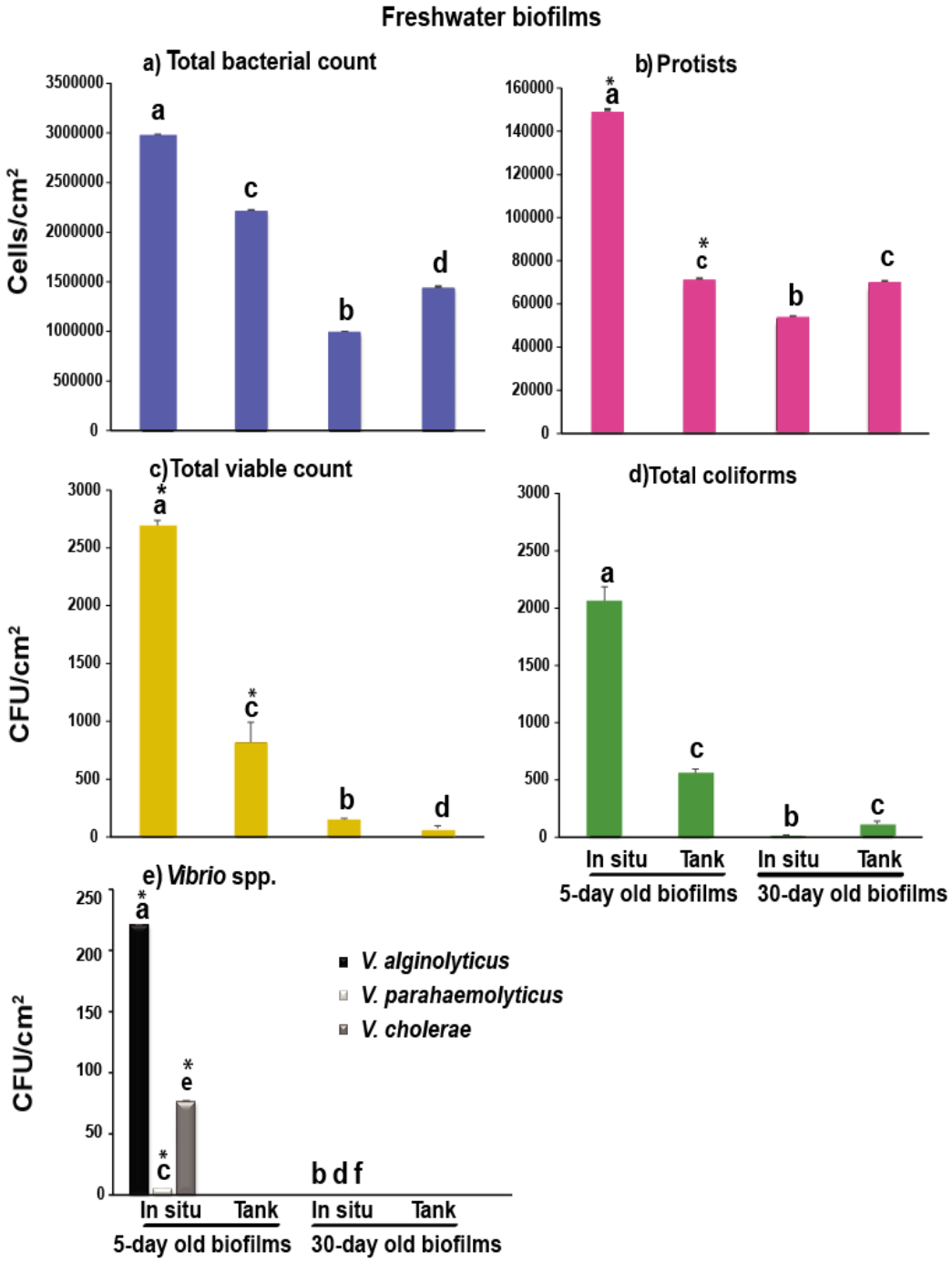


Figure 3A.6 Mean values of (a) total bacterial count, (b) protists, (c) total viable count, (d) total coliforms, and (e) *Vibrio* spp. (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*) in the in situ and tank freshwater biofilms during the sampling days (day 5 and day 30). The solid bars 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, $p < 0.05$, followed by Tukey's HSD post hoc test). The asterisk (*) above bars indicate significant differences between in situ and tank freshwater biofilms.

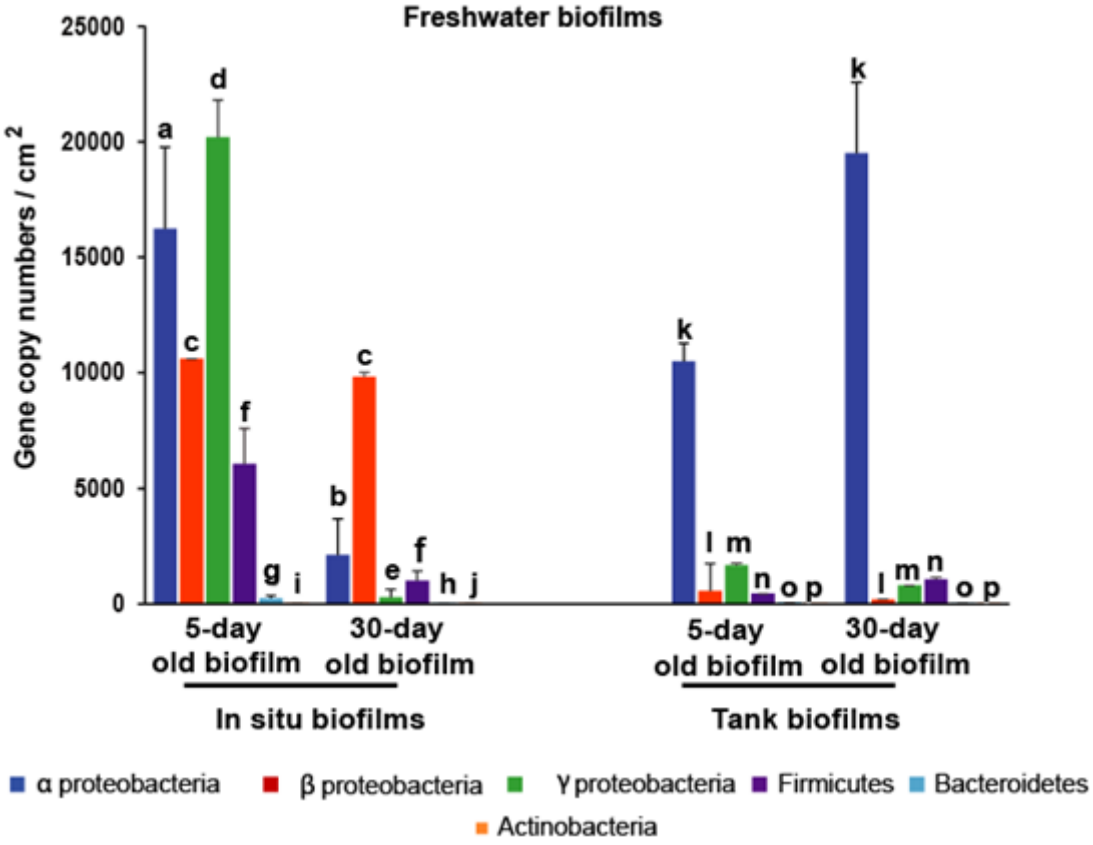


Figure 3A.7 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank freshwater biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples ± 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.

3A.3.2.2 Freshwater tank biofilms

Chlorophyll *a* (0.046 ± 0.0003 µg/cm^2) and the microbial parameters, i.e. TBC (2.2 × 10^6 Cells/cm^2, protists (6.98 × 10^4 Cells/cm^2), TVC (8.21× 10^2 CFU/cm^2), and TC (5.57× 10^2 CFU/cm^2) were low in the freshwater tank biofilms when compared to in situ biofilms (Figure 3A.6a-d). *Vibrio* spp. were not detected in the tank biofilms. Upon prolonged dark conditions, Chl *a* concentration (0.00731 ± 0.0003 µg/cm^2) and all the microbial parameters except for protists and TC resulted in a 10-fold reduction in their abundance ($p < 0.05$, Tukey's HSD, Figure 3A.6).

An increase in the nitrate levels (49.86 µM) was observed 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, wherein α-proteobacteria was dominant taxa (Figure 3A.7). However, no significant differences in bacterial biofilm community composition were observed in the case of freshwater tank biofilms upon prolonged darkness.

3B.3 Results

3B.3.1 Experiment IIIa: Mandovi estuarine biofilms

3B.3.1.1 In situ estuarine biofilms

Chl *a* was 1.2 ± 0.07 µg/cm^2 in the in situ Mandovi estuarine biofilms on day 5. However, the concentration decreased significantly with a minimum value of 0.99 ± 0.001 µg/cm^2 on day 30 ($p < 0.05$, Tukey's HSD), upon prolonged dark conditions.

The TBC and protist abundance declined significantly upon exposure to dark conditions on day 30 ($p < 0.05$, Tukey's HSD, Figure 3B.2a-b). Similarly, the TVC which were abundant in the in situ biofilms on day 5 (9.84×10^4 CFU/cm^2), also decreased significantly on exposure to prolonged darkness ($p < 0.001$, Tukey's HSD, Figure 3B.2c). An increase in the nitrate (26.57 µM) and phosphate (7.9 µM) levels observed at the end of the dark experimental period. TC were abundant in the in situ Mandovi estuarine biofilms on day 5 (7.1×10^1 CFU/cm^2) and decreased (6.2×10^1 CFU/cm^2) upon prolonged darkness ($p < 0.001$, 'Tukey's HSD, Figure 3B.2d). Among culturable *Vibrio* spp., *V. parahaemolyticus* were abundant (1.04×10^4 CFU/cm^2) followed by *V. alginolyticus* (8.83×10^3 CFU/cm^2) and *V. cholerae* (1.02×10^2 CFU/cm^2) in the in situ Mandovi estuarine biofilms on day 5 (Figure 3B.2e-g). It was observed that these culturable *Vibrio* spp., were not able to persist upon prolonged darkness on day 30, except for *V. alginolyticus,* which increased in numbers (2.68×10^4 CFU/cm^2) and showed a significant correlation with nitrite in the in situ Mandovi estuarine biofilms ($p < 0.05$). This was evident from the RDA plot as well (Figure 3B.3).

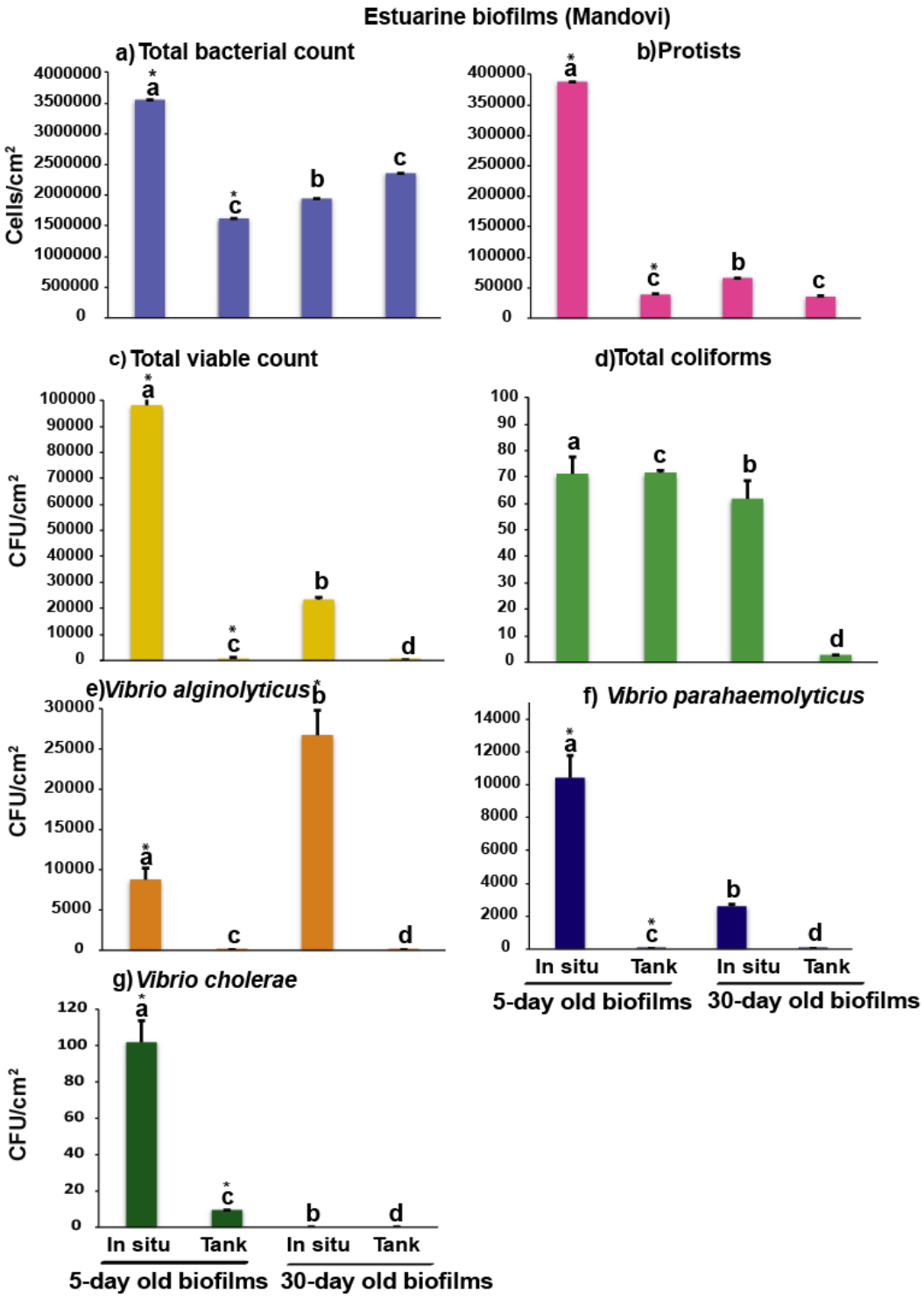


Figure 3B.2 Mean values of (a) total bacterial count, (b) protists, (c) total viable count, (d) total coliforms, (e) *V. alginolyticus*, (f) *V. parahaemolyticus*, and (g) *V. cholerae* in the in situ and tank Mandovi estuarine biofilms during the sampling days (day 5 and day 30). The solid bars represent 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 Mandovi estuarine biofilms (two-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). The asterisk (*) above bars indicate significant differences between in situ and tank Mandovi estuarine biofilms.

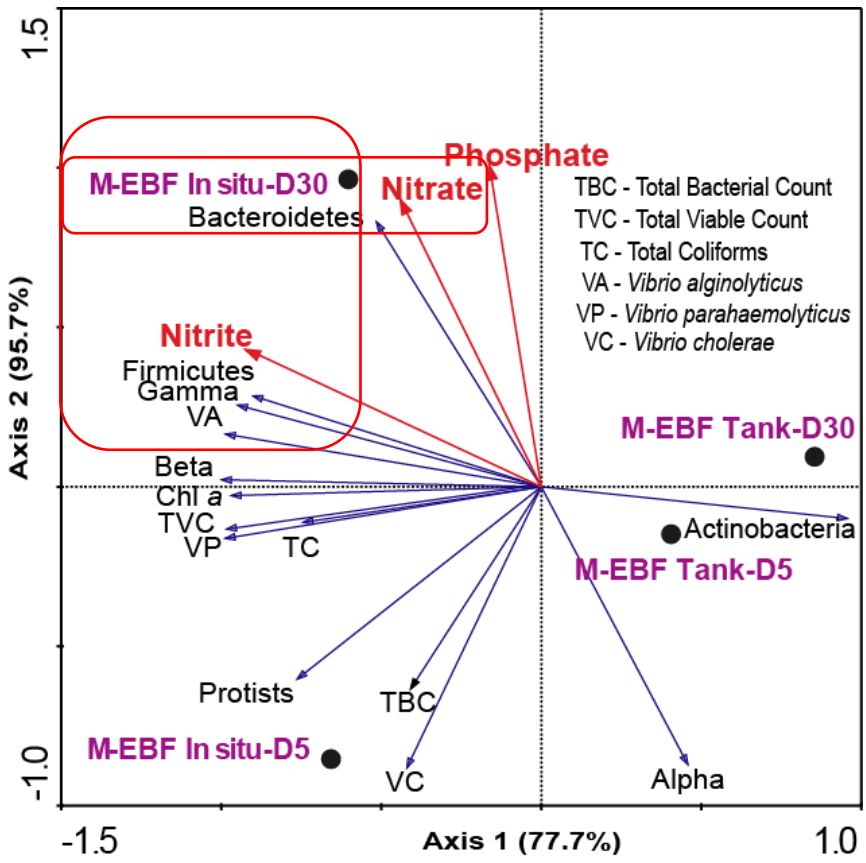


Figure 3B.3 Ordination plot of Redundancy analysis showing biotic variables (bacterial species, major bacterial taxa, protists) and their relationship with environmental variables in the in situ and tank Mandovi estuarine (●) biofilms during the sampling days (day 5 and day 30). M-EBF = Mandovi estuarine biofilms, In situ-D5 = 5-day old in situ Mandovi biofilms, M-EBF In situ-D5 = 30-day old in situ Mandovi biofilms, M-EBF Tank-D5 = 5-day old Mandovi tank biofilms, M-EBF Tank-D30 = 30-day old Mandovi tank biofilms, Chl *a* = Chlorophyll *a*, Alpha = α-proteobacteria, Beta = β-proteobacteria, Gamma = γ-proteobacteria. All data points are averages of several replicates. Red squares highlight significant results.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the estuarine biofilms are depicted in Figure 3B.4. The bacterial community composition was significantly different with respect to sampling days and type of biofilms (two-way ANOVA, $p < 0.0001$). γ-proteobacteria which were abundant in the in situ estuarine biofilms with high gene copy numbers (2.33×10^4 gene copy numbers/cm^2) on day 5, increased significantly upon the prolonged darkness ($p < 0.001$, Tukey's HSD, Figure 3B.4). Their abundance was significantly correlated with nitrite concentration ($p < 0.05$, Figure 3B.3). A similar trend was shown by Firmicutes and Bacteroidetes; wherein, both the taxa increased

significantly upon prolonged darkness on day 30 ($p < 0.001$, Tukey's HSD, Figure 3B.4). Bacteroidetes were significantly influenced by nitrate ($p < 0.05$, Figure 3B.3).

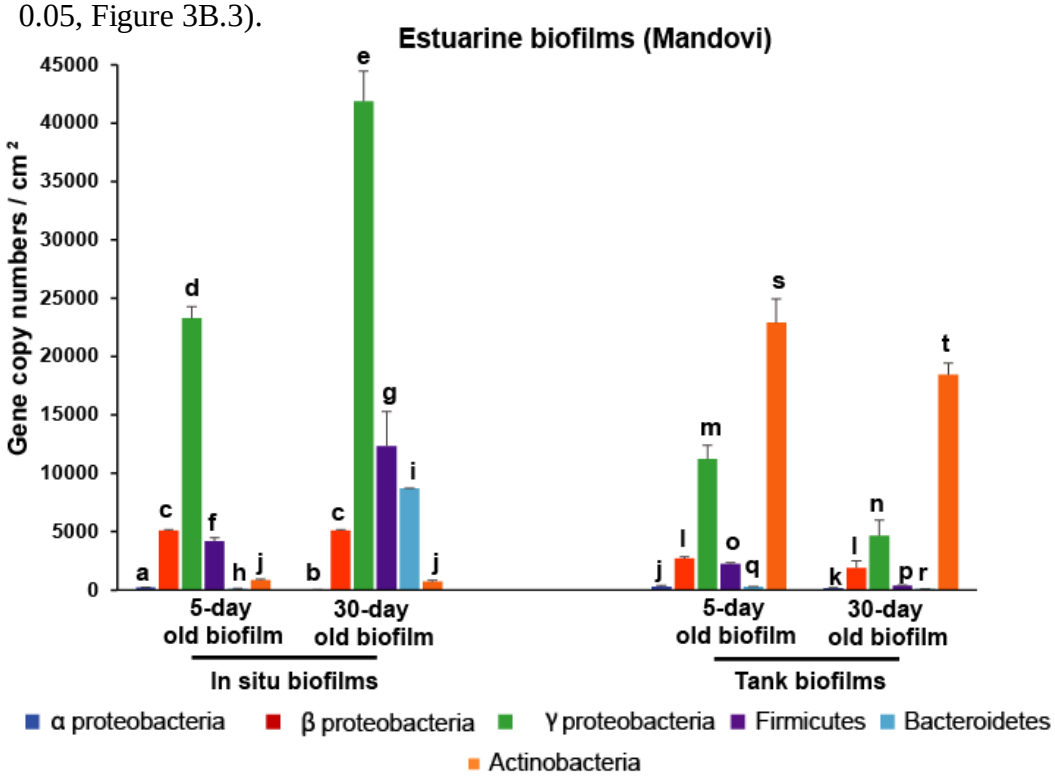


Figure 3B.4 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank Mandovi estuarine biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples ± SE. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the Mandovi estuarine 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.

3B.3.1.2 Estuarine tank biofilms

Chlorophyll *a* (0.0096 ± 0.0001 µg/cm^2) and the microbial parameters, i.e. TBC (1.94 × 10^6 Cells/cm^2), protists (6.53 × 10^4 Cells/cm^2), and TVC (2.22 × 10^4 CFU/cm^2) were low in the Mandovi estuarine tank biofilms (Figure 3B.2a-c). Upon prolonged dark conditions, all the microbial parameters resulted in a 10-fold reduction in their abundance ($p < 0.05$, Tukey's HSD, Figure 3B.2c-e), except for TBC and protists which did not show any significant changes.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the Mandovi estuarine tank biofilms are depicted in Figure 3B.4. The gene copy numbers of γ-proteobacteria (1.12

× 10^4 CFU/cm^2), and Actinobacteria (2.29 × 10^4 CFU/cm^2) which were abundant in the Mandovi estuarine tank biofilms on day 5, decreased significantly (4.67 × 10^3 CFU/cm^2 and 1.85 × 10^4 CFU/cm^2) upon prolonged darkness on day 30 ($p < 0.05$, 'Tukey's HSD, Figure 3B.4).

3B.3.2 Experiment IIIb: Zuari estuarine biofilms

3B.3.2.1 In situ estuarine biofilms

Chl *a* was 0.197 ± 0.0007 µg/cm^2 in the in situ estuarine biofilms on day 5, and with prolonged darkness, the concentration further decreased significantly with a minimum value of 0.067 ± 0.001 µg/cm^2 ($p < 0.05$, 'Tukey's HSD). The TBC and protists declined significantly upon exposure to dark conditions on day 30 ($p < 0.05$, Tukey's HSD, Figure 3B.5a-b). Similarly, TC numbers also reduced drastically upon prolonged dark conditions ($p < 0.05$, Tukey's HSD, Figure 3B.5d). An increase in the nitrate (9.38 µM) and phosphate (4.37 µM) levels were observed at the end of the dark experimental period. However, the TVC, *Vibrio alginolyticus* and *V. parahaemolyticus* abundance did not show any significant variations in the in situ Zuari estuarine biofilms when exposed to prolonged dark conditions on day 30 (Figure 3B.5c, 3B.5e).

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the estuarine biofilms are depicted in Figure 3B.6. The bacterial community composition was significantly different with respect to sampling days and type of biofilms (two-way ANOVA, $p < 0.05$). γ-proteobacteria were abundant in the in situ Zuari estuarine biofilms with high gene copy numbers (1.03 × 10^4 gene copy numbers/cm^2) on day 5 (Figure 3B.6). However, this group decreased drastically on day 30 upon prolonged darkness ($p < 0.05$, Tukey's HSD, Figure 3B.6) and showed a negative correlation with nitrate ($p < 0.12$). evident in the RDA plot as well (Figure 3B.7).

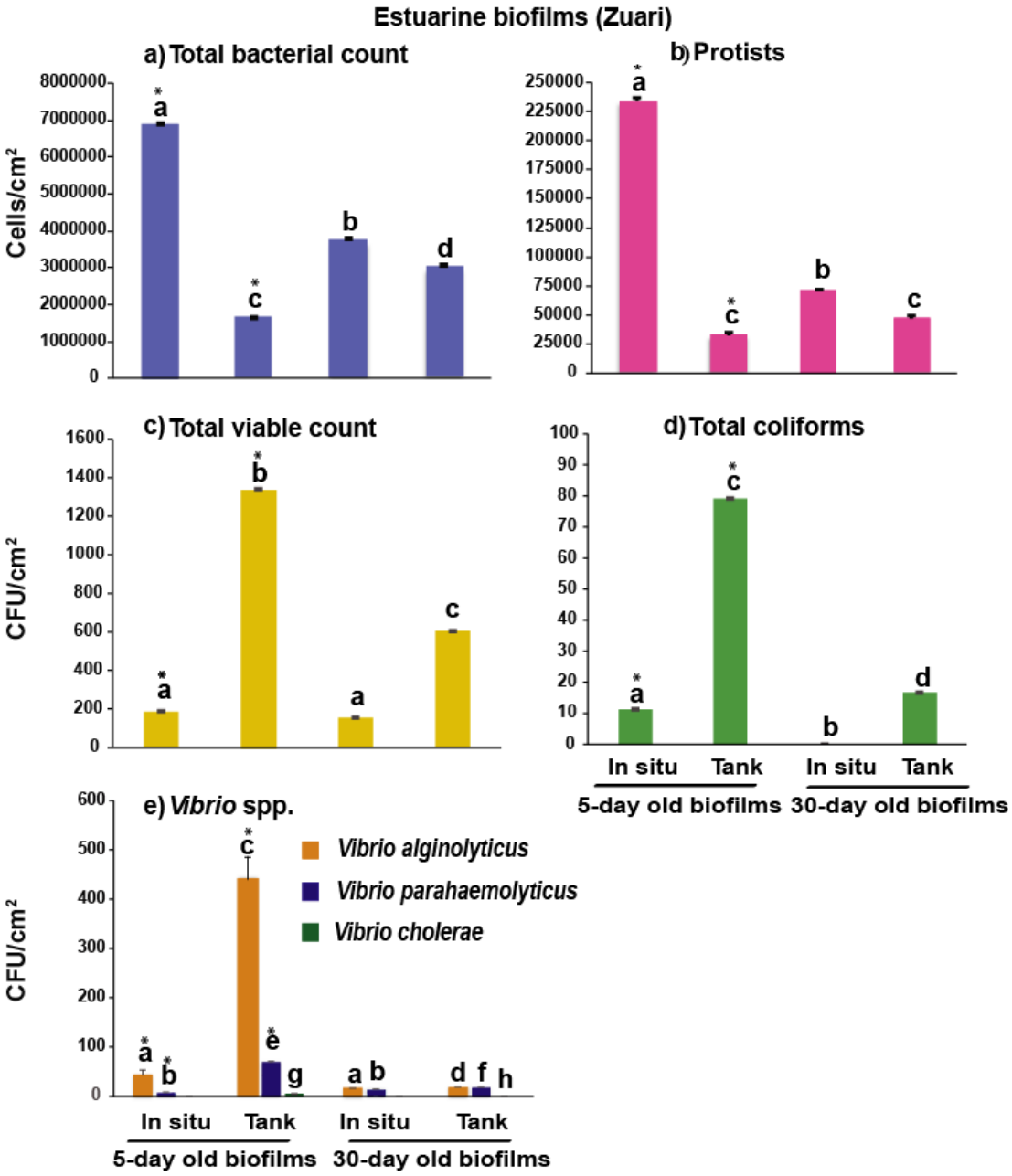


Figure 3B.5 Mean values of (a) total bacterial count, (b) protists, (c) total viable count, (d) total coliforms, and (e) *Vibrio* spp. (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*) in the in situ and tank Zuari estuarine biofilms during the sampling days (day 5 and day 30). The solid 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 Zuari estuarine biofilms (two-way ANOVA, $p < 0.05$, followed by Tukey's HSD post hoc test). Bars labelled by the same letters on the graph are not significantly different from each other. The asterisk (*) above bars indicate significant differences between in situ and tank Zuari estuarine biofilms.

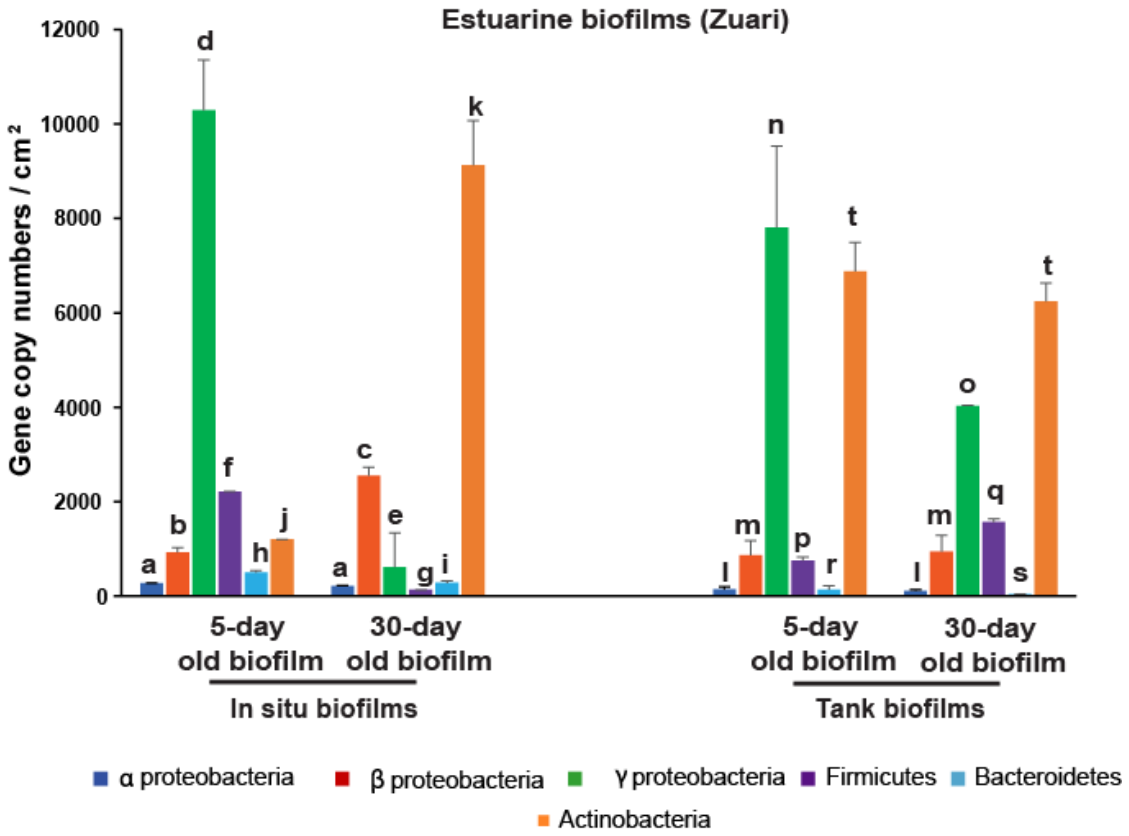


Figure 3B.6 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue) and Actinobacteria (orange) in the in situ and tank Zuari estuarine biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples ± SE. Different letters above the bars denote significant differences between the sampling days (day 5 and day 30) in the in situ and tank Zuari estuarine 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.

On the contrary, the gene copy numbers of β-proteobacteria increased significantly upon prolonged dark conditions (Figure 3B.6) and showed a significant correlation with nitrate concentration ($p < 0.05$, 3B.7). A similar trend was shown by Actinobacteria; wherein, gene copy numbers increased significantly upon prolonged darkness on day 30 ($p < 0.001$, Tukey's HSD, Figure 3B.5), and were influenced by phosphate ($p < 0.05$). This result was evident in the RDA plot as well (Figure 3B.7).

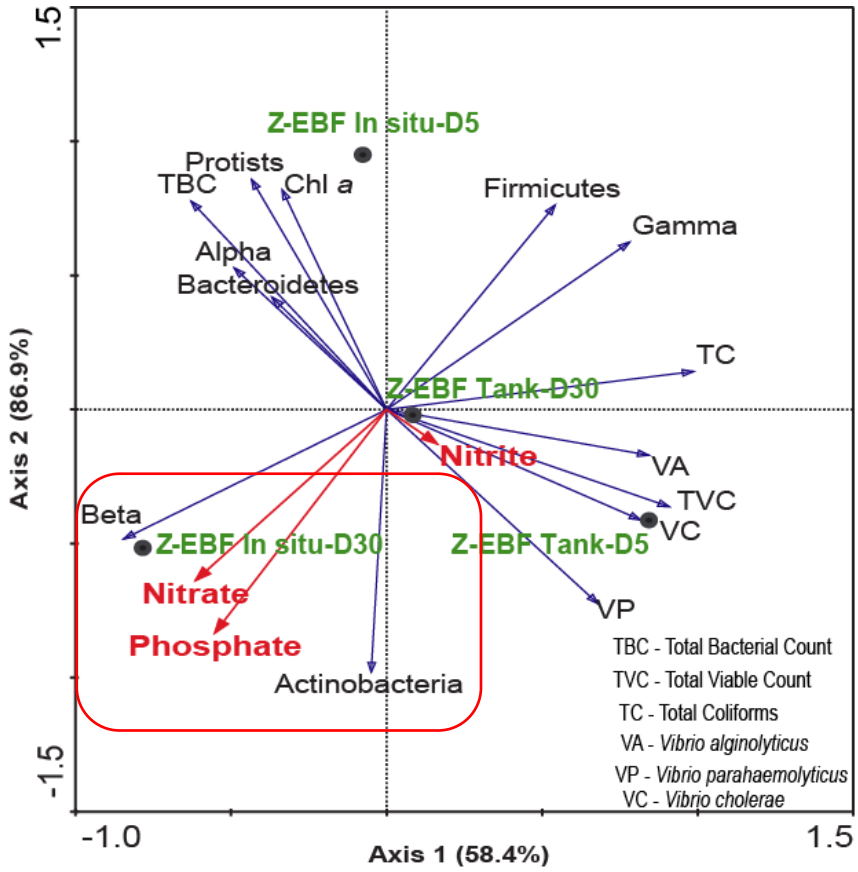


Figure 3B.7 Ordination plot of Redundancy analysis showing biotic variables (bacterial species, major bacterial taxa, protists) and their relationship with environmental variables in the in situ and tank Zuari estuarine (●) biofilms during the sampling days (day 5 and day 30). Z-EBF = Zuari estuarine biofilms, ZD5 In situ = 5-day old in situ Zuari biofilms, ZD30 In situ = 30-day old in situ Zuari biofilms, ZD5 Tank = 5-day old Zuari tank biofilms, ZD30 Tank = 30-day old Zuari tank biofilms, Chl *a* = Chlorophyll *a*, Alpha = α-proteobacteria, Beta = β-proteobacteria, Gamma = γ-proteobacteria. All data points are averages of several replicates.

3B.3.2.2 Estuarine tank biofilms

Chlorophyll *a* (0.018 ± 0.0007 µg/cm^2) and the abundance of the microbial parameters (TBC, protists) were low in the estuarine tank biofilms on day 5 (Figure 3B.5). On the other hand, TVC and pathogenic bacterial abundance, including *Vibrio* spp., and TC which were abundant in the tank biofilms on day 5 resulted in a 10-fold reduction in their abundance upon prolonged dark conditions on day 30 ($p < 0.05$, Tukey's HSD, Figure 3B.5), except for TBC and protists.

Variations in the bacterial community composition in terms of gene copy numbers per square centimeter in the estuarine tank biofilms are depicted in Figure 3B.6. γ-proteobacteria were abundant (7.81×10^3 CFU/cm^2) in the estuarine tank biofilms on day 5. However, this group decreased drastically (4.03×10^3 CFU/cm^2) on day 30 upon prolonged darkness ($p < 0.05$, Tukey's HSD, Figure 3B.6). On the other hand, the Actinobacteria which were dominant in the Zuari estuarine tank biofilms on day 5, did not show any significant changes upon prolonged dark conditions on day 30 (Figure 3B.6).

3.4 Discussion

Ballast water is known as a significant vector for microbial invasion and has been implicated in the introduction of aquatic planktonic organisms into novel areas, thereby causing a threat to aquatic biodiversity (Ruiz et al., 2000; Carlton et al., 2011; Khandeparker and Anil, 2017). Ballast-tank biofilms signify an additional risk of microbial invasion, yet their fate and invasion biology are poorly understood. The present study was carried out in the laboratory simulating ship's ballast tank conditions. 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 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. A similar trend was observed in the case of enriched marine biofilms, wherein a significant increase in the TBC and protist abundance was observed. These results supported the incubator hypothesis, which states that the ballast water tanks act as incubators for microorganisms (Drake et al., 2002). The dark conditions result in the death of the phytoplankton, thus influencing zooplankton, which feeds on them, leading to their mortality.

Subsequently, they are colonized and decomposed by ambient bacteria, which further fuel bacterial production (Drake et al., 2002; Tang et al., 2006). It is also reported earlier that a large number of bacteria emerge from the decomposing zooplankton contributing to increased bacterial production (Khandeparker and Anil, 2013). A study by Tomaru et al. (2014) suggested that this detritus could also fuel heterotrophic protists, which was also evident in these biofilms exposed to the prolonged dark condition.

In the case of freshwater biofilms developed in the in situ condition, chlorophyll *a* also decreased significantly upon prolonged dark conditions. An increase in the levels of nitrate was observed at the end of the dark experimental period, and this could be attributed to the cell lysis of biofilm-bound microalgae and their disintegration. A similar trend was also observed in the in situ estuarine (Mandovi/Zuari) biofilms upon prolonged dark conditions, which resulted in the increase in nitrate and phosphate levels at the end of the dark experimental period. The released cellular matter has been suggested to breakdown further into dissolved organic matter (DOM), which can support heterotrophic bacteria in the dark period (Carney et al., 2011). However, the number of TBC and protists decreased drastically in the freshwater and estuarine biofilms (5 days), after exposure to prolonged darkness (30 days), unlike marine biofilms. Thus, the results of our study for freshwater and estuarine biofilms indicate that both these biofilms did not support the incubator hypothesis, resulting in a decrease in the abundance of bacteria and protists with the ageing upon prolonged darkness conditions. The decline in the TBC and protist numbers in the case of in situ freshwater biofilms 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 (Parry, 2004; Huws et al., 2005; Dopheide et al., 2011; Weerman et al., 2011; Kanavillil and Kurissery, 2013). Moreover, the other probable reasons for the decrease in bacterial and protist abundance in the freshwater, as well as estuarine biofilm communities, could be that (1) the microbes might have entered an inactive or viable-but non-culturable state (VBNC) in response to environmental changes (a survival strategy) (Colwell,

2000; Villac et al., 2013) or (2) these biofilm communities were sensitive to unfavourable dark environmental conditions, which could have led to the elimination of less tolerant communities leaving behind the most adaptable ones (competition). Hence, it is expected that the tolerant bacterial communities with strategies to cope up with stress would survive and proliferate in the ballast tank conditions, thereby influencing the bacterial communities in the biofilms over a period of time.

In the present study, bacterial community composition 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 (De Gregoris et al., 2011). This technology merges the PCR chemistry with the use of fluorescent molecules (e.g., SYBR Green) to monitor the amplified products during each cycle of the PCR reaction (Navarro et al., 2015). In the current study, the composition of the biofilm bacterial community changed significantly over a period of time under dark condition. A similar trend was observed in the case of ballast water tank bacterial community, wherein a shift in the bacterial community was observed in the dark ballast tanks over a period of time during the voyage (Tomaru et al., 2010, Khandeparker et al., 2020).

A clear difference in the marine biofilm bacterial community composition was observed between in situ and tank biofilms during the study period. The in situ 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 (Nold and Zwart, 1998; Nogales et al., 2007; Neyland, 2009). In the present study, α-proteobacteria was the most stable taxonomic group in the enriched and unenriched marine biofilms over a period of time under the prolonged dark conditions. They are a cosmopolitan group of gram-negative bacteria found in both freshwater and marine ecosystems (Nold and Zwart, 1998; Nogales et al., 2007). This group possesses different metabolic strategies such as photosynthesis, nitrogen fixation, ammonia oxidation, etc. (Williams et al., 2007; Campagne et al.

2012), which could have aided in their survival under prolonged dark conditions.

This probably led to their dominance in the marine tank biofilms during the study period. An earlier study reported that the family Rhodospirillaceaens, 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 (Neyland, 2009). Moreover, α-proteobacteria are metabolically versatile, and capable of rapid growth, indicating that they can outcompete other bacteria in utilizing dissolved organic matter (DOM) (Azam and Malfatti, 2007; Newton et al., 2011; Brown et al., 2014; Yilmaz et al., 2016). A recent study by Khandeparker et al. (2020), also reported that the members of α-proteobacteria, belonging to family Rhodobacteraceae in the ballast water, were able to cope and survive in the dark conditions by expressing core metabolic functions that aided them to compete with the other members of the bacterial groups during the voyage that lasted for a short span of time (5 days).

A similar trend was observed in the case of freshwater biofilms developed in the dark tanks; wherein α-proteobacteria was an abundant taxon when exposed to a prolonged dark incubation. This group is poorly studied in the freshwater ecosystems. However, earlier studies have reported that although α-proteobacteria is known to be dominant taxa in the marine ecosystems, they can be even more abundant in the freshwater biofilms due to their competitive nature for utilizing DOM, ability to degrade humic substances, and resistant to grazing by forming grazing resistant morphologies that may favour certain members of α-proteobacteria in the freshwater biofilms (Anderson-Glenna et al., 2008; Newton et al., 2011; Romaní et al., 2014; Besemer, 2016; Yilmaz et al., 2016).

In the case of in situ freshwater biofilms developed for 5 days, α-proteobacteria, γ-proteobacteria and β-proteobacteria were dominant taxa. Earlier studies have 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 (Manz et al., 1999, Araya et al., 2003). They are dominant in freshwater

ecosystems, known for their versatile, degrading capabilities, whereas they are relatively low in the ocean (Nold and Zwart, 1998). This group is also found in the marine biofilms, but occur at lower levels compared to other Proteobacteria (Edwards et al., 2010; Walker and Keevil, 2015). In the present study, β-proteobacteria abundance was correlated with high nitrate concentration in the case of in situ freshwater biofilms when exposed to prolonged darkness for 30 days. They are known to play an important role in nitrogen fixation (Munn, 2011) 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 (Gao et al., 2005; Parveen et al., 2011). 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 (Gasol et al., 2002).

Moreover, the qPCR analyses showed that γ-proteobacteria, including most of the pathogenic genera, which were abundant in the in situ freshwater, and also in the case of in situ Zuari estuarine biofilms (Day 5), decreased significantly under prolonged dark conditions (Day 30). However, it was interesting to note that the relative abundance of this group increased significantly in the case of in situ Mandovi estuarine biofilms when subjected to prolonged dark conditions. A similar trend was shown by the culturable *Vibrio alginolyticus*, belonging to γ-proteobacteria, wherein the abundance increased significantly in the Mandovi estuarine biofilms. Both γ-proteobacteria and the culturable *V. alginolyticus* showed significant correlation with nitrite, which could be attributed to their role in the reduction of nitrate to nitrite. *Vibrio* spp. are known to play a significant role in nitrate reduction in marine environments (Philippot, 2005; Lara et al., 2011). Moreover, earlier studies have reported γ-proteobacteria as the predominant nasA-harbouring bacteria, possessing nasA genes, that encode for nitrate reductase which is responsible for nitrate assimilation in the marine environments (Cai and Jiao, 2008; Jiang et al., 2015; Ramos and Pajares, 2018 and references within). Apart from this, they are important pathogens for humans (ear infections) and fish (*V. alginolyticus*) (Pruzzo et al., 2005). Apart from γ-proteobacteria, which increased when exposed to prolonged dark conditions, the qPCR analyses also showed that the Bacteroidetes, also

known as *Cytophaga/Flavobacterium/Bacteroides* group, increased significantly in these biofilms. The genera belonging to this group are abundant in the coastal and nutrient-rich environments and belong to major biopolymer degrading bacteria (Gónzalez et al., 2008). Their ability to degrade carbohydrates and proteins suggests that they may play an important role as consumers of algal derived metabolites (Kirchman, 2002). They possess diverse extracellular enzymes for degradation of algal polysaccharides (Mann et al., 2013; Fernández-Gomez et al., 2013). These algal derived exudates are usually carbon-rich but poor in nitrogen content. Hence, apart from using carbon source derived from algal polysaccharides, Bacteroidetes may also need nitrogen content such as nitrate for growth (Bernhard et al., 2005; Jiang et al., 2015). This explains why Bacteroidetes showed a positive correlation with nitrate in the present study.

On the other hand, the culturable *V. parahaemolyticus,* and *V. cholerae* in the 5-day old in situ Mandovi biofilms, decreased significantly when exposed to prolonged dark conditions (30 days). Likewise, the *Vibrio* spp., also decreased in the case of enriched marine biofilms (except *V. cholerae*) when subjected to prolonged dark conditions. The possible reason for their decrease in abundance could be that these species can enter into a starvation mode of physiologically viable but non-culturable (VBNC) state under stressed conditions. It is a unique survival strategy acquired by many bacteria, especially *V. cholerae,* in response to harsh environmental conditions (Colwell, 2000). 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 (Colwell et al., 1985; Colwell, 2000; Ramamurthy et al., 2014). Hence, it is possible that the inactive *Vibrio* spp. can revert from the VBNC state to culturable forms on exposure to favourable environmental conditions for growth. Unlike in situ Mandovi estuarine biofilms, the culturable *Vibrio* spp. abundance did not show any significant change in the in situ Zuari estuarine biofilms upon prolonged dark condition. This suggests the unique ecology of *Vibrio* spp. and their response to prolonged dark conditions differed in both the estuaries.

In the case of Zuari estuarine biofilms, a clear shift in biofilm community composition was observed between in situ and tank biofilms. Among the bacterial communities, β-proteobacteria and Actinobacteria increased significantly in the in situ Zuari estuarine biofilms upon prolonged dark conditions. Both of these groups are known to play an important role in organic matter remineralization. In the present study, β-proteobacteria abundance showed a significant positive correlation with nitrate. β-proteobacteria are known to play an important role in nitrogen fixation (Munn, 2011) 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 (Gao et al., 2005; Parveen et al., 2011). 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 (Gasol et al., 2002), which was evident in the present study. Moreover, there is a possibility that the nitrate generated by this group was utilized by γ-proteobacteria during the study period. This was evident from the negative correlation between γ-proteobacteria and nitrate, which could have resulted in high nitrite concentration during the study period.

The other dominant taxon in the in situ Zuari estuarine biofilms was Actinobacteria. These are one of the largest bacterial phyla with high G+C content containing Gram-positive bacteria that are ubiquitously distributed in both aquatic and terrestrial ecosystems (Barka et al., 2016). The lifestyles and ecology of these bacteria are diverse, and thus can survive in most of the aquatic habitats, including open-ocean, coastal, estuarine, as well as within a ballast tank (Neyland, 2009). This taxon consists of large phylum and plays a significant role in the carbon cycling, recycling of organic matter and nutrient regeneration (Lacey, 1978; Glöckner et al., 2000; Ventura et al., 2007; Hill et al., 2011). This was evident from the significant correlation between Actinobacteria with phosphate concentrations during the study period. Moreover, this group are well documented for their ability to solubilize phosphate (Balakrishna et al., 2012; Dastager and Damare, 2015; Poovarasan et al., 2015).

In the case of both the estuarine biofilms developed in the tank unlike the in situ ones, it was observed that most of the microbial parameters and

bacterial community abundance decreased significantly upon exposure to prolonged dark conditions. It is known that microbes can survive hostile environments in an inactive state until they reach a suitable habitat with favourable conditions for growth (Ramette and Tiedje, 2007). Hence, there is a possibility that they can resuscitate back to culturable form if favourable conditions are met for growth, which is a cause of concern.

Our investigation revealed the prevalence of different bacterial groups in the biofilms (marine/freshwater/estuarine) that were able to thrive in dark environments. The resulting prevalence of bacterial taxa in the respective biofilms could also be attributed to two possible reasons (1) 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 (Matz and Kjelleberg, 2005; Gerea et al., 2013; Martinez-Garcia et al., 2012; Aguilera et al., 2013). 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 (Gude, 1989; Gerea et al., 2013; Khandeparker et al., 2018), and (2) the expression of core metabolic functions by bacteria, for e.g., biosynthesis of antimicrobials, carbohydrate metabolism, quorum sensing (including biofilm formation) etc. (Khandeparker et al., 2020). Thus, probably increasing the survival of several bacterial 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 marine, freshwater, and estuarine biofilms seemed to behave very differently when exposed to ageing and darkness, but this may not be true for all the marine, freshwater and estuarine biofilms. The biofilm bacterial community composition is niche specific and influenced by interactions with the surrounding environment (Arndt et al., 2003; Lee et al., 2016). The bacterial community composition of marine, estuarine and freshwater biofilms were distinctly different. These results are in agreement with the Baas-Becking hypothesis for microbial taxa which states that 'everything is everywhere, but the environment selects' (Baas

Becking, 1934). The composition of naturally occurring bacterial communities and biofilm bacterial communities exhibits marked biogeography, (Martiny et al., 2006; Langenheder and Ragnarsson, 2007; Lear et al., 2013), with distinct freshwater, coastal, and oceanic populations, which may further alter the community composition of the recipient community. Since the marine and estuarine 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. However, the ecological impact of ballast biofilm bacteria on the recipient communities may 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 (Litchman, 2010). All species transported in ballast tank biofilms may not be invasive nor will all survive the journey. 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 significant role as they can govern their ability to colonize and invade in the new environment. Further studies focused on the fate of biofilm communities upon discharge in the new environment, and their invasion potential is an important topic for future investigations.

3.5 Conclusions

The experimental observations (Chapters 3A and 3B) suggest that the transport conditions (i.e. darkness and ageing) can shape the biofilm microbial assemblages.

Overall, it can be concluded that the freshwater biofilms were more sensitive when exposed to the ballast tank conditions with an overall drastic decrease in the pathogenic bacterial abundance and bacterial diversity (approx. 40 %) in comparison to the estuarine and marine biofilms. However, this may not always be the truth, since the biofilm community characteristics

differ with the changing environmental conditions and depend on the inoculum characteristics and period of exposure.

In view of this, experiments were conducted addressing the effect of long-term ballast tank incubations on the freshwater biofilm communities developed in an inland port under varying environmental conditions. Furthermore, the impact of these biofilms, when released as a vector, on the estuarine water column communities was assessed using microcosm experiments, which has been discussed in the subsequent Chapter 3C.

3C. Ecological impact of biofilms as a vector of microbial invasion on the environment

3C.1 Introduction

Over the past many decades, ballast waters of either freshwater, estuarine or marine origin, have been disposed by ships in the ports, harbours, and coastal waters causing translocation of the diverse range of organisms from their native environment (Cohen and Carlton, 1998). The inadvertent introduction of species (alien) through the release of this ballast water has become a significant threat to aquatic biodiversity, and ecosystem functioning worldwide (Carlton et al., 2011; Khandeparker and Anil, 2017). However, the consequences of the biofilm communities formed on the interior walls of these ballast tanks are least explored, as their release through the ship's ballast water can cause or is likely to cause, harm to the environment (ecology), which is an additional concern.

The ballast tank biofilm community composition is niche specific and differs based on the ballast water taken on board, which can be either freshwater or marine origin, depending on the locality of the source port. It is well known that ships normally fill their ballast with seawater. However, apart from seaports, there are navigable inland ports located around several parts of the globe, which are exclusively freshwater in origin, wherein all commercial activities are undertaken. Hence, there is a possibility that ballast tanks are filled or flushed in such inland locations. Furthermore, all the species carried through the ballast tank biofilms may or may not survive the journey, especially the phototrophic biomass, which is affected by the lack of light within these tanks and thus can contribute to the heterotrophic mode of nutrition (Hede and Khandeparker, 2018). Moreover, the survival of biofilm communities also depends on several other factors such as the adaptation of tolerant communities, competition for resources, predation etc. (van Elsas et al., 2012; Hede and Khandeparker, 2018). The length of time that organisms can survive in ballast tanks varies, but densities are significantly lesser than at the beginning of the voyage (Verna et al., 2016). A recent study by Khandeparker et al. (2020), reported that the bacterial community and species richness in the ballast water tanks increased significantly during the short-term

voyage and the composition was different from that of the natural seawater (source water).

In general, the voyage duration differs and ranges from a few weeks to about 80 - 100 days or more (Seaplus, 2011). Thus, the age of interior ballast tank biofilms in the vessels also differ accordingly and can influence the composition of these biofilm microbial communities. A recent microcosm study on the freshwater biofilms developed in an inland port environment, addressing the influence of ballast tank conditions on these communities revealed that they were not able to sustain under dark conditions, and collapsed when exposed to short-term dark incubations, i.e., 30 days in the same environment (Hede and Khandeparker, 2018). However, this may not always be the reality, since the biofilm community characteristics differ with the changing environmental conditions, and depend on the inoculum characteristics of the source port and period of exposure (Khandeparker et al., 2017a). The present investigation addressed two independent case studies focused on the fate of freshwater biofilm communities when subjected to long-term dark incubations, and their impact when released in an estuarine environment. Such a scenario is expected when ballast tank filling occurs in inland port locations which are mostly of freshwater in origin. Subsequently, these biofilms can act as a vector in the form of suspended aggregates or movable biofilms, which may disperse communities (fragments or single cells) on maturation or are sloughed off as biofilm clusters by fluid-driven forces during ballasting operations. The fate of such dislodged biofilms if deballasted in the estuarine environment, was assessed in the present study. The rationale for considering the estuarine environment is based on the reports that many of the shipping ports are located in river mouths and estuaries characterized by broad temporal salinity changes (Keller et al., 2011; Paiva et al., 2018). These estuaries being delicate ecosystems are susceptible to anthropogenic disturbances by the introduction of alien species via major vectors and can pose a significant threat (NOAA, 2012; Crespo et al., 2018). The introduced biofilm species can potentially influence the ecology and composition of estuarine microbial communities, but to our knowledge, no previous research has specifically addressed this topic. With the development of culture-independent

methods such as quantitative polymerase chain reaction (qPCR), a rapid quantitative community-level assessment of microbes has become much easier (Bilodeau, 2011; Kim et al., 2013; Hede and Khandeparker, 2018), and was used in the present study.

Taking the above points into consideration, microcosm experiments were conducted on biofilms developed in inland freshwater port under varying environmental conditions to investigate the effect of long-term dark incubations on these biofilm communities, and their possible discharge effects on the estuarine water column microbial communities. We anticipated a change in the abundance of microbial assemblages and bacterial community structure in these biofilms with ageing. Subsequently, we hypothesized that the translocation of these aged biofilm communities in the estuarine water would result in modulation of estuarine water column characteristics, thereby causing an alteration in the community structure and introduction of new taxa which could be either beneficial or harmful to the environment.

3C.2 Materials and Methods

3C.2.1 Experimental design and sampling

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

3C.2.1.1 Fate of freshwater biofilm communities subjected to long-term dark incubations

Two independent studies were conducted on freshwater biofilms developed in the freshwater station located in Kidderpore Dock area (22°32.45994' N, 088°18.95370' E) across the Hooghly River in the West Bengal, India. A schematic illustration of the experimental workflow is depicted in Figure 3C.1. Biofilms were initially developed on standard pre-treated glass slides (25.4 mm x 76.2 mm) by deploying them in the station (subsurface layer) for five days for the case study I and II, during February 2015 and December 2015, respectively. This initial period of biofilm development was used to attain a heterogeneous biofilm community with sufficient biomass (Hede and Khandeparker, 2018). Subsequently, the 5-day-old biofilms were evaluated for different microbiological parameters as described below.

Since the voyage duration differs and lasts for about 80 - 100 days or more, the 5-day old freshwater biofilms were exposed to 80 and 100 days' incubation to evaluate the influence of long-term dark incubation on these biofilm microbial communities. These biofilms were further transferred to the large polycarbonate rectangular cage microcosms (43 x 27 x 18 cm, Tarsons, 15 l) containing the same field water for 100 days (case study I) and 80 days (case study II), which were incubated and aged in the dark. These microcosms were static during incubations and covered with a thick black cover to avoid entry of light. After incubation, these 100-day old and 80-day old biofilms were evaluated for different microbiological parameters as described below.

A set of seventeen slides were randomly chosen and collected for microbiological analyses. Two slides 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 subsample was used for the analysis of total viable count (TVC), and pathogenic bacterial abundance while 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, set of ten individual slides were scraped and subsequently pooled using 20 ml of 0.22 µm filtered autoclaved seawater. Lastly, five slides were scraped using 20 ml of 0.22 µm filtered autoclaved seawater for chlorophyll *a* content analysis.

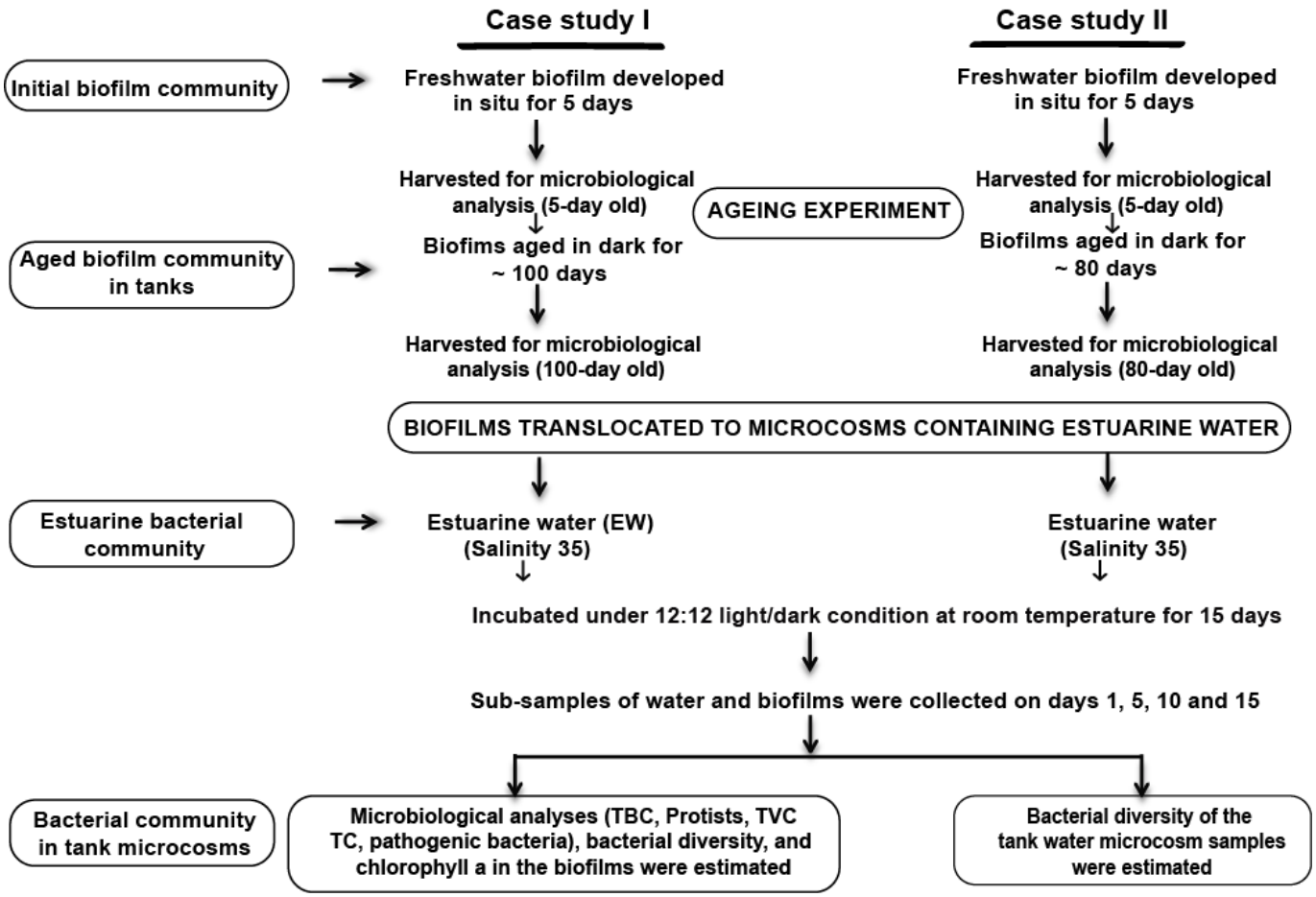


Figure 3C.1 Schematic diagram illustrating the experimental workflow. TBC = Total Bacterial Count, TVC = Total Viable Count, TC = Total Coliforms, VA = *Vibrio alginolyticus*, VP = *Vibrio parahaemolyticus.*

3C.2.1.2 Microcosm experiments

The microcosm experiments were carried out to evaluate the microbial dynamics of the aged biofilms (100-day old and 80-day old) and their impact when released in the estuarine water and aged seawater, used as control.

For this, the 100-day old and 80-day old aged biofilms were subjected to microcosms, containing the freshly collected estuarine water (EW), from at the Dona Paula Bay situated at the mouth of the Zuari estuary (salinity ~35), along the central west coast of India, in May 2015 and March 2016 for case study I and II, respectively. Besides, the aged biofilms were also subjected to the aged seawater (ASW), used as representative of aged water in ballast tanks (control) for both the experiments, with salinity similar to that of estuarine water (~35), and expected to contain a minimal amount of bacterial cells, dissolved organic matter and nutrients (Mou et al., 2015) in comparison to coastal estuarine water. ASW (aged seawater) was prepared by storing fresh seawater in the dark for several months or longer to age. The microbiological analyses were also conducted for the freshly collected estuarine water and aged seawater to determine their initial microbial community.

For each treatment, two microcosms were established, and the individual microcosm was considered as a replicate containing sixty-eight slides each. The glass slides were placed on slide holders and deployed in each of the sterile polycarbonate rectangular cage microcosms (43 x 27 x 18 cm, Tarsons, 15l), which were filled with estuarine water and aged seawater (12 litres). 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. Besides this, 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.

3C.2.2 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 the experiments. Salinity was measured using Salinometer.

The measurement of nutrient concentrations (mainly nitrate, phosphate, nitrite, ammonia) in the estuarine water, aged seawater, and microcosm samples were carried out by SKALAR SANplus ANALYSER, using skalar methods.

3C.2.3 Chlorophyll a concentration in biofilms

The protocol details are described previously in Chapter 2, subsection 2.2.3.

3C.2.4 Microbiological analysis

3C.2.4.1 Total Viable Count (TVC)

The protocol details are described previously in Chapter 2, subsection 2.2.4. In addition, the Zobell Marine Agar (ZMA) 2216 was used to count marine bacteria from the biofilms subjected to microcosm experiments.

3C.2.4.2 Total Bacterial Count (TBC) and Protists

The protocol details are described previously in Chapter 2, subsections 2.2.5 and 2.2.6.

3C.2.5 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 MO BIO PowerBiofilm™ DNA extraction kit, as stated by the manufacturer's protocol.

3C.2.6 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.

3C.2.7 Bacterial diversity of water and biofilm samples by quantitative PCR (qPCR)

The dominant bacterial taxa, namely α-proteobacteria, β-proteobacteria, γ-proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes were quantified in different samples by real-time quantitative polymerase chain reaction (qPCR) using group-specific primers, as described earlier, with slight modifications (Hede and Khandeparker, 2018). The universal primers (926F - AAACTCAAAKGAATTGACGG and 1062R - CTCACRRCACGAGCAGAC) were chosen for amplification of the 16S rRNA genes (De Gregoris et al., 2011). The corresponding annealing temperatures for the universal and group-specific primers were standardized in the laboratory. The qPCR assays were carried out using Rotor-Gene Q (Qiagen) to measure universal and group-specific bacteria.

The Fast SYBR® Green PCR Master Mix (Applied Biosystems) and the appropriate PCR primers were used to run qPCR reactions. All the qPCR mixtures were run in triplicates, contained in the total volume of 20 µl, 10 µl of the master mix, 2 µM of each primer except for β-proteobacteria, and Actinobacteria (1.0 µM of each primer), 1 µl of DNA template (~1.0 - 5.0 ng of environmental DNA), and made up with nuclease-free water (AmbionTM). The protocol for qPCR amplification using universal and 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 α-proteobacteria, annealing at 60°C), and the final extension at 72°C for 30s. Melt curve analysis (Tm) was done to determine the specificity of amplification for each qPCR reaction. Data were further processed using Rotor-Gene software (v.2.3.1, Qiagen) and PCR amplification efficiency (E) was evaluated using the software. The water, as well as biofilm data, were expressed as the relative abundance of the bacterial taxa (%) based on the sum of the absolute abundances of all group taxa.

3C.2.8 Statistical analysis

The abiotic data were normalized, and the biotic data were log (x+1) transformed. To fulfil the criteria for parametric tests, the data were checked for the normality and homogeneity of variances using the Statistica 6.0 program (Stat Soft Inc.,

Tulsa, OK, USA). One-way analysis of variance (ANOVA) was done to determine the statistical significance between the control and the experimental data, followed by post hoc Tukey's HSD test (Bonferroni corrected) (IBM statistics, SPSS version 16.0). Changes in bacterial communities 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 (Clarke and Warwick, 1994). Also, Simpson's diversity index, which is commonly used as a measure of diversity, was determined 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).

3C.3 Results

3C.3.1 Initial microbial communities before microcosm experiments

3C.3.1.1 Initial biofilm assemblages and their survival during long-term dark incubations

When the 5-day old in situ freshwater biofilms with distinct communities were subjected to prolonged dark incubation, the abundance of microbial assemblages in the aged biofilms differed between case study I (100 days) and case study II (80 days), as depicted in Table 3C.1a (One-way ANOVA, $p < 0.001$). In the case of 100-day old biofilms, all the microbial parameters declined significantly (Table 3C.1a, Tukey's HSD, $p < 0.001$). On the contrary, most of the microbial parameters, i.e. Chl *a*, TVC (marine), *Vibrio parahaemolyticus*, total coliforms, TBC and protists, were abundant and thrived in the 80-day old biofilms (Table 3C.1a). Moreover, the Simpsons diversity index pointed a higher diversity (0.553) in the 80-day old biofilms and were dominated by β-proteobacteria, γ-proteobacteria, Bacteroidetes and Firmicutes, when compared to 100-day old biofilms (0.087), wherein Actinobacteria was the dominant taxa. Thus, these aged biofilms were termed as more diverse and less diverse freshwater biofilms, respectively.

3C.3.1.2 Estuarine water (EW) and aged seawater (ASW) communities

The communities in the estuarine and aged seawater were significantly different between case study I and II, as depicted in Table 3C.1b-c (One-way

ANOVA, $p < 0.001$). In the case of estuarine water, the Simpsons diversity index showed higher diversity (0.71) during case study II and was dominated by Bacteroidetes, Firmicutes, and β-proteobacteria. On the other hand, the diversity index was lower (0.45) during the case study I, wherein Proteobacteria were dominant taxa. Moreover, the culturable *Vibrio* spp. and total coliforms were also abundant (Table 3C.1b).

Although the viable pathogenic bacteria were not detected in the aged seawater, the metagenomic analysis elucidated through quantitative PCR revealed that the bacterial diversity in the aged seawater was dominated by α-proteobacteria, and γ-proteobacteria during the case study I. Whereas, in the case of case study II, β-proteobacteria were dominant taxa (Table 3C.1c). The nutrient concentrations in the estuarine and aged seawater were significantly different between case study I and II, shown in Table 3C.1b-c (One-way ANOVA, $p < 0.001$, Tukey's HSD, $p < 0.001$).

Table 3C.1a Mean values of different biotic variables in the in situ and aged freshwater biofilms. All the data points are averages of several replicates.

S. No		5-day old in situ biofilm	100-day old aged biofilm	5-day old in situ biofilm	80-day old aged biofilm
		Case study I		Case study II	
1	Chl *a* (µg / cm^2) *	0.351	ND	0.754	0.036
2	TVC (freshwater)	2.7×10^3	1.2×10^2	6.9×10^2	2.6×10^2
(CFU/ cm^2)	TVC (marine)*	NS	1.7×10^1	1.0×10^3	3.8×10^2
	Vibrio alginolyticus	2.2×10^2	ND	1.4×10^2	ND
	*V. parahaemolyticus**	6.0×10^0	ND	8.3×10^1	1.1×10^2
	V. cholerae	7.6×10^1	ND	ND	ND
	Total coliforms*	2.1×10^3	ND	6.2×10^1	8.2×10^1
(Cells/ cm^2)	*TBC	3.0×10^6	3.4×10^5	2.5×10^6	3.7×10^6
	*Protists	1.5×10^5	9.5×10^2	9.8×10^4	1.2×10^5
4 (Gene copy numbers / cm^2)	Universal*	3.3×10^4	3.0×10^3	1.3×10^2	8.5×10^2
	*Alphaproteobacteria**	1.6×10^4	4.0×10^2	3.8×10^2	6.5×10^2
	*Betaproteobacteria**	1.1×10^4	3.0×10^1	8.0×10^3	2.4×10^4
	*Gammaproteobacteria**	2.0×10^4	1.2×10^3	3.2×10^3	1.2×10^4
	Bacteroidetes*	2.4×10^2	1.9×10^2	1.2×10^1	1.3×10^3
	Firmicutes*	6.0×10^3	3.2×10^1	1.3×10^1	2.1×10^3
	Actinobacteria*	$1.0 \times 10^{2-}$	3.9×10^4	4.6×10^2	2.8×10^2
	Simpson's Diversity of Index (D)		0.087		0.553
	→		Less diverse biofilms		More diverse biofilms

S. No = Serial number, ND = Not detected, TVC = Total Viable Count, TBC = Total Bacterial Count, Chl *a* = Chlorophyll *a*, NS = Not sampled. The asterisk (*) indicates significant differences observed between case study I and II aged biofilms (Tukey's HSD, $p \leq 0.05$).

Table 3C.1b continued

Table 3C.1b Mean values of different abiotic and biotic variables in the estuarine water. All the data points are averages of several replicates.

(b) Estuarine water

S. No		(Case study I)	(Case study II)
1	Chl *a* (µg / L) *	0.18	0.37
2 (CFU/mL)	TVC (marine) *	4.5×10^4	3.8×10^3
	Vibrio alginolyticus *	2.2×10^3	ND
	V. parahaemolyticus *	2.1×10^2	1.0×10^1
	V. cholerae	ND	ND
	Total coliforms *	7.5×10^1	1.0×10^1
3 (Cells/mL)	TBC	1.3×10^6	1.9×10^6
	Protists	4.1×10^4	5.2×10^4
4 (Gene copy numbers /µL)	Universal*	1.0×10^5	7.6×10^4
	*Alphaproteobacteria**	1.4×10^5	9.9×10^2
	*Betaproteobacteria**	1.3×10^4	2.5×10^4
	*Gammaproteobacteria**	3.2×10^4	2.7×10^3
	Bacteroidetes *	3.5×10^3	3.9×10^4
	Firmicutes *	5.7×10^3	3.5×10^4
	Actinobacteria *	9.4×10^2	5.6×10^3
5 (µmol)	Nitrate *	8.5	17.4
	Nitrite *	0.9	1.2
	Silicate*	4.5	3.5
	Phosphate*	0.87	0.69
	Ammonia*	17.3	13.4
Simpson's Diversity of Index (D)		0.45	0.71

ND = Not detected, TVC =Total Viable Count, TBC = Total Bacterial Count, Chl *a* = Chlorophyll *a*. The asterisk (*) indicates significant differences observed between case study I and II at the level of $p < 0.05$.

Table 3C.1c continued

Table 3C.1c Mean values of different abiotic and biotic variables in the aged seawater. All the data points are averages of several replicates.

(c) Aged Seawater (Control)

S. No		Case study I	Case study II
1 (Cells/mL)	TBC*	9.9×10^5	1.9×10^6
	Protists*	6.1×10^4	8.1×10^4
2 (Gene copy numbers / µL)	Universal*	1.5×10^5	2.0×10^6
	*Alphaproteobacteria**	1.6×10^4	4.8×10^4
	*Betaproteobacteria**	5.9×10^2	1.4×10^5
	*Gammaproteobacteria**	1.8×10^4	3.0×10^4
	Bacteroidetes*	8.7×10^3	3.1×10^4
	Firmicutes*	1.8×10^2	1.8×10^4
	Actinobacteria*	4.4×10^3	2.1×10^4
3 (µmol)	Nitrate*	41.87	28.45
	Nitrite*	0.08	0.97
	Silicate*	7	10.2
	Phosphate*	0.8	0.9
	Ammonia*	13.2	13.1

TBC = Total Bacterial Count. The asterisk (*) indicates significant differences observed between case study I and II at the level of $p < 0.05$.

3C.3.2 Dynamics of aged biofilms when released in the estuarine and aged seawater

3C.3.2.1 Case study I tank microcosms: Less diverse biofilms exposed to EW and ASW

All the microbial parameters in the less diverse biofilms, i.e., TBC, protists, TVC (marine bacteria), total coliforms and abundance of pathogenic bacteria, i.e. *Vibrio* spp., increased significantly when exposed to EW, except on Day 15 ($p < 0.001$, Tukey's HSD, Figure 3C.2a-h). On the other hand, significant variations in the Chl *a* were observed in the biofilms when subjected to EW (Wilks' Lambda, $p < 0.001$), wherein the concentration peaked on Day 15 in the less diverse biofilms (0.1 ± 0.0002 µg/cm^2, $p < 0.001$, Tukey's HSD). Overall, the microbial parameters in the biofilms were significantly different between the treatments (EW vs ASW), as visualized using NMDS ordination (Figure 3C.3a-b). Analysis of Similarity (ANOSIM) also revealed these differences (by species and diversity: $R = 0.965$, $p < 0.03$). The biofilm bacterial communities were dominated by γ-proteobacteria and Bacteroidetes in the less diverse biofilms when exposed to EW (Figure 3C.4a). While, Actinobacteria and Firmicutes were abundant when subjected to aged seawater (ASW) during the study period (Figure 3C.4c).

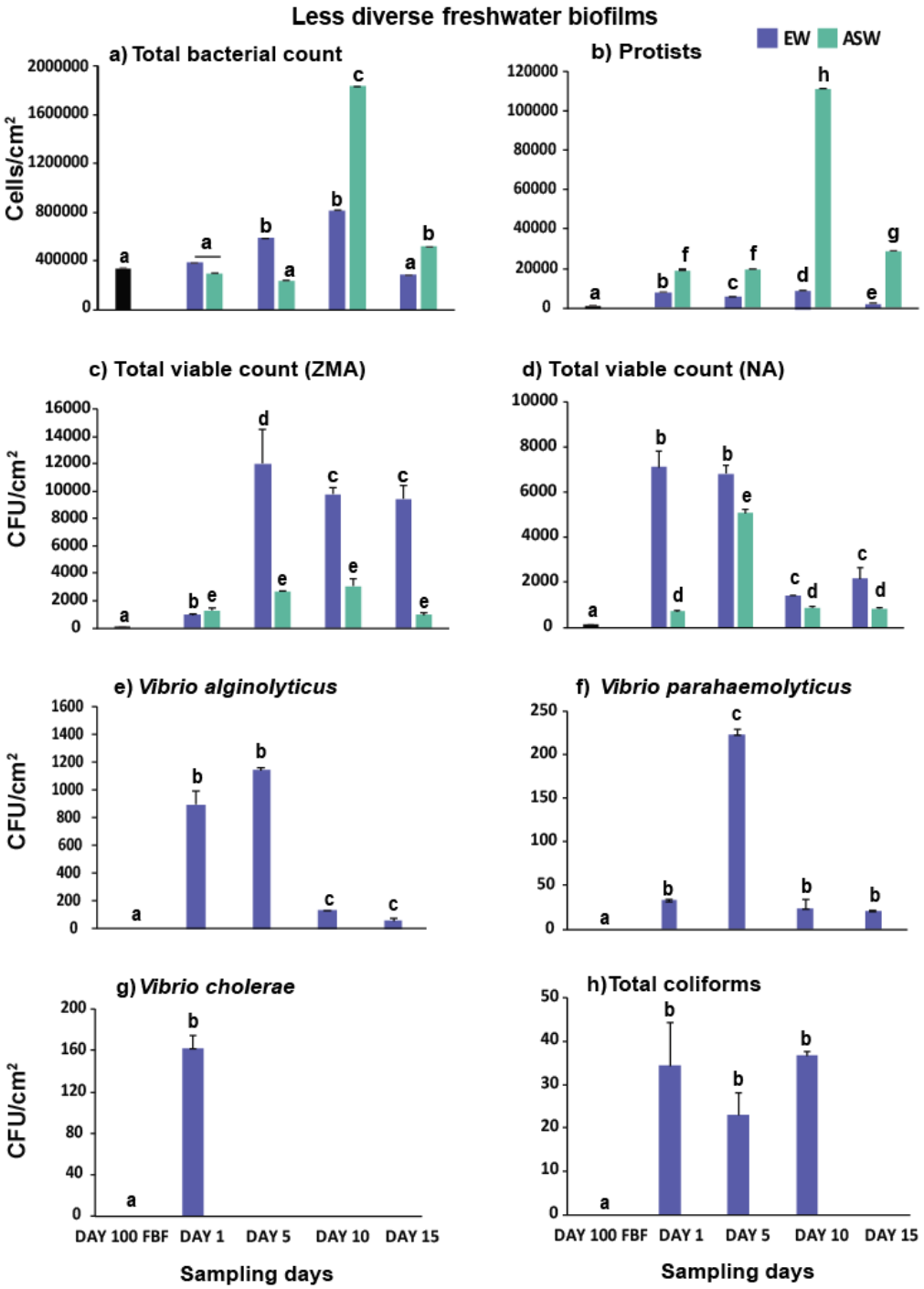


Figure 3C.2 Mean values of (a) total bacterial count, (b) protists, (c) total viable count (ZMA), (d) total viable count (NA), (e) *V. alginolyticus*, (f) *V. parahaemolyticus*, (g) *V. cholerae*, and (h) total coliforms in the less diverse freshwater biofilms exposed to estuarine water (EW), and aged seawater (ASW) in the tank microcosms during the sampling days. FBF = freshwater biofilm, ZMA = Zobell Marine Agar, and NA = Nutrient agar. 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 control and treatments (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.

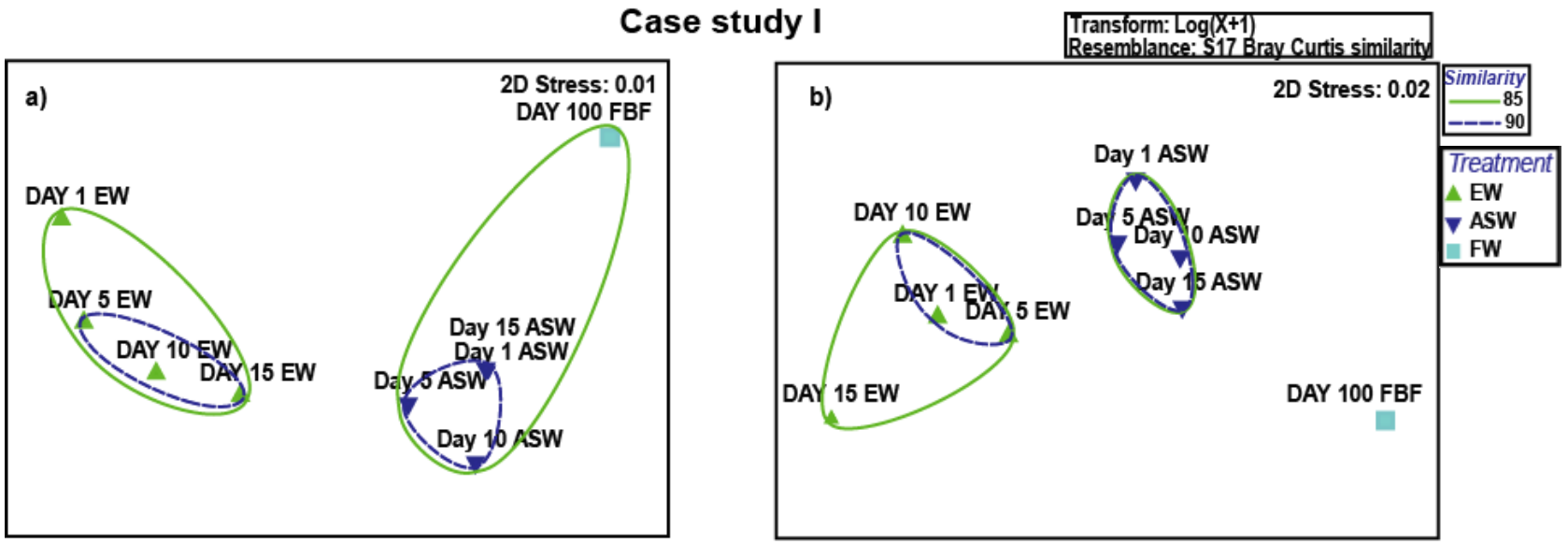


Figure 3C.3 Non-metric multidimensional scaling (NMDS) analysis of (a) culturable (including TVC, pathogenic bacteria, TC), TBC, and protists; and (b) biofilm bacterial communities in less diverse freshwater biofilms (case study I) subjected to microcosm experiments. Samples clustered together with 85 % and 90 % similarity in the dendrogram are circled by green, and blue ovals, respectively, in the plot. Symbols indicate the treatments (EW = Estuarine water, ASW = Aged seawater, F = Freshwater, and FBF = Freshwater biofilm), whereas Day 1, Day 5, Day 10, and Day 15 indicate the days of sampling in the EW/ASW. Day 100 FBF = 100-day old freshwater biofilms.

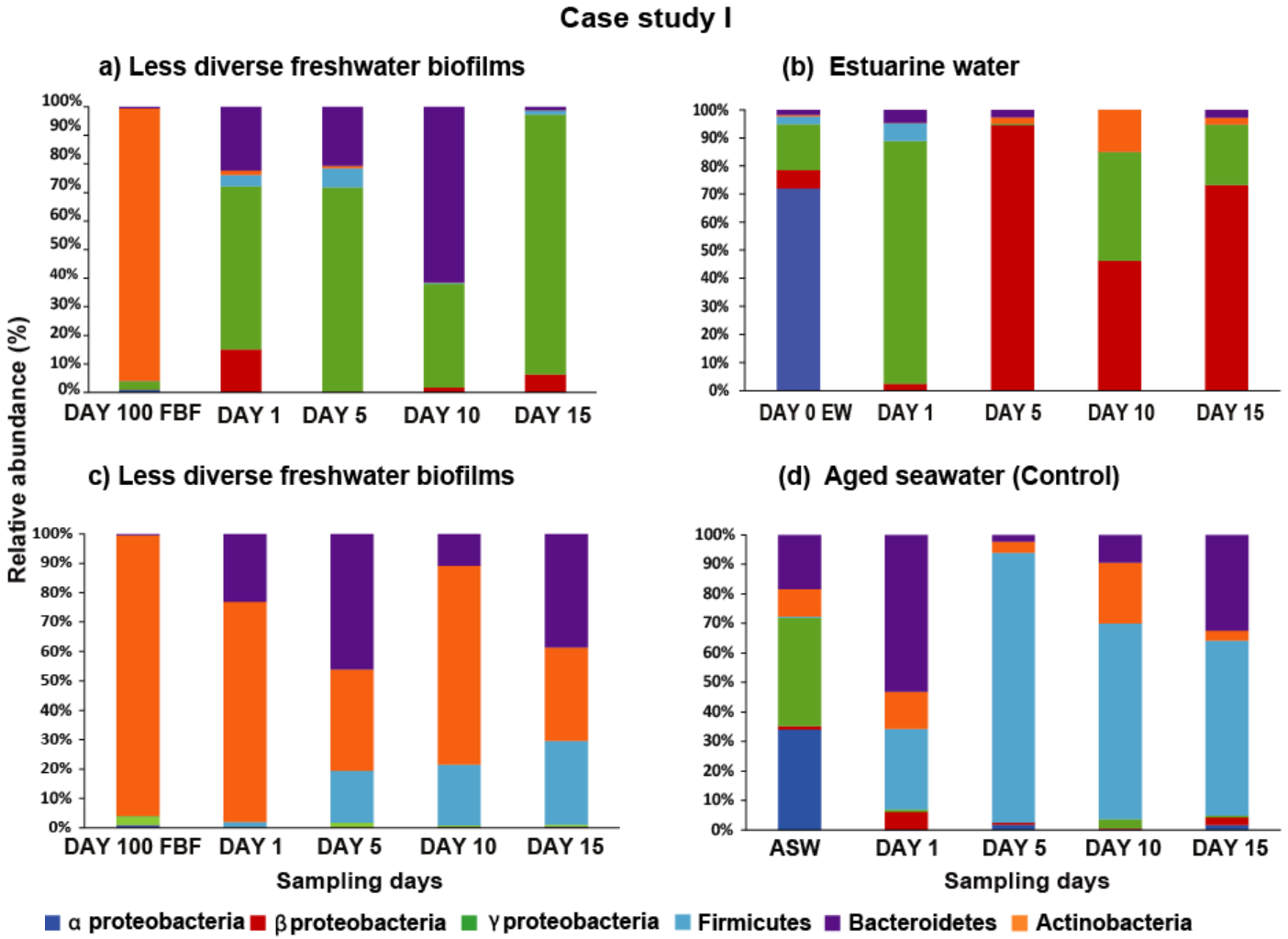


Figure 3C.4 Bacterial community composition demonstrating changes in the (a) less diverse freshwater biofilms exposed to the (b) estuarine water, and (c) less diverse freshwater biofilms exposed to the (d) aged seawater (ASW). α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (blue), Bacteroidetes (purple) and Actinobacteria (orange). FBF = Freshwater biofilm, EW = Estuarine water, ASW = Aged seawater, % = percentage.

3C.3.2.2 Case study II tank microcosms: More diverse biofilms exposed to EW and ASW

In the case of more diverse biofilms, no significant changes were observed in the TBC and protist abundance when exposed to EW (Figure 3C.5a-b). However, TVC (both marine and freshwater) peaked significantly on Day 1, followed by a drastic decrease in its abundance by the end of the experimental period (Figure 3C.5c-d). Among *Vibrio* spp., primarily, *Vibrio alginolyticus* and *Vibrio cholerae* appeared in the biofilms when subjected to EW (Figure 3C.5e, 3C.5g). It was interesting to note that the culturable *Vibrio* spp. (*V. alginolyticus* - 1.03×10^4 CFU/mL, *V. parahaemolyticus* - 8.0×10^2 CFU/mL, and *V. cholerae* - 1.06×10^3 CFU/mL) also appeared and proliferated into the surrounding water column (Day 1), which was devoid of it. However, their abundance decreased significantly in the water column by the end of the experiment ($p < 0.001$, Tukey's HSD) and was influenced by protists populations ($p < 0.05$).

Vibrio parahaemolyticus and the total coliforms, which were initially dominant in the more diverse biofilms decreased significantly upon exposure to EW and ASW ($p < 0.001$, Tukey's HSD, Figure 3C.5f, 3C.5h). Moreover, Chl *a* concentration also decreased significantly in the biofilms, except on Day 5 (Wilks' Lambda: $p < 0.001$, data not shown) and showed a significant positive correlation with *Vibrio parahaemolyticus* ($p < 0.05$). Overall, the microbial parameters in the biofilms were significantly different between the treatments (EW vs ASW) (Figure 3C.6a-b). Analysis of Similarity (ANOSIM) also revealed these differences (by species: $R = 0.931$, $p < 0.003$; by diversity: $R = 0.486$, $p < 0.006$). The biofilm bacterial communities were dominated by γ-proteobacteria and Bacteroidetes (Figure 3C.7a). On the other hand, when the biofilms were subjected to the aged seawater, a slimy EPS layer was observed on the slides at the end of the experimental period, and these biofilms were dominated by Bacteroidetes (Figure 3C.7c).

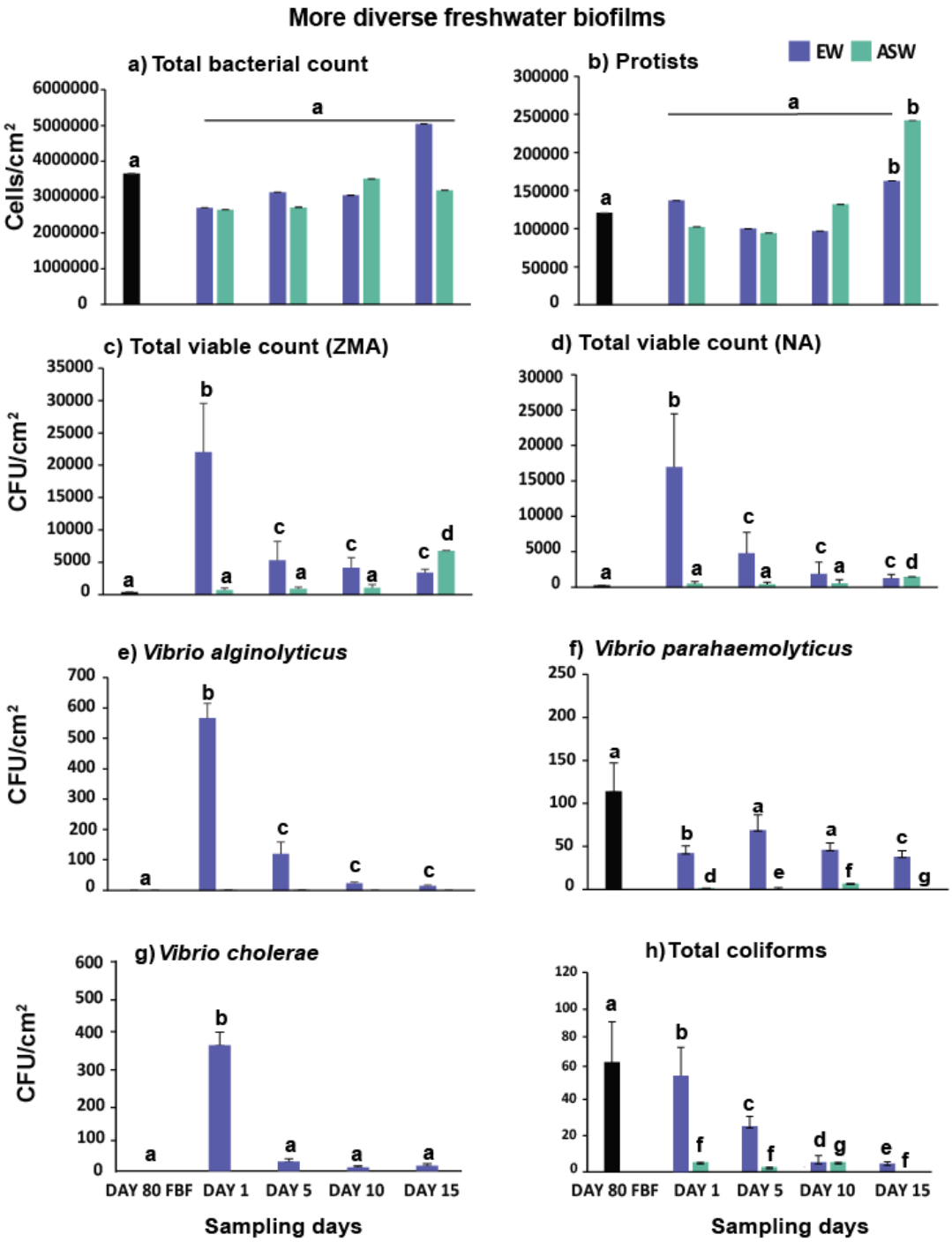


Figure 3C.5 Mean values of (a) total bacterial count, (b) protists, (c) total viable count (ZMA), (d) total viable count (NA), (e) *V. alginolyticus*, (f) *V. parahaemolyticus*, (g) *V. cholerae*, and (h) total coliforms in the more diverse freshwater biofilms exposed to estuarine water (EW) and aged seawater (ASW) in the tank microcosms during the sampling days. FBF = Freshwater biofilm, ZMA = Zobell Marine Agar, and NA = Nutrient Agar. 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 control and treatments (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.

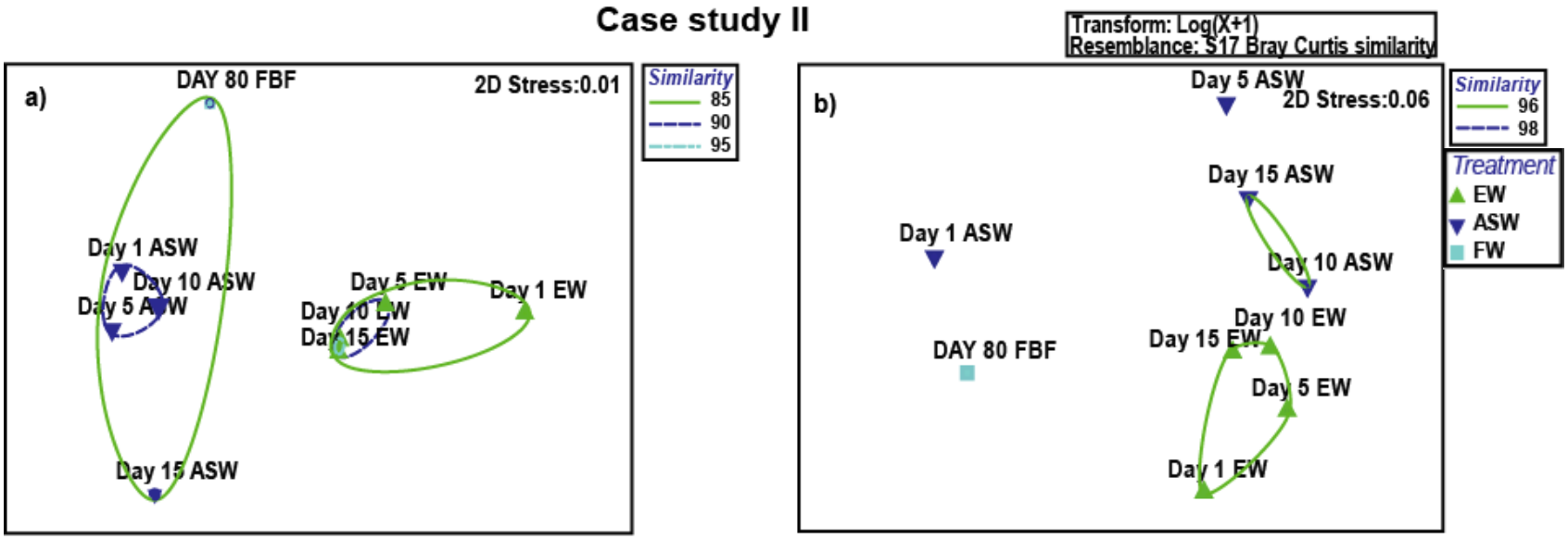


Figure 3C.6 Non-metric multidimensional scaling (NMDS) analysis of (a) culturable (including TVC, pathogenic bacteria, TC), TBC, and protists; and (b) biofilm bacterial communities in the more diverse freshwater biofilms (case study II) subjected to microcosm experiments. Samples clustered together with respective similarity percentages in the dendrogram are circled by green, blue and aqua ovals, in the plot. Symbols indicate the treatments (EW = Estuarine water, ASW = Aged seawater, FW = Freshwater, and FBF = Freshwater biofilm), whereas Day 1, Day 5, Day 10, and Day 15 indicate the days of sampling in the EW/ ASW (Day 80 FBF = 80-day old freshwater biofilms).

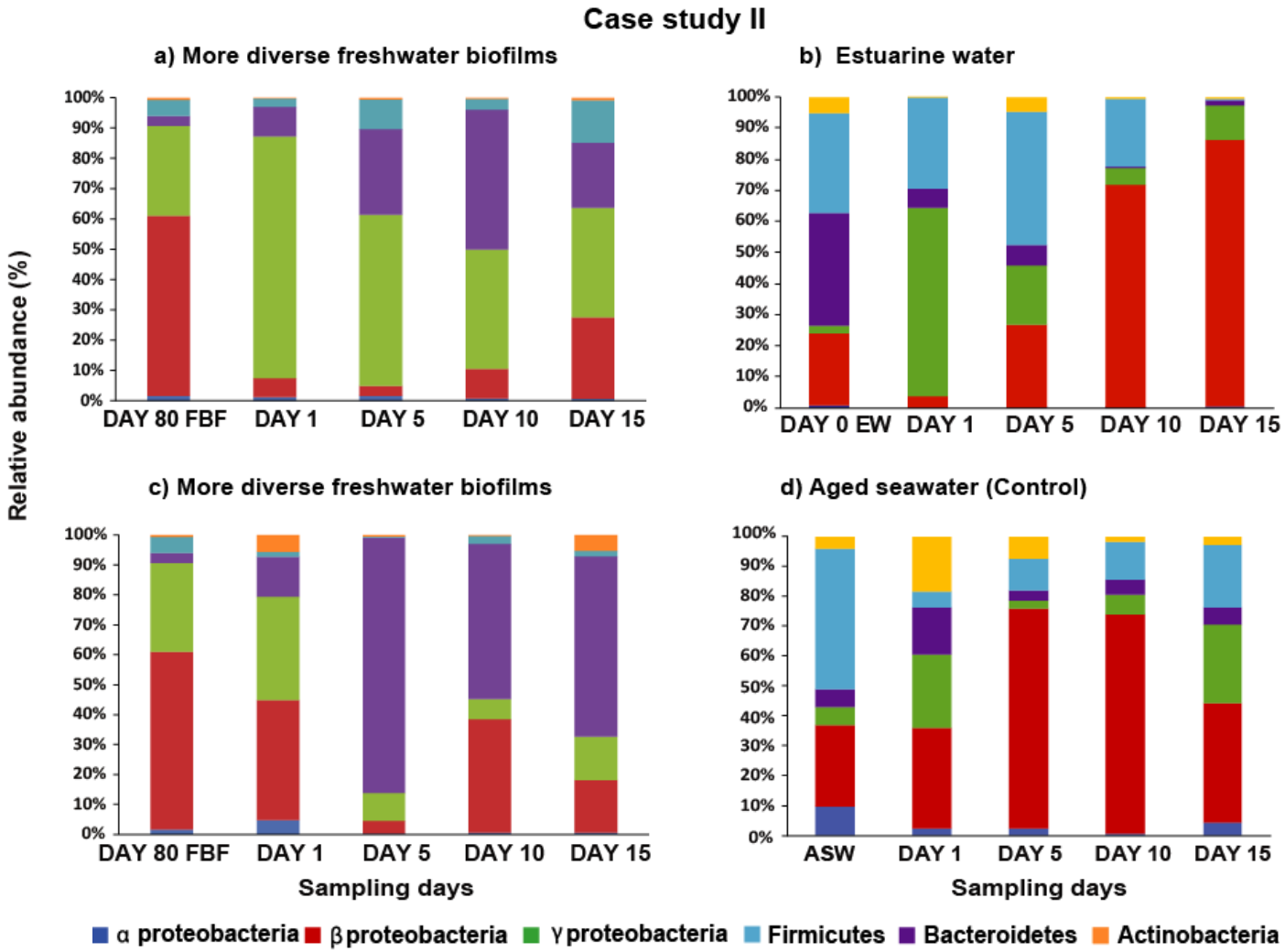


Figure 3C.7 Bacterial community composition demonstrating changes in the a) more diverse freshwater biofilms exposed to the (b) estuarine water, and the c) more diverse biofilms exposed to the (d) aged seawater (ASW). α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (blue), Bacteroidetes (purple) and Actinobacteria (orange).

3C.3.2.3 Influence of aged freshwater biofilms on the estuarine and aged seawater in the tank microcosms

α-proteobacteria and Firmicutes decreased significantly ($p < 0.001$, Tukey's HSD) in the estuarine water exposed to the less diverse biofilms. Moreover, γ-proteobacteria peaked on Day 1, followed by β-proteobacteria, which increased drastically at the end of the experimental period (Figure 3C.4b), and was influenced by ammonia ($p < 0.05$). A similar trend was observed in the estuarine water exposed to the more diverse freshwater biofilms, wherein, γ-proteobacteria and β-proteobacteria taxa dominated (Figure 3C.7b). Drastic and significant changes were observed in the estuarine water column communities, regardless of the type of biofilms exposed, as visualized using NMDS ordination (Figure 3C.4b, 3C.7b). Analysis of Similarity (ANOSIM) also confirmed these differences (by diversity: $R = 0.52$, $p < 0.008$; by diversity: $R = 0.24$, $p < 0.032$, respectively). On the other hand, the significant differences in the water column bacterial communities were also observed on the exposure of biofilm types to aged seawater (ASW) (Figure 3C.4d, 3C.7d), as visualized using NMDS ordination (Figure 3C.8a, 3C.8b). Moreover, the aged seawater exposed to less diverse biofilms was dominated by Firmicutes, while β-proteobacteria peaked in the aged seawater exposed to the more diverse biofilms (Figure 3C.4d, 3C.7d).

3C.4 Discussion

The present study showed a temporal variation in the freshwater biofilm microbial communities, resulting in the less diverse and more diverse biofilms. A pictorial illustration of the response of the less diverse and more diverse freshwater biofilms exposed to estuarine water is represented in Figure 3C.9. When less diverse biofilms were exposed to estuarine water with comparatively higher diversity index, 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 the establishment and proliferation of the water column communities.

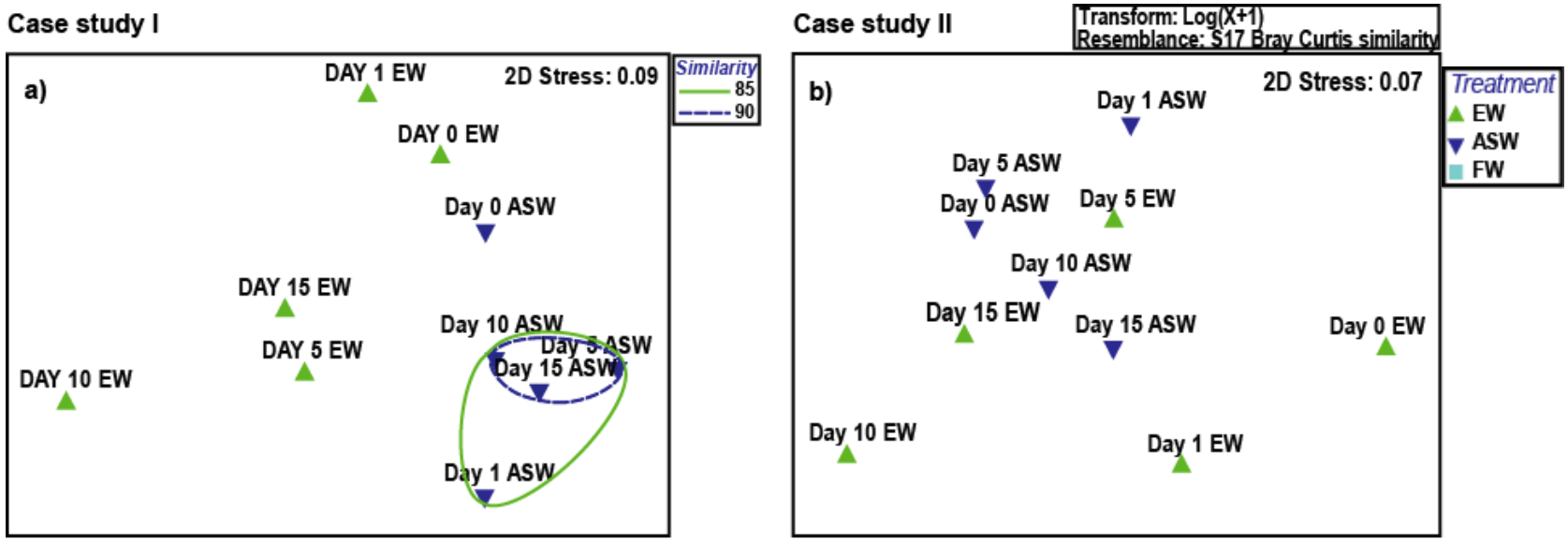


Figure 3C.8 Non-metric multidimensional scaling (NMDS) analysis of water column bacterial communities in the tank microcosms when exposed to (a) less diverse freshwater biofilms (case study I), and b) more diverse freshwater biofilms (case study II) during the microcosm experiments. Samples clustered together with respective similarity percentages in the dendrogram are circled by green and aqua ovals, in the plot. Symbols indicate the treatments (EW = Estuarine water, ASW = Aged seawater, and FW = Freshwater) whereas Day 0, Day 1, Day 5, Day 10, and Day 15 indicate the days of sampling in the EW/ASW.

These results point out the lack of competition between the less diverse communities in the biofilm niche and the estuarine communities, which led to the successful establishment of water column microbial communities in these biofilms.

Likewise, in the case of the bacterial community composition, the less diverse biofilms initially dominated by Actinobacteria resulted in a significant reduction in their numbers and were colonized by surrounding estuarine communities comprising of γ-proteobacteria and Bacteroidetes. The dominance of γ-proteobacteria and Bacteroidetes in the biofilms could be attributed to their close association with the diatoms. Several studies have demonstrated that the Proteobacteria and Bacteroidetes are the main heterotrophic bacterial phyla associated with the diatoms (Grossart et al., 2005; Sapp et al., 2007; Guannel et al., 2011; Amin et al., 2012). Within these phyla, specific genera (e.g., *Vibrio, Alteromonas*, and *Flavobacterium*) appear to be strongly associated with the diatoms (Rehnstam-Holm et al., 2010; Buchan et al., 2014; Khandeparker et al., 2014; Dang and Lovell, 2016). On the other hand, when the less diverse biofilms were subjected to aged seawater, with different inoculum characteristics, which was used as a control, Gram-positive spore-producing Actinobacteria and Firmicutes were the dominant taxa. Thus, it seems that the less diverse biofilms had plenty of empty spaces that resulted in the colonization of the microbial communities from the surrounding water.

Unlike certain estuarine communities which colonized the less diverse biofilms, the resulting shifts in the ambient water column bacterial communities might have influenced other genera of the water column communities. This was evident from a relative decrease in the abundance of α-proteobacteria and Firmicutes during the study period. On the other hand, the abundance of β-proteobacteria increased in the water column when subjected to less diverse biofilms. The ammonia concentrations had a positive influence on β-proteobacteria abundance during the study period. This could be attributed to the proliferation and dominance of ammonia-oxidizing β-proteobacteria (AOB) that seems to play a significant role in the biogeochemical cycling of nitrogen by converting ammonium to nitrite (Schleper and Nicol, 2010; Damashek and Francis, 2018). This result was consistent with the previous study (Veettil et al., 2015).

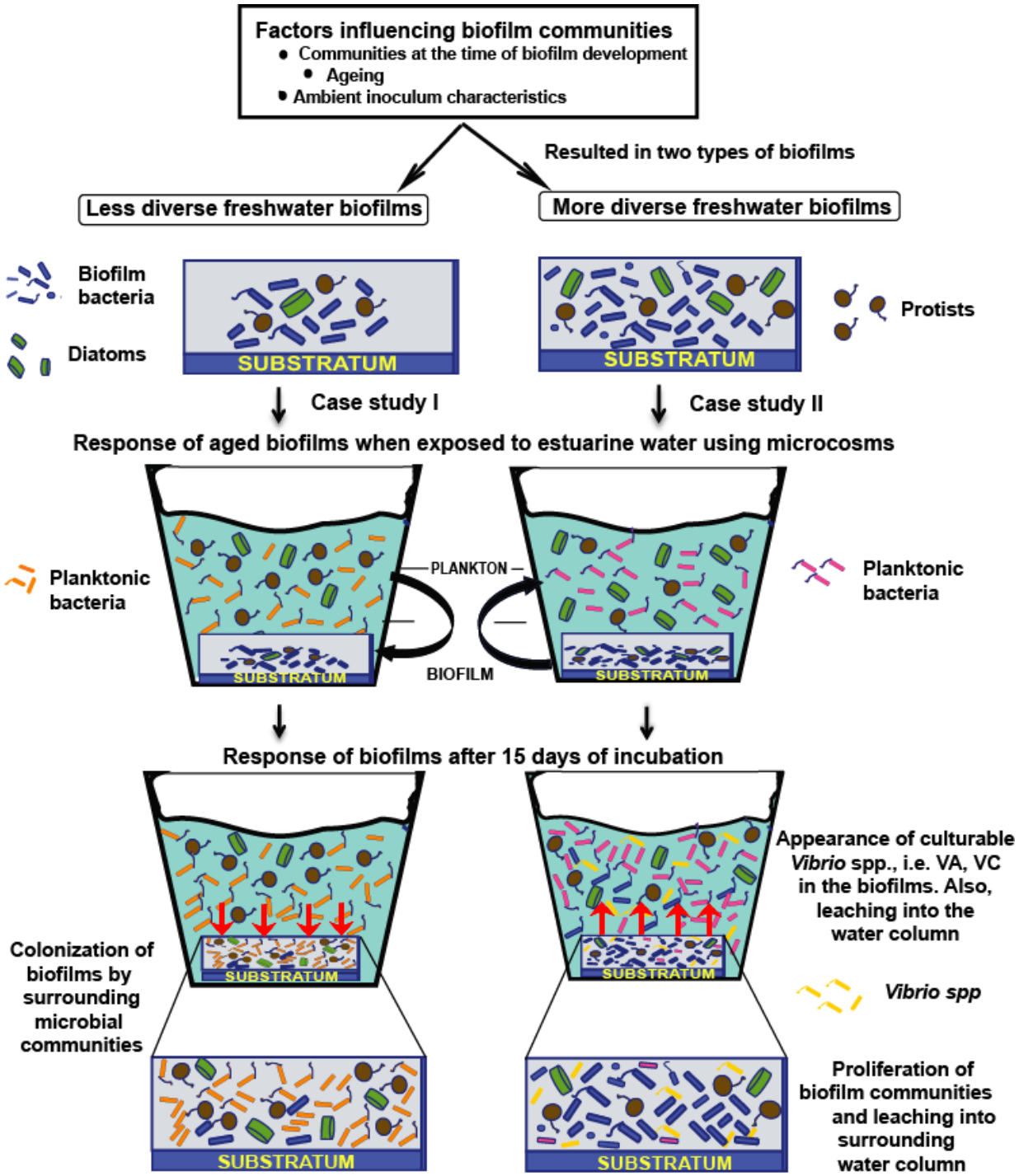


Figure 3C.9 Pictorial representation of the response of the less diverse and more diverse freshwater biofilms exposed to estuarine water. VA = *Vibrio alginolyticus*, VC = *V. cholerae*.

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 (Griffiths, 1989; Verhagen et al., 1993; Bonkowski, 2004; Pogue and Gilbride, 2007; Rosenberg et al., 2009). These high levels of ammonia are believed to favour the growth

of *Nitrosomonas* spp. (Hiorns et al., 1995). These results suggest that such conditions, wherein the waters are enriched with ammonia-oxidizing β-proteobacteria, known for their crucial role in the biogeochemical cycling of nitrogen, could be beneficial if translocated to the new environment by contributing to nitrite and nitrate production. 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 and possibly the translocation of ammonia-oxidizing β-proteobacteria from ballast waters may or may not have a real impact in the biogeochemical cycling of nitrogen in the recipient environment.

On the other hand, the more diverse biofilms, dominated by γ-proteobacteria, which includes most of the pathogenic bacteria, and Bacteroidetes, although initially increased in biofilms under the influence of the estuarine water with high salinity (~35), their sloughing off resulted in their release into the surrounding water column. These results point out the biotic interactions between the established biofilm communities and estuarine water column communities, especially the ability of the resident biofilm members to competitively exclude outsiders (estuarine communities) from entering the biofilms. Such mechanisms mediating the competition in the biofilms have been broadly reviewed (Rendueles and Ghigo, 2015; Stubbendieck and Straight, 2016). Another important factor influencing their dynamics is salinity, which is known as an important physiological stress factor for riverine bacteria (Logares et al., 2009; Székely et al., 2013). Moreover, it is also responsible for the distribution of bacterial communities in the aquatic ecosystems (Painchaud et al., 1995). However, this seemed less applicable to the more diverse freshwater biofilm bacterial communities exposed to estuarine water with higher salinity, wherein the salinity did not influence the freshwater biofilm species richness. This is in congruence with a similar study on water column freshwater bacteria from the same port environment, which could withstand salinity stress conditions (Kuchi and Khandeparker, 2020). Moreover, this result is also consistent with the previous experimental study reported by Zhang et al. (2014). On the other hand, when these biofilms were exposed to aged seawater, with different inoculum characteristics, the slimy EPS layers were observed on the slides. Bacteroidetes dominated these biofilms at the end

of the experimental period. Members of this group mainly, *Flavobacterium* class are known for the production of slime layer of exopolysaccharides and tolerate a wide range of salinities from freshwater to seawater (Bernardet and Bowman, 2006; Buchan et al., 2014). Moreover, species belonging to this genus are known to dominate attached microbial communities and are the major contributors to the utilization and degradation of EPS (Zhang et al., 2014; Zhang et al., 2015). Therefore, it seems that this increase in polymeric matrix concentration surrounding these biofilms might have helped the communities in maintaining the osmotic pressure between the matrix and the exterior environment.

Whereas, the culturable *Vibrio* spp. appeared in the more diverse biofilms when subjected to the estuarine water during the study period. Among *Vibrio* spp., *V. alginolyticus* and *V. cholerae* belonging to γ-proteobacteria appeared in the more diverse biofilms. 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 more diverse biofilms. Their existence in these aged biofilms is anticipated from high gene copy numbers of γ-proteobacteria as detected by metagenomic analyses using qPCR. It is a survival strategy attained by many bacteria, especially *V. cholerae*, in response to harsh environmental conditions (Colwell, 1985; Colwell et al., 2000; Ramamurthy et al., 2014). Subsequently, the reappearance of culturable *Vibrio* spp. in the biofilms when subjected to the estuarine condition can be attributed to VBNC fraction of *Vibrio* spp. Thus, it seems that *Vibrio* spp. reverted from the VBNC state to culturable forms under these favourable environmental conditions. This phenomenon is widely referred to as resuscitation, and there are only a few reports on the potential of *Vibrio* spp. to revert back to culturable form (Kahla-Nakbi et al., 2007; Mishra et al., 2012; Fernández-Delgado et al., 2015).

It was also interesting to note that the *Vibrio* spp. in the more diverse biofilms were released into the surrounding waters, which were devoid of it. As biofilms mature, dispersal becomes a choice. Hence, bacteria can sense whether it is beneficial to reside within the biofilms or its time to resume the planktonic lifestyle (Kostakioti et al., 2013). Although these *Vibrio* spp., are known to be ubiquitous and survive in a wide range of salinities, it is suggested

that the release of these *Vibrio* spp., especially, *V. cholerae* from the biofilms could be a response to a sudden change in salinity from the freshwater to the estuarine environment. The significance of salinity in the distribution of *V. cholerae* in the aquatic environment has been reported earlier (Lutz et al., 2013; Khandeparker et al., 2015; Khandeparker et al., 2017b). Moreover, the sustenance of *Vibrio* spp. in the estuarine water could also be attributed to the availability of nutrients, especially high levels of nitrate during the study period. Other than salinity and nutrients, temperature and planktons are also significant for the ecology of *Vibrio* spp. (De Magny et al., 2008; Urquhart et al., 2016; Khandeparker et al., 2017a; Taneja et al., 2019), which might have also favoured their growth during the initial days of the experiment. Other than environmental factors, predation by grazers (heterotrophic protists) is another important factor faced by these bacteria in the environment (Matz and Kjellberg, 2005). In the present study, it seems that the *Vibrio* spp., released from the more diverse biofilms, were rapidly grazed by the protists in the water column by the end of the study period. A significant positive correlation was observed between the *Vibrio* spp., i.e. *V. alginolyticus* and *V. cholerae* and the protists abundance in the water column. Earlier studies have demonstrated that the planktonic *Vibrio* spp. are predated by protists populations more efficiently than the ones encased in a biofilm matrix (Martínez Pérez et al., 2004; Matz et al., 2005; Worden et al., 2006; Lutz et al., 2013). These results showed that heterotrophic protists played an important role in controlling *Vibrio* spp. populations in the estuarine conditions of the tank microcosms. However, a recent study has also revealed that the protists release some undigested bacteria (*Vibrio cholerae*) present in their food vacuoles leading to their escape back into the water column (Espinoza-Vergara et al., 2019). Thus, the contamination of water bodies with *Vibrio* spp., and the harmful effects of their pathogenic forms, causing cholera will depend upon the environmental characteristics, which may result in the expression of virulence genes (Khandeparker et al., 2017b).

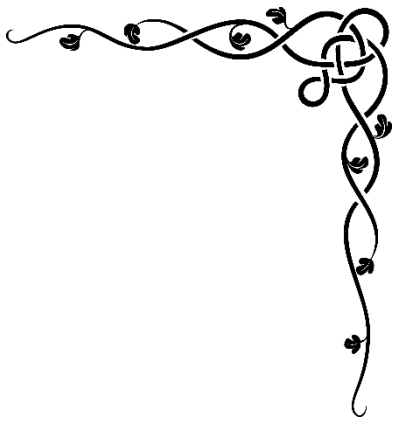
Unlike other communities which proliferated or sloughed off from the more diverse biofilms, the total coliforms and *Vibrio parahaemolyticus* which were initially dominant in these biofilms decreased drastically upon exposure to estuarine water. The decrease in the total coliform numbers could be attributed

to their freshwater origin and intolerance to high saline conditions. The high salinity habitats do not favour colonization for many freshwater species upon exposure, reflecting the importance of osmotic adaptation (Smith et al., 1999; Besemer, 2016). On the other hand, the probable reason for the decline in the *Vibrio parahaemolyticus* abundance could be due to their close association with biofilm-bound microalgae. This was evident from a strong positive correlation of *Vibrio parahaemolyticus* abundance with biofilm-bound microalgal biomass, i.e. chlorophyll *a*, as indicated by the decrease in *Vibrio parahaemolyticus* abundance with a subsequent decline in chlorophyll *a* concentration. These results were consistent with the previous studies (Oberbeckmann et al., 2012; Urquhart et al., 2016; Khandeparker et al., 2017a; Marcinkiewicz et al., 2017). Although coliforms and *Vibrio parahaemolyticus* are known to prevail and survive in the estuarine conditions, their response was different in the microcosm experiment during the present study. Therefore, it can be concluded that the dispersal barriers do exist for some microbes, and not all biofilm-associated pathogenic bacteria may survive when exposed to the new environment. It is also important to note that ballast tanks are generally made up of metals with protective coatings (hydrophobic), and the colonized surfaces used in the present study were glass slides (hydrophilic). Hence, it is expected that the biofilm community composition would differ based on the surface properties (hydrophobic versus hydrophilic) (Pringle and Fletcher, 1983; Mc Eldowney and Fletcher, 1988; Pederson, 1990) and has to be taken into consideration while extrapolating these results to natural conditions.

3C.5 Conclusions

The aged freshwater biofilms (80 and 100 days) on exposure to estuarine water resulted in the modification of bacterial community structure, reduction in some bacterial genera, and the emergence of microbes with different metabolic capabilities not present earlier, which can influence the ecology of that environment. High salinity (~35) of the estuarine water did not influence the more diverse freshwater biofilm species richness upon exposure, thus highlighting the importance of EPS in maintaining the osmotic pressure (evident from an overall increase in freshwater biofilm communities after exposure to estuarine water), but did impact their composition (evident from

the decline in culturable total coliforms and *Vibrio parahaemolyticus*). Interestingly, the dormant *Vibrio* spp., i.e. *V. alginolyticus* and *V. cholerae*, existing in aged more diverse freshwater biofilms could resuscitate back to culturable form when subjected to estuarine water and subsequently, were released into the ambient water column. 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 reduction or proliferation of some genera with different metabolic potential, thus influencing the biogeochemistry, and (2) changes in the water quality, if the species emerged turns out to be pathogenic, and thus have a cascading effect on the environment. Overall, this study provides some fundamental insights into the issues related to ballast tank biofilm management, and this research is anticipated as an initial step towards understanding possible discharge effects of biofilms on the environment when the ballast tanks are filled or flushed in different locales. For implementing the ballast biofilm management strategies, quantifying these effects in the field using advanced molecular tools is needed, which will help in understanding traits that enable them to adapt to changing environment and is an important topic for future investigation.



Chapter 4

Objective 3: Characterize specific compounds produced by chosen marine fouling strains and to identify their role in the biofilm formation

4. Characterize specific compounds produced by chosen marine fouling strains and to identify their role in the biofilm formation

4.1 Introduction

In the aquatic environments, biofilms are ubiquitous and can develop on the number of surfaces (biotic or abiotic), which are rapidly colonized by microorganisms (Salta et al., 2013 and references within). The formation of the biofilms involves the multistage process, starting from an initial attachment on the conditioned surface, which eventually leads to the release of individual bacterial cells on maturation or slough off aggregates as a part of dispersal process, thereby resulting in colonization of new habitats (Stoodley et al., 2001; Sauer et al., 2002; Purevdorj-Gage et al., 2005; Kragh et al., 2016). Subsequently, these biofilms are responsible for a major cause of deleterious effects such as biofouling in the marine environment, leading to the deterioration of materials, including ship hulls, interior ballast tanks, power plants etc., and other aquatic establishments, which has gained considerable attention (Callow and Callow, 2002; Donlan, 2002, Drake et al., 2007, 2008; Huq et al., 2008, Schultz et al., 2011; Salta et al., 2013, Trepos et al., 2014; Dang and Lovell, 2016; Procópio, 2019).

The ability of bacterial cells to aggregate or form biofilms is an adaptive process, wherein they cooperate and improve their chances of survival on sensing and responding to environmental stress (de Carvalho, 2018). Many studies have reported that the formation of biofilms, whether it is cell-surface or cell-cell interactions, involves different molecules (e.g. lipids, polysaccharides, proteins, nucleic acids, and some other biomolecules), and processes (e.g. coaggregation and quorum sensing) which contribute to the development of biofilms (Rickard et al., 2000; Watnick and Kolter, 2000; Stoodley et al., 2001; Sauer et al., 2002; Purevdorj-Gage et al., 2005; Irie and Parsek, 2008; Jain and Bhosle, 2009; Rumbaugh and Armstrong, 2014; Kviatkovski and Minz, 2015; Dang and Lovell, 2016; Abisado et al., 2018 etc.). Moreover, recent studies have focused on the importance of coaggregation, a form of cell-cell interaction, characterized by highly specific recognition and adherence of potential partners mediated by lectin-saccharide interactions between cell surface molecules, thereby possibly resulting in the formation of

biofilms, originating from these pre-formed cell aggregates (Rickard et al., 2000; Rickard et al., 2003; Melaugh et al., 2015; Kragh et al., 2016).

Coaggregation was first reported by Gibbons and Nygaard, between different species of human dental plaque bacteria in the 1970s. It is not a random process but highly specific recognition and adherence of the bacterial cells that employ specific lectins (protein adhesins or carbohydrate moieties) present on their cell surface to recognize partner bacteria (Rickard et al., 2002; Kolenbrander et al., 2006). This process is a key mechanism and phenomenon in the biofilm formation, which also facilitates interaction among different bacterial species in the biofilms (Rickard et al., 2003). These interactions are known to occur between members of the same genus (intra-generic), same species (intra-species) or with different bacteria (inter-species) (Rickard et al., 2002; Katharios-Lanwermeyer et al., 2014). Moreover, the coaggregation process along with autoaggregation, viz. self-aggregation or adherence of identical bacteria to one another (Trunk et al., 2018), depends not only on environmental conditions but also on the distinct bacterial species, that attract potential partners through specific molecules either located on their cell surfaces (including polysaccharide content and the expression of adhesins) or associated with external appendages, thus forming an integral part responsible for the development and formation of multispecies biofilms (Ellwood and Tempest, 1972; Rickard et al., 2000; Rickard et al., 2003; Min et al., 2010).

So far, this coaggregation process and the molecules involved have been extensively studied with regard to oral biofilms (Gibbons and Nygaard, 1970; Cisar et al., 1979; Kolenbrander et al., 1993; Palmer et al., 2003; Rickard et al., 2003; Ledder et al., 2009), and is also known to occur within several clinical and non-clinical environments (Adav et al., 2008; Basson et al., 2008; Malik et al., 2003; Simões et al., 2008; Vornhagen et al., 2013; Cheng et al., 2014; Stevens et al., 2015; Kumar et al., 2019). Relatively few studies are reported on coaggregation between aquatic biofilm bacteria, e.g. in the freshwater biofilms (Buswell et al., 1997; Rickard et al., 2000, 2002), and marine biofilms (Saravanan et al., 2014). These studies have focused on understanding the role of coaggregation in

aquatic biofilm development because of its potential to support or hinder colonization, retention of pathogens within biofilms in shear environments, microbial induced corrosion, and biofouling of surfaces (Stewart and Costerton, 2001; Kerr et al., 2003; Min and Rickard, 2009; Katharios-Lanwermeyer et al., 2014).

The importance of bacterial biofilms and associated biopolymers in 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). Moreover, it has been reported that parentally associated biofilms are unique and play an important role in providing important signals for the settlement of marine invertebrate larvae by producing different types of compounds (Wahl et al., 2012; De Gregoris et al., 2012). Although, it is well known that biochemical cues expressed by the microbial aggregates or biofilms play a very important role in aquaculture and the settlement of the macrofouling organisms (Qian et al., 2007; Hung et al., 2009; Dobretsov and Rittschof, 2020), little is known about the mechanisms by which the aquatic biofilm-forming bacteria from different environments aggregate. Moreover, the molecules involved in the process and their relevance in the biofilm formation are least studied, and the same was addressed in the present study.

Biofilms present in all the aquatic environments including freshwater ecosystems (e.g. lakes, inland port, rivers) and marine environments (e.g. estuaries, open ocean), are influenced by several environmental factors such as salinity, nutrient levels etc. (Mora-Gómez et al., 2016). The biofilm bacterial composition and microbial communities in these environments differ (Hede and Khandeparker, 2018; Khandeparker et al., 2017a). Hence, it is expected that the composition of exopolymers and mechanisms involved in the formation of biofilms by the aquatic biofilm bacteria would also be different. These aquatic biofilm bacteria range from stenohaline bacteria tolerating only a small range of environmental salinities (marine bacteria) to euryhaline ones with their ability to switch from freshwater or saline water (estuarine bacteria), and the freshwater bacteria which cannot tolerate any salinity changes. Among these, the estuarine biofilm-forming bacteria belong to the most dynamic environment, which is influenced by tides and experiences continuous influx

of freshwater, resulting in the changes in salinity (Smyth and Elliott, 2016). Therefore, it is also very essential to understand how the changing environmental conditions such as translocation of biofilm bacteria from one habitat to other (e.g. salinity changes) would play a role in the biofilm formation, from the perspective of microbial invasion. The same scenario can be expected for e.g., in the case of biofilms formed on the interior walls of ship's ballast tanks, when they release individual cells or slough off aggregates during ballasting operations or multiple flushings, which can further contribute to new biofilm formation. Hence, in order to initiate biofilm formation, bacteria either needs to attach to a surface or form co-aggregates, a phenomenon contributing to biofilm formation (Rickard et al., 2003, 2004). Some environments might not favour for colonization, such as if freshwater species are translocated to high salinity habitats or vice versa (Stachowicz et al., 1999; Smith et al., 1999; Verling et al., 2005). Since salinity is regarded as an environmental master factor in the distribution of these aquatic organisms and shaping the bacterial community composition (Stratil et al., 2014; Smyth and Elliott, 2016; Lymperopoulou and Dobbs, 2017; Kuchi and Khandeparker, 2020), any changes in salinity due to environmental conditions or anthropogenic inputs (e.g. via ballast water tanks) may influence their biofilm formation. Hence, the present investigation elucidated the coaggregation capability of biofilm bacteria from different aquatic environments with different salinities. Such studies are crucial in understanding microbial behaviour and their environmental responses during the key stages of biofilm formation (Dang and Lovell, 2016). Moreover, the cues expressed if altered with changing environmental conditions will be perceived differently, either by supporting or hindering the settlement of the macrofouling organisms.

Taking the above points into consideration, we examined (1) the biofilm-forming potential of the bacterial strains isolated from biofilms developed in different aquatic environments (freshwater, estuarine, and marine habitats), and (2) characterized the cell-bound extracellular polymeric substances (CB-EPS) of chosen biofilm-forming bacterial strains (freshwater, estuarine, marine) for functional groups and sugar

composition via Fourier-transform infrared spectroscopy (FT-IR) and high-pressure liquid chromatography (HPLC). The role of cell-bound EPS was then evaluated by assessing the coaggregation potential of these biofilm-forming bacterial strains in the absence of CB-EPS. The bacterial pairs which could retain their aggregation ability even in the absence of CB-EPS were selected, and the aggregates were characterized using FT-IR. It was hypothesized that the ability of these bacterial strains to cope up with other stressor would be more owing to their high coaggregation potential. These bacterial isolates were exposed to one of the important environmental stressor, i.e. abrupt change in salinity, using laboratory experiments. It was expected that the exposure of these biofilm-forming bacteria to salinity stress would either hinder their ability to aggregate or the tolerant bacterial strains might retain their aggregation capability by expressing specific molecules.

4.2 Materials and methods

4.2.1 Sampling site and isolation of bacteria from biofilms

The bacteria were isolated from the in situ biofilms developed on pre-treated glass slides which were deployed for a period of five days in different aquatic environments, namely freshwater (Kolkata Kidderpore dock area- 22° 32.45994′ N, 088° 18.95370′ E), and marine environment, including estuarine (Mandovi estuary-15° 30' 17.442" N, 73° 49' 56.2392" E, west coast of India), and open ocean (the Arabian Sea, 15° 51.482′ N, 072° 43.511′ E). The biofilms were then scraped using a sterile cell scraper (BD Biosciences) in 10 mL of 0.85% saline water (prepared using sodium chloride) for freshwater biofilms, and 0.22 µm filtered autoclaved seawater (~35 salinity) for the remaining two biofilms. After sonication (30W for 60 secs), 100µl of the sample was spread plated on Nutrient Agar (NA, Himedia) for freshwater bacteria and Zobell Marine Agar 2216 (ZMA, Himedia) for marine bacteria. In total, twenty-three successfully isolated bacterial strains from these biofilms were obtained and referred to as freshwater, estuarine and marine biofilm bacteria which were purified, subcultured and maintained at 4 ºC.

4.2.2 Experimental design

The summarised methodological flow chart is illustrated in Figure 4.1, and the experimental study was divided into two experiments as described below.

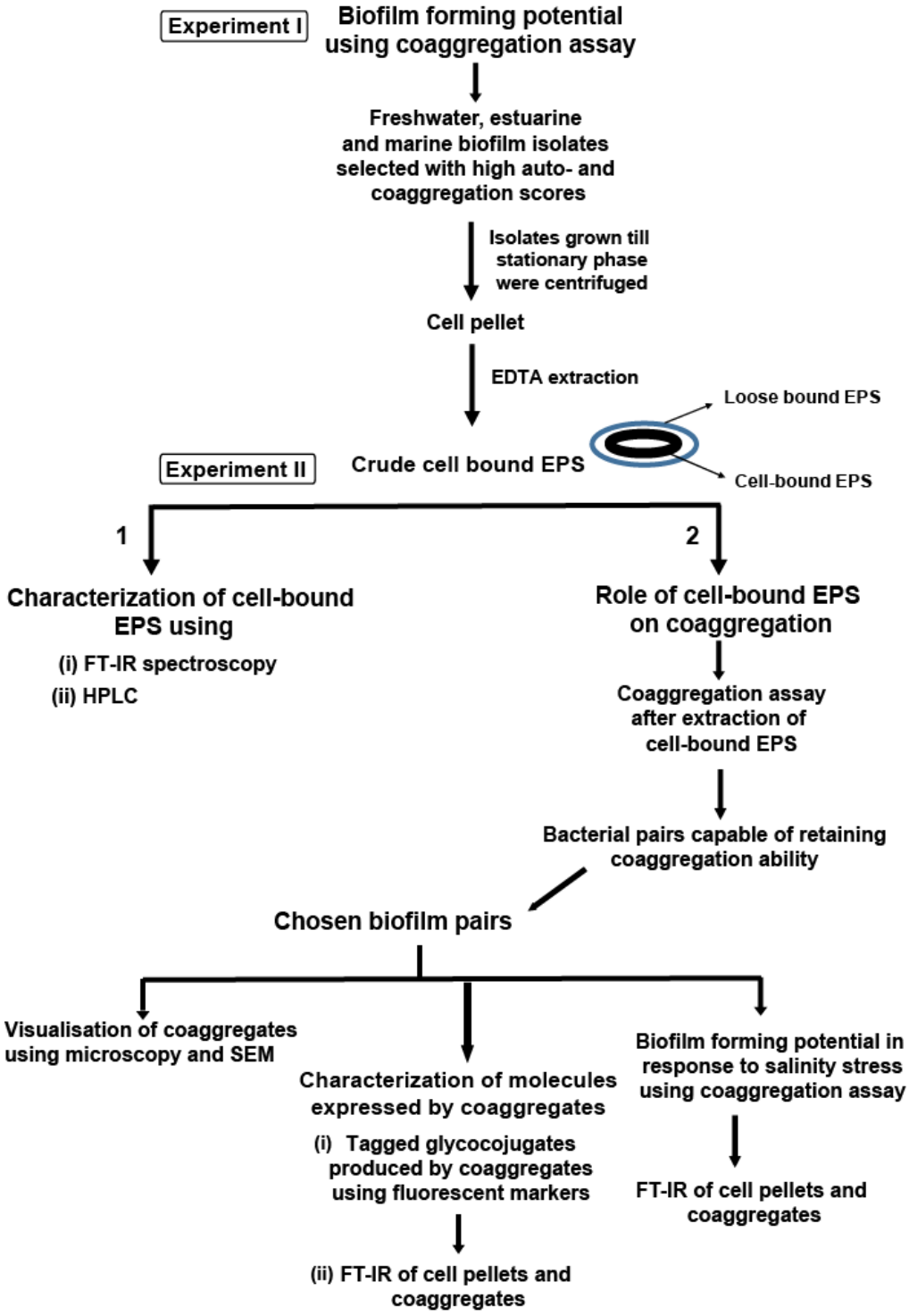


Figure 4.1 The schematic illustration of the experimental design. EPS = Extracellular polymeric substances, FT-IR = Fourier-transform infrared spectroscopy, SEM = Scanning Electron Microscopy, HPLC = High-pressure liquid chromatography.

Experiment I: Aggregation ability and identification of biofilm-forming bacteria

4.2.3 Biofilm-forming potential using coaggregation assay

The biofilm formation potential of the bacterial strains was assessed using well-established coaggregation assay. The bacterial isolates were cultured in the nutrient broth (NB) and Zobell marine broth 2216 (ZMB) for freshwater and marine/estuarine biofilm bacteria, respectively and kept on the rotary shaker (Remi, India) at 100 rpm (30ºC). The average period of stationary phase for the isolates was found as 48hrs and 72hrs for freshwater bacteria and marine bacteria, respectively. Cultures (50mL) in their respective stationary growth phases were centrifuged at 4500 × g for 20 min and washed twice with phosphate-buffered saline (PBS), pH 7.4 (Sigma-Aldrich). The cell pellets were then resuspended in sterile PBS, and the working stock (bacterial cell density: 10^9 cells/mL) was used to determine the aggregation properties of the bacterial isolates. Visual coaggregation assays were carried to determine the qualitative analysis of auto-aggregation and coaggregation between the biofilm bacteria as described below.

4.2.4 Visual aggregation assay

The auto-aggregation and coaggregation were carried out in pairwise combinations between the biofilm bacterial isolates as described by Cisar et al., (1979), with slight modifications. Briefly, 1000 µl of two different strains of bacteria in PBS (bacterial cell density: 10^9 cells/mL) were mixed in a test tube and then incubated at room temperature for 24hrs. After incubation, the scores for the bacterial aggregation were assigned from “0” to “4” as described by Rickard et al., (2002). Based on the 4-point scale, the turbid supernatant of bacterial culture was assigned “0”, whereas “1” for small uniform aggregates in turbid suspension, “2” for easily visible aggregates with turbid suspension, “3” indicated large settled coaggregates leaving some turbidity in the supernatant and “4” depicted large settled coaggregates with clear supernatant. On the other hand, the auto-aggregation or self-aggregation was determined by mixing equal volumes of the same bacterial suspension, incubated at room temperature for 24hrs and scored from “0” to “4” as mentioned above. The bacterial strains showing strong auto-aggregation and coaggregation scores

were identified as biofilm-forming bacteria, and ten strains with high biofilm-forming potential were selected for sequencing as well as characterization of cell-bound EPS. In addition, the aliquots were lyophilized and desiccated for characterization using Fourier-transform infrared spectroscopy (FT-IR) as described below.

4.2.5 Identification of selected biofilm-forming bacteria using 16S rRNA sequencing

For identification of selected biofilm-forming bacteria, the marine/estuarine and freshwater bacterial colonies were inoculated into ZMB and NB, respectively. They were incubated at 30°C overnight (approximately 18hrs) in an incubator shaker at 80 rpm. After the incubation period, overnight grown cultures were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 13,000 × g for 5 min to pellet the cells. The genomic DNA was extracted from the bacterial isolates using Thermofisher ® Purelink Genomic DNA Mini Kit according to the manufacturer's instructions and stored at – 20ºC until further analysis.

Further, the bacterial 16S rRNA gene was amplified by PCR in a 50 µl reaction mixture using Platinum® Blue PCR SuperMix (Invitrogen, USA), 0.25 µM of each primer (8F-5′-AGAGTTTGATCCTGGCTCAG and 1492R (l) - GGTTACCTTGTTACGACTT) and template. The PCR products were checked by electrophoresis (2% w/v agarose gel) and purified using the GenElute PCR purification kit (Sigma), according to the manufacturer's protocol. These purified products were analyzed using a DNA sequencer (Applied Biosystems) for sequencing by Bioserve Biotechnologies Private Limited (Hyderabad, India). The results obtained were assembled using DNA Baser and aligned using the Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). Sequences were aligned with Clustal W, and the phylogenetic tree was built by using the MEGA 6.0 software (Tamura et al., 2013). These sequences obtained from the present study have been deposited in the

NCBI GenBank under accession numbers from MH429953 to MH429961 and MH620809.

Experiment II: Importance of cell-bound extracellular polymeric substances (CB-EPS) in the coaggregation process

4.2.6 Isolation and characterization of CB-EPS from selected biofilm-forming bacteria

For the isolation of CB-EPS, the freshwater and estuarine/marine bacteria were cultured in NB and ZMB for 48hrs and 72hrs respectively, i.e. upto the stationary phase. The cultures were then centrifuged at 4500 × g for 20 min, and the cell-bound EPS were extracted using the EDTA (10mM) as described by Tallon et al. (2003). After centrifugation, an aliquot of the EPS was stored at -20 °C for the sugar analysis by high-performance liquid chromatography (HPLC). The remaining aliquot of the EPS was lyophilized and desiccated for the analysis of Fourier-transform infrared spectroscopy (FT-IR).

4.2.7 FT-IR analysis of CB-EPS

A pellet was prepared by grinding approximately 1 mg of lyophilized sample and mixed thoroughly with 2.5 mg of potassium bromide (KBr). KBr was used as a background reference. The spectrum was recorded as an average of 20 scans in the mid-infrared (mid-IR) range of 600-4000 cm^{-1} using Fourier-transform infrared spectrophotometer (IR Affinity-1, Shimadzu, Singapore) at a resolution of 4 cm^{-1}.

4.2.8 Characterization of sugar composition in CB-EPS by high-performance liquid chromatography

The sugar composition of the CB-EPS was analyzed by reverse-phase high-performance liquid chromatography (HPLC, Agilent 1200 series) equipped with a refractive index detector (RID). The sugars were separated isocratically on a Zorbax Carbohydrate column (4.6 mm I.D. x 150 mm, silica particle size 5 µm, Agilent) using acetonitrile: water (85:15) mobile phase at a flow rate of 1.4 mL/min with a column temperature at 30°C for 35 min. The injection volume of 10 µL was used for each sample and run in duplicates.

Before running the samples, the column was calibrated with 19 different HPLC grade sugar standards, i.e. mannose, galactose, rhamnose, sucrose, ribose, glucose, fucose, ribose, arabinose, maltose, N-acetyl-D-glucosamine, fructose, xylose, lactose, inositol, cellobiose, mannitol, sorbitol, melibiose, as described by Sahoo and Khandeparker, (2018). Sugars were identified according to their retention times by comparing with sugar standards (Appendix I A1-A2). The calibration curves were prepared for each sugar by diluting stock solutions (10 mg/mL) at multiple concentrations. HPLC analysis was then performed for all standard solutions, and the peak area against the retention time for each sugar was recorded. Calibration curves were obtained using the software by plotting peak area versus the amount injected (concentration). The determination coefficients (R^2) were >0.99, indicating a linear relationship between the chromatographic response areas and the concentrations for all the sugars. The calibration table was then created using this data in the Agilent Chemstation data analysis software. Sugars for samples were identified by comparing with retention times of sugar standards and calculated by the data analysis software. Furthermore, these sugars were subjected to NMDS (non-metric multidimensional scaling) to visualize similarities among the bacterial species and confirmed with Analysis of similarity (ANOSIM) test using PRIMER version 6.0 software (Clarke and Warwick, 1994). Also, the similarity percentage (SIMPER) analysis was carried to identify sugars that significantly contributed to the differences among the biofilm types using PRIMER version 6.0 software.

4.2.9 Role of CB-EPS in biofilm formation

A separate coaggregation assay was conducted after the extraction of CB-EPS (using EDTA method as per the protocol described in subsection 4.2.6) for the ten bacterial strains with high biofilm-forming potential to identify the role of cell-bound EPS in the formation of coaggregates. After EPS extraction, the bacterial strains were washed using PBS (pH 7.4). Further, pairs of bacterial strains were mixed (1000 µL of each strain with equal cell density) in a test

tube and incubated at room temperature for 24hrs. Subsequently, the score was assigned based on the coaggregation using the 4-point scale, as mentioned earlier. The estuarine and freshwater bacterial pairs which retained their ability to coaggregate even in the absence of CB-EPS were further characterized as described below.

4.2.10 Visualization of coaggregates using epifluorescence microscopy

The coaggregates of selected bacterial pairs were observed by using epifluorescence microscopy (Olympus, Tokyo Japan). The protocol was standardized, wherein 1000 µL of each strain in the PBS were stained with either DAPI (50 µg/mL) or SYBR Green I (1:10000 final concentration). Subsequently, each of the bacterial suspension was incubated at room temperature in the dark for 20 min to allow staining of the cells. After incubation, the cells were centrifuged at maximum speed (13,000 × g for 5 min) and washed thoroughly with phosphate-buffered saline (pH 7.4) to remove excess stain. Each strain was then resuspended in fresh PBS solution and mixed to allow them to coaggregate. After 1-2hrs, 50µL of the coaggregate was taken on a glass slide for microscopic observation of coaggregates using epifluorescence microscopy. Further, both the SYBR Green I and DAPI fluorescence images (green and blue) were overlayed using cellSens standard imaging software (Olympus, Tokyo Japan) and Image J software (v. 1.52a).

4.2.11 Scanning electron microscopy (SEM) of coaggregates

The selected coaggregated bacteria were visualized using a scanning electron microscope (SEM), which allows imaging and investigation of surface topographic features. The samples were fixed using 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4), subsequently kept in the refrigerator (4°C) for 1hr and dehydrated in the ethanol series (70%, 80%, 90% ethanol for 10 min each and 100% ethanol for 1hr). Then, samples were allowed to air dry in a laminar flow chamber for 30 min. After drying, the samples were examined using a Hitachi Tabletop Scanning Electron Microscope (TM3000).

Characterization of freshwater and estuarine bacterial pairs and their coaggregates before (control) and after the extraction of CB-EPS

4.2.12 Characterization of glycoconjugates on coaggregate surfaces using fluorescent markers

The fluorescent markers (lectins) obtained from Sigma-Aldrich were used to identify surface glycoconjugates of the coaggregated bacterial pairs. The preliminary characterization of bacterial cell-bound EPS using HPLC showed that D-mannose and galactose were dominant sugars, and therefore we hypothesised their possible involvement in the biofilm formation. Hence, the specific lectins tagged with either FITC/TRITC were used in the present study (1) Concanavalin A (Con A) – FITC conjugated lectin to tag D-mannose/D-glucose residues (Strathmann et al., 2002; Guillonneau et al., 2018), (2) Wheat germ agglutinin (WGA) - FITC conjugated lectin to tag N-acetyl-D-glucosamine and sialic acids, (3) Glycine max (Soybean agglutinin) – TRITC conjugated lectin for tagging N-acetyl-D-galactosamine. Moreover, these glycoconjugates are also known to serve as cues for settlement of macrofouling organisms.

The protocol was standardized for this, wherein the coaggregates formed by the selected bacterial strains after mixing them for coaggregation assay (approx. 1-2 hrs) were stained and incubated with DAPI (50 µg/mL) (Himedia) for 15 min in the dark. Subsequently, the coaggregates were rinsed with PBS to remove excess stain. The coaggregates were then tagged with lectins (1 mg/mL) and visualized under an epifluorescence microscope (Olympus, Tokyo Japan). Similarly, the above protocol was repeated on coaggregates formed after the extraction of cell-bound EPS. The microscopic images of DAPI for aggregates and FITC fluorescence for lectins appeared as blue and green colour, respectively, except in the case of Glycine max, which was TRITC conjugated (orange). Furthermore, both the images of FITC and DAPI were overlayed and prepared using cellSens standard imaging software (Olympus, Tokyo Japan) and Image J

software (v. 1.52a). Likewise, the images of TRITC-labelled conjugate and DAPI (orange and blue combination) were processed.

4.2.13 FT-IR analysis of freshwater and estuarine bacterial pairs and their coaggregates before and after the extraction of CB-EPS

In order to identify the molecules which are probably involved in retaining coaggregation ability in the absence of CB-EPS, the aliquots of the selected estuarine and freshwater biofilm bacterial cell pellets and their coaggregates (formed before and after the extraction of CB-EPS) were lyophilized and subjected to the Fourier-transform infrared spectroscopy (FT-IR) as described above. This analysis has been proven as a powerful tool to understand structural changes occurring in the bacterial cell surfaces in response to environmental changes (Kamnev, 2008). In addition, laboratory experiments were conducted on the bacterial pairs which retained their ability to coaggregate even in the absence of CB-EPS by subjecting them to salinity-stress to assess the impact of salinity-stressed biofilm bacteria on the aggregation process. Briefly, the selected freshwater strains were translocated and grown in ZMB (exposed to high salinity, i.e. 34) and estuarine bacteria in NB (exposed to low salinity, i.e. 5) upto the stationary phase. Furthermore, the coaggregation assays were performed in duplicates as described above, and the aliquots of these coaggregates were lyophilized and desiccated to assess the response of these bacteria to environmental changes using FT-IR.

4.3 Results

4.3.1 Experiment I

4.3.1.1 Biofilm-forming potential using coaggregation assay

Among the total twenty-three strains, ten strains with high biofilm-forming potential were selected based on their strong autoaggregation and coaggregation scores, as depicted in Appendix II B1. It was observed that the coaggregation process was growth-phase dependent, i.e., the isolates showed maximum coaggregation during the stationary phase, irrespective of the type of biofilm. The average period of the stationary phase was found to be 48hrs and 72hrs for freshwater and estuarine/marine biofilm bacteria, respectively.

Out of twenty-three strains, nine isolates (4-freshwater, 5-estuarine) were chosen based on their strong auto-aggregation (i.e. “3” and “4”) and coaggregation scores (i.e. in range of “3” to “4”). Most of the isolates within these freshwater and estuarine, coaggregated with more than one strain with high coaggregation scores. However, in the case of marine biofilm bacteria, the majority of pairwise combinations demonstrated weak coaggregation scores (i.e. “1”), except only one marine biofilm bacteria, which showed the highest auto-aggregation score (i.e. “3”) and coaggregated with most of the strains (Appendix II B1). Overall, the coaggregation potential based on pairwise combinations was highest in the estuarine biofilm bacteria (62.5%), followed by freshwater (44%) and minimum for marine biofilm bacteria (28%). Further, the ten bacterial strains with high biofilm-forming potential based on the strong auto-aggregation and coaggregation scores were further selected for their identification.

4.3.1.2 Identification of the biofilm-forming isolates with high biofilm-forming potential

In general, the 16S rRNA sequencing data of the selected biofilm-forming bacterial isolates and their comparison with the gene bank database indicated that most of the isolates belonged to *Bacillus* genera followed by *Exiguobacterium* and *Staphylococcus* (accession numbers: MH429953-MH429961, MH620809) (Figure 4.2).

Most of the biofilm-forming bacteria from estuarine biofilms showed > 99% similarities with *Bacillus* spp. (MH429953.1), *Bacillus indicus* (MH429954.1), *Bacillus cereus* (MH429955.1), and *Staphylococcus lentus* (MH429956.1), except for one estuarine bacteria, which showed < 98% similarity with *Bacillus cereus* (MH620809.1). On the other hand, freshwater biofilm-forming bacteria showed > 99% similarities with *Exiguobacterium* spp. (MH429958.1 and MH429959.1), *Bacillus subtilis* (MH429960.1) and *Bacillus cereus* (MH429960.1). While, marine biofilm-forming bacteria showed > 99% similarity with *Staphylococcus lentus* (MH429957.1).

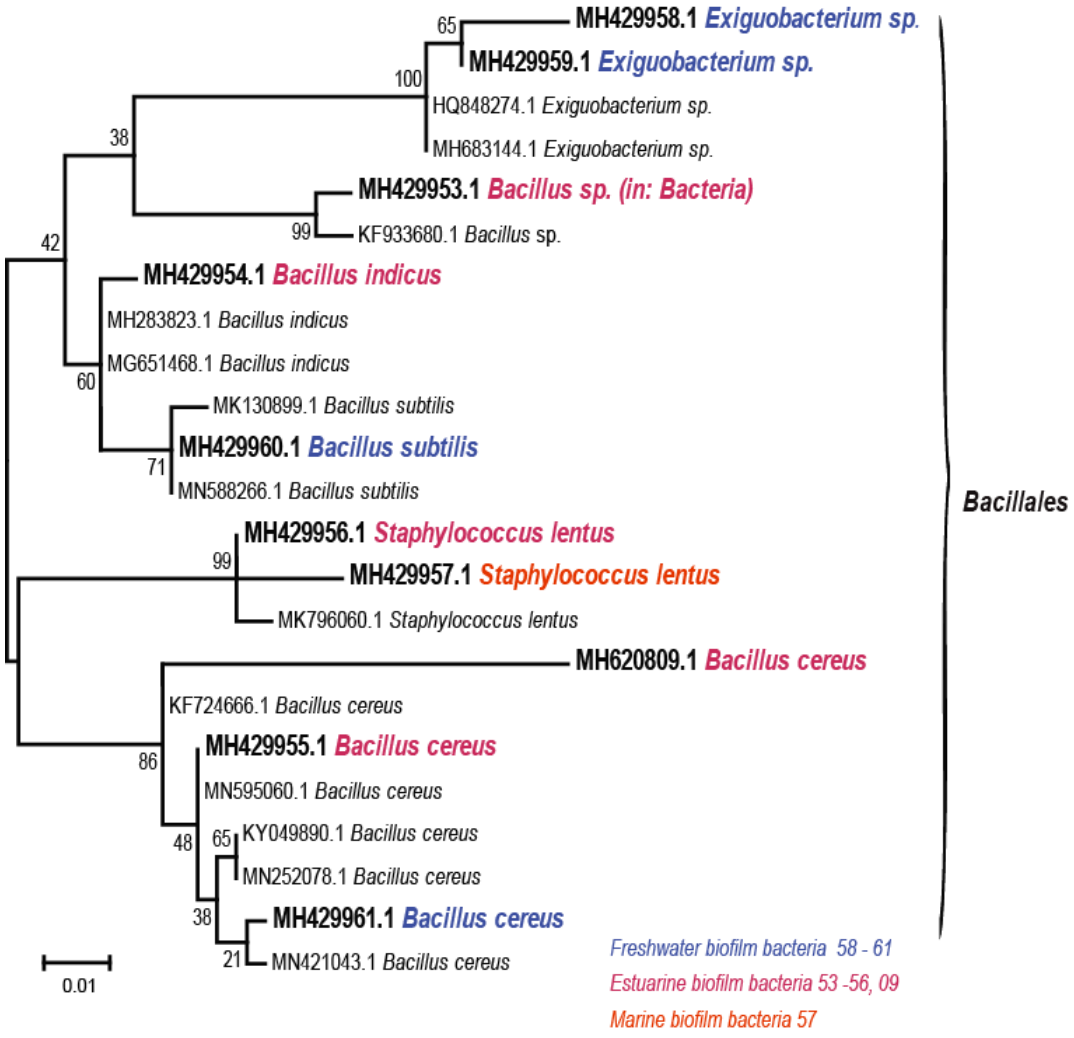


Figure 4.2 Phylogenetic relationship of the chosen biofilm isolates from the freshwater, estuarine, and marine biofilms based on 16S rRNA gene sequence analysis. The sequences obtained from the present study are highlighted in blue for freshwater, pink for estuarine and orange colour for marine. Scale bar, 0.01 substitutions per nucleotide position. sp. = species

4.3.2 Experiment II

4.3.2.1 Characterization of CB-EPS from selected biofilm-forming isolates with high biofilm-forming potential using FT-IR

The Fourier-transform infrared spectra of cell-bound EPS extracted from the biofilm bacteria is illustrated in Fig. 4.3. The possible assignments of the absorption bands and their references are tabulated in Table 4.1. The spectral patterns of freshwater and marine bacterial EPS differed from the estuarine bacterial EPS (Fig. 4.3a-c). The pattern was similar for the rest of the

freshwater and estuarine biofilm-forming bacteria (Appendix III C1-C2), except for one of the estuarine (*Staphylococcus lentus*), which showed a spectral pattern similar to the freshwater bacteria (Appendix III C3). The spectra's of the freshwater, estuarine, and marine bacterial CB-EPS displayed a broad peak at around 3600-3200 cm^{-1} indicating the presence of hydroxyl groups, followed by an asymmetrical stretching peak in the range of 1593-1629 cm^{-1} corresponding to ring stretching of mannose or galactose (Fig. 4.3a-c, Table 4.1). Moreover, the absorption peaks in the range from 1,000 cm^{-1} to 1,200 cm^{-1} were also noted linked to the presence of C-O-H side groups and C-O-C glycosidic bond vibrations which are characteristics of all sugar derivatives (Fig. 4.3a-c, Table 4.1). The presence of carboxylic acids COO^{-} group (strong peak at 1402-1408 cm^{-1}), uronic acids (1111-1112 cm^{-1}), and ester sulphate groups (810-816 cm^{-1}) were also evident in the spectra (Fig. 4.3a-c, Table 4.1). The freshwater and marine bacterial EPS spectrum contained, in addition to spectral bands noted above, the presence of terpenoids and steroids which were evident from peaks at 1475/1473 and 1363/1365 cm^{-1}, respectively (Fig. 4.3a, 4.3c, Table 4.1). Overall, the spectroscopic results were suggestive of carbohydrate nature of CB-EPS in all the biofilm bacteria, irrespective of types. Therefore, it was further characterized for its sugar content by using HPLC.

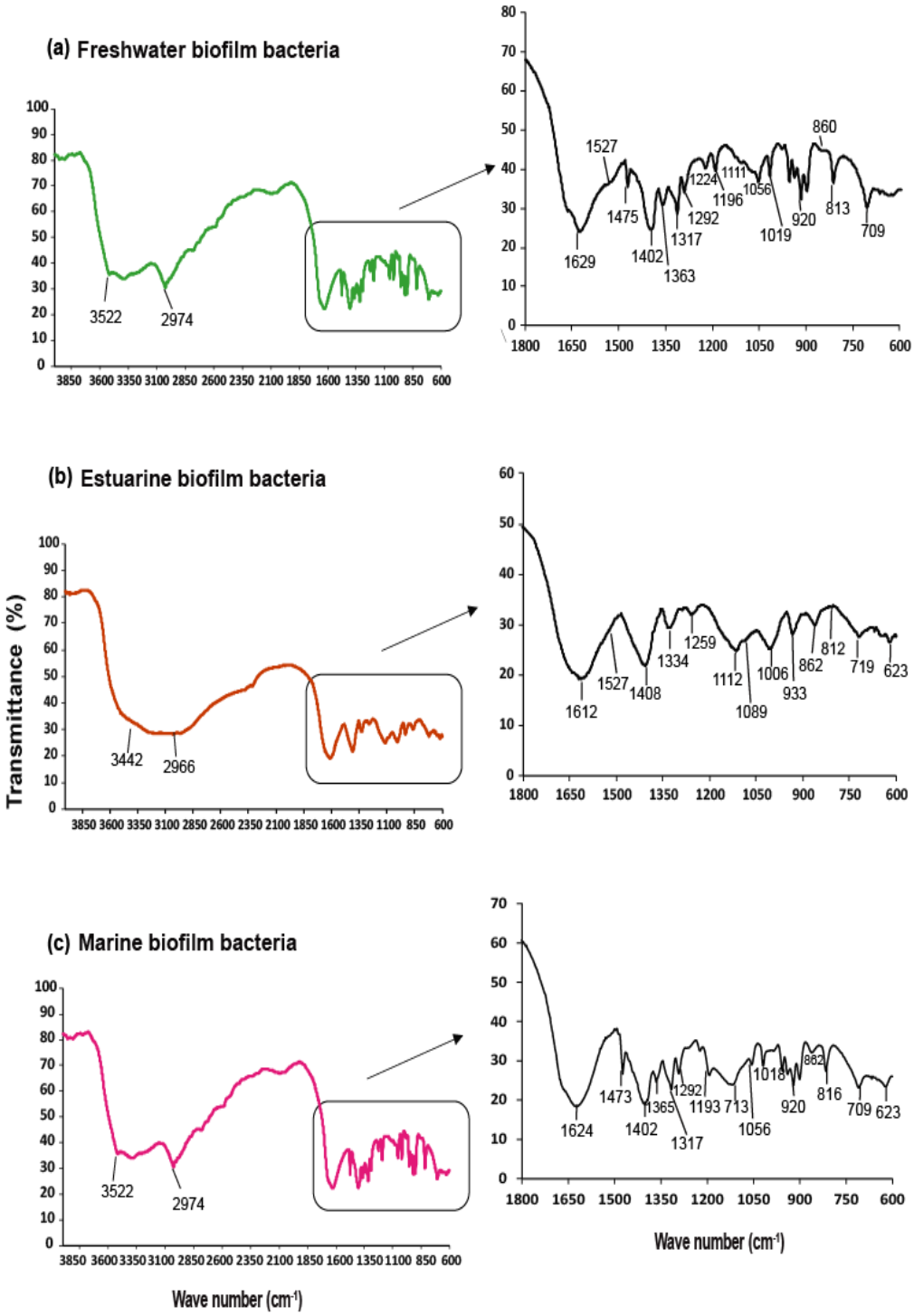


Figure 4.3 Fourier-transform infrared (FT-IR) spectra's of cell-bound bacterial EPS, wherein representative spectra of (a) freshwater (b) estuarine, and (c) marine biofilm-forming bacteria is presented. The freshwater bacterial EPS spectra are represented in green colour, estuarine bacterial EPS spectra in orange colour and marine bacterial EPS spectra in pink colour. The zoomed infrared (1800 - 600 cm^{-1}) spectral section is shown in black colour. The pattern for other freshwater and estuarine biofilm-forming bacteria was similar, except for one of the estuarine bacteria (MH429956.1), which showed a spectral pattern similar to the freshwater bacteria.

Table 4.1 Interpretation of IR spectra and peak assignments of the cell-bound EPS

Wave number (cm^{-1})	Molecular vibrations of functional groups and biomolecule contributor	References
3600-3200	Broad O–H stretching absorption peak indicating the presence of hydroxyl groups, which are characteristics for carbohydrates	Khandeparker et al., 2002; Seedevi et al., 2013; Zeng et al., 2016; Elnahas et al., 2017
3000-2800	C-H stretching vibration band of the aliphatic CH_2 group by fatty acids and lipids	Cheng et al., 2013; Fang et al., 2014
1593-1629	presence of an asymmetrical stretching peak corresponding to ring stretching of mannose or galactose	Freitas et al., 2009; Kavita et al., 2011, 2013, 2014
Peaks at 1527 and 1533	C-N stretching vibrations in combination with N-H bending of proteins (Amide I and Amide II bands)	Lorite et al., 2011; Fang et al., 2014
1402-1408	symmetric stretching of the carboxylic acids COO^- group	Zhao et al., 2007; Lorite et al., 2011
Peaks at 1475 and 1363	presence of terpenoids and steroids	Khandeparker et al., 2002
1000 -1125	O-acetyl ester linkage bonds indicating the presence of uronic acids	Bramhachari and Dubey, 2006; Kavita et al., 2014
1000-1200	C-O-H side groups and C-O-C glycosidic bond vibrations which are characteristics of all sugar derivatives	Suh et al., 1997; Sheng et al., 2005; Bramhachari and Dubey, 2006; Cai et al., 2013; Kavita et al., 2013
Peaks at 810-816	Presence of ester sulphate groups	Lloyd et al., 1961; D'Souza, 2004; Seedevi et al. 2013; Guezennec et al., 1998
858-862	Presence of α-glycosidic linkages between individual glycosyl residues	Kodali et al., 2009

4.3.2.2 Sugar characterization (%) of CB- EPS from biofilm bacteria

The sugar composition (%) of the bacterial cell-bound EPS belonging to different biofilm types is given in Table 4.2. The sugar compositions were subjected to NMDS to cluster different biofilm bacterial types on the basis of their sugar composition (Figure 4.4). Nearly all of the freshwater bacterial cell-bound EPS showed the dominance of galactose with a minor

contribution of mannose, xylose, and others (Figure 4.4, Table 4.2). On the other hand, the estuarine bacterial cell-bound EPS were diverse and distinct, wherein mannose was dominant along with galactose, rhamnose, ribose, arabinose and others (Figure 4.4, Table 4.2). On the contrary, the marine bacterial cell-bound EPS composition differed, wherein fructose and sorbitol were dominant sugars, along with ribose, and galactose (Figure 4.4, Table 4.2). The components of the sugars varied with the biofilm bacterial types; moreover, the relative proportions of the individual saccharides differed, suggesting that the exopolymer is a heteropolysaccharide (Figure 4.4). Analysis of Similarity (ANOSIM) also revealed these differences ($R = 0.56$, $p < 0.05$). The results of SIMPER analysis showing overall dissimilarities in the sugar composition and sugars that significantly contributed to differences between biofilm types are tabulated in Table 4.3. The average dissimilarity was comparable between freshwater and estuarine (43.63 %) and freshwater and marine (43.84 %), while it was 52.84 % between the estuarine and marine group. The mannose, melibiose, galactose, fructose, and D-sorbitol significantly contributed to the dissimilarity between the biofilm types (refer contribution % in Table 4.3).

4.3.3 Role of CB-EPS in biofilm formation

Most of the biofilm bacteria, including marine, lost their ability to coaggregate in the absence of CB-EPS, which was evident from turbid supernatant after mixing the bacterial suspensions. For those bacterial pairs, i.e. *Exiguobacterium* spp., (MH429959) and *Bacillus cereus* (MH429961) from freshwater, and *Bacillus indicus* (MH429954) and *Bacillus cereus* (MH620809) from the estuarine biofilms, which were able to coaggregate and showed high coaggregation scores (“3”), even in the absence of CB-EPS, were chosen for further characterization.

Table 4.2 Composition (%) of sugars in the cell-bound EPS extracted from biofilm bacteria with high biofilm-forming potential from different aquatic environments.

	Rha	Rib	Xyl	Arab	GlcNAc	Fruc	Man	Glc	Sorb	Mann	Gal	Suc	Ino	Cell	Mal	Lac	Mel	Tre
Freshwater																		
F1 (*Exiquobacterium* spp.)	0.00	3.73	10.23	3.22	0.00	0.00	22.40	0.00	12.04	0.00	18.02	1.80	2.40	2.20	7.00	3.92	9.10	3.94
F2 (*Exiquobacterium* spp.)	0.00	2.70	6.90	0.00	0.00	0.00	24.50	0.00	3.10	0.60	47.00	1.60	0.40	1.70	3.70	2.30	3.30	2.20
F3 (*Bacillus subtilis*)	0.00	6.04	9.82	2.97	0.00	4.23	14.17	0.00	7.36	0.00	24.37	0.44	3.20	2.60	4.50	4.00	9.03	7.27
F4 (*Bacillus cereus*)	0.16	0.89	5.75	7.36	0.00	12.11	14.37	0.00	8.06	1.70	20.46	2.88	1.73	4.65	7.00	4.27	5.36	3.25
Estuarine																		
E1 (*Bacillus* spp.)	0.00	0.72	0.00	0.35	0.07	0.00	61.43	0.00	4.49	0.37	5.36	1.15	2.17	3.14	3.88	5.95	7.92	3.00
E2 (*Bacillus cereus*)	3.29	4.70	0.00	0.00	0.00	0.00	0.00	0.61	5.39	0.00	19.58	3.29	3.28	4.08	5.88	8.02	37.65	4.23
E3 (*Bacillus indicus*)	1.76	5.45	4.11	6.13	0.00	2.79	19.08	0.00	4.42	0.85	12.90	1.54	2.63	3.29	4.37	6.87	20.09	3.72
E4 (*Bacillus cereus*)	0.00	2.39	5.88	2.95	1.38	0.00	59.37	0.00	0.00	0.34	6.05	1.61	1.23	1.47	1.99	3.95	9.00	2.39
E5 (*Staphylococcus lentus*)	0.00	10.50	0.00	7.60	1.00	5.70	0.00	0.00	0.00	0.40	14.60	2.50	6.30	5.60	8.50	11.30	21.50	4.50
Marine																		
M1 (*Staphylococcus lentus*)	0.00	10.32	0.00	0.00	0.00	19.76	0.00	0.00	22.54	1.75	20.61	1.36	1.25	2.63	4.59	5.30	7.65	2.24

F = Freshwater, E = Estuarine, M = Marine, spp. = species, Rha = Rhamnose, Rib = Ribose, Xyl = Xylose, Arab = Arabinose, GlcNAc = N-acetyl-D-glucosamine, Fruc = Fructose, Man = Mannose, Glc = Glucose, Sorb = Sorbitol, Gal = Galactose, Mann = Mannitol, Suc = Sucrose, Ino = Inositol, Cell = Cellobiose, Mal = Maltose, Lac = Lactose, Mel = Melibiose, Tre = Trehalose.

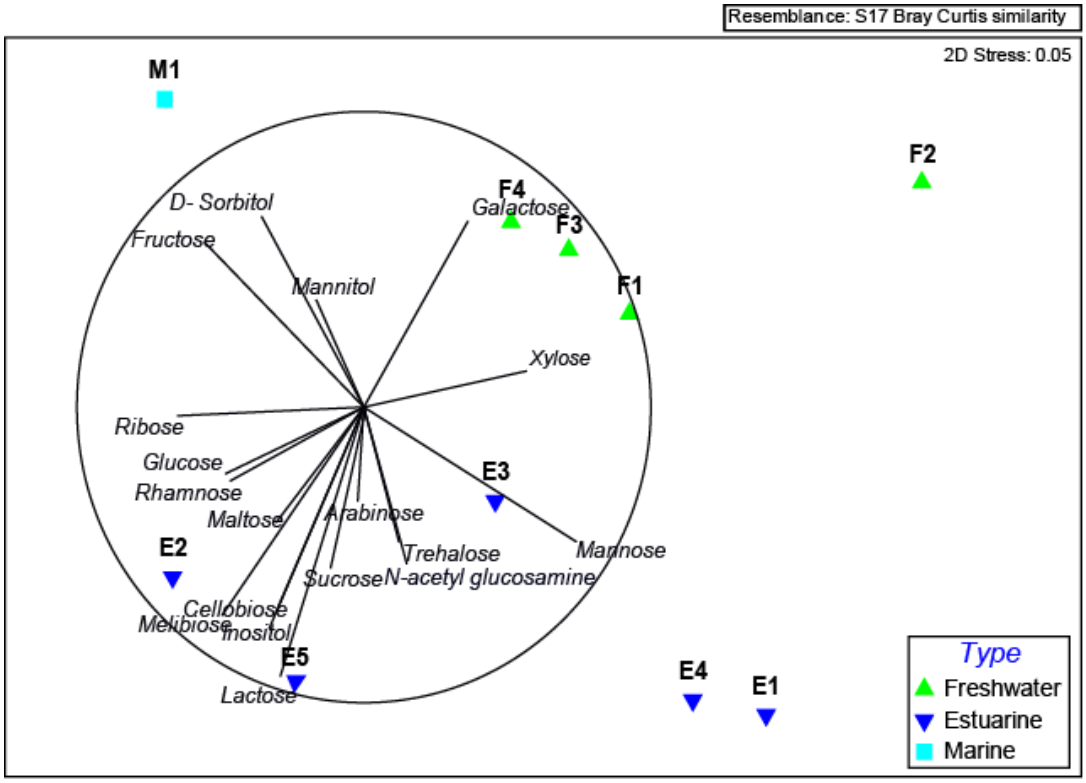


Figure 4.4 Non-metric multidimensional scaling (NMDS) analysis of sugars in the cell-bound EPS extracted from biofilm-forming bacteria. F = Freshwater (green up triangle), E = Estuarine (dark blue down triangle), M = Marine (blue square). The circle represents the length of a vector with a perfect correlation (R = 1).

The visualisation of the selected freshwater and estuarine biofilm bacterial coaggregates were observed by using epifluorescence microscopy, which showed that two different species were closely associated with one another (Figure 4.5a-b). Moreover, this distribution was confirmed by scanning electron microscopy, which revealed the presence of highly dense coaggregates covered by an extracellular EPS, which helped in holding the bacteria together (Figure 4.5c-d).

4.3.4 Characterization of freshwater and estuarine bacterial pairs and their coaggregates before (control) and after the extraction of CB-EPS

4.3.4.1 Characterization of glycoconjugates on coaggregate surfaces using fluorescent markers

The selected coaggregation pairs showed strong and specific binding to mannose and glucose residues (alpha polysaccharides as detected by Concanavalin A) as well as N-acetyl-D-glucosamine and sialic acids (as detected by wheat germ agglutinin) (Figure 4.6). On the other hand, a very low-

level of N-acetyl-D-galactosamine residues (as detected by Glycine max) were associated with coaggregation pairs during the study period. A similar type of expression of these glycoconjugates was observed by these aggregates even after extraction of CB-EPS (Figure 4.6).

Table 4.3 SIMPER analysis showing sugars that contributed to the differences among the biofilm types. a) Freshwater and Estuarine, b) Freshwater and Marine, and c) Estuarine and Marine.

a)	**Average dissimilarity = 43.63**					
Group				**Freshwater**	**Estuarine**	
Sugars	Av. Diss	Contrib%	Cum.%	Av. Abund	Av. Abund	Diss/SD
Mannose	11.15	25.55	25.55	18.88	27.98	1.79
Melibiose	7.65	17.53	43.09	6.7	22.84	1.71
Galactose	6.25	14.32	57.4	27.48	14.16	1.1
Xylose	3.25	7.45	64.85	8.15	3.72	2.56
Lactose	2.54	5.83	70.68	3.63	9.12	3.02
D-Sorbitol	2.3	5.28	75.96	7.65	3.42	1.47
Fructose	1.97	4.53	80.49	4.08	1.7	1.01
Arabinose	1.68	3.84	84.33	3.4	4.32	1.43
Ribose	1.57	3.59	87.92	3.33	5.56	1.22
Inositol	0.91	2.09	90.01	1.93	3.76	1.13

b)	**Average dissimilarity = 43.84**					
Group				**Freshwater**	**Marine**	
Sugars	Av. Diss	Contrib%	Cum.%	Av. Abund	Av. Abund	Diss/SD
Mannose	9.44	21.54	21.54	18.88	0	9.44
Fructose	7.87	17.95	39.49	4.08	19.8	7.87
D-Sorbitol	7.43	16.95	56.44	7.65	22.5	7.43
Galactose	4.11	9.39	65.82	27.48	20.6	4.11
Xylose	4.08	9.3	75.12	8.15	0	4.08
Ribose	3.49	7.96	83.08	3.33	10.3	3.49
Arabinose	1.7	3.88	86.96	3.4	0	1.7
Melibiose	1.18	2.68	89.64	6.7	7.6	1.18
Trehalose	0.98	2.23	91.87	4.15	2.2	0.98

c)	**Average dissimilarity = 52.84**					
Group				**Estuarine**	**Marine**	
Sugars	Av. Diss	Contrib%	Cum.%	Av. Abund	Av. Abund	Diss/SD
Mannose	11.97	22.65	22.65	27.98	0	0.94
D-Sorbitol	8.86	16.77	39.42	3.42	22.5	5.54
Fructose	8.38	15.87	55.28	1.7	19.8	7.61
Melibiose	7.23	13.69	68.97	22.84	7.6	1.54
Galactose	2.96	5.61	74.58	14.16	20.6	1.66
Ribose	2.21	4.18	78.76	5.56	10.3	1.52
Arabinose	1.99	3.76	82.52	4.32	0	1.15
Lactose	1.76	3.33	85.86	9.12	5.3	2.15
Xylose	1.53	2.89	88.75	3.72	0	0.63
Inositol	1.21	2.3	91.04	3.76	1.2	1.58

Av. Diss = average dissimilarity, Contrib% = Contribution, Cum. % = Cumulative %, Av. Abund = average abundance.

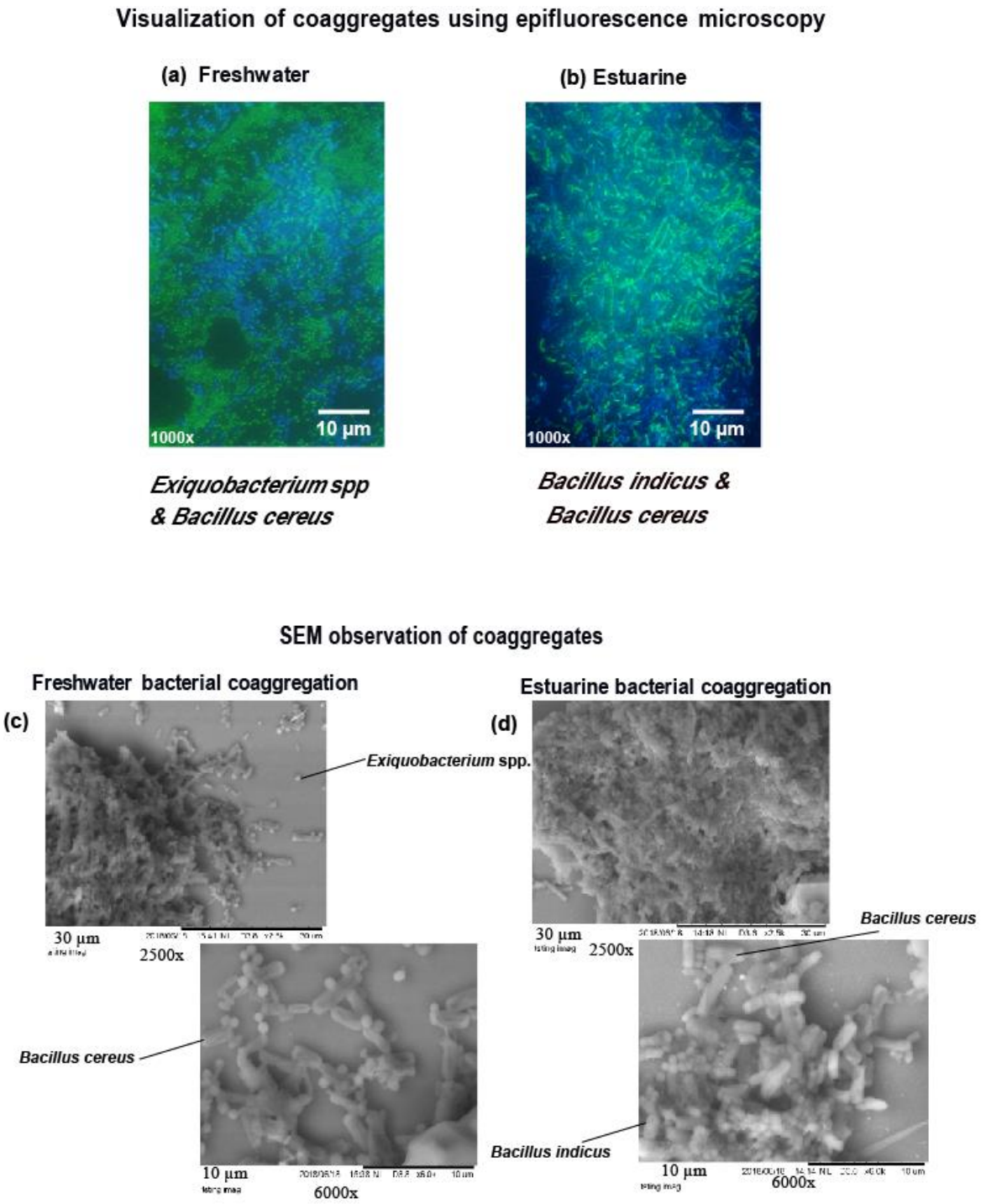


Figure 4.5 Visualization of coaggregates using epifluorescence microscopy (1000x magnification) from (a) freshwater, and (b) estuarine biofilm bacterial pairs (cultures grown till stationary phase), stained either with DAPI (blue) or SYBR green nucleic acid (green) nucleic acid stain and scanning electron microscopy (SEM) images of coaggregates (2500x and 6000x magnification) formed by (c) freshwater, and (d) estuarine biofilm bacterial pairs (cultures grown till stationary phase).

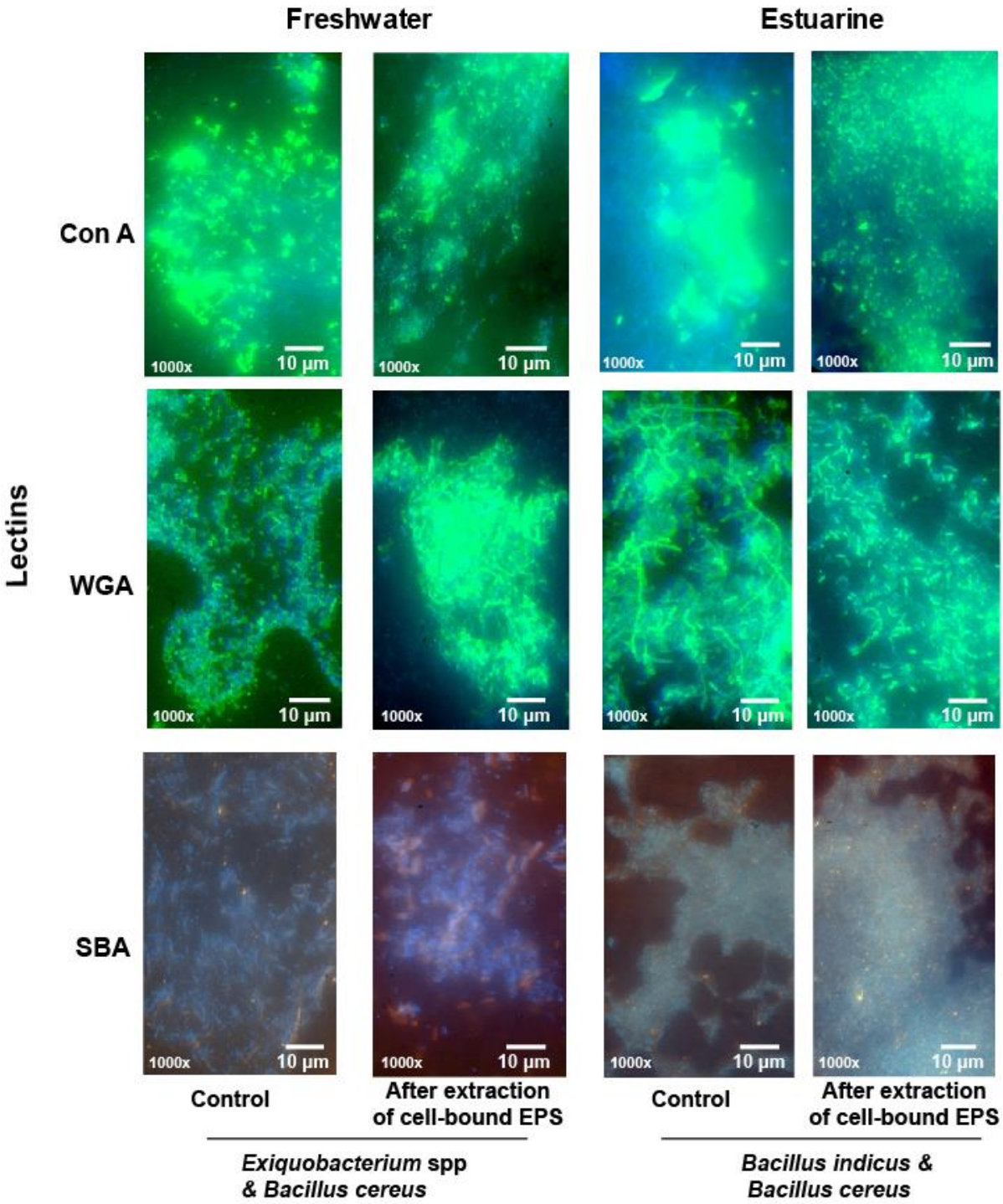


Figure 4.6 Fluorescently tagged glycoconjugates associated with coaggregates using epifluorescence microscopy (1000x magnification). Con A: Concanavalin A, WGA: Wheat germ agglutinin, and Glycine Max (Soya bean agglutinin, SBA). Bacterial coaggregation pairs are visible as blue while D-Mannose/ D-glucose residues (detected by Con A) and N-acetyl-D-glucosamine and sialic acids (detected by WGA) are visible as green. In the case of Glycine Max, bacterial coaggregation pairs are visible as blue and N-acetyl-D-galactosamine as orange.

4.3.4.2 FT-IR analysis of freshwater and estuarine bacterial strains and their coaggregates

The infrared spectra of the freshwater and estuarine bacterial strains and coaggregates exhibited marked changes in the spectral regions (as tabulated in Table 4.4) before and after the extraction of cell-bound EPS (Figure 4.7a-b, 4.8a-b).

Table 4.4 Interpretation of IR spectra of the bacterial cell pellets

Wave number (cm^{-1})	Molecular vibrations of functional groups and biomolecule contributor	References
3000 – 2800	Membrane lipids and fatty acids	Naumann, 2000
1739 – 1725	Stretching C=O of ester functional groups from the membrane lipids and fatty acids	Ojeda et al., 2009 Deepika et al.,2012
1700 – 1500	Proteins and peptides containing Amide I and II bonds	Naumann, 2000
1500 – 1200	Mixed region which includes (1) Fatty acids around 1468, 1455 (2) P=O stretching of $PO4^-$ (phosphodiesters, the backbone of nucleic acids) around 1250-1200 (3) Amide III band of proteins around 1350-1240	Naumann, 2000
1200 – 900	Carbohydrate region	Naumann, 2000
900 – 600	Fingerprint region	Naumann, 2000

The FT-IR of freshwater coaggregates showed distinct peaks at 2957, 1658, and 1537 cm^{-1} attributed to –CH asymmetric stretching of CH_3 in the fatty acids, amide I and II regions of proteins respectively (Figure 4.7a). On the other hand, a weak peak pattern was observed in the case of the estuarine coaggregated bacteria (Figure 4.8a). One common trend observed in both were notable changes in the peaks between 1058-1072 cm^{-1}, attributed to alterations in the carbohydrate region.

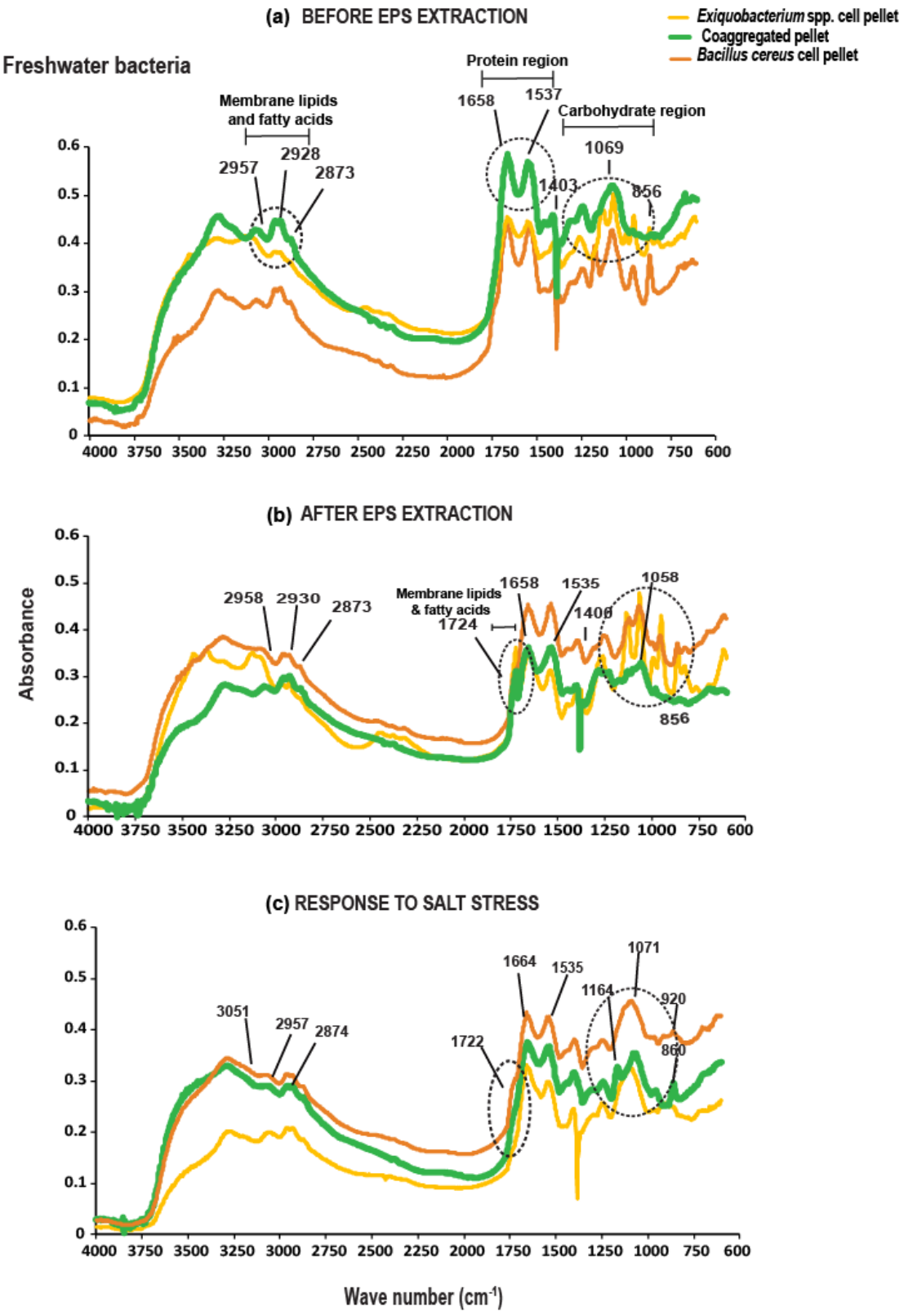


Figure 4.7 Fourier-transform infrared (FT-IR) spectroscopy of bacterial cell pellets and coaggregated freshwater biofilm bacteria (a) before extraction of cell-bound EPS, (b) after extraction of cell-bound EPS, and (c) response to salt-stress. Encircles (black) indicate notable changes in the spectral patterns between cell pellets and freshwater coaggregates.

However, the opposite trend was observed in the expression pattern by these coaggregates after extraction of cell-bound EPS. In the case of estuarine bacteria, the coaggregates showed the involvement of lipids and proteins which was evident from the emergence of peaks at 2956, 1664 (amide I), and at 1535 cm^{-1} (amide II) respectively, as observed in the spectra (Figure 4.8b). On the other hand, a weak peak pattern was observed in the case of the freshwater coaggregated bacteria (Figure 4.7b). Except for, the appearance of a peak at 1724 cm^{-1} corresponding to stretching C=O of ester functional groups from the membrane lipids and fatty acids (Figure 4.7b). Moreover, notable alterations were also observed in the carbohydrate spectral region (1200 to 900 cm^{-1}) of both the biofilm bacteria, which could be attributed to compositional alterations in the cell wall or cell membrane. A similar type of spectral expression pattern was observed by these coaggregates, formed after exposure of these bacterial pairs to salt stress. The spectra observed were indicative of changes that occurred in the surfaces of the individual and coaggregated bacteria when subjected to environmental changes (Figure 4.7c, 4.8c). The estuarine biofilm bacteria expressed lipids and proteins which was evident from the emergence of peaks at 2957, 1664 (amide I), and 1535 cm^{-1} (amide II) respectively, different from the freshwater spectra (Figure 4.7c, 4.8c). The most remarkable modifications were observed in the carbohydrate spectral region of both the bacterial spectra (1200 to 900 cm^{-1}) due to alterations in the cell membrane along with the appearance of a peak at 1722 cm^{-1} corresponding to the membrane lipids and fatty acids (Figure 4.7c, 4.8c).

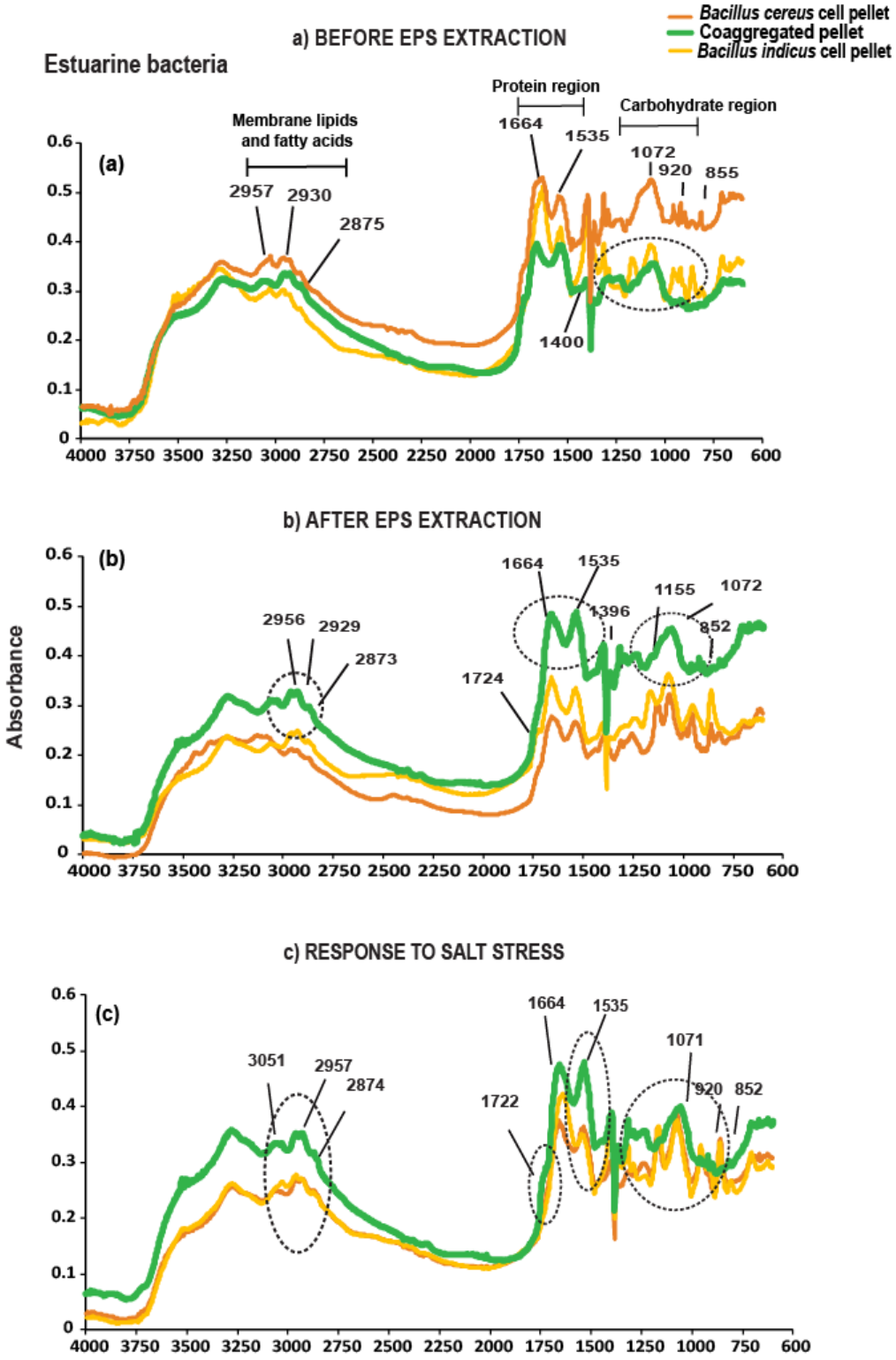


Figure 4.8 Fourier-transform infrared (FT-IR) spectroscopy of bacterial cell pellets and coaggregated estuarine bacteria (a) before extraction of cell-bound EPS, (b) after extraction of cell-bound EPS, and (c) response to salt stress. Encircles (black) indicate notable changes in the spectral patterns between cell pellets and estuarine coaggregates.

4.4 Discussion

In the present study, the estuarine and freshwater biofilm bacteria showed higher autoaggregation and coaggregation potential as indicated by strong auto- and coaggregation scores, compared to marine biofilm bacteria. This coaggregation potential was growth phase-dependent, i.e., aggregation was observed at the stationary phase. This finding corroborates well with earlier studies which reported growth phase-dependent aggregation in the biofilm bacteria from freshwater and marine environments (Rickard et al., 2000, 2002; Saravanan et al., 2014). The bacteria possessing the aggregation capabilities, i.e. auto-aggregation and coaggregation, imparts selective advantage over non-coaggregating ones, thereby playing an important role in the development of enhanced biofilm formation via interbacterial interactions (Rickard et al., 2004). On the other hand, weak coaggregation was observed among most of the marine biofilm bacterial strains isolated in this study, except for one marine biofilm-forming bacteria, which showed the highest coaggregation scores. An earlier study by Buswell et al. (1997), reported that low coaggregation scores do not imply weak interactions. Moreover, these coaggregation properties may depend on the relative size or morphologies of bacteria and the density of interacting ligands present on the cell surface (Buswell et al., 1997). Overall, these variations observed in the coaggregation potential among biofilm bacteria within different aquatic environments based on their salinity differences could be attributed to the diverse bacterial species and differences in the mechanisms mediating this process. Besides, the role of pH in influencing this coaggregation process cannot be ruled out for the reason that the pH profiles in the aquatic environments differ, and such changes in physicochemical factors can have a profound effect on the composition and viscosity of the bacterial adhesive exopolymers as well as cell surface molecules, which in turn may influence the coaggregation process but this needs further validation.

Although the bacterial strains used in the present study represented only a small proportion of total biofilm bacteria, the coaggregation appeared to be a significant phenomenon within these culturable biofilm-associated bacteria. The 16S rRNA sequencing data of the selected biofilm bacterial isolates and

their comparison to the gene bank database indicated that most of the isolates were identified as *Bacillus* genera followed by *Exiguobacterium* and *Staphylococcus,* which are Gram-positive bacteria belonging to Firmicutes. These genera are predominantly found in the oral infections and dental caries as well (Helgason et al., 2000; Raju and Anitha, 2015; Rani et al., 2016; Sakthivel et al., 2016). Moreover, members of Firmicutes have been reported as the most frequent isolates identified from the culturable bacteria diversity in the saline conditions (Kalwasinska et al., 2017; Remonsellez et al., 2018). Phylogenetic analysis revealed that all the *Bacillus* species clustered together differed in the rRNA sequence compositions. Members of genus *Bacillus* are ubiquitous in the terrestrial, freshwater as well as in the seawater habitats (Ruger, 1989). This genus includes several species which can colonize different specialized niches and adapt to changing environmental conditions such as salinity (Khandeparker et al., 2011; Sravankumar et al., 2014). This suggests that *Bacillus* spp. may be one of the many bridging organisms which facilitate the coaggregation with other bacteria, which are incapable of aggregating but needs further validation. Likewise, in the case of oral biofilms, it is known that the pathogenic bacteria will attach only to biofilms formed by initial colonizers, which are non-pathogenic ones (mostly Gram-positive bacteria) that contribute to coaggregation process and form a suitable favourable environment by producing metabolic end products such as lactate, N-acetyl muramic acid, p-amino benzoic acid etc., for pathogenic ones (mostly Gram-negative bacteria) to adhere (Bowden et al., 1979; Vasudevan, 2017). It is thought-provoking whether such a scenario exists in aquatic biofilm bacteria with *Bacillus* spp., as one of the many other species involved in creating a favourable environment for pathogens to adhere.

As the coaggregation process includes the interaction of different bacteria at the cell surface and involves cell-cell interactions, the cell-bound EPS were characterized to identify their role in the coaggregation. The nature of cell-bound EPS was characterized in terms of chemical functional groups using FT-IR. The cell-bound EPS were rich in carbohydrates showing the presence of hydroxyl, carbonyl groups, mannose and uronic acids, which has great relevance in the adhesion, aggregation and cohesion processes (Decho, 1990; Jain and Bhosle, 2008; Casillo et al., 2018). The HPLC analysis revealed the

freshwater cell-bound EPS to be a sulphated heteropolysaccharide, wherein galactose was dominant sugar along with terpenoids and steroids as evident from spectra (Figure 4.3a, Table 4.1a, Table 4.2). These terpenoids are known as the largest class of natural products, which serve as a medium of communication among species, thereby playing a significant role in antagonistic and beneficial interactions among organisms (Gershenzon and Dudareva, 2007). Whereas, steroids are mostly chemical signalling compounds which trigger phenotypic changes in microbes via quorum sensing and enhance their adherence to surfaces (Patt et al., 2018). This suggests that these compounds might play an important role in the biofilm formation of these freshwater strains.

On the other hand, the estuarine bacterial EPS, also a sulphated heteropolysaccharide, were dominated by mannose. Although the relative proportions of the other individual sugars differed between the estuarine bacterial strains, it was not-significant. This result suggests that diversity in sugar composition could further possibly result in variation in functionality among the estuarine strains. It has been reported that the sugars found in the bacterial EPS facilitate and enhances the bacterial adhesion (van loosdrecht et al., 1990; Azeredo and Oliveira, 2000; Tsuneda et al., 2003; Cavalcante et al., 2014). The presence of the sulphate moieties in the bacterial EPS provides flexibility to EPS, impart gel-like consistency and thus help in stabilising the polymer, and might also be useful in the aggregation of cells (Hoagland et al., 1993; Bhasker, 2003). Whereas, the cell-bound EPS extract of marine strain showed fructose and sorbitol as dominant sugars along with terpenoids and steroids. Overall, the sugar composition of estuarine bacterial EPS was more diverse, followed by freshwater and least was observed in the marine. Hence, the sugar composition of the CB-EPS seems to be crucial for strong coaggregation capabilities. However, since the present study included only one marine isolate, it makes the comparison across different aquatic systems rather difficult and requires further validation.

Most of the biofilm bacteria, including marine bacteria used in the present study, lost their ability to coaggregate after extraction of the cell-bound EPS, suggesting the importance of cell-bound EPS in the aggregation process. For those bacterial pairs, i.e. *Exiguobacterium* spp., and *Bacillus cereus* from

freshwater, and *Bacillus indicus* and *Bacillus cereus* from the estuarine biofilms, which still retained their ability to coaggregate even after extraction of the cell-bound EPS, were chosen for further studies. The scanning electron micrographs of these coaggregated bacterial pairs revealed that highly dense bacterial aggregates were embedded in an abundant extracellular material. Hence, it was evident that the presence of some specific molecules produced by the coaggregates helped in holding the bacteria together. Earlier studies have investigated the nature of bacterial cell surface molecules using the sugar reversal tests, i.e. by comparing the coaggregation capability in the presence of sugars (Kolenbrander et al., 1993; Rickard et al., 2000; Kolenbrander et al., 2006; Stevens et al., 2015). Lactose or N-acetyl-D-galactosamine were major sugars reported in blocking coaggregation between bacteria from human oral biofilms (Katharios-Lanwermeyer et al., 2014 and references with). However, this does not provide information on the type of the surface molecules associated with the coaggregates. Hence, these coaggregated pairs were characterized for the presence of glycoconjugates using specific fluorescent lectins.

Lectin molecules specifically bind to carbohydrates and thus are powerful tools used for analyzing glycidic structures of microbial origin aggregates (Cavalcante et al., 2014). In the present study, both the estuarine (*Bacillus indicus* and *Bacillus cereus*) and freshwater (*Exiguobacterium* spp., and *Bacillus cereus*) coaggregates showed high levels of specific binding to D-mannose, D-glucose residues and N-acetyl-D-glucosamine/sialic acids and these molecules were expressed even after the extraction of the cell-bound EPS. The mannose is a hexose sugar which possesses more number of hydroxyl groups than a pentose sugar and is considered as a promotory sugar (Khandeparker and Anil, 2011; Sahoo and Khandeparker, 2018). Both D-glucose and D-mannose serve as important cues for the settlement of *Balanus amphitrite* cyprids (Khandeparker et al., 2002, 2003; Khandeparker and Anil, 2011). The interactions between these sugars and cypris temporary adhesive are most likely to be affected via polar groups. The higher number of hydroxyl groups influence stronger attachment with polar groups of cyprid antennules (Neal and Yule, 1996; Sahoo and Khandeparker, 2018). On the other hand, sialic acids are sugars, which play an important role in cellular recognition,

cell-cell attachment, and signaling (Sigma-Aldrich, 2009). It is well known that several pathogenic bacteria or parasites use surface sialic acids for attachment to the host cell (Sigma-Aldrich, 2009). Moreover, relatively high amounts of these molecules (N-acetyl-D-glucosamine, D-glucose and D-mannose) have been detected in the algal cell wall surfaces as well (Tien et al., 2005). Earlier studies have also reported that N-acetyl-D-glucosamine also plays a significant role in the attachment of fouling diatoms (Bahulikar and Kroth, 2008; Khodse and Bhosle, 2010). A recent study demonstrated the importance of N-acetyl-D-glucosamine expressed by diatoms and its interaction with barnacle shell proteins in facilitating cyprid metamorphosis (Sahoo and Khandeparker, 2018). N-acetyl-D- galactosamine, along with D-glucose and D-mannose produced by haemocytes have been reported as cues for the settlement of *Balanus amphitrite* (Khandeparker et al., 2019). Thus, the results from the present study indicate the involvement of N-acetyl-D-glucosamine/sialic acid, D-mannose, and D-glucose in the bacterial cell-cell interactions, which are similar to the cues reported for the attachment of fouling diatoms and recruitment of larger macrofouling organisms.

FT-IR analysis has been recognized as a powerful tool to understand the structural changes occurring in the bacterial cell surfaces in response to environmental changes (Kamnev, 2008). The biomolecules, mainly sugars, proteins, and lipids in the bacterial membrane have distinct infrared (IR) vibrations that indicate their conformation and physical state (Pan et al., 2017). The results revealed that before the extraction of cell-bound CB-EPS, some peculiar proteins and lipids were expressed by the freshwater coaggregates, as evident from the spectra, probably to carry out cell-cell interactions. On the other hand, after EPS extraction, the proteins and lipids were expressed by the estuarine coaggregates, which possibly helped them in retaining the biofilm-forming capability. Alternatively, the same was not the case with the freshwater bacteria, which coaggregated by expression of lipids in the absence of CB-EPS. An earlier study by Andrews et al. (2010), confirmed the importance of lipids, which can influence and facilitate the attachment of different bacteria. Moreover, in this study, the alterations in the carbohydrate region of the cell membrane were also observed, irrespective of the biofilm types, thus highlighting the importance of carbohydrates in the cell-cell

attachment (coaggregation) by biofilm bacterial cells over individual bacterial cells. This result is in accordance with the previous studies by Bengtsson, (1991); Vandervivere and Kirchman, (1993); Cheung et al., (2000).

Formation of biofilms is a quorum-sensing controlled process (involving cell-cell communication and gene regulation); which involves various bacterial physiological activities, including EPS production (Dobretsov et al., 2009; Hmelo, 2017; Jemielita et al., 2018). Moreover, the secretion of EPS depends on the type of bacteria and environmental factors, which further influences their chemical composition (Sonak, 1998; Khandeparker et al., 2002, 2003; Camilli and Bassler, 2006; Decho and Gutierrez, 2017). As environmental conditions change rapidly, biofilm-forming bacteria possess different mechanisms for sensing and adapting to these changes. One of the important environmental stressors for bacteria is salt stress, i.e. exposure to either high or low salt concentration, which can cause physiological changes in the bacteria and subsequently influence the biofilm formation. Earlier studies have reported that enhanced production of carbohydrates and proteins in the EPS was used as a protective adaptation by bacteria to cope up with salinity stress (Zhao et al., 2016; Kim and Chong, 2017).

The present study revealed that the exposure of biofilm-forming bacterial cells (*Bacillus indicus*, *Bacillus cereus* from estuarine and *Exiguobacterium* spp., *Bacillus cereus* from freshwater) to abrupt changes in the salt concentrations (salinity stress) resulted in different physiological response among these bacteria and the subsequent alterations in the surface chemistry of the coaggregates were detected using FT-IR. The estuarine biofilm bacteria could retain their biofilm-forming capability, i.e. by forming aggregates through the expression of proteins and lipids in response to salt-stress. Whereas, the freshwater biofilm bacteria also coped up with the salinity stress and formed aggregates through the expression of lipids. Moreover, this type of expression pattern was also observed by the estuarine and freshwater aggregates in the absence of CB-EPS, and probably this is the mechanism by which these bacteria still retained their biofilm-forming ability under unfavourable conditions. The response and mechanism of *Exiguobacterium* spp., under varying salinities, have been reported earlier, wherein the salt-dependent alterations in the cell membranes were reflected in the concentration

of phospholipids that dominated it (Remonsellez et al., 2018). Whereas, the involvement of fatty acids and their composition in the *Bacillus* spp., has been reported in response to a wide variety of adaptations (Diomandé et al., 2015 and references within). Unlike *Bacillus* spp., the exposure of salt-stress had an influence on the intensity of orange colour pigmentation of *Exiguobacterium* strains during the study period (data not shown), indicating some relation between salt-tolerance and pigmentation. One common trend observed in both the biofilm types were notable changes in the carbohydrate region, indicating the significance of carbohydrates in the coaggregation process. Thus, it seems like the salt stress conditions effectively induced quorum sensing in the *Exiguobacterium* spp., and *Bacillus* spp., irrespective of biofilm types, resulting in the alterations in their cell surface chemistry in response to environmental changes, thus retaining their coaggregation potential and these modifications aided in their survival.

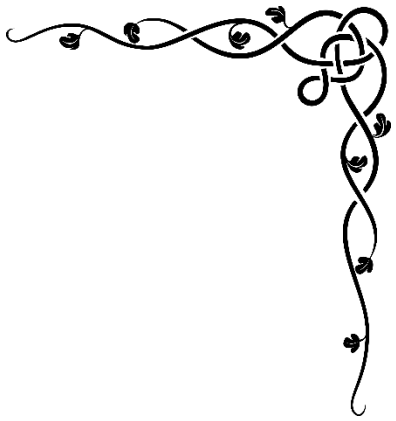
4.5 Conclusions

The present study is a first report that demonstrated the coaggregation potential of biofilm-forming bacteria from different aquatic environments (freshwater, estuarine, and marine), and identified the molecules involved in their aggregation. The biofilm-forming potential via cell-cell interactions of estuarine and freshwater biofilm bacteria was higher, attributed to cell-bound EPS rich in carbohydrates with distinct sugar composition, compared to marine. The carbohydrate content of the cell-bound EPS was influenced by the environment and varied among the biofilm bacteria. Most of the biofilm bacteria, including marine, lost their ability to coaggregate in the absence of cell-bound EPS suggesting its importance in the aggregation process. The estuarine (*Bacillus indicus* and *Bacillus cereus*) and freshwater (*Exiguobacterium* spp. and *Bacillus cereus*) biofilm bacterial pairs, which retained their ability to coaggregate even in the absence of cell-bound EPS, produced specific biomolecules (D-mannose, D-glucose, N-acetyl-D-glucosamine on the cell surface) in common. However, the underlying mechanism of the formation of estuarine coaggregates differed from freshwater ones, by expression of proteins and lipids. A similar expression pattern was also observed by the estuarine coaggregates, with high biofilm-

forming potential, after subjecting to salt-stress conditions, and probably this is the mechanism by which estuarine bacteria switch over and still retain their biofilm-forming capability under changing environmental conditions. Thus, it seems that the capability to coaggregate is influenced by the interplay of the environmental factors, biofilm bacterial species and molecules expressed at the cell surface. As biofilms serve as important settlement cues for the recruitment of macrofouling organisms, understanding the role of the molecules expressed by these biofilms via cell-cell interactions and their influence on the larval settlement and recruitment of macrofoulers under different environmental settings is an important topic for future investigation.

Appendix

Supplementary data associated with this chapter can be found in Appendix I-III.



Chapter 5

Summary

5. Summary

Biofilms are ubiquitous and can develop on a number of surfaces in the aquatic environments. Just like any other submerged surface, biofilms are also formed on the interior walls of the ship's ballast tanks, which are closed dark environments. These ballast tank biofilms are rich microbial communities harbouring pathogenic bacteria and have been implicated in the transport of microorganisms, including pathogens, into new environments, termed as microbial invasions (Drake et al. 2005; Drake et al. 2007). The present study evaluates the ballast tank biofilms as a vector of microbial invasions and their impact when released in a new environment using microcosm experiments. Such introductions can cause or likely to cause detrimental effects on the environment (ecology), economy (e.g. fouling of water supply systems) and human health (i.e. potential for introducing toxic dinoflagellates, cholera and other pathogenic organisms), indirectly by affecting the fisheries. Experimenting with biofilms gives an opportunity to understand the influence of changing environmental conditions on the biofilm communities and their impact when released in a new environment.

The formation of ballast tank biofilms depends on the type of inoculum which can be either freshwater, estuarine, or marine origin, depending on the locality of the source port. In this study, the biofilm bacterial communities from different aquatic environments, with varying salinities and nutrients were evaluated. The biofilms were developed on glass slides for five days in the three different aquatic environments (marine, freshwater and estuarine) under darkness, reflecting conditions which occur in the ballast tanks and compared with the biofilms developed in the in situ (natural light/dark cycle) conditions. The observations revealed that the biofilms developed under in situ conditions predominantly consisted of algae (as chlorophyll *a* biomass), and bacteria, irrespective of the biofilm types. Whereas, biofilms developed in the dark conditions were dominated by bacteria. The bacterial community composition in the biofilms in terms of gene copy numbers differed with the environmental conditions, as well as in the in situ and dark conditions.

Our investigation revealed the prevalence of different bacterial groups in the in situ marine, freshwater and estuarine biofilms, suggesting that the biofilm bacterial composition is niche-specific and the environment plays a

very important role in determining the community composition. γ-proteobacteria, which includes most of the pathogenic bacteria, were dominant in all the three biofilm types but was dominant in the estuarine biofilms. The culturable *Vibrio* spp., belonging to γ-proteobacteria, were also abundant in the estuarine biofilms. Total coliforms were dominant in the freshwater biofilms.

A shift in bacterial communities was observed in all the biofilms developed under the dark tanks, wherein α-proteobacteria were dominant in the marine and freshwater biofilms. Whereas, Actinobacteria were abundant in the estuarine biofilms. This points out that α-proteobacteria and Actinobacteria coped up with the stress through different mechanisms that probably allowed them to compete with the other taxa and were able to survive under unfavourable conditions. Geographically proximal estuarine biofilm bacterial communities (Mandovi and Zuari estuaries, Goa), when subjected to dark conditions, showed contrasting responses in the case of culturable *Vibrio* spp. The in situ Mandovi estuarine biofilms, had high numbers of *V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*, whereas, when they were developed in the dark condition a reduction in these bacterial species was evident, and vice-versa was observed in the case of Zuari estuarine biofilms. This points out the unique ecology of *Vibrio* spp. with different response to dark conditions in both these estuaries, in spite of being geographically closer. These studies helped in unravelling the patterns of biofilm bacterial communities across the environmental gradients (marine, estuarine, and freshwater). The response of these biofilms to changing environmental conditions was then assessed by mimicking ballast tank conditions (i.e. prolonged darkness and ageing), and the 5-day old biofilms were aged further in the dark for 30 days using microcosm experiments.

Observations revealed that the transport conditions (i.e. prolonged darkness and ageing) could shape the biofilm microbial assemblages. It was interesting to note that the response of the biofilms developed in different aquatic environments (marine, freshwater, and estuarine) was different when exposed to ageing and prolonged darkness for a period of 30 days. Overall, the freshwater biofilms were more sensitive and collapsed when exposed to the ballast tank conditions with an overall drastic decrease in the pathogenic

bacterial abundance and bacterial diversity (approx. 40 %) in comparison to the estuarine and marine biofilms. But this may not always be the reality because the biofilm community composition differs with the changing environmental conditions and depends on inoculum characteristics as well as period of exposure (Khandeparker et al., 2017a). Therefore, the freshwater biofilms were aged further and exposed to long-term dark incubations, taking into consideration the maximum possible voyage duration, which ranges from about 80 - 100 days or more (Seaplus, 2011). The ecological consequences of these aged freshwater biofilm communities, when released as a vector, on the estuarine water column communities was assessed using microcosm experiments, as many of the shipping ports are located at the river mouths and estuaries.

The results revealed that distinct microbial communities at the time of biofilm development had a significant role in modulating biofilm composition when exposed to prolonged dark incubations, resulting in less and more diverse aged biofilms (as indicated by diversity indices). Furthermore, both these biofilm types were translocated in the estuarine water using microcosm experiments. On exposure of these biofilms to estuarine water, the more diverse biofilms were resistant to environmental changes when compared to less diverse ones, suggesting that diversity confers community stability. The observations revealed that less diverse biofilms were more receptive towards colonization by surrounding estuarine communities, comprising of γ-proteobacteria, which includes most of the pathogenic bacteria, and Bacteroidetes, and resulted in reduction or proliferation of some genera in the ambient water. Interestingly, the dormant *V alginolyticus* and *V cholerae*, existing in the more diverse freshwater biofilms could resuscitate back to culturable form in the biofilms when subjected to estuarine water and subsequently, were released into the ambient water. However, their degree of pathogenicity or virulence will depend on environmental conditions. Thus, the activity of the microbial communities including pathogenic and non-pathogenic organisms, either within the biofilms or those released from the biofilms may (1) result in alterations in the water column community composition resulting in either reduction or proliferation of some genera with different metabolic potential, thus

influencing the water biogeochemistry, and (2) can lead to alteration in the water quality if the emerged species turns out to be pathogenic, thereby causing a cascading effect to the ecosystem functioning,

Many individual biofilm-forming bacteria tend to aggregate in the planktonic phase, thereby possibly resulting in biofilms originating from these pre-formed cell aggregates (Melaugh et al., 2016). The same scenario can be expected to happen with biofilms formed on the interior walls of ballast tanks when they release individual cells or slough off aggregates during ballasting operations or multiple flushings, which could further contribute to new biofilm formation. The earlier experimental studies (Chapter 2) revealed that the biofilm bacterial community composition varied in the different aquatic environments. Moreover, their response to changing environmental conditions, with reference to darkness and ageing, was also different (Chapter 3). Therefore, it was expected that the mechanisms involved in the formation of biofilms by these aquatic biofilm bacteria would also be different. Hence, the successfully isolated freshwater, estuarine, and marine biofilm bacteria were evaluated for the biofilm-forming potential via aggregation capabilities, and their cell-bound extracellular polymeric substances (CB-EPS), known to play an important role in biofilm formation, were characterized for identifying the specific molecules mediating this process.

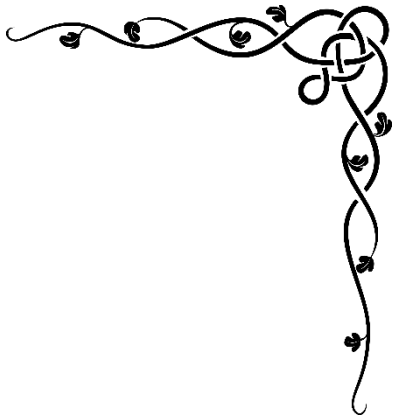
The present study is a first report demonstrating the aggregation potential of the biofilm bacteria from different aquatic environments, and the results revealed that the estuarine and freshwater biofilm bacteria showed high biofilm-forming potential via cell-cell interactions (evident from high coaggregation scores), attributed to CB-EPS with distinct sugar types, compared to marine. The role of cell-bound EPS was evaluated by assessing the coaggregation potential of these biofilm-forming bacterial strains in the absence of CB-EPS. Most of the biofilm bacteria lost their ability to coaggregate after removal of CB-EPS, indicating its importance in coaggregation process. The estuarine (*Bacillus indicus*, *Bacillus cereus*), and freshwater (*Exiguobacterium* spp., *Bacillus cereus*) bacterial pairs, retained their aggregation capability, probably via expression of lipids

and proteins, suggesting their ability to rebuild themselves by expressing specific biomolecules under stressed conditions. A similar expression pattern was observed when these strains, with high biofilm-forming potential, were exposed to abrupt salinity-change (an important environmental stressor), indicating modulation of cell surface chemistry as a strategy to protect biofilm bacteria in harsh conditions. Future studies should aim at targeting the pathways involved in such a modulation. As biofilms serve as important settlement cues for the recruitment of macrofouling organisms, unravelling the role of the molecules expressed by these biofilms via cell-cell interactions and their influence on the larval settlement of macrofoulers under different environmental settings is an important topic for future investigation and to develop suitable control for preventing fouling.

In conclusion, this thesis has addressed one of the important vectors by which the translocation of organisms occurs across the globe, i.e. by ballast tank biofilms. This study enhances our understanding of biofilm ecology and also provides some fundamental insights into the issues related to ballast tank biofilm management. The results of the present investigation suggest that the risk of microbial invasion and the extent of success or failure of the introduced biofilm communities depends on the diversity. It is evident that the possibility of less diverse biofilms if present, e.g. in the interior ballast tanks, to get colonized by freshly loaded inoculum, comprising of highly diverse communities, is more during the process of ageing. Whereas, the more diverse aged biofilms if present, giving refuge to pathogenic bacteria, can contribute to their leaching into the freshly loaded inoculum and can cause a threat if discharged elsewhere, suggesting that both the biofilm types can pose a threat to the environment. This research is an initial step towards understanding the possible discharge effects of biofilms on the environment when the ballast tanks are filled or flushed in different locales. Much work still needs to be done to determine the implications and invasion potential of these hitchhikers.

Future studies should focus on studying the biofilm bacterial diversity and its fate during a voyage which could provide a better understanding of the invasive potential of the biofilm organisms and develop an efficient management strategy. Moreover, a metagenomic database of ballast tank

biofilm bacteria using advanced molecular tools is needed and characterizing different biofilm species and elucidating their functional potential is an important topic for future investigations.



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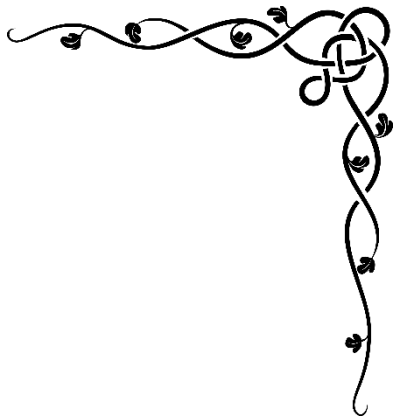
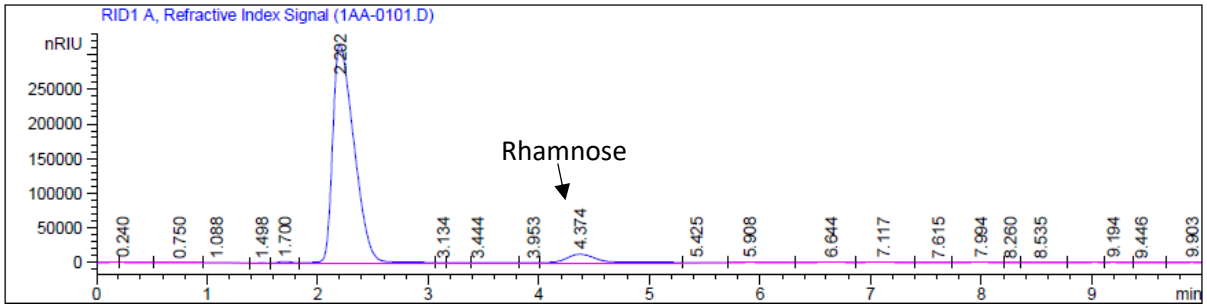


Appendix

Appendix I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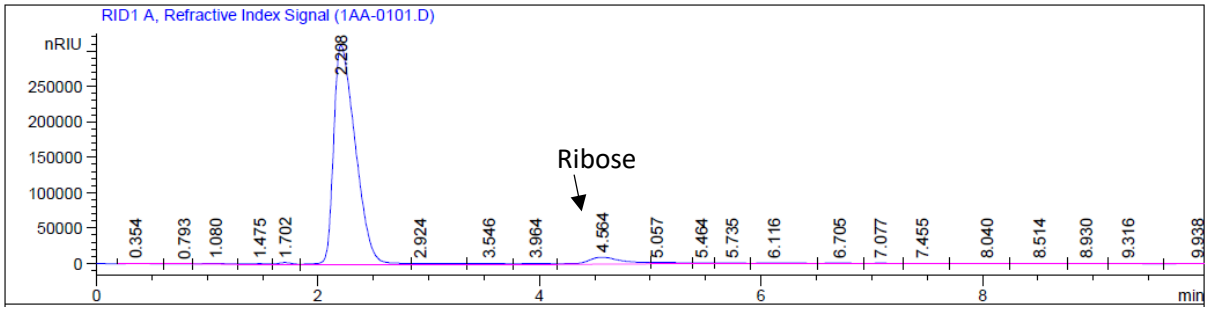
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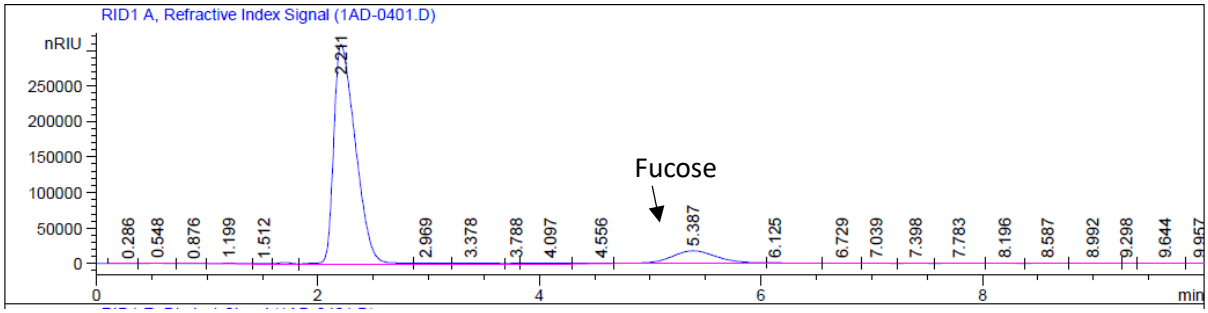
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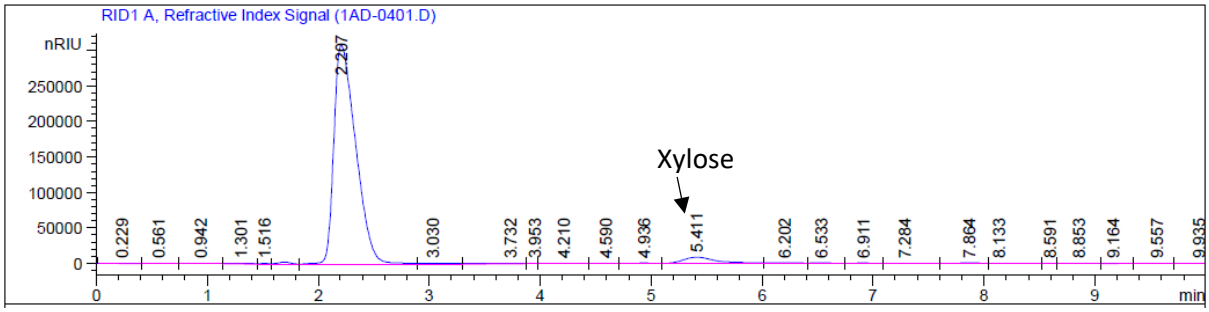
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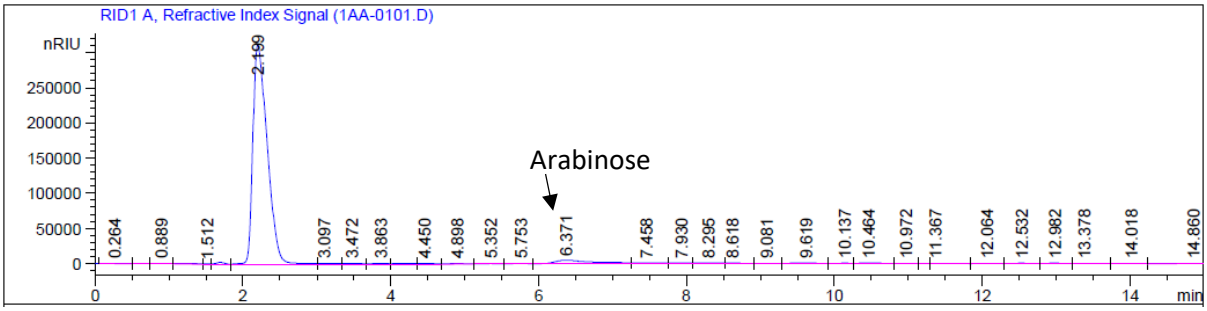
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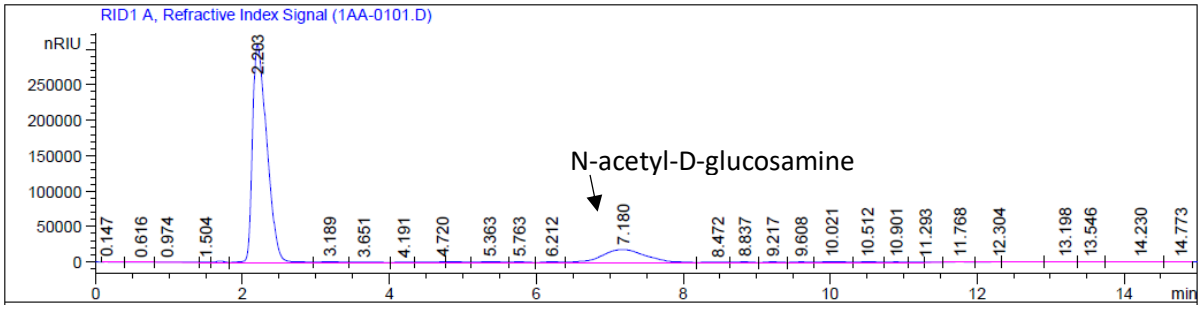
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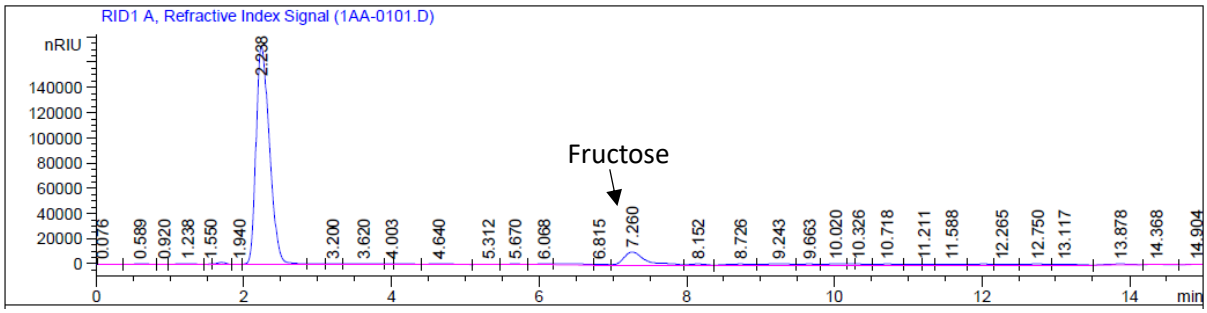
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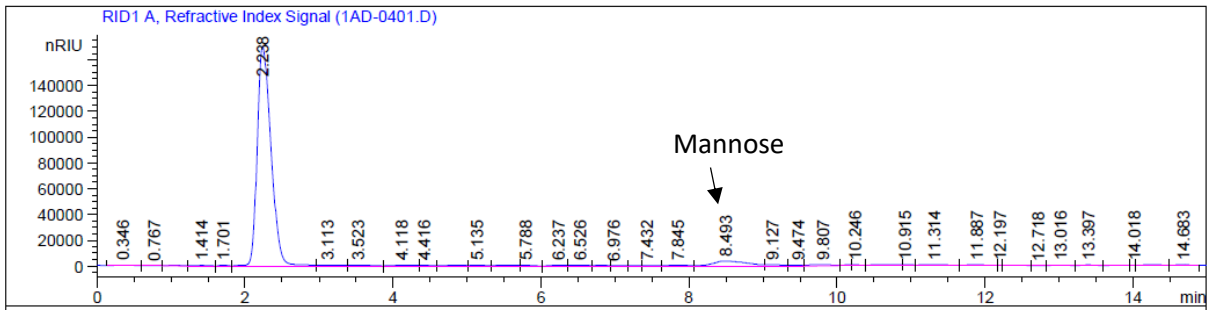
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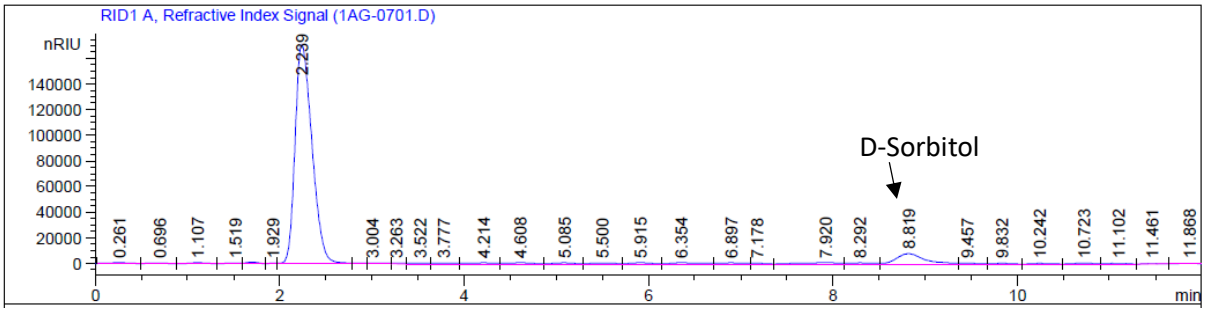
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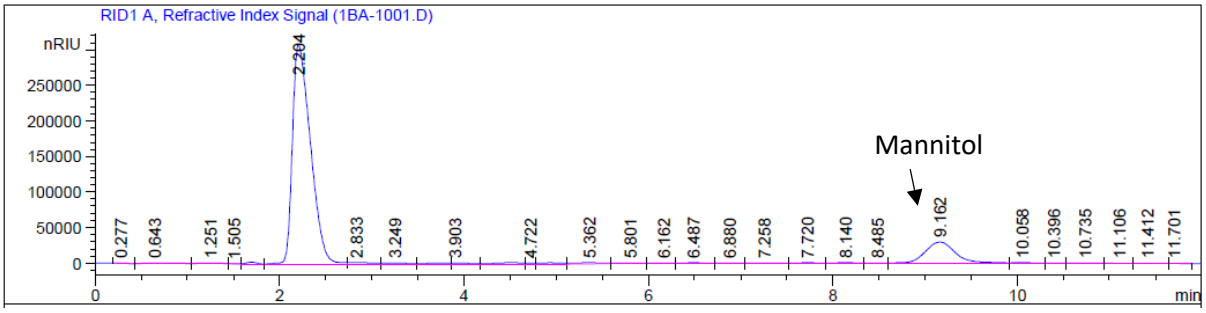
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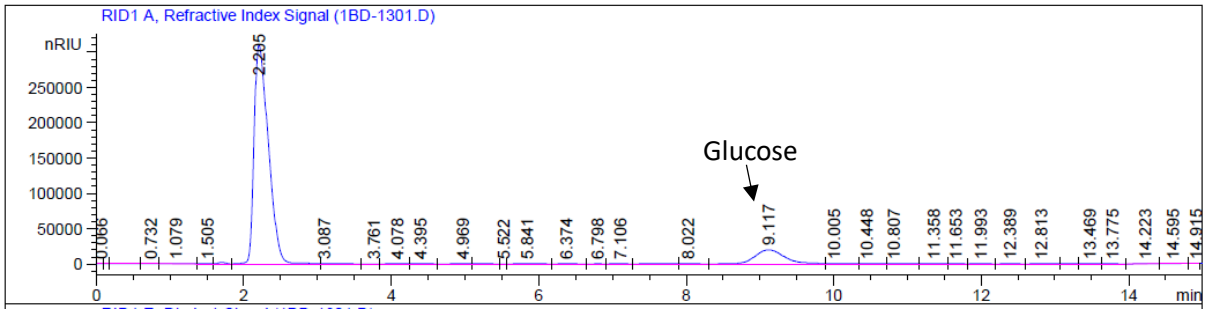
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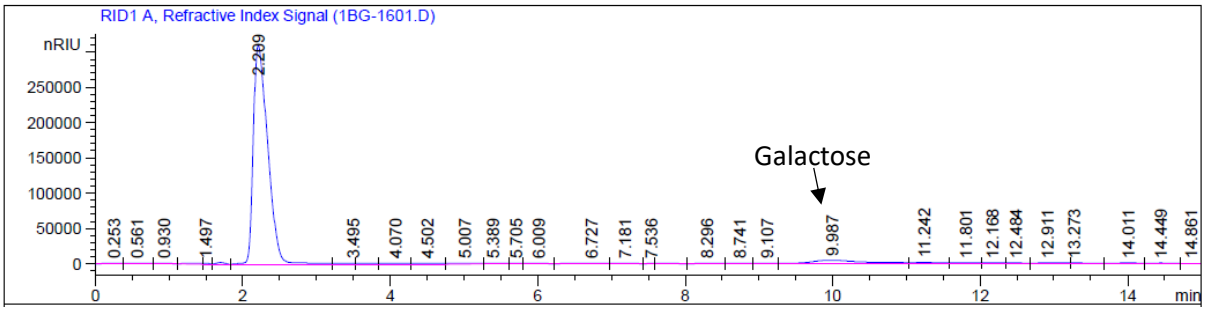
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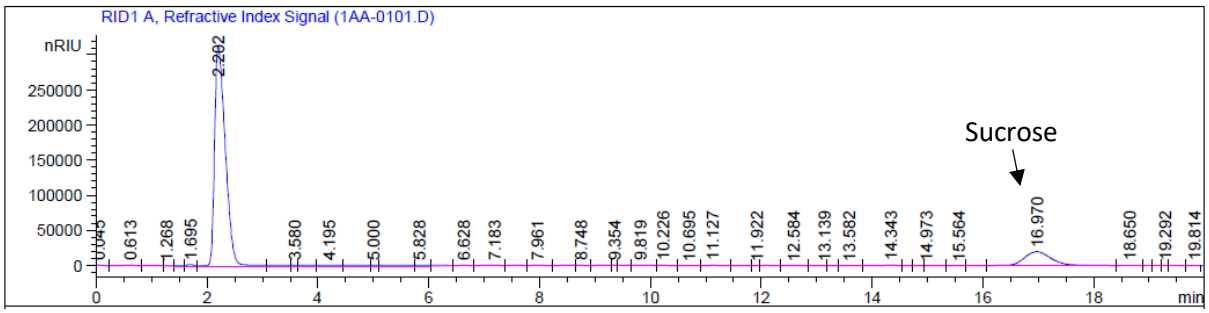
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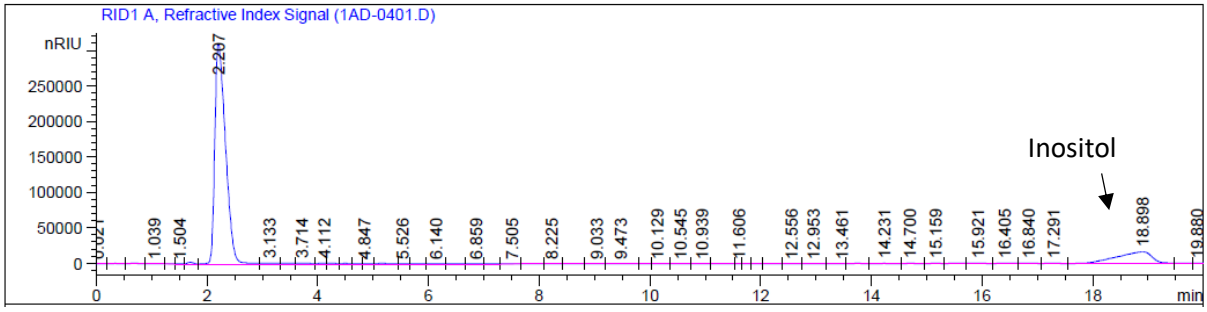
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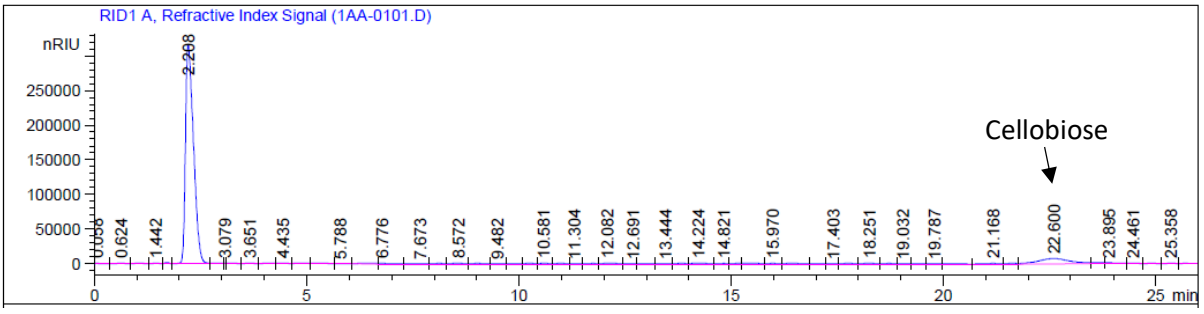
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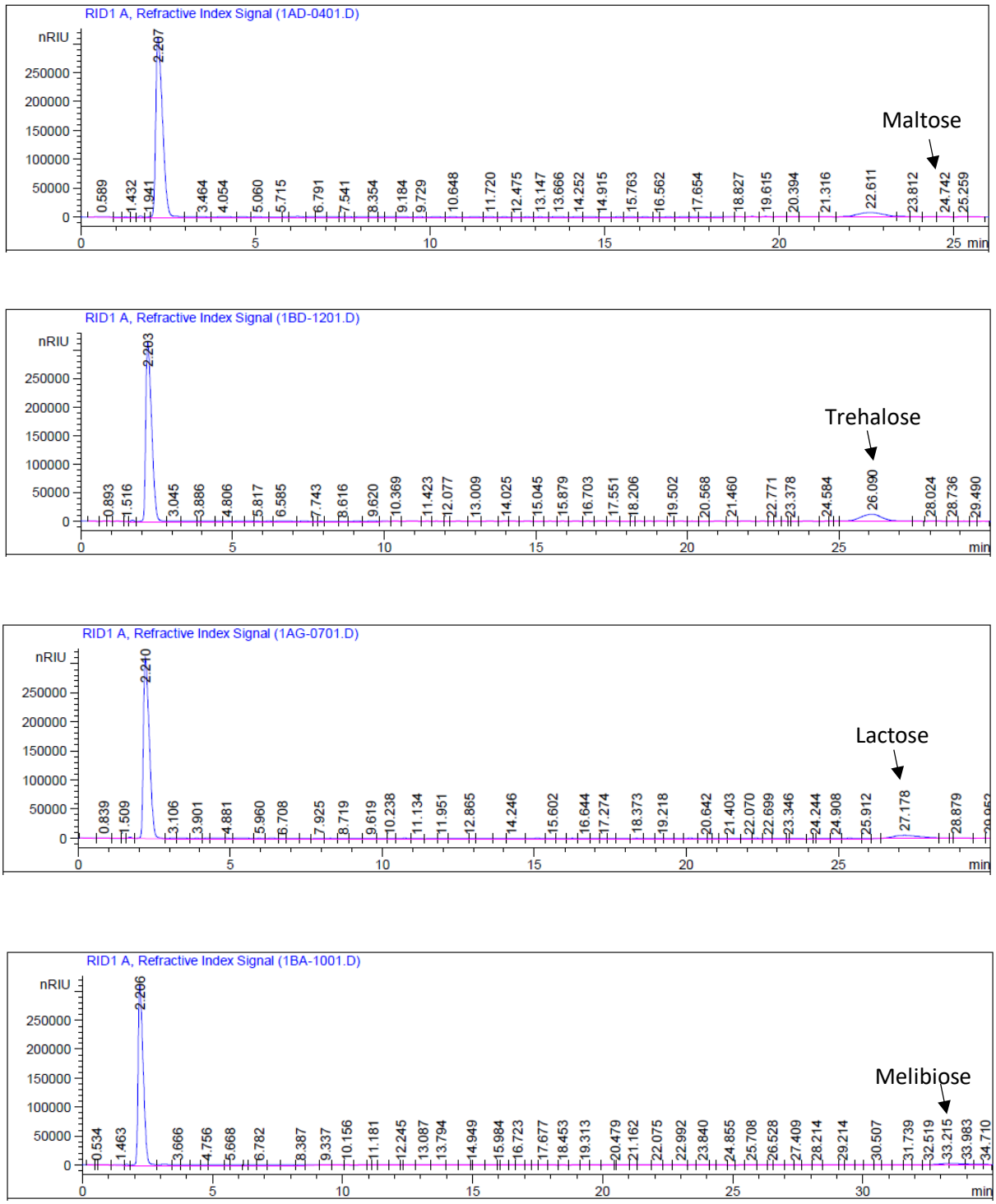
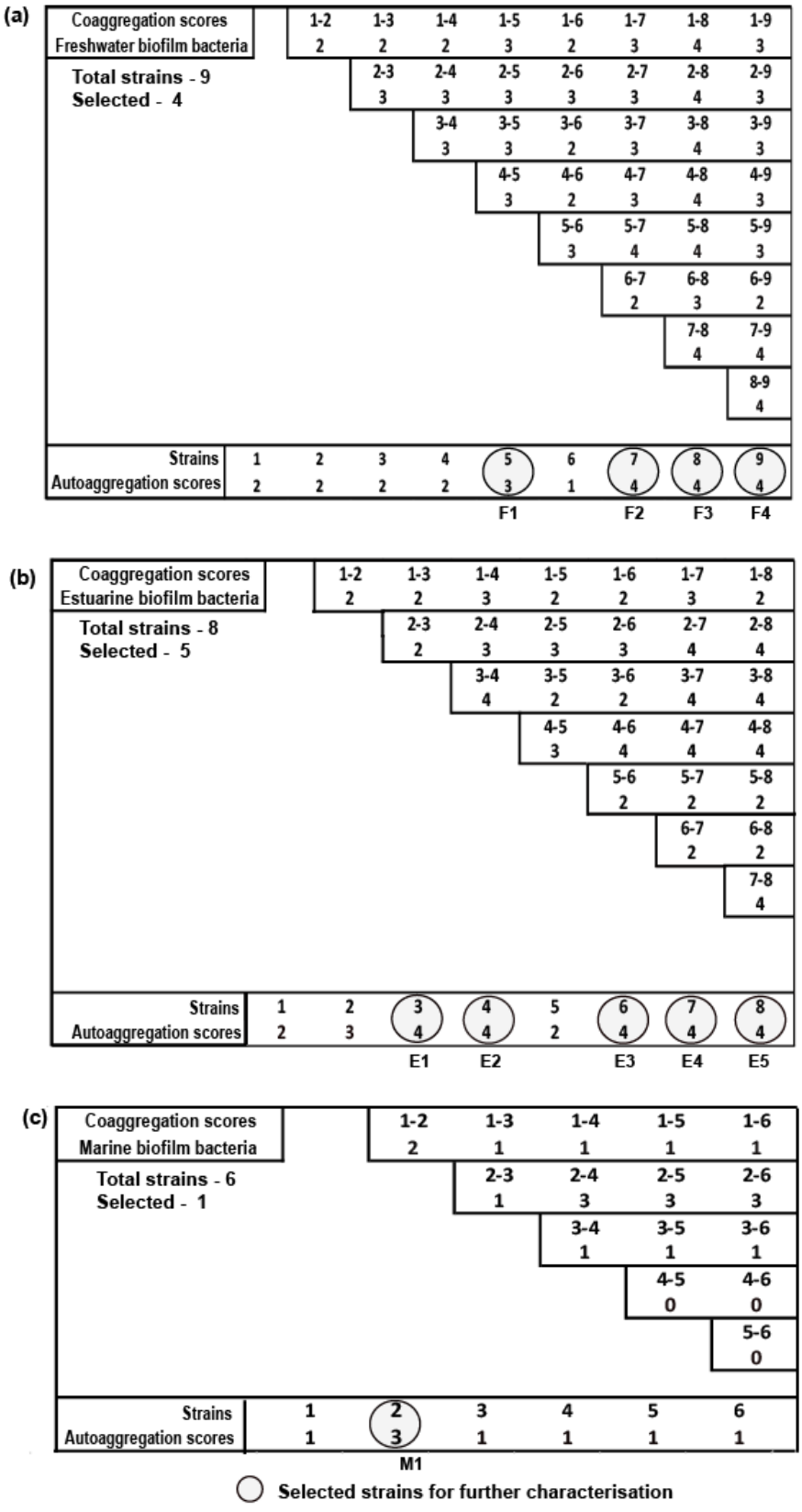


Appendix A1 Chromatograms of 19 sugar standards (Concentration: 10mg/mL)

		SUGARS	Retention Time (RT)
1	Monosaccharides	Rhamnose	4.374
2		Ribose	4.564
3		D Fucose	5.387
4		Xylose	5.411
5		Arabinose	6.371
6		N-acetyl-D-glucosamine	7.18
7		Fructose	7.26
8		Mannose	8.493
9		D- Sorbitol	8.819
10		Glucose	9.117
11		Mannitol	9.162
12		Galactose	9.987
13	Disaccharides	Sucrose	16.97
14		Inositol	18.898
15		Cellobiose	22.6
16		Maltose	22.611
17		Trehalose	26.09
18		Lactose	27.178
19		Melibiose	33.215

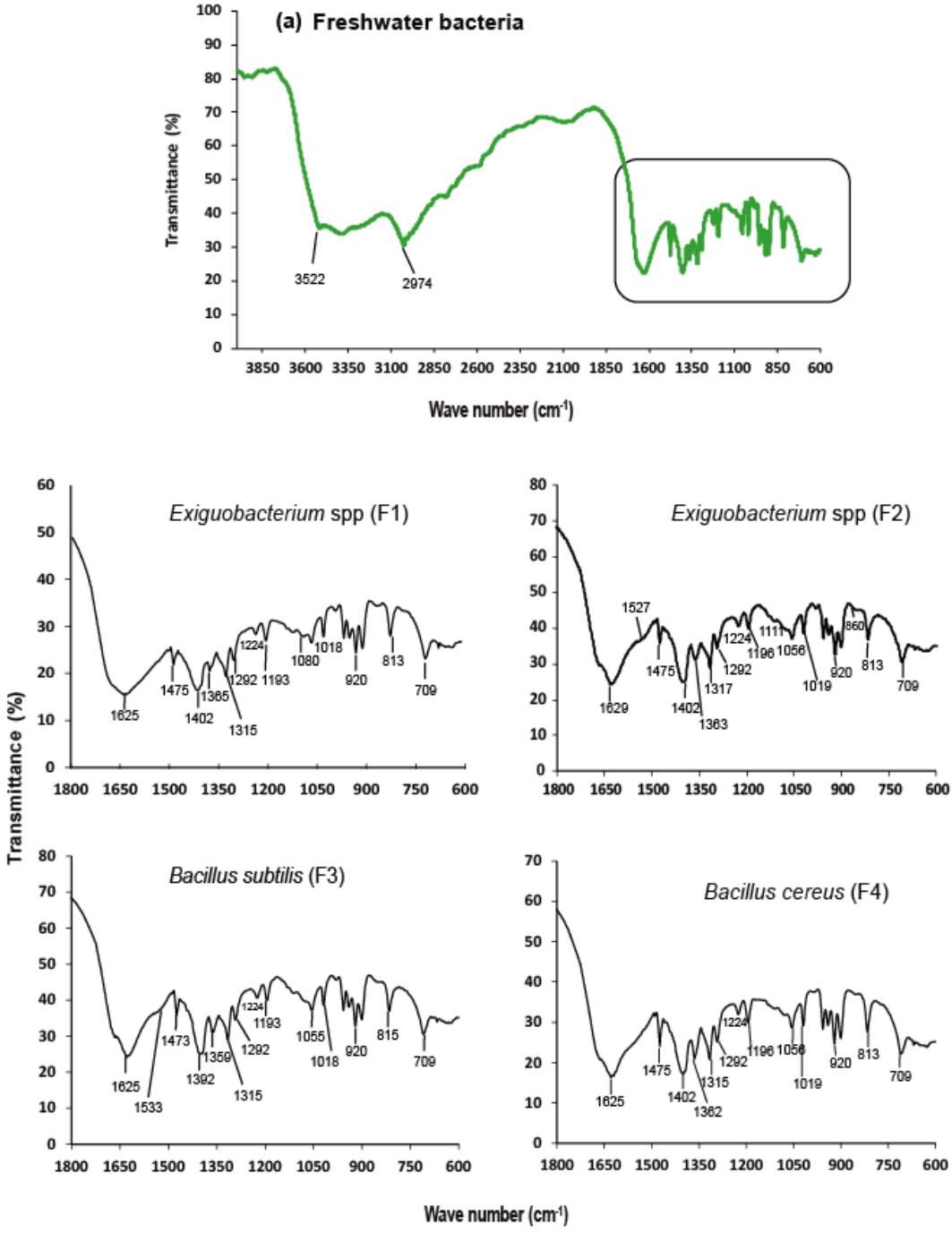
Appendix A2 Sugars standards with their retention times

Appendix II

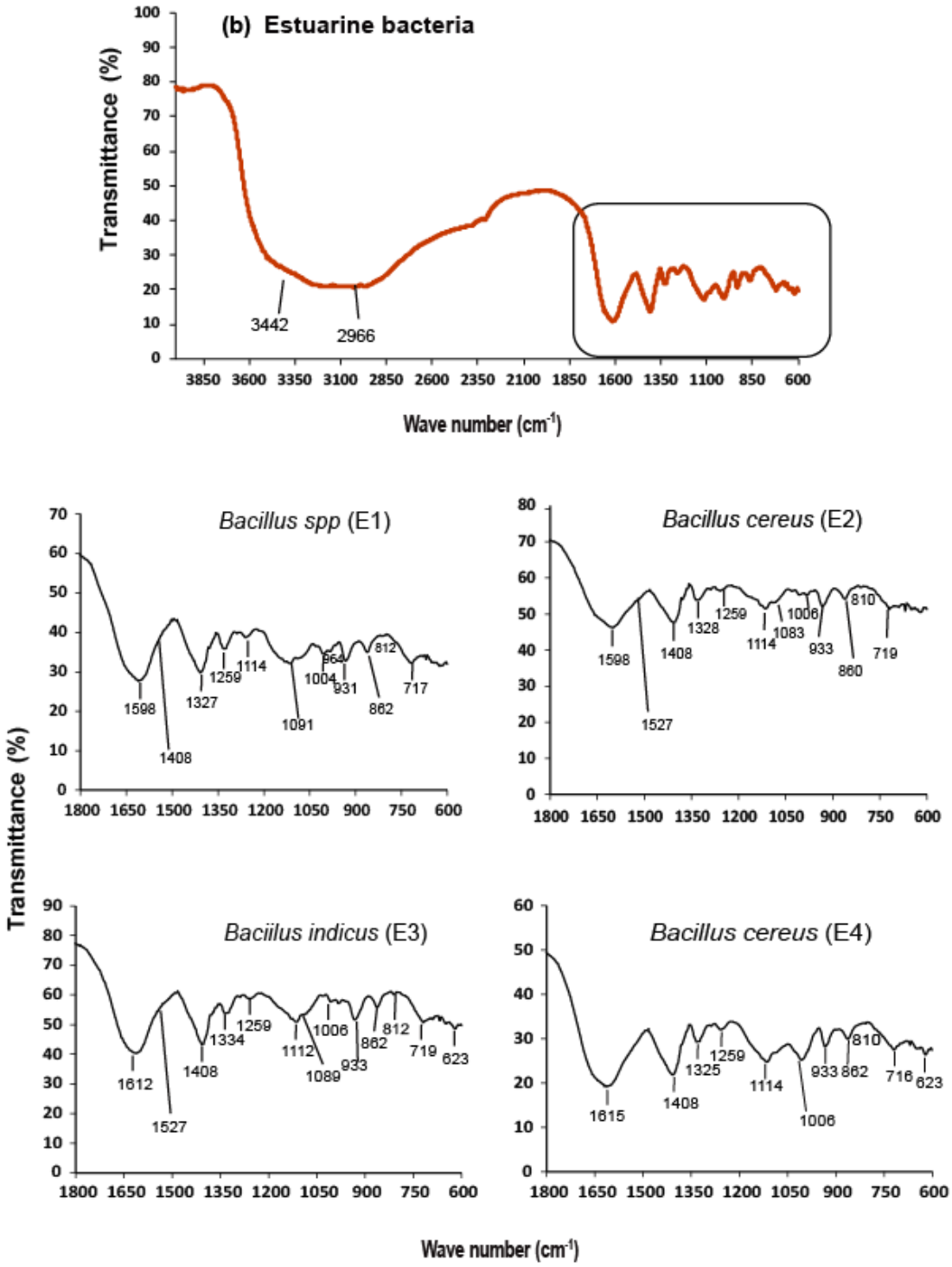


Appendix B1 Visual aggregation scores exhibited by biofilm bacteria at stationary phase during auto- and coaggregation assay carried out in pairwise combinations between the (a) freshwater, (b) estuarine, and (c) marine biofilm bacterial isolates. Values are noted after 24 hours of incubation. E = Estuarine, F = Freshwater, M = Marine.

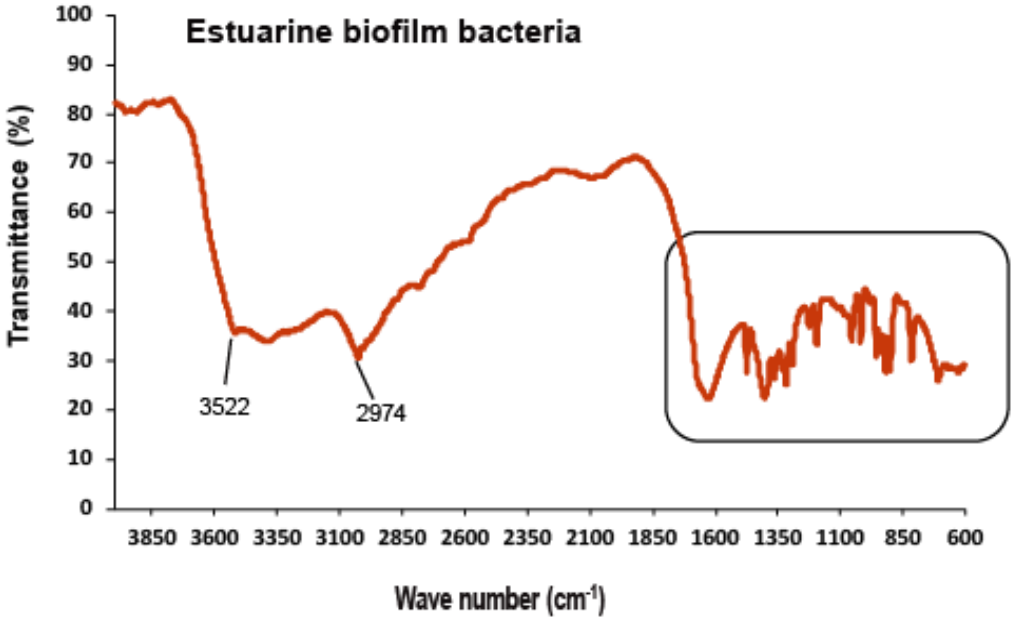
Appendix III

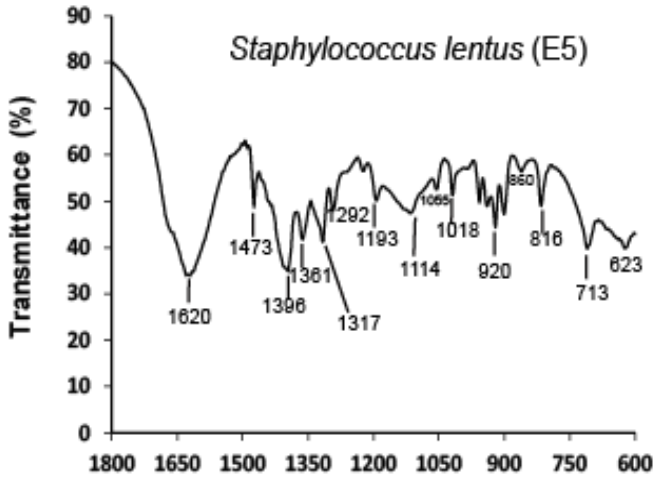


Appendix C1 Fourier-transform infrared (FT-IR) spectra's of cell-bound bacterial EPS extracted from (a) freshwater biofilm-forming bacteria. The freshwater bacterial EPS spectra is represented in green colour. The zoomed infra-red (1800 - 600 cm^{-1}) spectral section is shown in black colour. F= Freshwater.

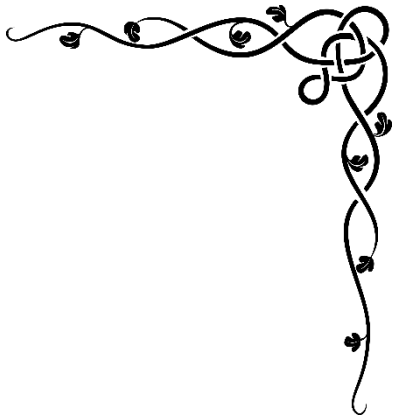


Appendix C2 Fourier-transform infrared (FT-IR) spectra's of cell-bound bacterial EPS extracted from estuarine biofilm-forming bacteria. The estuarine bacterial EPS spectra in orange colour. The zoomed infra-red (1800 - 600 cm^{-1}) spectral section is shown in black colour. E = Estuarine. One of the estuarine (MH429956.1), showed a spectral pattern similar to the freshwater bacteria as shown below.





Appendix C3 Fourier-transform infrared (FT-IR) spectra's of cell-bound bacterial EPS extracted from estuarine biofilm-forming bacteria depicting spectral pattern similar to the freshwater bacteria. The estuarine bacterial EPS spectra in orange colour. The zoomed infra-red (1800 - 600 cm^{-1}) spectral section is shown in black colour. E = Estuarine.



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Microbial dynamics in a tropical monsoon influenced estuary: Elucidation through field observations and microcosm experiments on biofilms

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Periphyton
Bacteria
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Vibrio spp.
Estuary

ABSTRACT

The changes that occur in biofilms reflect the influence of the environment. In a case study, the biofilms developed along a salinity gradient from marine to freshwater in a tropical monsoon influenced estuary (Zuari, west coast of India) were evaluated through in situ observations, and effect of translocation through microcosm experiments. The bacterial abundance was tide driven and high at the mouth of the estuary, whereas periphyton biomass, in terms of chlorophyll *a* was higher in the freshwater to brackish zone. These results point out decoupling of bacteria and phototrophic organisms in estuarine early stage biofilms. *Vibrio* spp. and faecal indicator bacteria were abundant in marine and brackish biofilms. The translocation experiments revealed that at times when marine biofilms with high total bacterial abundance were transferred to either brackish or freshwater, the abundance decreased. However, a significant increase in the bacterial abundance was observed when freshwater biofilms were transferred to either brackish or marine conditions. The microcosm experiments revealed that the influence of grazers differed with the type of biofilm and played an important role in modulating the composition of biofilms. The diagnostic pigments provided considerable insights into spatial and seasonal dynamics of periphyton groups in different biofilms and were determined by the surrounding water column communities and the availability of nutrients. Decay of freshwater periphyton in biofilms when translocated to marine and brackish conditions lead to remineralisation and higher bacterial population.

1. Introduction

Biofilms are complex structured communities of autotrophic and heterotrophic organisms surrounded by the matrix of exopolysaccharides. Bacteria and diatoms are dominant components of biofilms and their interplay, influence the composition of biofilms (Khandeparker et al., 2014). The biofilm community composition is determined by its ecosystem, mainly the seasonal, and geographical variations including biogeochemical and physical interactions (Chiu et al., 2005; Lau et al., 2005; Bellou et al., 2012; Briand et al., 2012; Battin et al., 2016; Oberbeckmann et al., 2014, 2016). In coastal areas, research on the biofilm communities is vast but very little is known about biofilm communities in the estuarine environments. They are regarded as environmentally naturally stressed areas because of variability in physicochemical properties (Elliott and Quintino, 2007; Elliott and Whitfield, 2011). Many of these parameters differ over daily light and tidal cycles, rainfall events and seasons (Moss et al., 2006).

Along the subcontinent of India, majority of the peninsular rivers originating in the Western Ghats along the west coast of India experience maximal rainfall during the south-west monsoon. During this period, freshwater influx from land runoff and precipitation is added to the estuary resulting in large fluctuations in the physicochemical characteristics of the water (Devassy and Goes, 1988). The physical processes in some estuarine environments differ significantly between the monsoon season, when runoff is high, and the dry season when runoff is negligible, besides the tide dominates circulation and mixing in the estuaries. Therefore, such estuaries are referred to as monsoonal estuaries (Shetye et al., 2007). They are highly unique and do not reach steady state at any time when compared to the temperate estuarine systems (Vijith et al., 2009). Zuari is one of the tropical monsoon influenced estuaries of Goa located along the central west coast, where runoff, rainfall, tides and salinity play an important role in determining its environment in comparison to the other estuaries in different parts of the world (Vijith et al., 2009).

Pollution of coastal waters due to anthropogenic activity has been a global issue (Nogales et al., 2011; Malham et al., 2014). The bacterial load, especially potential pathogens in the coastal waters are generally higher than the open sea (Brettar et al., 2007). Monitoring tropical

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monsoon influenced environment for pathogenic bacteria and sewage indicators have focused on the water column (Nagvenkar and Ramaiah, 2009; Rodrigues et al., 2011; Khandeparker et al., 2015). Biofilms on submerged surfaces also act as a reservoir for pathogens such as *Escherichia coli* and *Vibrio cholerae* (Shikuma and Hadfield, 2010). Studies relevant to bacterial pathogens associated with biofilms in estuaries is least studied except for a recent work (Khandeparker et al., 2014), which addressed their importance in marine biofilms.

Estuaries are dynamic environments, with a regular physical exchange of water with different properties. In this context, floating or moving objects experience different environmental ambience. Biofilms in such environments will also experience such a change and insights to such influence provide an opportunity to study the response of different biofilm communities. The response studies are sometimes difficult to evaluate by only using field based studies (Sabater and Borrego, 2015). The use of microcosms allows for investigation of the responses of the biofilms to specific environmental conditions such as salinity, nutrients under controlled conditions when compared to in situ observations (Craig et al., 2004; Kästner and Richnow, 2010). Other than environmental variables, protozoan grazing is also one of the important factors in controlling and altering the biofilm composition in aquatic ecosystems, which can influence marine invertebrate larval settlement (Pederson, 1990; Skov et al., 2010; Shimeta et al., 2012). It is often prey-selective, wherein the protozoans exhibit species-specific responses and thus significantly alter the biofilm population dynamics (Huws et al., 2005; Pernthaler, 2005; Dopheide et al., 2011; Yang et al., 2013). Protozoans have been demonstrated as important components of biofilms, which can rapidly colonise new substrata and occur in high abundances on biofilms (Arndt et al., 2003).

Taking the above points into consideration, we examined (1) impact of monsoon influenced estuarine conditions on the microbial dynamics of biofilms along a salinity gradient, (2) the response of biofilms developed under specific habitats to changes in the environmental conditions (survival in marine, brackish and freshwater habitats), and inoculum therein through laboratory microcosm experiments. The influence of grazing was indirectly evaluated by filtering the water used for translocation, which enabled removal of fresh inoculum and unfiltered water provided fresh inoculum and grazers.

2. Materials and methods

2.1. Description of study sites

This study was carried out in the Zuari estuary (15°27.5′ N, 73°48′ E) located in Goa, west coast of India (Fig. 1). The main channel of the Zuari estuary is about 50 km long and 5 km wide with a depth of ~5 m. The cross section area decreases towards the riverine end (0.5 km) (Shetye et al., 1995; Sundar and Shetye, 2005). Many streams and rivers are freshwater sources to this estuary (Shetye et al., 1995). During the monsoon season (June to September) freshwater influence is comparatively higher than the summer season (March to May), wherein there is less river discharge and is vertically mixed throughout the estuary till riverine region (Shetye et al., 1995). In this study, the stations were chosen along the estuary experiencing different tidal influence. Tides in this estuary are of the mixed semidiurnal type normally occurring twice a day and tide ranges are approximately 2.3 and 1.5 m during the spring and neap tide respectively (Shetye et al., 2007). The tidal influence is more at the mouth of the estuary when compared to the upstream end of the estuary. These tides as well as monsoon runoff are responsible for the mixing and circulation of water and hence are seasonal in nature (Shetye et al., 2007). Based on physico-chemical characteristics, three different stations were selected for developing biofilms, which are (1) Dona Paula (15°27.5′ N, 73°48′ E) which is located at the mouth of the estuary with salinity ranging from 15 to 35 psu representing marine condition. (2) Cortalim (15°24′32.0′ N, 73°54′50.2′ E) located at 13.7 km from the mouth of the estuary with salinity ranging from 2 to 32 psu representing brackish condition and (3) Sanvordem (15°16′01.1′ N, 74°06′36.0′ E) station which, is located at the upstream of the estuary with 0 psu salinity representing freshwater condition. These stations are hereafter referred as marine (Dona Paula), brackish (Cortalim) and freshwater (Sanvordem) stations.

2.2. Simultaneous sampling of biofilms from the study sites

Before deployment, glass slides (25.4 mm × 76.2 mm) were cleaned as described by Bhosle et al. (2005). For each season, the treated glass slides were deployed at marine, brackish and freshwater stations simultaneously during spring (n = 210) and neap tide (n = 210), for the development of marine, brackish and freshwater biofilms for 24 h. The slides were suspended from trawlers that were anchored midstream in the Zuari estuary at each station. These stations were sampled simultaneously for physicochemical parameters and biofilm samples twice during the premonsoon (PreM, 19th May 2011 and 27th April 2011) followed by monsoon (MoN, 1st August 2011 and 9th August 2011) and post monsoon season (PostM, 29th October 2011 and 23rd October 2011) over spring and neap cycles respectively. For control samples, set of thirty slides were collected for microbiological analysis during each season and tide. Three slides were sampled (n = 1 × 3) and each one was scraped separately into 10 ml of 0.22 μm filtered autoclaved seawater for microbial counts. 1 ml of subsample was used for the analysis of viable and pathogenic bacterial abundance while 5 ml of subsample was fixed with formalin for enumeration of total bacterial counts. For pigment analysis, twenty seven slides were sampled (n = 9 × 3) and nine slides were scraped separately in 25 ml filtered seawater. The remaining six sets of thirty slides (n = 180) with the biofilms developed at different stations were used to carry out microcosm experiments as described in Section 2.4.

2.3. Physicochemical parameters

All the physicochemical parameters (temperature, salinity, and dissolved nutrients) were collected in duplicates each time (seasons and tides) during the sampling period. Water temperature and salinity were recorded at the sampling stations by using conductivity-temperature-depth (CTD) probe (Sea bird, USA). Tide and flow were recorded using a current meter. Nutrients in the water column, namely nitrate (NO_3), nitrite (NO_2), phosphate (PO_4) and silicate (SiO_4) were analysed using autoanalyser (Skalar SAN PLUS 8505 Interface v3.31, Netherland). The nutrient samples were analysed within 30 days of sampling date.

2.4. Microcosm experiments

The microcosm experiments were carried out to assess the response of biofilm communities to changes in the environmental conditions. The experimental protocol used for performing the microcosm experiment relied on an earlier investigation used to study bacteria-diatom interactions using an antibiotic approach (Khandeparker et al., 2014). Briefly, biofilms were developed at three different stations for 24 h in the field. After this, one set of slides (n = 30) from each station was kept as control as described above in Section 2.2 and remaining six sets of thirty slides (n = 180) were translocated to the thirty six microcosm units (5 slides per microcosm) in the laboratory, containing unfiltered and filtered water from (marine/brackish/freshwater) stations. For example, the slides with marine biofilms were translocated to unfiltered marine (n = 30), brackish (n = 30), freshwater (n = 30), and filtered (0.22 μ pore size filters) marine (n = 30), brackish (n = 30) and freshwater (n = 30). Experiments with filtered seawater enabled the removal of fresh inoculum and grazers whereas unfiltered seawater conditions provided fresh inoculum and grazers. The glass slides were placed in each of the sterile polycarbonate square jars (75 × 74 × 138 mm, 450 ml, Himedia). All the jars (or microcosms) were closed and static during the incubation. The experimental units

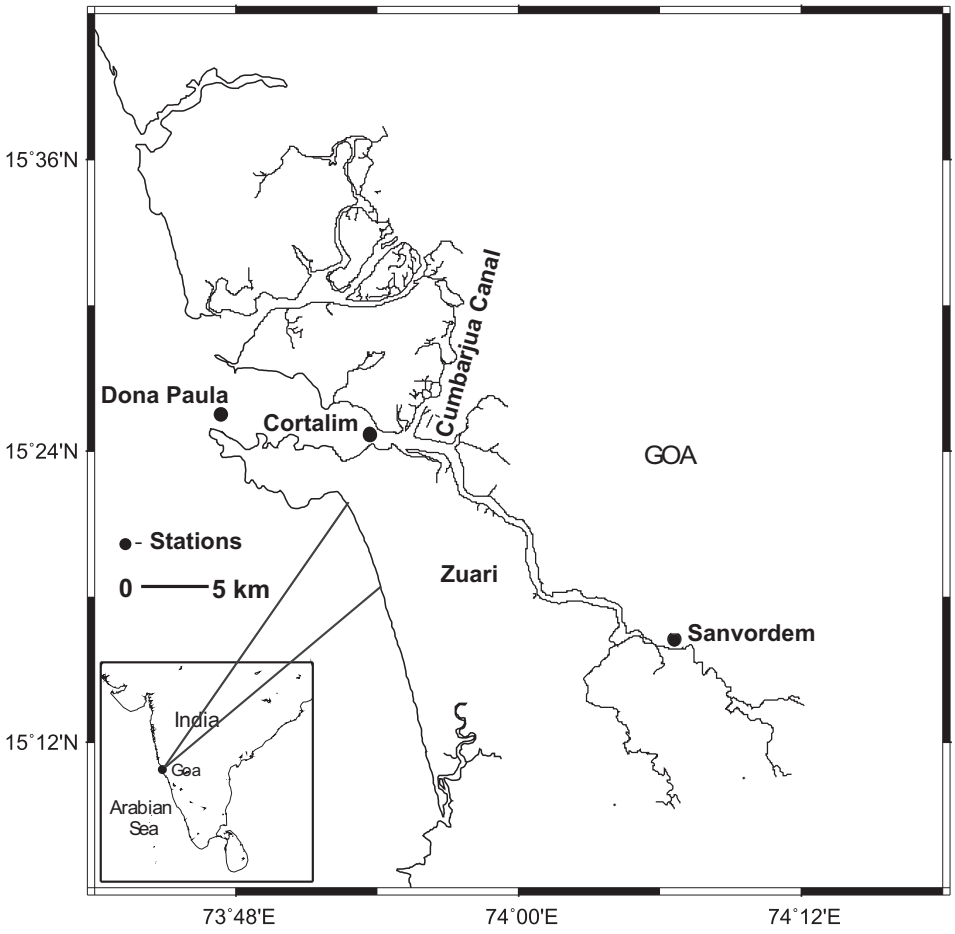


Fig. 1. Map showing sampling locations along the Zuari estuary.

were incubated for 5 days under 12 h light: 12 h dark cycle at room temperature. At the end of the incubation, a set of thirty slides were collected for microbiological analysis from each treatment and the 5-day-old biofilms were analysed for the microbiological analysis and periphyton pigments as described below.

2.5. Total bacterial counts in biofilms

For the total bacterial counts, 5 ml of subsample was fixed with 0.22 μm filtered formalin at 0.2% final concentration and kept in − 20 °C until further analysis. The samples were analysed within 30 days of sampling date. For the flow cytometric analysis, 1 ml of subsample was sonicated (30 W for 60 s) and subsequently passed through BD cell strainer cap (Cat no: 352235) to remove larger particles. The samples were then stained with DAPI (Sigma) at 1 μg/ml final concentration (Troussellier et al., 1995) and incubated for 30 mins in the dark at room temperature. After incubation, samples were analysed using a BD FACSAria™ II flow cytometer equipped with a nuclear UV laser 375 nm which can differentiate blue fluorescence excited by UV light. Emitted light was collected through the following filters sets 488/10 band pass (BP) for right angle light scatter (SSC) and 450/20 band pass (BP) for blue fluorescence. Fluorescent beads (1 μm, Polysciences) were used as internal standards and for calibration of the above parameters. Gating was done against SSC versus blue fluorescence. Flow cytometry data was processed using BD FACS Diva software. Total Bacterial Counts (TBC) is expressed as cells/cm^2 of the slide surface area sampled.

2.6. Enumeration of viable and pathogenic bacteria in biofilms

To enumerate Total Viable Counts (TVC) and pathogenic bacteria, 1 ml of subsample was serially diluted and spread plated on different agar medium as per manufacturer's instructions [Zobell Marine Agar (ZMA) 2216 (Total viable bacteria), Mac Conkey agar (Total Coliforms), Thiosulphate Citrate Bile Salt Agar (*Vibrio* spp.)]. *Vibrio* spp. in the TCBS agar were differentiated as described by Pfeffer and Oliver (2003). In short, sucrose fermenting, yellow, < 2 mm diameter colonies were counted as *Vibrio cholerae*, > 2 mm diameter colonies as *Vibrio alginolyticus* while greenish colonies were counted as *Vibrio parahaemolyticus*. Identification of these bacterial species has been verified previously by protein profiling using MALDI-TOF MS Biotyping (MTB) (Khandeparker et al., 2015). The selective agar plates were incubated at 37 °C for 24 h before the colonies were enumerated. ZMA plates were incubated at room temperature for enumerating culturable bacteria. After 24 h, viable colonies were counted and expressed as CFU/cm^2 of the slide surface area sampled.

2.7. Analysis of periphyton pigments in biofilms

For pigment analysis, 1 day old (natural) and 5 day old aged (natural translocated) biofilms (n = 9 × 3) were scraped and pooled in 25 ml of 0.22 μm filtered autoclaved seawater. Subsequently, the pooled samples were filtered on GF/F 25 mm Whatman filters in dark condition and kept in − 20 °C until further analysis. The samples were analysed within 30 days of sampling date. For pigment analysis, filters were extracted using 90% HPLC grade acetone and extracts were filtered through 0.22 μm PTFE sterile syringe filters (Millipore). Subsequently, filtered extracts were analysed using Reverse Phase High Performance Liquid Chromatography (Agilent 1200 series) equipped with the auto start kit as described by Wright et al. (1991). Briefly, pigment separation was done using C-18 column and the eluent gradient program. Pigments were distinguished by their absorbance peaks

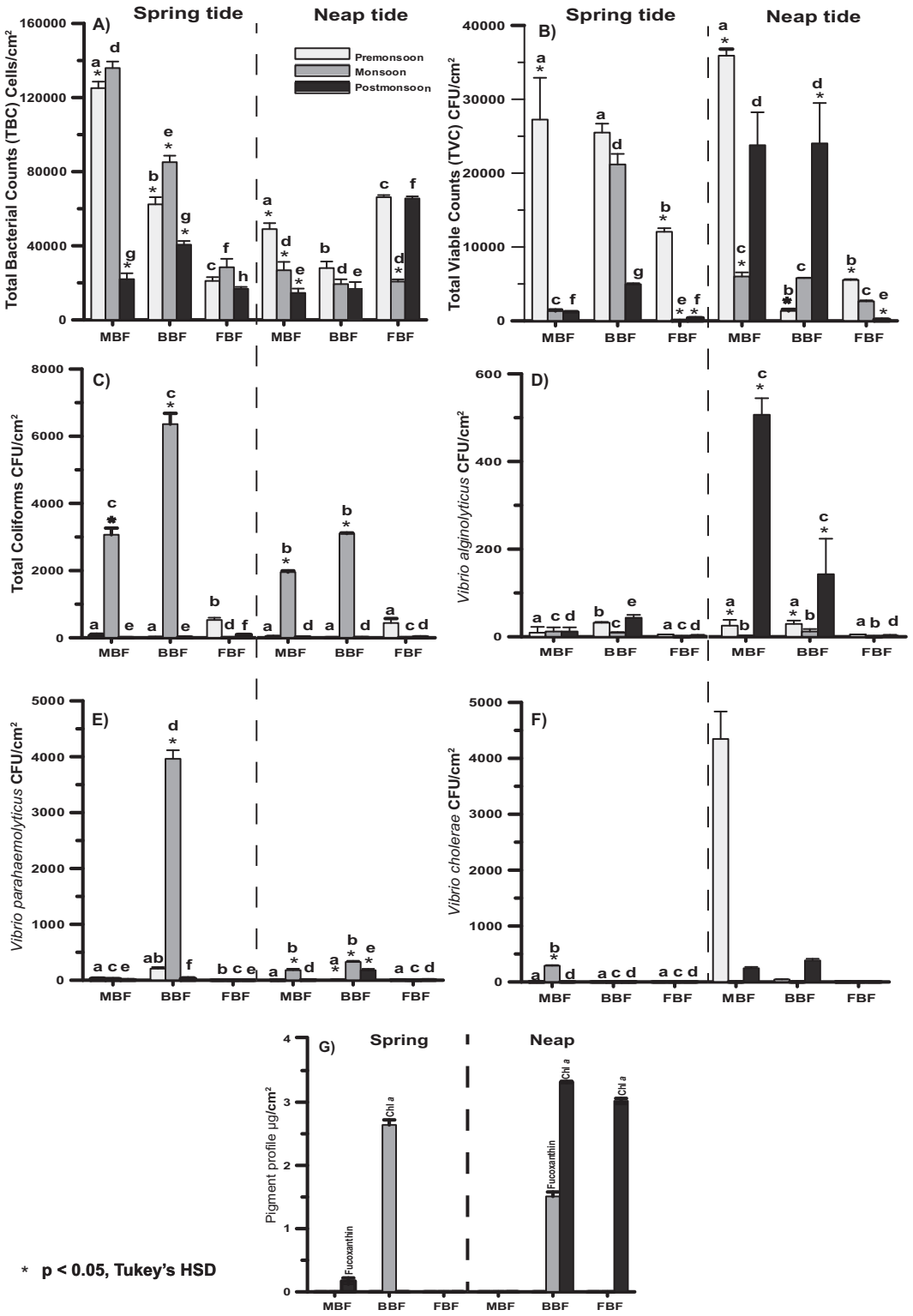
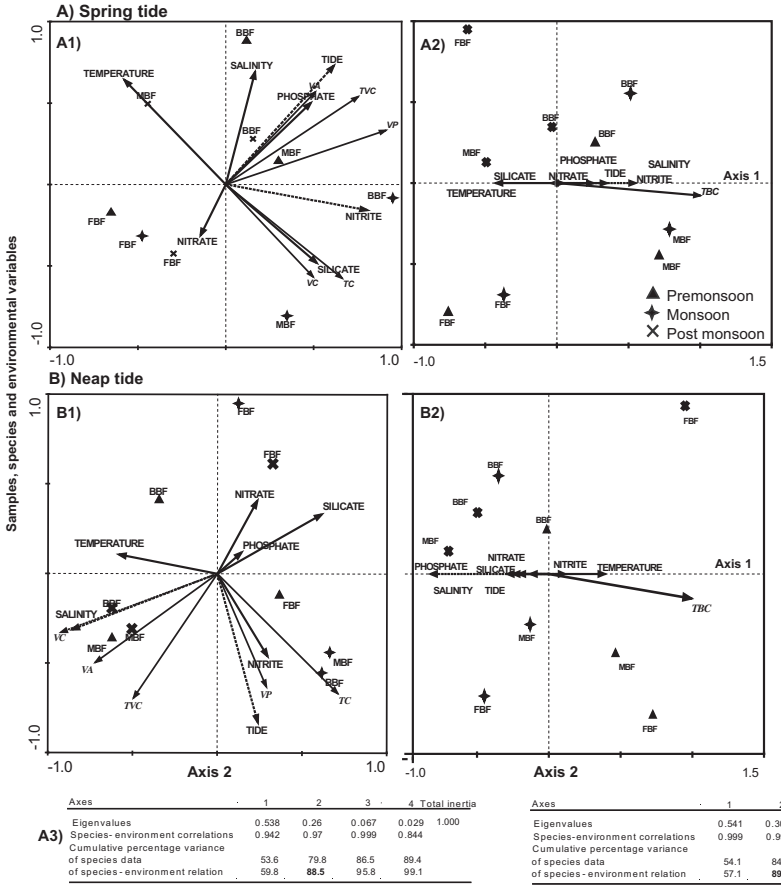


Fig. 2. Spatio-temporal and tidal variations of (A) total bacterial counts, (B) total viable counts, (C) total coliforms, (D) *Vibrio alginolyticus*, (E) *Vibrio parahaemolyticus*, (F) *Vibrio cholerae* abundance, and (G) Periphyton pigment profile in one-day old biofilms developed along Zuari estuarine stretch over spring and neap cycles. MBF – marine biofilm, BBF – brackish biofilm, FBF – freshwater biofilm. The solid bars represent averaged data (n = 3) of the samples. Error bars indicate the standard deviation of the mean based on three replicate samples. The asterisk (*) above bars indicate significant differences within the samples. Different letters indicate significant seasonal differences between the groups (Tukey's HSD, $p < 0.05$).

at 436 nm and identified by comparison with the retention times of standard pigments. The following pigments were used as standards: chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl b), chlorophyll *c*2 (Chl c2), β–carotene, fucoxanthin (Fuco), diadinoxanthin (Diad), peridinin (Peri), zeaxanthin (Zea), alloxanthin (Allo), violaxanthin (Vio), prasinoxanthin (Pras), pheophytin, 19′hexanoyloxyfucoxanthin (19′hex), 19′butanoyloxyfucoxanthin (19′but), lutein (Lut), neoxanthin (Neo) and myxoxanthophyll (Myxo); All pigments from DHI, Denmark.



A4)

Conditional Effects - Spring tide				
Variables	Var.N	λ I	P	F
Nitrite	**7**	**0.36**	**0.032**	**3.96**
Tide	**3**	**0.27**	**0.004**	**4.5**
Temperature	1	0.14	0.098	2.84
Salinity	2	0.04	0.415	0.99
Silicate	4	0.03	0.508	0.55
Phosphate	5	0.06	0.405	1.16
Nitrate	6	0	0.95	0.02

TBC - Total Bacterial Count
TVC - Total Viable Count
TC - Total Coliforms
VA - *Vibrio alginolyticus*
VP - *Vibrio parahaemolyticus*
VC - *Vibrio cholerae*

B4)

Conditional Effects - Neap tide				
Variable	Var.N	λ I	P	F
Salinity	**2**	**0.43**	**0.019**	**5.21**
Tide	**3**	**0.24**	**0.017**	**4.53**
Nitrite	7	0.09	0.154	1.92
Silicate	4	0.06	0.291	1.33
Temperature	1	0.04	0.508	0.87
Nitrate	6	0.05	0.472	0.97
Phosphate	5	0.04	0.574	0.76

A3)

Axes	1	2	3	4	Total inertia
Eigenvalues	0.538	0.26	0.067	0.029	1.000
Species-environment correlations	0.942	0.97	0.999	0.844	
Cumulative percentage variance of species data	53.6	79.8	86.5	89.4	
of species-environment relation	59.8	**88.5**	95.8	99.1	

B3)

Axes	1	2	3	4	Total inertia
Eigenvalues	0.541	0.304	0.067	0.027	1.000
Species-environment correlations	0.999	0.999	0.886	0.969	
Cumulative percentage variance of species data	54.1	84.5	91.2	93.9	
of species-environment relation	57.1	**89.2**	96.2	99.1	

Fig. 3. Redundancy analysis (RDA) of one-day old biofilms showing the relationship between bacteria (viable, pathogens, total coliforms and total bacterial counts) and environmental variables during (A) Spring, and (B) Neap tide. MBF - marine biofilms, BBF - brackish biofilms, FBF - freshwater biofilms. All data points are averages of several replicates. A1, B1 – RDA triplot with viable bacteria; A2, B2 – RDA triplot with total bacteria; A3 – Eigenvalues for RDA axes for spring tide analysis; B3 – RDA axes for neap tide analysis; A4, B4 - Lambda (λ I) is the eigenvalue explained by the environment variables for spring and neap tides respectively. $p \leq 0.05$ are highlighted in bold.

2.8. Statistical analysis

Bacterial abundance data were log(x + 1) transformed to normalise the data because data needs to be balanced to down-weight the influence of the highly abundant species and to raise the profile of the less abundant species. Statistical analyses were preceded by checking for normality and homogeneity of variances with Shapiro-Wilk's W test using the STATISTICA 6 program (Stat Soft Inc., Tulsa, OK, USA). The datasets fulfilling the assumptions for parametric analysis were analysed through two-way analysis of variance (ANOVA), for evaluating significant variations in the biofilm communities, with seasons and tides as factors among one-day old biofilms. Whereas, in the case of five-day old biofilms, treatment was taken as an independent factor since the inoculum in the marine/brackish/freshwater was different. Tukey's HSD post hoc test was performed to determine statistically significant differences between the groups. This statistical analysis was carried out by using the SPSS statistical package (Version 16). The relationship between the environmental variables and bacteria in the field was identified by means of Redundancy Analysis (RDA) using the CANOCO version 4.5 (ter Braak and Verdonschot, 1995). 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 linear variation in the data (ter Braak and Smilauer, 2002). 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 performed on the set of environmental variables that explained most of the variances and Monte Carlo permutation test was done to test the statistical significance of each variable by running 999 number of permutations. The position of sampled sites in RDA triplot reflects the biofilm bacteria and their association with the environmental variables. The length of the arrows (environmental variables) and their orientation indicates their relative importance and approximate correlations to the axes (Lepš and Šmilauer, 2003). Further, correlation analysis was performed between abiotic and bacterial components in the field using STATISTICA 6 statistical package at a significance level of ≤ 0.05 (Stat Soft, OK, USA).

3. Results

3.1. Physicochemical parameters

The surface water temperature was higher in the PreM season and varied from 30.2–31.2 °C during spring tide and 30.8–32.3 °C during neap tide. The variation in the mean salinity was minimal (~1) in the marine water during PreM and PostM season irrespective of tides whereas it was low in the MoN (15–12 during spring and neap tides respectively). Large salinity variations were observed in the brackish water, the salinity varied between 25.2 and 31.2 (spring tide) and 24.4–28.7 (neap tide) during PreM and PostM followed by 0.06–2.1 during MoN. The salinity was low in freshwater (0.036–0.65) throughout the study period. The nutrients in the surface water were high during the spring tide. The NO_3 (PostM (50.6 μmol) > PreM (12.5 μmol) > MoN (6.7 μmol)) and PO_4 (PostM (12.3 μmol) > MoN (6.2 μmol) > PreM (4.2 μmol)) concentrations were higher at the upstream end and decreased towards the mouth of the estuary during spring tide. SiO_4 concentration was high at all the three stations; freshwater (222.9 μmol), marine (173.4 μmol) and brackish

(146.9 μmol) especially in the MoN season (Supp. Table 1).

3.2. Bacteria in 1-day old biofilms (field study)

The two-way ANOVA revealed that both seasons and tides, individually and in combination, significantly influenced the biofilm parameters at all the three stations (Wilks' Lamba: $p < 0.001$). During spring tide, TBC was significantly high in the marine biofilms (1.25×10^5–1.36×10^5 cells cm^{-2}) followed by brackish (6.24×10^4–8.51×10^4 cells cm^{-2}) and least in the freshwater biofilms (2.10×10^4–2.84×10^4 cells cm^{-2}) during PreM and MoN seasons (Fig. 2A, Tukey's HSD, $p \leq 0.05$). The TBC was relatively low during the PostM at all three stations. Whereas, TVC in the biofilms (Fig. 2B) was significantly higher during the neap tide (3.59×10^4–1.80×10^2 CFU cm^{-2}). Marine biofilms harboured high TVC during PreM season compared to other biofilms irrespective of tides (Fig. 2B). The viable abundance was least during MoN season (Fig. 2B). In the RDA triplot, marine and brackish biofilms were distinctly different across the seasons along the Axis 1 (Fig. 3A1–B1). The RDA Axis 1 and 2 explained 88.5% and 89.2% of the cumulative variance of the relation between the species and environmental variables during spring and neap tide respectively (Fig. 3A3–B3).

Total coliforms were abundant in marine and brackish biofilms during MoN, irrespective of tides (Fig. 2C). They were influenced by SiO_4 during spring tide and NO_2 during the neap tide (Fig. 3A1). Among *Vibrio* spp., *V. cholerae* and *V. alginolyticus* were dominant in marine biofilms during neap tide and were significantly influenced by salinity ($r = 0.90$, $p \leq 0.05$; $r = 0.77$, $p \leq 0.05$) in the PreM and PostM respectively (Fig. 2D, F). Other than salinity, *V. cholerae* were also related to SiO_4 concentration during MoN spring tide. Whereas, *V. parahaemolyticus* were dominant in brackish biofilms during MoN spring tide and their abundance showed positive relation with tide ($r = 0.80$, $p \leq 0.05$) and nutrients such as NO_2 ($r = 0.70$, $p \leq 0.05$) and PO_4 ($r = 0.68$, $p \leq 0.05$) (Figs. 2E, 3A1).

3.3. Response of bacteria in 5-day old biofilms upon translocation (microcosm study)

3.3.1. Marine biofilms

A schematic illustration of the response of biofilm communities to translocation studies is depicted in Fig. 4. When 1-day old marine biofilms developed during the PreM and MoN seasons with high TBC were translocated, the abundance significantly decreased in both unfiltered/filtered (marine/brackish/fresh) water (Fig. 5A, Tukey's HSD, $p \leq 0.001$). The response was significant and prominent in unfiltered freshwater and depended on the tide (spring/neap tide) during which they were developed. Whereas, during PostM, TBC increased significantly in filtered/unfiltered brackish water (Tukey's HSD, $p \leq 0.001$). However, TVC, total coliforms and the abundance of *V. cholerae* significantly decreased in unfiltered waters upon translocation as stated in Fig. 4 (Figs. 5B–C, 6C, Tukey's HSD, $p \leq 0.05$).

3.3.2. Brackish biofilms

TBC in the brackish biofilms increased when exposed to unfiltered (marine/brackish/fresh) water during PreM season (Fig. 5D, Tukey's HSD, $p \leq 0.001$). Whereas, total coliforms and *V. parahaemolyticus* decreased significantly when translocated to unfiltered brackish water during MoN season (Fig. 5F, 6E, Tukey's HSD, $p \leq 0.05$). However, TVC increased significantly (Tukey's HSD, $p \leq 0.001$) in the unfiltered marine and brackish as well as filtered freshwater during PostM season.

3.3.3. Freshwater biofilms

One-day-old freshwater biofilms with low bacterial numbers when translocated to both unfiltered/filtered (marine/brackish/fresh) water, showed a significant increase in the TBC during PreM season (Fig. 5G, Tukey's HSD, $p \leq 0.001$). The TVC in the freshwater biofilms increased significantly in all the treatments during MoN neap tide (Fig. 5H, Tukey's HSD, $p \leq 0.001$). Total coliforms increased significantly when exposed to unfiltered/filtered (marine/brackish/freshwater) during PostM season (Fig. 5I). When one-day old freshwater biofilms developed during neap tide of MoN season, with low numbers of *Vibrio* spp., were translocated in filtered marine and freshwater, a significant increase in the abundance of *V. parahaemolyticus* was observed (Fig. 6H, Tukey's HSD, $p \leq 0.001$).

3.4. Periphyton pigments in 1-day old biofilms (field study)

In the case of biofilms developed during the PreM, no pigments were detected on day 1. The periphyton biomass in terms of Chl *a* was detected in brackish (MoN and PostM) and freshwater biofilms (PostM), but not the marine biofilms. In the case of marine biofilms, Fuco which is a chemotaxonomic biomarker of diatoms was present; Fuco was also present in brackish biofilms (Fig. 2G). In summary, 1-day old marine biofilms were poor in periphyton.

3.5. Periphyton pigments in 5-day old biofilms upon translocation (microcosm study)

3.5.1. Marine biofilms

Marine biofilms developed during PreM neap tide, had Chl *a*, b and Fuco pigments specific to green flagellates and diatoms, when exposed to unfiltered marine and freshwater (Fig. 7) but not in brackish water. During the MoN season, no pigments were detected after translocation except for Chl b detected upon exposure to unfiltered marine water (Fig. 7). In the case of PostM, marine biofilms exposed to filtered fresh water showed Vio and Allo pigments with the predominance of cryptophytes, and prasinophytes respectively (Fig. 7).

3.5.2. Brackish biofilms

Not much variability was observed when brackish biofilms were translocated in marine/brackish except for the presence of Diad and Allo pigments indicating the predominance of euglenophytes and cryptophytes upon exposure to unfiltered and filtered freshwater during PreM (Fig. 8).

3.5.3. Freshwater biofilms

In the case of freshwater biofilms developed during the PreM, wherein no pigments were detected on day 1, after translocation, the biofilms were dominated by diatoms, cyanobacteria, haptophytes, nanoflagellates, dinoflagellates prasinophytes, cryptophytes, euglenophytes, green algae and land plants. The response was prominent in unfiltered marine, freshwater and filtered brackish water (Fig. 9) indicating a shift in the periphyton communities. High concentrations of pheopigments were observed when freshwater biofilms were translocated to marine and brackish water during PreM and MoN seasons. There was no change in the periphyton community after translocation in the case of the freshwater biofilms developed during the PostM season. Not much variability was observed when freshwater biofilms were exposed to neap tide waters (Fig. 10).

4. Discussion

The present study addressed the interactive effects of tides and environmental factors on the biofilm development in a tropical monsoon influenced estuarine environment. Although nutrients were higher at the freshwater end, same was not reflected in the bacterial abundance in the biofilms especially during PostM. The TBC was tide driven and higher in the marine biofilms during spring tide wherein water was well mixed and suspended load was high (Khandeparker et al., 2017). An earlier study in the York River estuary reported that bacterial abundance covaried with spring-neap tide induced changes in the mixed layer depth (Eldridge and Sieracki, 1993). These changes usually

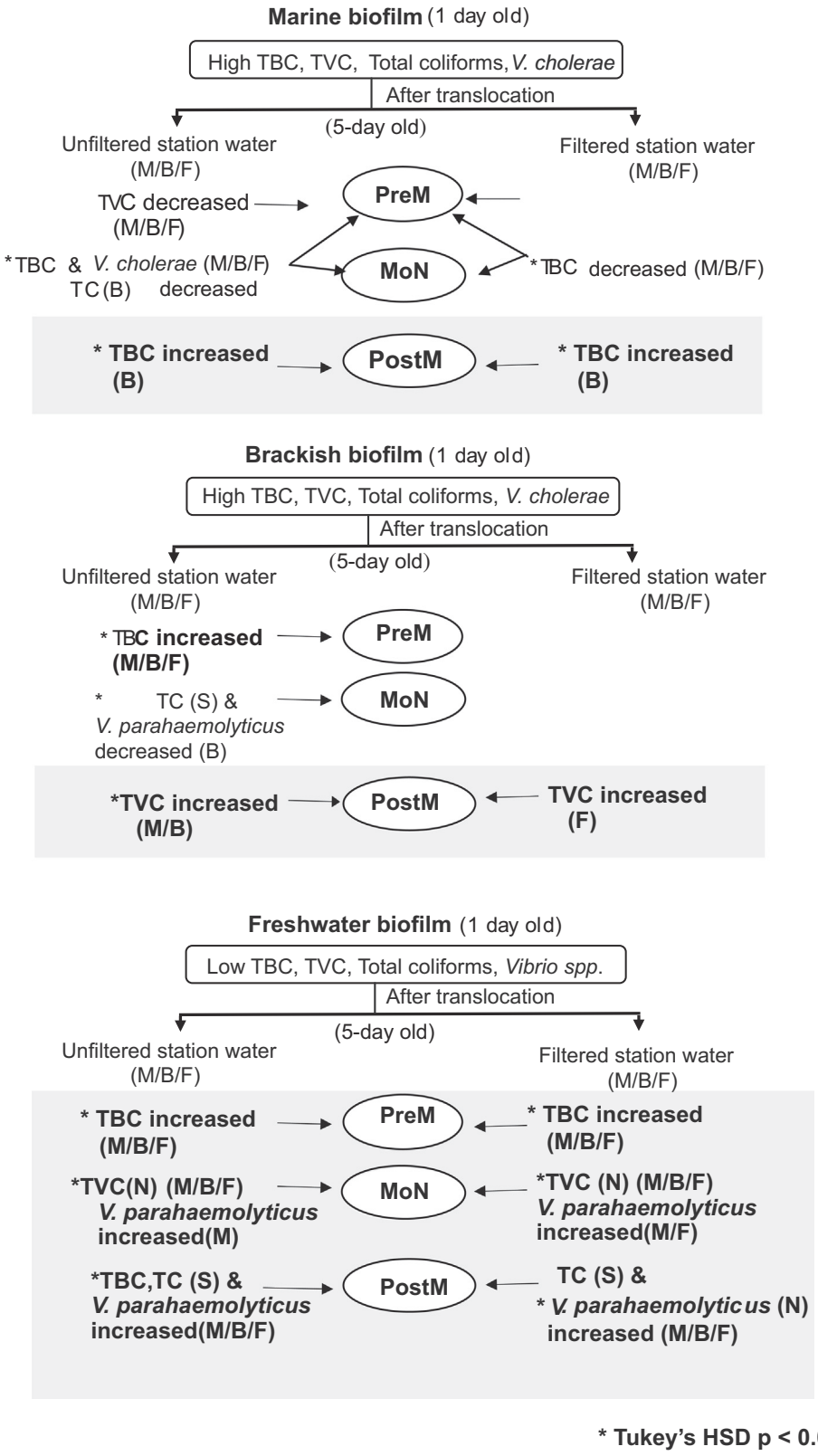


Fig. 4. Schematic illustration of the response of biofilm bacterial communities after 5 days incubation in unfiltered and filtered station waters. M - marine, B - brackish, F - freshwater, PreM - pre monsoon, MoN - monsoon, PostM - post monsoon, S - spring tide, N - neap tide, TBC - total bacterial counts, TVC - total viable counts, TC - total coliforms. The shaded grey area represents prominent response of bacteria after translocation. The asterisk (*) represent significant response (Tukey's HSD, $p < 0.05$) and arrows represent the seasonal response of biofilm bacteria to environmental changes.

control the mixing of the water column in the estuaries. As tidal energy increases, mixing of the water column increases and vice versa (Lalli and Parsons, 1997). Weithoff et al. (2000), reported that water-column mixing not only supports phytoplankton growth but also enhances the growth of bacteria, which represents the basis of the microbial food chain. High suspended load can often mean high concentrations of bacteria in the water column. The TBC was lower in the brackish biofilms as compared to marine, where water was more stratified during the spring tide (Sundar et al., 2015). These results point out the influence of tide on the bacterial abundance in an estuarine system.

The abundance of viable bacteria in one-day old biofilms was significantly higher during the neap tide, especially in the marine biofilms,

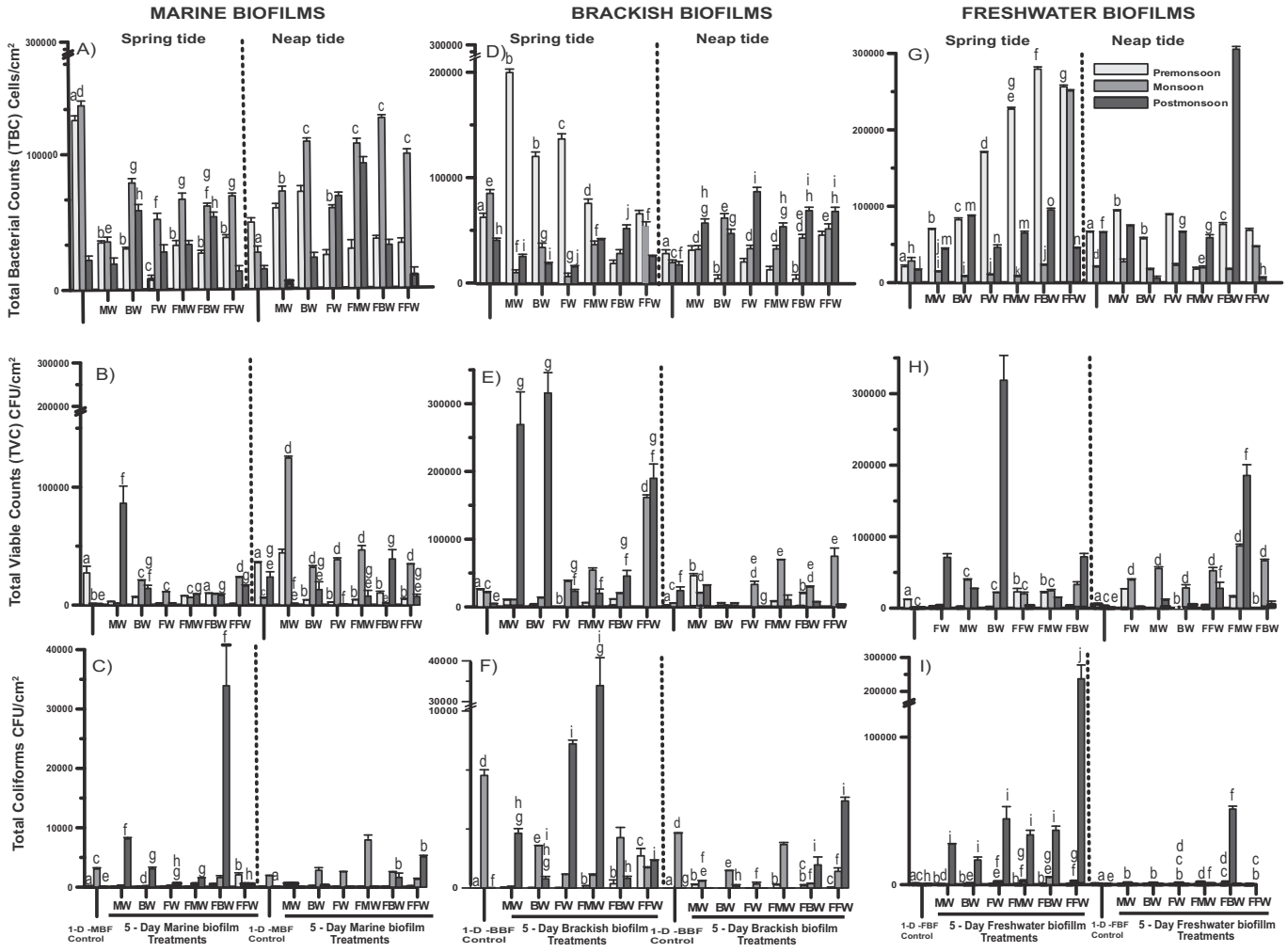


Fig. 5. Response of total bacteria counts, total viable counts and total coliforms in one-day old biofilm bacterial communities (A–C) marine, (D–F) brackish, (G–I) freshwater, after five day incubation in the laboratory using microcosm experiments. 1 – D- MBF - 1 day marine biofilm, 1 – D - BBF - 1 day brackish biofilm, 1 – D - FBF - 1 day freshwater biofilm, MW – marine water, BW – brackish water, FW – freshwater, FMW – filtered marine water, FBW – filtered brackish water, FFW – filtered freshwater. The solid bars represent averaged data (n = 3) of the samples. Error bars indicate the standard deviation of the mean based on three replicate samples. Different letters indicate significant differences between the control and treatments (Tukey's HSD, $p < 0.05$).

whereas the periphyton biomass was more in freshwater and brackish biofilms. Previous studies have reported coupling between algae and heterotrophic bacteria in the biofilms (Rier and Stevenson, 2002; Stewart and Fritsen, 2004; Barranguet et al., 2005). However, the present study showed decoupling between bacteria and phototrophic organisms in the estuarine biofilms. Estuarine ecosystems are complex and vary both spatially and temporally, and this could be one of the probable reasons for the nature of algal-bacterial interactions in the biofilms. Another possible reason for the decoupling seems to be the biological top down control of microbial biomass involving grazing of bacteria and algae by protists and microzooplankton (Stewart and Fritsen, 2004) but needs validation. Moreover, many studies carried out in the water column of Massachusetts (USA) estuaries, Hudson River plume (USA), Delaware Estuary (USA), Fraser River plume (W Canada) and Upper St. Lawrence estuary, also reported negative correlation between the estuarine bacteria and phytoplankton biomass (Albright, 1983; Coffin and Sharp, 1987; Painchaud and Therriault, 1989).

Faecal coliforms which are known as severe indicators of water pollution were higher in both marine and brackish biofilms during MoN season. Earlier studies have pointed out the influence of sewage in the Zuari estuary (Nagvenkar and Ramaiah, 2009) and the seaward flow during low tide that increases the amount of effluents leading to higher concentrations of sewage pollution indicator bacteria (Rodrigues et al., 2011; Shirodkar et al., 2012). Khandeparker et al. (2015), also reported dominance of total coliforms in the marine station during MoN period and indicated that during this period, environmental changes could be responsible for variation in pathogenic bacteria abundance. During MoN, a significant positive correlation was observed between total coliforms and SiO_4 suggesting its allochthonous input. A considerable amount of anthropogenic silicate gets into coastal waters through sewage disposal (Owili, 2003). A similar relation observed during MoN season was also reported by Khandeparker et al. (2017), in the concurrent study carried out in the Zuari estuary.

V. cholerae which are ubiquitous in brackish or limnic waters (Yildiz and Visick, 2009), were also abundant in the marine biofilms. Their high abundance could also be attributed to high numbers of *V. cholerae* in the water column as reported earlier by Khandeparker et al. (2015). RDA analysis pointed out that *V. cholerae* abundance was significantly influenced by the salinity. A strong correlation between salinity and *Vibrio* spp. has been reported in temperate, tropical and coastal ecosystems (Eiler et al., 2006; Farmer et al., 2005; Khandeparker et al., 2017). Other than salinity, nutrients are also significant for *Vibrio* spp. (Sudhanandh et al., 2010; Holm et al., 2010) and the influence is dependent on the type of *Vibrio* spp. In the present study, *V. parahaemolyticus* which are known as intermediates of seafood–borne disease and infections in the estuaries (Johnson et al., 2012), were

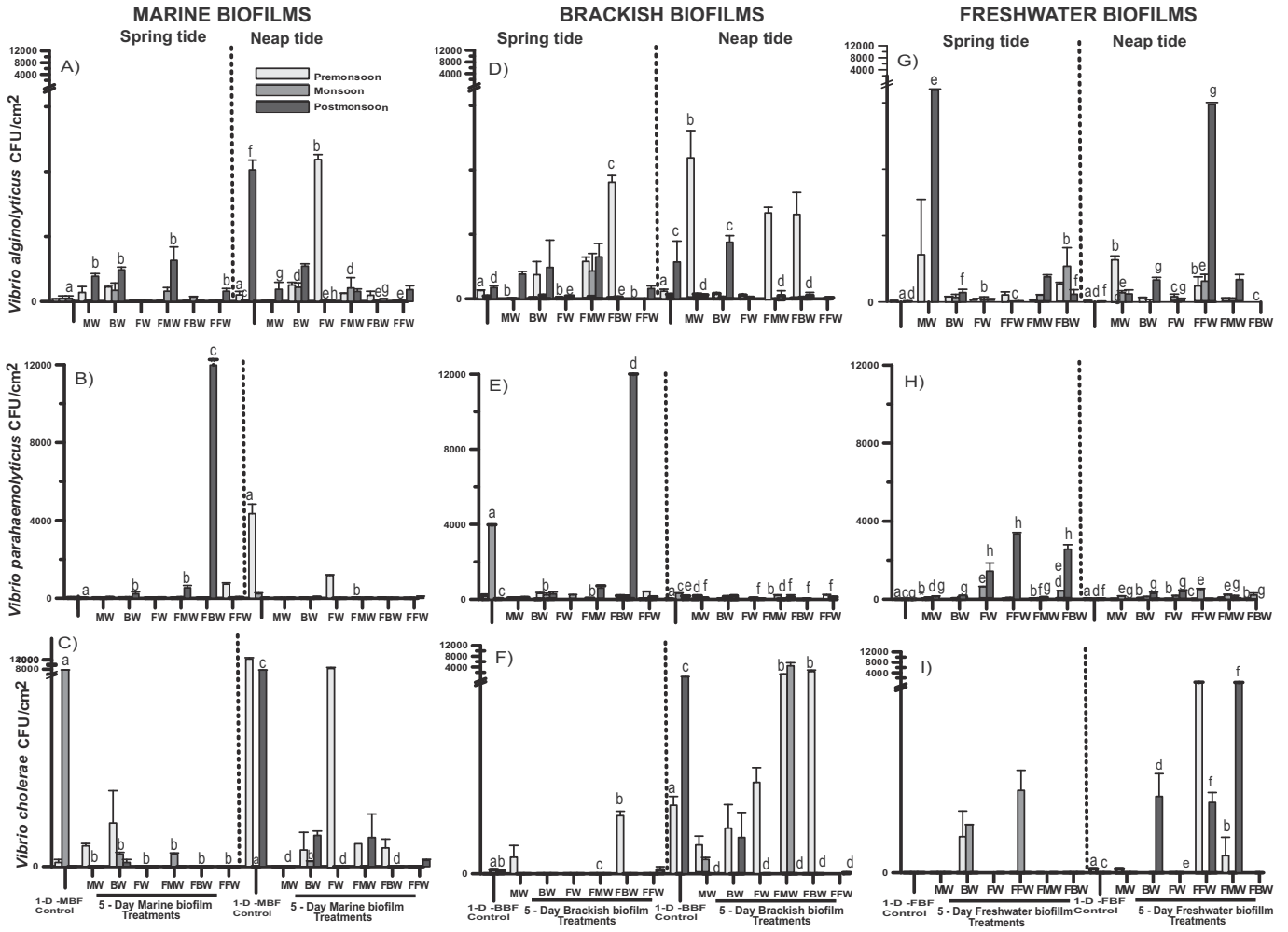


Fig. 6. Response of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Vibrio cholerae* abundance in one-day old biofilm bacterial communities (A–C) marine, (D–F) brackish, (G–I) freshwater, after five day incubation in the laboratory using microcosm experiments. 1 – D- MBF - 1 day marine biofilm, 1 – D - BBF - 1 day brackish biofilm, 1 – D - FBF - 1 day freshwater biofilm, MW – marine water, BW – brackish water, FW – freshwater, FMW – filtered marine water, FBW – filtered brackish water, FFW – filtered freshwater. The solid bars represent averaged data (n = 3) of the samples. Error bars indicate the standard deviation of the mean based on three replicate samples. Different letters indicate significant differences between the control and treatments (Tukey's HSD, $p < 0.05$).

positively correlated with high NO_2 which could be due to nitrate reduction, common in the *Vibrio* spp. (Lara et al., 2011). Since SiO_4 is an important nutrient for diatoms, this suggests the close link of this bacterium with the diatoms *Vibrio* spp. are associated with the phytoplankton, as free living or directly attached to algae or living inside algae (Holm et al., 2010). Thus, other than tides and salinity, the availability of nutrients seems to play a major role in the distribution of pathogenic bacteria in the estuarine biofilms.

The studies on influence of biofilm communities to changing environmental conditions using microcosms are scarce (Moss et al., 2006; Nocker et al., 2007). Moreover, the response of young biofilms to changing environment in a tropical monsoon influenced estuarine conditions is still not known. The translocation experiments carried out to address this, revealed that when marine biofilms with high TBC, viable and total coliforms were translocated to unfiltered (marine/brackish/fresh) water, the abundance decreased significantly during PreM, MoN and increased during the PostM season. The unfiltered water contains a gamut of organisms, including grazers which could play an important role in controlling the bacterial numbers. The effects of predation on bacterial diversity depend on both predator and prey characteristics and abiotic factors, such as nutrient supply (Matz and Jürgens, 2003; Dopheide et al., 2011). Besides, the availability of dissolved organic matter (DOM) varies with the season in this estuary (De Souza, 2000; Khodse and Bhosle, 2011). The water column bacteria utilises DOM generated from phytoplankton via processes like rupture and degradation of cells during grazing, viral lysis or direct extracellular release (Jensen, 1983; Larsson and Hagstrom, 1979; Wolter, 1982). The availability of biofilm bacteria during a particular season could also play an important role in controlling the bacterial abundance in biofilms. On the contrary, an increase in the bacterial abundance when exposed to brackish water during the PostM season could be attributed to high bacterial abundance and suspended particulate matter in the station.

However, the same trend was not observed with the brackish biofilms, TBC increased when exposed to different unfiltered waters. An earlier study by Berdjeb et al. (2011), reported that the presence of grazers didn't have any influence on the bacterial communities during the experiments. Although grazers are known to feed on bacteria present in the biofilms, it also depends on different grazer types with species-specific food preferences (Dopheide et al., 2011; Aguilera et al., 2013). It might be of great interest to gain deeper insights into the different grazers in biofilms with different feeding habits.

In the case of freshwater biofilms, an increase in the TBC was observed when exposed to filtered (marine/fresh/brackish) water. Many bacteria can survive for extended periods without nutrients, these are termed as ultramicro-bacteria and their reduced size allows them to pass through 0.22 μm membrane filters (Li and Dickie, 1985). They have developed starvation-survival strategies to survive prolonged

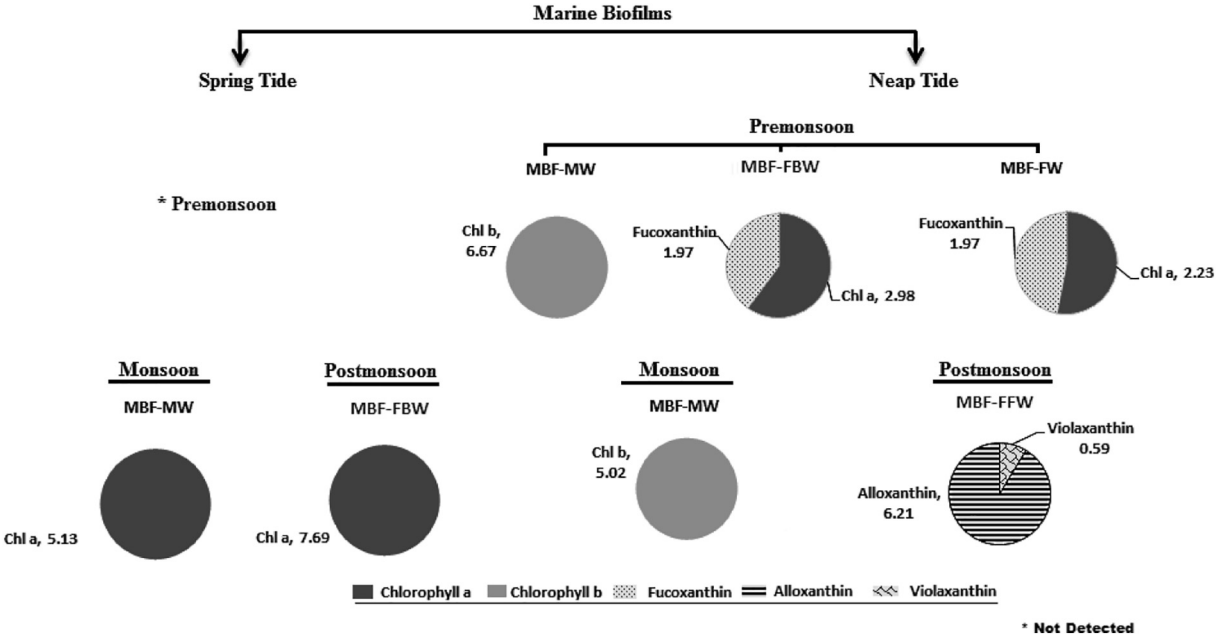


Fig. 7. Response of periphyton pigment composition in one-day old marine biofilm communities, after five day incubation in the laboratory using microcosm experiments. MBF – marine biofilms, BBF – brackish biofilms, FBF – freshwater biofilms, MW – marine water, BW – brackish water, FW – freshwater, FBW – filtered brackish water and FFW - filtered freshwater. * - No pigment signatures were detected irrespective of conditions during the premonsoon spring (pigment concentrations ($\mu g \cdot cm^{-2}$) are indicated in the figures by the side of the pie charts).

periods of starvation, enabling them to persist in the environment until conditions become favorable for growth (Watson et al., 1998). These filterable bacteria have been detected in various aquatic systems and include marine and freshwater ecosystems and have been also referred as nanobacteria or starvation forms (Elsaied et al., 2001; Haller et al., 2000; Wang et al., 2007). When conditions become favorable, they start growing rapidly. An increase in the bacterial abundance due to the disintegration of periphyton rich freshwater biofilms cannot be ruled out.

The periphyton pigments have been used increasingly in oceanography for quantifying the major taxonomic groups of phytoplankton using high pressure liquid chromatography (HPLC) (Giraud et al., 2016; Jeffrey et al., 1997; Moreno et al., 2012) (Supp. Table 2). This is especially important for very small phytoplankton species, which are not easily determined by light microscopic methods. Since diagnostic pigments provide quick information on periphyton groups in the

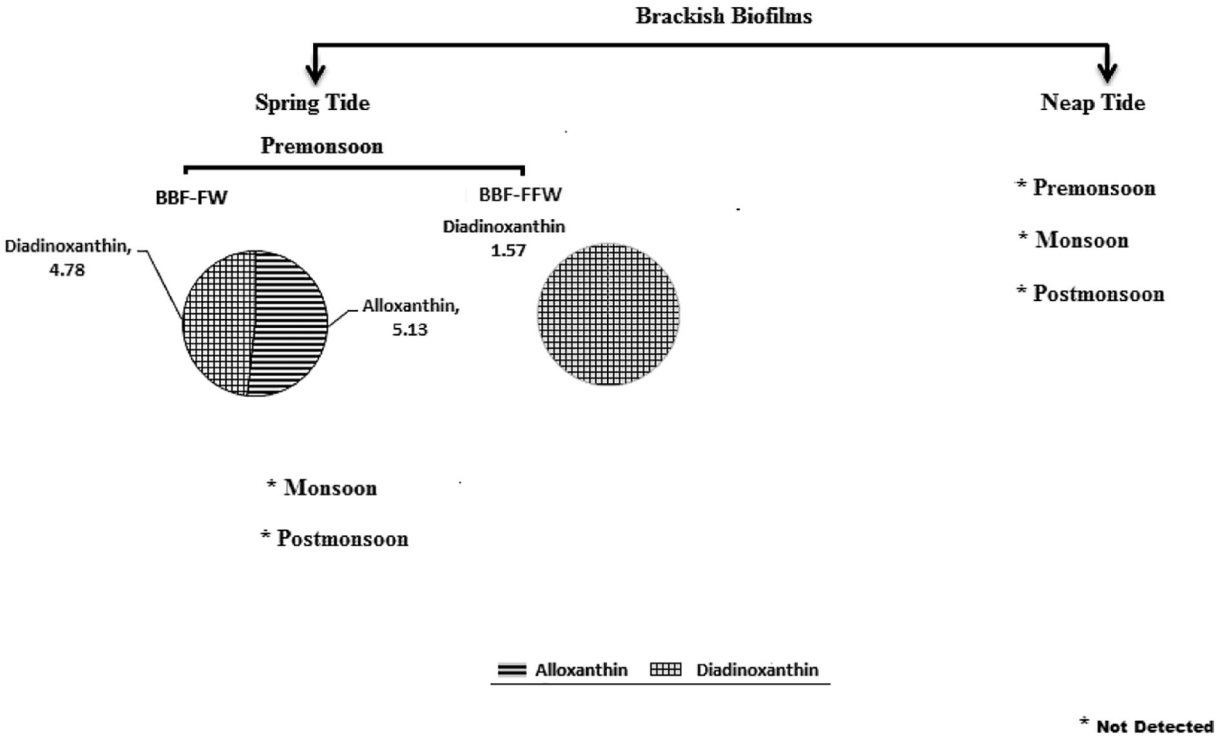


Fig. 8. Response of periphyton pigment composition in one-day old brackish biofilm communities, after five day incubation in the laboratory using microcosm experiments. MBF – marine biofilms, BBF – brackish biofilms, FBF – freshwater biofilms, MW – marine water, BW – brackish water, FW – freshwater and FFW - filtered freshwater. * - No pigment signatures were detected irrespective of conditions during the post monsoon spring and premonsoon neap (pigment concentrations ($\mu g \cdot cm^{-2}$) are indicated in the figures by the side of the pie charts).

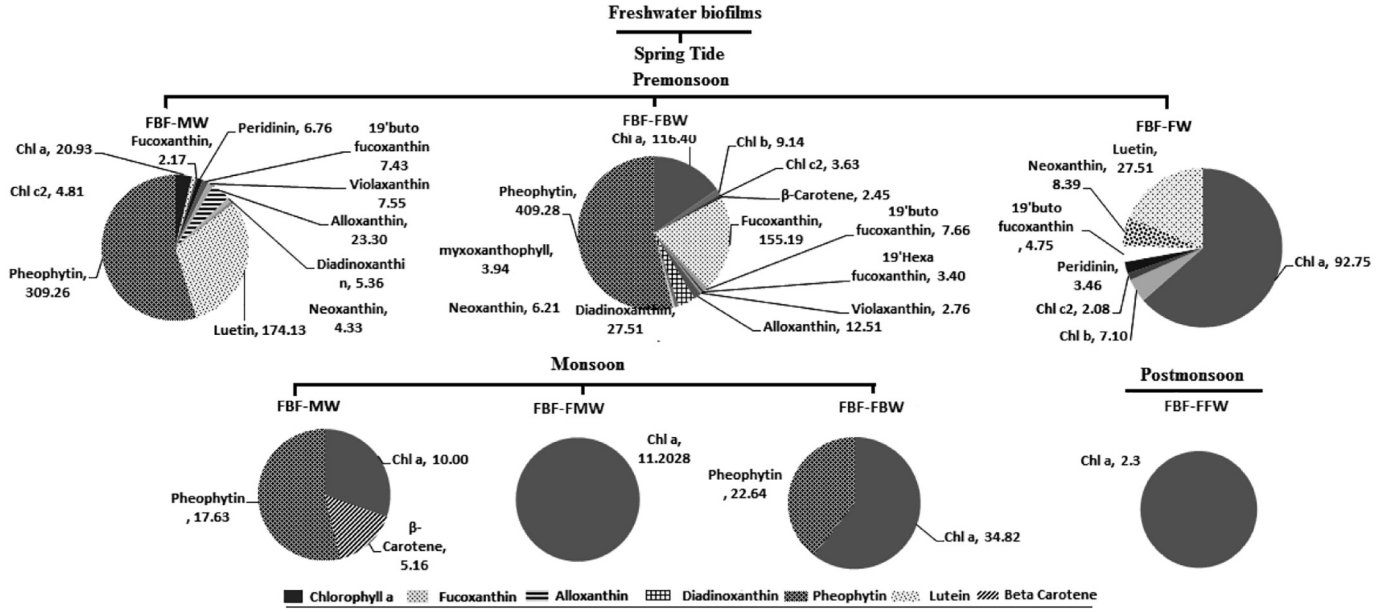


Fig. 9. Response of periphyton pigment composition in one-day old freshwater biofilm communities developed during spring tide, after five day incubation in the laboratory using microcosm experiments. MBF – marine biofilms, BBF – brackish biofilms, FBF – freshwater biofilms, MW – marine water, BW – brackish water, FW – freshwater, FMW - filtered marine water, FBW – filtered brackish water and FFW- filtered freshwater (pigment concentrations ($\mu g \cdot cm^{-2}$) are indicated in the figures by the side of the pie charts).

biofilms, they were used. In the present study, when freshwater biofilms were exposed to unfiltered marine and freshwater during PreM spring tide, pigments such as Fuco, Peri, Diad, Allo, Zea, Lut, Neo, Vio, and 19′ but, dominated 5 day old biofilms indicating a shift in the periphyton community structure (Fig. 9). These pigments are contributed by diatoms, dinoflagellates, cryptophytes, cyanobacteria, chlorophytes, green algae, prasinophytes, and nanoflagellates. Other accessory pigments such as 19′hexanoyloxyfucoxanthin, myxoxanthophyll, β carotene along with Chl b, Chl c2, 19′but, Fuco, Neo, Diad, Vio, Allo pigments which couldn't be detected initially in one-day old biofilms, appeared on day 5 upon translocation in filtered brackish water during PreM. 19′-hexanoyloxyfucoxanthin and 19′butanoyloxyfucoxanthin are used as pigment signatures for nanoflagellates and some prymnesiophytes (haptophytes) (Jeffrey and Wright, 1994). β-Carotene is reported as a minor (1–10%) marker pigment of cyanobacteria and is synthesised as protective carotenoids (Chakraborty et al., 2011). The appearance of these pigments on day 5 could be attributed to two reasons, one is the availability of these periphyton groups in the water column to which they were translocated, and the other reason could be their non-detection in the 1-day old biofilms due to very low concentration which was evident from distinct periphyton community structure when translocated to filtered brackish water.

It was also observed that during PreM spring tide, the pheopigment concentration increased in freshwater biofilms when translocated to marine and brackish water indicating decay of freshwater periphyton and remineralisation of this phytobiomass can be an important source of nutrients for bacteria and this could be the possible reason for the increased bacterial abundance in 5 day aged biofilms during PreM spring tide. Pheophytin is an indicative of the physiological status of phytoplankton biomass and its transformation (Ahel et al., 1996).

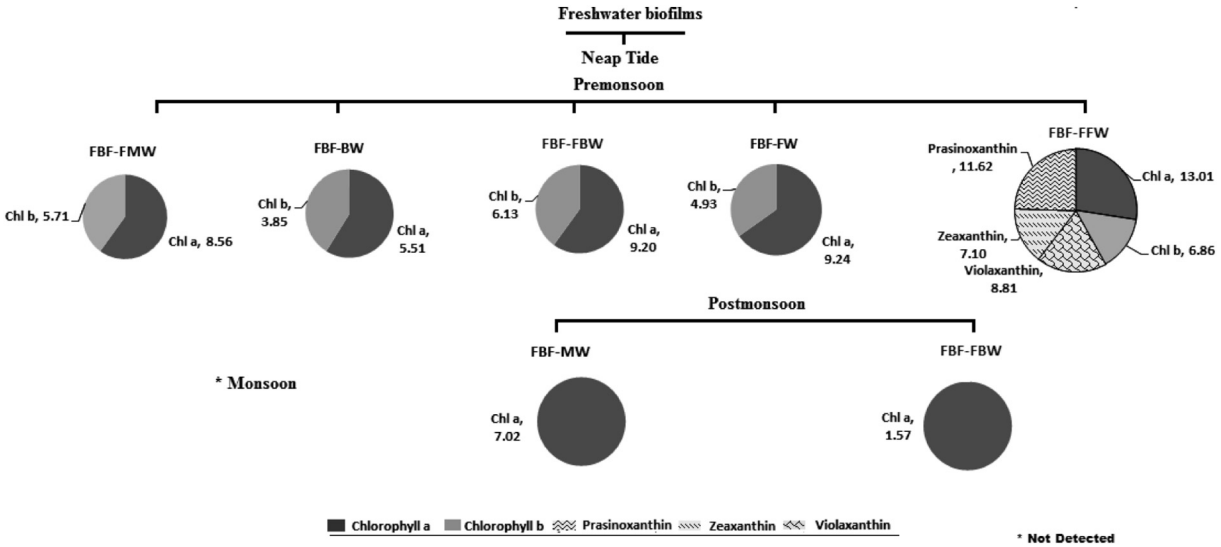


Fig. 10. Response of periphyton pigment composition in one-day old freshwater biofilm communities developed during neap tide, after five day incubation in the laboratory using microcosm experiments. MBF – marine biofilms, BBF – brackish biofilms, FBF – freshwater biofilms, MW – marine water, BW – brackish water, FW – freshwater, FMW - filtered marine water, FBW – filtered brackish water, and FFW - filtered freshwater.* - No pigment signatures were detected irrespective of conditions during the monsoon (pigment concentrations ($\mu g \cdot cm^{-2}$) are indicated in the figures by the side of the pie charts).

Earlier studies have reported that phytodetrital aggregates are degraded and remineralised by bacteria (Dale, 2003; Descy et al., 2002; Morris et al., 1978).

High numbers of *V. parahaemolyticus*, known to be associated with phytoplankton, were also noted in the freshwater biofilms upon translocation to marine or brackish water. They were able to sustain and proliferate in the freshwater biofilms compared to other two biofilms. Several mechanisms may conclude the presence of pathogens in the biofilms exposed to anthropogenically influenced station waters as well as its transport and incorporation into the biofilms. Such biofilms can form an important shelter for the pathogenic bacteria and provide chemical resistance against antibiotics, pesticides, etc. (Donlan, 2000; Hoiby et al., 2010). Earlier studies have indicated that this biofilm lifestyle can protect microbial pathogens when attached to the wetted hulls of ships while in transit overseas (Shikuma and Hadfield, 2010). The disintegration of periphyton rich freshwater biofilms as indicated by an increase in the pheopigments probably lead to increased bacterial abundance and *V. parahaemolyticus* numbers growing on decaying planktonic cells.

The use of culture-dependent techniques possesses severe limitations as it favours only a fraction of the inhabiting bacterial community (Al-Awadhi et al., 2013; Dickson et al., 2014). Also, it does not offer a complete picture of bacterial diversity and fail to grow all biofilm cells on the selective media (Vandecandelaere et al., 2012). The use of molecular techniques to characterise biofilm bacterial community structure in this estuary would provide better understanding and is an important topic for future investigation.

5. Conclusions

This investigation elucidated that the estuarine microbial dynamics can be assessed through changes in the biofilm communities. In this tropical monsoon influenced estuary, early stage biofilms were dominated by bacteria in marine conditions and periphyton in fresh and brackish water conditions. The translocation studies further indicated that these changes in their community characteristics varied temporally and were influenced by the inoculum characteristics. The decay of sensitive freshwater periphyton in the biofilms when translocated to marine and brackish conditions leads to higher bacterial and pathogenic abundance. The insights from this study will pave ways for understanding the influence of biofilm vector routed translocation of organisms from one habitat to another.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2017.09.014.

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MICROBIOLOGY OF AQUATIC SYSTEMS

Influence of Darkness and Aging on Marine and Freshwater Biofilm Microbial Communities Using Microcosm Experiments

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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 aging 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* 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 · Aging · Protists

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 harbor pathogenic bacteria [20, 21]. They act as seed banks, releasing microorganisms and signifying an

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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, aging, 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 unfavorable 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 hypothesized that the decline in phytoplankton and zooplankton populations, due to unfavorable 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 aging 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 unfavorable 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 × 76.2 mm) were cleaned as described by Bhosle et al. [35]. Briefly, the slides were treated with the chromic acid solution for 5 h, then washed with UV-treated Milli-Q water, followed by methanol, and dried in an oven. They were then covered in aluminum foil and ashed at 450 °C for 5 h 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 to 29.1 °C and salinity ranged from 35.1 to 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 5 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), and the final concentration of nitrate was ~ 7 μM, 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 5-day-old marine tank biofilms were harvested for different microbiological analysis as described below.

A set of 49 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 ($n = 1$ slide × 4 replicates). One milliliter 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, 30 slides were sampled ($n = 10$ slides × 3 replicates), and 10 slides were scraped and pooled into 20 mL of 0.22 μm filtered

Fig. 1 Sampling site

autoclaved seawater. Lastly, 15 slides were sampled ($n = 5$ slides × 3 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 5 days. These 5-day-old biofilms were evaluated for different microbiological analysis as described above.

Translocation Experiment

After 5 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 unfavorable environment. The glass slides were then placed in each of the

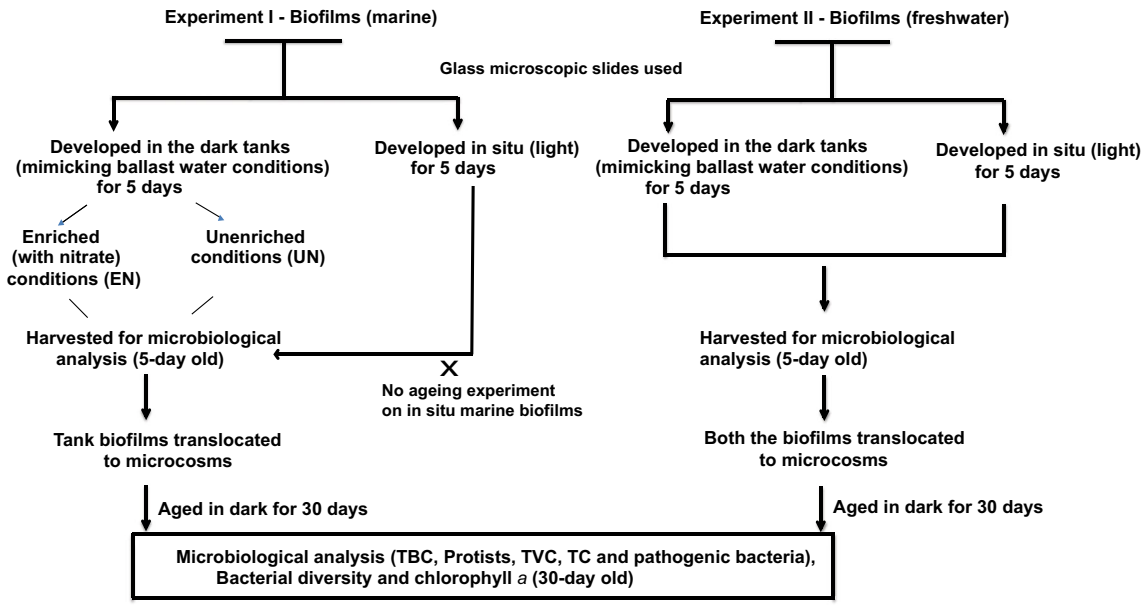

Fig. 2 Schematic representation of experiments I and II. *TBC* total bacterial counts, *TVC* total viable counts, *TC* total coliforms

sterile polycarbonate square jars (75 × 74 × 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 aging 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 to 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 up to 5 days. These biofilms are termed as in situ freshwater biofilms. Nutrient concentrations were higher in the freshwater, especially nitrate with 27.35 μM. 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 5-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 5 days of incubation, these 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 unfavorable 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 microgram per square centimeter (μg/cm^2) of the slide surface area sampled.

Total Viable Counts and Pathogenic Bacteria in Biofilms

Enumeration of total viable counts (TVC) and pathogenic bacteria was 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), Xylose Lysine Deoxycholate (XLD) Agar (*Salmonella/Shigella* spp.), and Thiosulphate Citrate Bile Salts (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 > 2 mm diameter were counted as *V. alginolyticus*, those with < 2 mm 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 centimeter (CFU/cm^2) of the slide surface area sampled.

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 (1:10,000 final concentration, Molecular Probes, USA) and incubated in the dark for 15 min before measurement. After incubation, samples were analyzed by BD 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 (v.6.2). The total bacterial counts (TBC) is expressed as cells per square centimeter (Cells/cm^2) 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 (1:10,000 final concentration, Molecular Probes, USA), and incubated in the dark for 15 min before measurement. After incubation, samples were analyzed by a flow cytometer (BD 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 (v.6.2). The protist abundance is expressed as cells per square centimeter (Cells/cm^2) 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 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 tenfold, ranging from 10^6 to 10^9 copies.

One microliter of DNA from the biofilm samples (~ 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 μL, 10 μL of master mix, 0.25 μM of each primer, and 1 μL 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 15 s, primer annealing at 53 °C for 20 s and primer extension at 72 °C for 20 s. 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 per square centimeter 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

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	[42]
β-Proteobacteria	53	908R: CCCCGTCAATTCCTTTGAGTT	[43]
γ-Proteobacteria	53	Beta359f: GGGGAATTTTGGACAATGGG	[42]
		Beta682r: ACGCATTTCACTGCTACACG	
		1080F: TCGTCAGCTCGTGTYGTGA	
		1202R: CGTAAGGGCCATGATG	
Bacteroidetes	53	798cfbF: CRAACAGGATTAGATACCCT	[42]
Firmicutes	53	cfb967R: GGTAAGGTTCCTCGCGTAT	[42]
Actinobacteria	53	928F: TGAAACTYAAAGGAATTGACG	[42]
		1040FirmR: ACCATGCACCACCTGTC	
		Act920F3: TACGGCCGCAAGGCTA	
		Act1200R: TCRTCCCCACCTTCCTCCG	

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 pathogenic bacteria as well as bacterial diversity in the in situ and laboratory experiments was determined by using CANOCO version 4.5 [44]. 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 [45]. Due to the linear character of the data, redundancy analysis (RDA) 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 [46]. 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 ± 0.0001 μg/cm^2) and UN marine tank biofilms (0.00213 ± 0.0001 μg/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) and 0.0024 ± 0.0002 (EN) μg/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 up to tenfold 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 axes 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/cm^2) followed by *V. parahaemolyticus* (1.22×10^2 CFU/cm^2) 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

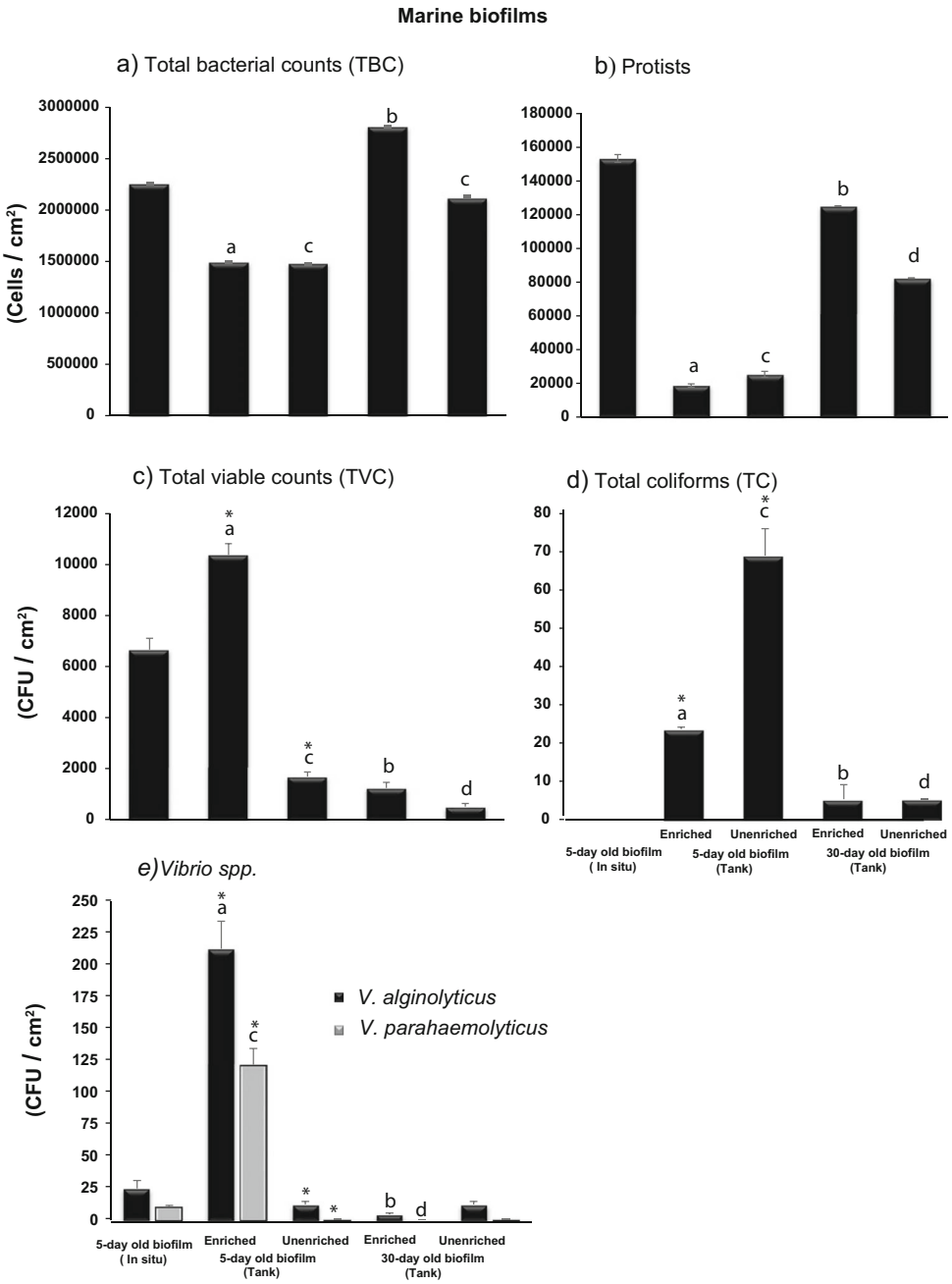

Fig. 3 Mean values of **a** total bacterial counts, **b** protists, **c** total viable counts, **d** total coliforms, and **e** *Vibrio* spp. (*V. alginolyticus* and *V. parahaemolyticus*) in the in situ and tank (enriched and unenriched) marine biofilms during the sampling days (day 5 and day 30). The 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 labeled 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

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 μg/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 μg/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

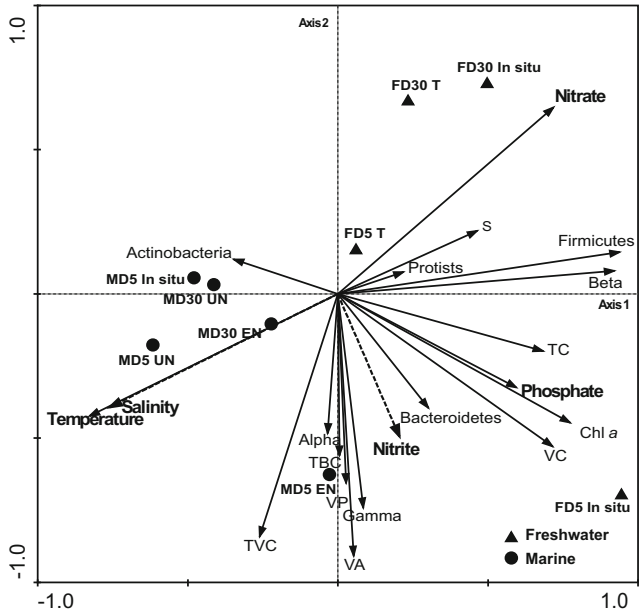

Fig. 4 RDA analysis of marine (●) and freshwater (▲) biofilms during the study period. *MD5 In situ* 5-day-old in situ marine biofilms, *MD5 UN* 5-day old unenriched marine biofilms, *MD5 EN* 5-day old enriched marine biofilms, *MD30 UN* 30-day old unenriched marine biofilms, *MD30 EN* 30-day old enriched 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

decreased significantly with a minimum value of 0.033 ± 0.005 μg/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.

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/cm^2), also decreased significantly on exposure to prolonged darkness ($p < 0.001$, Tukey's HSD, Fig. 6c). An increase in the nitrate levels (64.03 μM) was 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/cm^2) 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/cm^2) followed by *V. cholerae* (7.6×10^1 CFU/cm^2) and *V. parahaemolyticus* (6.0 CFU/cm^2) 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.

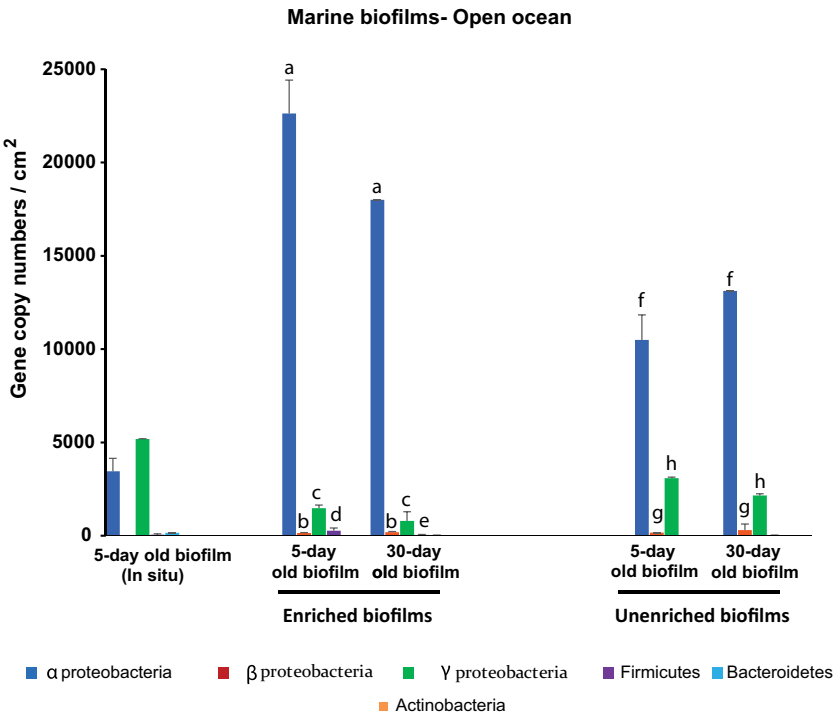

Fig. 5 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue), and Actinobacteria (orange) in the in situ and tank (enriched and unenriched) marine biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples ± 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 labeled by the same letter on the graph are not significantly different from each other

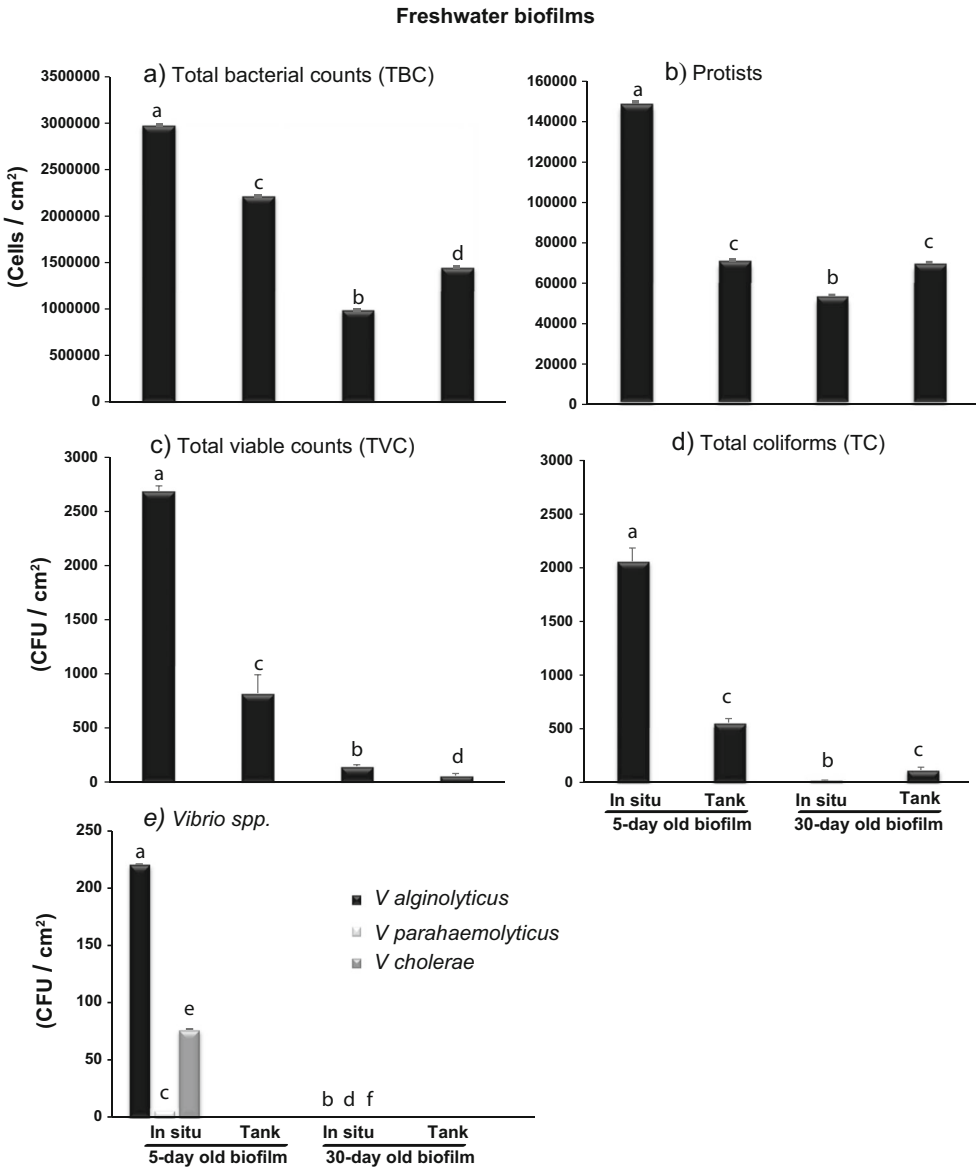

Fig. 6 Mean values of **a** total bacterial counts, **b** protists, **c** total viable counts, **d** total coliforms, and **e** *Vibrio* spp. (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae)* in the in situ and tank freshwater biofilms during the sampling days (day 5 and day 30). The solid 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, $p < 0.05$, followed by Tukey's HSD post hoc test). The asterisk (*) above bars indicate significant differences between in situ and tank freshwater 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 tenfold reduction in their abundance ($p < 0.05$, Tukey's HSD, Fig. 6). An increase in the nitrate levels (49.86 μM) was observed at the end of the dark experimental period. Tank biofilms showed a different pattern of bacterial communities in comparison to the in situ

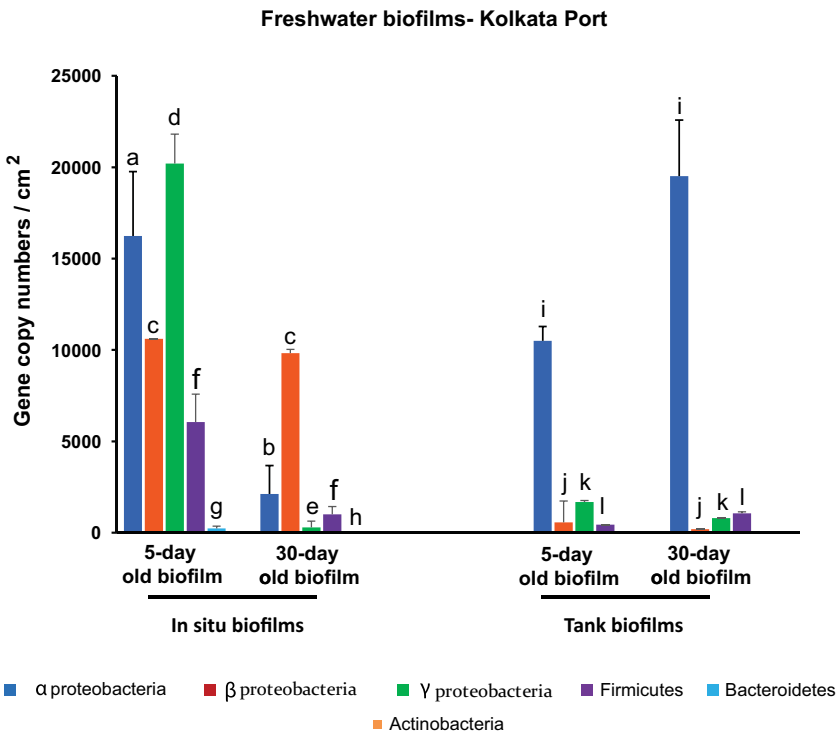

Fig. 7 Gene copy numbers of α-proteobacteria (dark blue), β-proteobacteria (dark red), γ-proteobacteria (green), Firmicutes (purple), Bacteroidetes (blue), and Actinobacteria (orange) in the in situ and tank freshwater biofilms during the sampling days (day 5 and day 30). Values are means of triplicate samples ± 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 labeled by the same letter on the graph are not significantly different from each other

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 aging 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, 47]. 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. [22] 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 products during each cycle of the PCR reaction [48]. 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 [49, 50].

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 [50–52]. 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 [50, 51]. This group possesses different metabolic strategies such as photosynthesis, nitrogen fixation, ammonia oxidation, etc. [53, 54]. An earlier study reported that the family Rhodospirillaceaens, 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 [52].

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 aging 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 [55].

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 [56, 57]. They are dominant in freshwater ecosystems, known for their versatile, degrading capabilities, whereas they are relatively low in the ocean [50]. This group is also found in the marine biofilms, but occur at lower levels compared to other Proteobacteria [58, 59]. 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 [60] 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 [61, 62]. 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 [63].

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 [64–66] 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 favorable conditions for growth [67].

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 [68]. 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 [69]. 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 [69–71].

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 [72–75]. 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 [73, 76], 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 aging 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 [77, 78]. The bacterial community composition of marine and freshwater biofilms was 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 [79, 80], 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 [81]. 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 [81]. 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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PRIMARY RESEARCH PAPER

Extracellular polymeric substances mediate the coaggregation of aquatic biofilm-forming bacteria

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Abstract Coaggregation, a phenomenon contributing to biofilm formation, occurs among biofilm bacteria from different aquatic environments. However, not much is known about molecules involved in aggregation. In this study, freshwater, estuarine and marine biofilm bacteria were evaluated for aggregation capabilities, and their cell-bound extracellular polymeric substances (CB-EPS), known to play an important role in biofilm formation, were characterized for functional groups, and sugar composition via Fourier-transform infrared spectroscopy and high-pressure liquid chromatography. Biofilm-forming potential of estuarine and freshwater biofilm bacteria was higher as indicated by their coaggregation scores, attributed to CB-EPS with distinct sugar types, compared to marine. Most of the biofilm bacteria lost their ability to coaggregate after removal of CB-EPS, indicating its importance in coaggregation. Estuarine (*Bacillus indicus*, *Bacillus cereus*), and freshwater (*Exiguobacterium* spp., *B. cereus*) bacterial pairs, retained their aggregation capability probably via expression of lipids and proteins, suggesting their ability to rebuild themselves by expressing specific biomolecules under stressed conditions. A similar expression pattern was observed when these strains were exposed to abrupt salinity change (environmental stressor), indicating modulation of cell surface chemistry as a strategy to protect biofilm bacteria in harsh conditions. Unravelling role of these biomolecules as cues for settlement of macrofoulers is a step ahead.

Keywords Bacteria · FT-IR · Carbohydrates · Biomolecules · 16S rDNA sequencing · *Bacillus* spp.

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Introduction

The ability of bacterial cells to aggregate or form biofilms is an adaptive process, wherein they cooperate and improve their chances of survival on sensing and responding to environmental stress (de Carvalho, 2018). Many studies have reported that the formation of biofilms, whether it is cell–surface or cell–cell interactions, involves different molecules (e.g. lipids, polysaccharides, proteins, nucleic acids and some other biomolecules) and processes (e.g. coaggregation

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and quorum sensing) which contribute to the development of biofilms (Rickard et al., 2000; Watnick & Kolter, 2000; Stoodley et al., 2001; Sauer et al., 2002; Purevdorj-Gage et al., 2005; Irie & Parsek, 2008; Jain & Bhosle, 2009; Rumbaugh & Armstrong, 2014; Kviatkovski & Minz, 2015; Dang & Lovell, 2016; Abisado et al., 2018 etc.). Moreover, recent studies have focused on the importance of coaggregation, a form of cell–cell interaction, characterized by highly specific recognition and adherence of potential partners mediated by lectin–saccharide interactions between cell surface molecules, thereby possibly resulting in the formation of biofilms, originating from these pre-formed cell aggregates (Rickard et al., 2000, 2003; Kragh et al., 2016; Melaugh et al., 2015).

Coaggregation was first reported by Gibbons and Nygaard, between different species of human dental plaque bacteria in the 1970s. It is not a random process but highly specific recognition and adherence of the bacterial cells that employ specific lectins (protein adhesins or carbohydrate moieties) present on their cell surface to recognize partner bacteria (Rickard et al., 2002; Kolenbrander et al., 2006). This process is a key mechanism and phenomenon in the biofilm formation, which also facilitates interaction among different bacterial species in the biofilms (Rickard et al., 2003). These interactions are known to occur between members of the same genus (intra-generic), same species (intra-species) or with different bacteria (inter-species) (Rickard et al., 2002; Katharios-Lanwermeyer et al., 2014). Moreover, the coaggregation process along with autoaggregation, viz. self-aggregation or adherence of identical bacteria to one another (Trunk et al., 2018), depends not only on environmental conditions but also on the distinct bacterial species that attract potential partners through specific molecules either located on their cell surfaces (including polysaccharide content and the expression of adhesins) or associated with external appendages, thus forming an integral part responsible for the development and formation of multispecies biofilms (Ellwood & Tempest, 1972; Rickard et al., 2000, 2003; Min et al., 2010). So far, this coaggregation process and the molecules involved have been extensively studied with regard to oral biofilms (Gibbons & Nygaard, 1970; Cisar et al., 1979; Kolenbrander et al., 1993; Palmer et al., 2003; Rickard et al., 2003; Ledder et al., 2009) and is also known to occur within several clinical and non-clinical environments (Malik et al., 2003; Adav et al., 2008; Basson et al., 2008; Simões et al., 2008; Vornhagen et al., 2013; Cheng et al., 2014; Stevens et al., 2015; Kumar et al., 2019). Relatively few studies have been reported on coaggregation between aquatic biofilm bacteria, e.g. in the freshwater biofilms (Buswell et al., 1997; Rickard et al., 2000, 2002) and marine biofilms (Saravanan et al., 2014). These studies have focused on understanding the role of coaggregation in aquatic biofilm development because of its potential to support or hinder colonization, retention of pathogens within biofilms in shear environments, microbial-induced corrosion and biofouling of surfaces (Stewart & Costerton, 2001; Kerr et al., 2003; Min & Rickard, 2009; Katharios-Lanwermeyer et al., 2014). The importance of bacterial biofilms and associated biopolymers in the settlement of macrofoulers and their implications in biofouling has been well studied (Khandeparker et al., 2002, 2003; Hadfield, 2011; Dobretsov & Rittschof, 2020 and references within). Moreover, the parentally associated biofilms are unique and play an important role in providing important signals for the settlement of marine invertebrate larvae by producing different types of compounds (De Gregoris et al., 2012; Wahl et al., 2012). Although, it is well known that biochemical cues expressed by the microbial aggregates or biofilms play a very important role in aquaculture and the settlement of the macrofouling organisms (Qian et al., 2007; Dobretsov & Rittschof, 2020), little is known about the mechanisms by which the aquatic biofilm-forming bacteria from different environments aggregate. Moreover, the molecules involved in the process and their relevance in the biofilm formation are least studied, and the same was addressed in the present study.

Biofilms present in all the aquatic environments including freshwater ecosystems (e.g. lakes, inland port, rivers) and marine environments (e.g. estuaries, open ocean) are influenced by several environmental factors such as salinity, nutrient levels, etc. (Mora-Gómez et al., 2016). The biofilm bacterial composition and microbial communities in these environments differ (Khandeparker et al., 2017; Hede & Khandeparker, 2018). Hence, it is expected that the composition of exopolymers and mechanisms involved in the formation of biofilms by the aquatic biofilm bacteria would also be different. These aquatic biofilm bacteria range from stenohaline bacteria tolerating only a small

range of environmental salinities (marine bacteria) to euryhaline ones with their ability to switch from freshwater or saline water (estuarine bacteria), and the freshwater bacteria which cannot tolerate any salinity changes. Among these, the estuarine biofilm-forming bacteria belong to the most dynamic environment, which is influenced by tides and experiences continuous influx of freshwater, resulting in the changes in salinity (Smyth & Elliott, 2016). Since salinity is regarded as an environmental master factor in the distribution of these aquatic organisms and shaping the bacterial community composition (Stratil et al., 2014; Smyth & Elliott, 2016), any changes in salinity due to environmental conditions or anthropogenic inputs may influence their biofilm formation. Hence, this factor was used in the present investigation to elucidate how the coaggregation capability varies with respect to salinity changes. Such studies are crucial in understanding microbial behaviour and their environmental responses during the key stages of biofilm formation (Dang & Lovell, 2016). Moreover, the cues expressed if altered with changing environmental conditions will be perceived differently, either by supporting or hindering the settlement of the macrofouling organisms.

Taking the above points into consideration, we examined (1) the biofilm-forming potential of the bacterial strains isolated from biofilms developed in different aquatic environments (freshwater, estuarine and marine habitats), and (2) characterized the cell-bound extracellular polymeric substances (CB-EPS) of chosen biofilm-forming bacterial strains (freshwater, estuarine, marine) for functional groups and sugar composition via Fourier-transform infrared spectroscopy (FT-IR) and high-pressure liquid chromatography (HPLC). The role of cell-bound EPS was then evaluated by assessing the coaggregation potential of these biofilm-forming bacterial strains in the absence of CB-EPS. The bacterial pairs which could retain their aggregation ability even in the absence of CB-EPS were selected, and the aggregates were characterized using FT-IR. It was hypothesized that the ability of these bacterial strains to cope up with other stressor would be more owing to their high coaggregation potential. These bacterial isolates were exposed to one of the important environmental stressor, i.e. abrupt change in salinity, using laboratory experiments. It was expected that the exposure of these biofilm-forming bacteria to salinity stress would either hinder their ability to aggregate or the tolerant bacterial strains might retain their aggregation capability by expressing specific molecules.

Materials and methods

Sampling site and isolation of bacteria from biofilms

The bacteria were isolated from the in situ biofilms developed on glass slides which were deployed for a period of 5 days in different aquatic environments, namely freshwater (Kolkata Kidderpore Dock Area 22° 32.45994′ N, 088° 18.95370′ E), and marine environment, including estuarine (Mandovi Estuary 15° 30′ 17.442″ N, 73° 49′ 56.2392″ E, west coast of India), and open ocean (the Arabian Sea, 15° 51.482′ N, 072° 43.511′ E). The biofilms were then scraped using a sterile cell scraper (BD Biosciences) in 10 ml of autoclaved 0.85% saline water (prepared using sodium chloride) for freshwater biofilms, and 0.22 µm filtered autoclaved seawater (~ 35 salinity) for the remaining 2 biofilms. After sonication (30 W for 60 s), 100 µl of the sample was spread plated on Nutrient Agar (NA, Himedia) for freshwater bacteria and Zobell Marine Agar 2216 (ZMA, Himedia) for marine bacteria. In total, twenty-three successfully isolated bacterial strains from these biofilms were obtained and referred to as freshwater, estuarine and marine biofilm bacteria which were purified, subcultured and maintained at 4°C.

Experimental design

The summarized methodological flow chart is illustrated in Fig. 1 and the experimental study was divided into two experiments as described below.

Experiment I: Aggregation ability and identification of biofilm-forming bacteria

Biofilm-forming potential using coaggregation assay The biofilm formation potential of biofilm bacterial strains was assessed using well-established coaggregation assay. The bacterial isolates were cultured in the nutrient broth (NB) and Zobell marine broth 2216 (ZMB) for freshwater and marine/estuarine biofilm bacteria, respectively, and

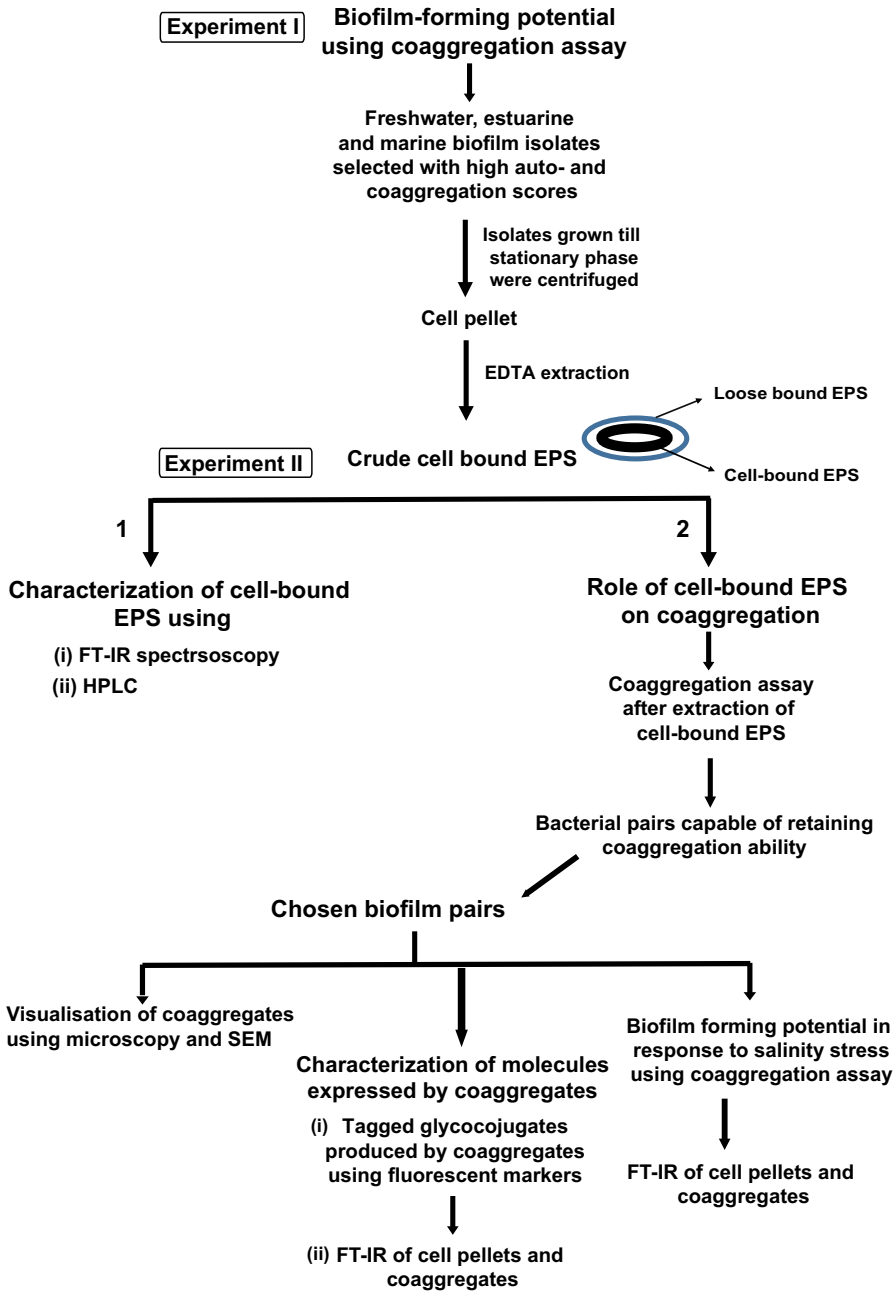


Fig. 1 The schematic illustration of the experimental design. *EPS* extracellular polymeric substances, *FT-IR* Fourier-transform infrared spectroscopy, *SEM* scanning electron microscopy, *HPLC* high-pressure liquid chromatography

kept on the rotary shaker (Remi, India) for 100 rpm at 30°C. The average period of stationary phase for the isolates was found as 48 h and 72 h for freshwater bacteria and marine bacteria, respectively. Cultures (50 ml) in their respective stationary growth phases were centrifuged at 4,500 × *g* for 20 min and washed twice with phosphate-buffered saline (PBS), pH 7.4 (Sigma-Aldrich). The cell pellets were then resuspended in sterile PBS, and the working stock (bacterial density 10^9 cells/ml) was used to determine the aggregation properties of the bacterial isolates. Visual coaggregation assay was carried to determine the qualitative analysis of auto-aggregation and coaggregation between the biofilm bacteria as described below.

Visual aggregation assay

The auto-aggregation and coaggregation were carried out in pairwise combinations between the biofilm bacterial isolates as described by Cisar et al. (1979), with slight modifications. Briefly, 1,000 µl of two different strains of bacteria in PBS (bacterial cell density 10^9 cells/ml) were mixed in a test tube and then incubated at room temperature for 24 h. After incubation, the scores for the bacterial aggregation were assigned from "0" to "4" as described by Rickard et al. (2002). Based on the 4-point scale, the turbid supernatant of bacterial culture was assigned "0", whereas "1" for small uniform aggregates in turbid suspension, "2" for easily visible aggregates with turbid suspension, "3" indicated large settled coaggregates leaving some turbidity in the supernatant and "4" depicted large settled coaggregates with clear supernatant. On the other hand, the auto-aggregation or self-aggregation was determined by mixing equal volumes of the same bacterial suspension, incubated at room temperature for 24 h and scored from "0" to "4" as mentioned above. The bacterial strains showing strong auto-aggregation and coaggregation scores were identified as biofilm-forming bacteria, and ten strains with high biofilm-forming potential were selected for sequencing as well as characterization of cell-bound EPS. In addition, the aliquots of these coaggregates were lyophilized and desiccated for characterization using Fourier-transform infrared spectroscopy (FT-IR) as described below.

Identification of selected biofilm-forming bacteria using 16S rDNA sequencing

For identification of selected biofilm-forming bacteria, the marine/estuarine and freshwater bacterial colonies were inoculated into ZMB and NB, respectively. They were incubated at 30°C overnight (approximately 18 h) in an incubator shaker at 80 rpm. After the incubation period, overnight grown cultures were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 13,000 × *g* for 5 min to pellet the cells. The genomic DNA was extracted from the bacterial isolates using Thermofisher® Purelink Genomic DNA Mini Kit, according to the manufacturer's instructions and stored at − 20°C until further analysis. Further, the bacterial 16S rDNA gene was amplified by PCR in a 50 µl reaction mixture using Platinum® Blue PCR SuperMix (Invitrogen, USA), 0.25 µM of each primer (8F: 5′-AGAGTTTGATCCTGGCTCAG and 1492 R (l)-GGTTACCTTGTTACGACTT) and template. The PCR products were checked by electrophoresis (2% w/v agarose gel) and purified using the GenElute PCR purification kit (Sigma). These purified products were then sequenced (Bioserve Biotechnologies Private Limited Hyderabad, India). The sequencing results obtained were assembled using DNA Baser and aligned using the Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). These sequences have been deposited in the NCBI GenBank under accession numbers from MH429953 to MH429961 and MH620809. Sequences were aligned with Clustal W, and the phylogenetic tree was built by using the MEGA 6.0 software (Tamura et al., 2013).

Experiment II: Importance of cell-bound extracellular polymeric substances (CB-EPS) in the coaggregation process

Isolation and characterization of CB-EPS from selected biofilm-forming bacteria For the isolation of CB-EPS, the freshwater and estuarine/marine bacteria were cultured in NB and ZMB for 48 h and 72 h, respectively, i.e. upto the stationary phase. The cultures were then centrifuged at 4,500 × *g* for 20 min, and the cell-bound EPS were extracted using the EDTA (10 mM) as described by Tallon et al. (2003). After centrifugation, an aliquot of the EPS was stored at − 20°C for the sugar analysis by high-performance liquid chromatography (HPLC). The remaining aliquot of the EPS was lyophilized and desiccated for the analysis of different types of biomolecules by FT-IR.

FT-IR analysis of CB-EPS A pellet was prepared by grinding approximately 1 mg of lyophilized samples and mixed thoroughly with 2.5 mg of potassium bromide (KBr). KBr was used as a background reference. The spectrum was recorded as an average of 20 scans in the mid-infrared (mid-IR) range of 600–4,000 cm^{-1} using Fourier-transform infrared spectrophotometer (IR Affinity-1, Shimadzu, Singapore) at a resolution of 4 cm^{-1}.

Characterization of sugar composition in CB-EPS by high-performance liquid chromatography The sugar

composition of the CB-EPS was analysed by reverse-phase high-performance liquid chromatography (HPLC, Agilent 1200 series) equipped with a refractive index detector (RID). The sugars were separated isocratically on a Zorbax Carbohydrate column (4.6 mm ID × 150 mm, silica particle size 5 µm, Agilent) using acetonitrile:water (85:15) mobile phase at a flow rate of 1.4 ml/min with a column temperature at 30°C for 35 min. The injection volume of 10 µl was used for each sample and run in duplicates. Before running the samples, the column was calibrated with 19 different HPLC grade sugar standards, i.e. mannose, galactose, rhamnose, sucrose, ribose, glucose, fucose, ribose, arabinose, maltose, *N*-acetyl-D-glucosamine, fructose, xylose, lactose, inositol, cellobiose, mannitol, sorbitol, melibiose, as described by Sahoo & Khandeparker (2018). Sugars were identified according to their retention times by comparing with sugar standards (ESM_1). The calibration curves were prepared for each sugar by diluting stock solutions (10 mg/ml) at multiple concentrations. HPLC analysis was then performed for all standard solutions, and the peak area against the retention time for each sugar was recorded. Calibration curves were obtained using the software by plotting peak area versus the amount injected (concentration). The determination coefficients (R^2) were > 0.99, indicating a linear relationship between the chromatographic response areas and the concentrations for all the sugars. The calibration table was then created using this data in the Agilent Chemstation data analysis software. Sugars for samples were identified by comparing with retention times of sugar standards and calculated by the data analysis software. Furthermore, the sugar compositions (%) were subjected to NMDS (non-metric multidimensional scaling) to visualize similarities among the bacterial species and confirmed with Analysis of similarity (ANOSIM) test using PRIMER version 6.0 software (Clarke & Warwick, 1994). Also, the similarity percentage (SIMPER) analysis was carried to identify sugars that significantly contributed to the differences among the biofilm types using PRIMER version 6.0 software.

Role of CB-EPS in biofilm formation

A separate coaggregation assay was conducted after the extraction of CB-EPS (using EDTA method as per the protocol described above) for the selected 10 bacterial strains with high biofilm-forming potential to identify the role of cell-bound EPS in the formation of coaggregates. After EPS extraction, the bacterial strains were washed using PBS (pH 7.4). Further, pairs of bacterial strains were mixed (1,000 µl of each strain with equal cell density) in a test tube and incubated at room temperature for 24 h. Subsequently, the score was assigned based on the coaggregation using the 4-point scale, as mentioned earlier. The estuarine and freshwater bacterial pairs which retained their ability to coaggregate even in the absence of CB-EPS were further characterized as described below.

Visualization of coaggregates using epifluorescence microscopy The coaggregates of selected bacterial pairs were observed by using epifluorescence microscopy (Olympus, Tokyo, Japan). The protocol was standardized, wherein 1,000 µl of each strain in the PBS were stained with either DAPI (50 µg/ml) or SYBR Green I nucleic acid (1:10,000 final concentration). Subsequently, each of the bacterial suspension was incubated at room temperature in the dark for 20 min to allow staining of the cells. After incubation, the cells were centrifuged at maximum speed (13,000 × *g* for 5 min) and washed thoroughly with phosphate-buffered saline (pH 7.4) to remove excess stain. Each strain was then resuspended in fresh PBS solution and mixed to allow them to coaggregate. After 1–2 h, 50 µl of the coaggregate was taken on a glass slide for microscopic observation of coaggregates using epifluorescence microscopy. Further, the SYBR Green I and DAPI fluorescence images (green and blue) were overlayed using cellSens standard imaging software (Olympus, Tokyo, Japan) and Image J software (v. 1.52a).

Scanning electron microscopy (SEM) of coaggregates The selected coaggregated bacteria were visualized using a scanning electron microscope (SEM), which allows imaging and investigation of surface topographic features. The samples were fixed using 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4), then subsequently kept in the refrigerator (4°C) for 1 h and dehydrated in the ethanol series. After drying, the samples were examined using a Hitachi Tabletop Scanning Electron Microscope (TM3000).

Comparative characterization of freshwater and estuarine bacterial pairs and their coaggregates before (control) and after the extraction of CB-EPS

Characterization of glycoconjugates on coaggregate surfaces using fluorescent markers The fluorescent markers (lectins) obtained from Sigma-Aldrich were used to identify surface glycoconjugates of the coaggregated bacterial pairs. The specific lectins used were (1) Concanavalin A (Con A)—FITC-conjugated lectin to tag D-mannose/D-glucose residues (Strathmann et al., 2002; Guillonneau et al., 2018), (2) wheat germ agglutinin (WGA)—FITC conjugated lectin to tag *N*-acetyl-D-glucosamine and sialic acids, and (3) *Glycine max* (soybean agglutinin)—TRITC-conjugated lectin (SBA) for tagging *N*-acetyl-D-galactosamine.

The protocol was standardized for this, wherein the coaggregates formed by the selected bacterial strains after mixing them for coaggregation assay (approx. 1–2 h) were stained and incubated with DAPI (50 µg/ml) (Himedia) for 15 min in the dark. Subsequently, the coaggregates were rinsed with PBS to remove excess stain. The coaggregates were then stained with one of the fluorescent markers (1 mg/ml) and visualized under an epifluorescence microscope (Olympus, Tokyo, Japan). Similarly, the above protocol was repeated on coaggregates formed after the extraction of cell-bound EPS. The microscopic images of DAPI for aggregates and FITC fluorescence for lectins appeared as blue and green colour, respectively, except in the case of *G. max*, which was TRITC conjugated (orange). Furthermore, both the images of FITC and DAPI were overlayed and prepared using cellSens standard imaging software (Olympus, Tokyo, Japan) and Image J software (v. 1.52a). Likewise, the images of TRITC-labelled conjugate and DAPI (orange and blue combination) were processed.

FT-IR analysis of freshwater and estuarine bacterial pairs and their coaggregates before and after the extraction of CB-EPS In order to identify the molecules which are probably involved in retaining coaggregation ability in the absence of CB-EPS, the aliquots of the selected estuarine and freshwater biofilm bacterial cell pellets and their coaggregates (formed before and after the extraction of CB-EPS) were lyophilized and subjected to the FT-IR as described above. This analysis has been proven as a powerful tool to understand structural changes occurring in the bacterial cell surfaces in response to environmental changes (Kamnev, 2008). In addition, laboratory experiments were conducted on the bacterial pairs which retained their ability to coaggregate even in the absence of CB-EPS by subjecting them to salinity stress to assess the impact of salinity-stressed biofilm bacteria on the aggregation process. Briefly, the selected freshwater strains were translocated and grown in ZMB (exposed to high salinity, i.e. 34) and estuarine bacteria in NB (exposed to low salinity, i.e. 5) upto the stationary phase. Furthermore, the coaggregation assays were performed in duplicates as described above, and the aliquots of these coaggregates were lyophilized and desiccated to assess the response of these bacteria to environmental changes using FT-IR.

Results

Experiment I

Biofilm-forming potential using coaggregation assay

Among the total 23 strains, 10 strains with high biofilm-forming potential were selected based on their strong autoaggregation and coaggregation scores, as depicted in the Electronic Supplementary Material (ESM_2). It was observed that the coaggregation process was growth-phase dependent, i.e. the isolates showed maximum coaggregation during the stationary phase, irrespective of the type of biofilm. The average period of the stationary phase was found to be 48 h and 72 h for freshwater and estuarine/marine biofilm bacteria, respectively. Out of 23 strains, nine isolates (4-freshwater, 5-estuarine) were chosen based on their strong auto-aggregation (i.e. "3" and "4") and coaggregation scores (i.e. in range of "3" to "4"). Most of the isolates within these freshwater and estuarine, coaggregated with at least 1 other strain with high coaggregation scores. However, in the case of marine biofilm bacteria, the majority of pairwise combinations demonstrated weak coaggregation scores (i.e. "1"), except only one marine biofilm bacteria (M1), which showed the highest auto-aggregation score (i.e. "3") and coaggregated with most of the strains (ESM_2). Overall, the coaggregation potential based on pairwise combinations was highest

in the estuarine biofilm bacteria (62.5%), followed by freshwater (44%) and minimum for marine biofilm bacteria (28%).

Identification of the selected biofilm-forming isolates with high biofilm-forming potential

From the 16S rDNA sequencing data of the selected biofilm-forming bacterial isolates and their comparison with the gene bank database indicated that most of the isolates belonged to *Bacillus* genera followed by *Exiguobacterium* and *Staphylococcus* (accession numbers MH429953–MH429961, MH620809). Phylogenetic analysis revealed that the *Bacillus* species were clustered together, and several isolates differed in the rDNA sequence composition (Fig. 2). Most of the biofilm-forming bacteria from estuarine biofilms showed > 99% similarities with *Bacillus* spp. (MH429953.1), *Bacillus indicus* (MH429954.1), *Bacillus cereus* (MH429955.1) and *Staphylococcus lentus* (MH429956.1), except for one estuarine bacteria, which showed < 98% similarity with *B. cereus* (MH620809.1). On the other hand, freshwater biofilm-forming bacteria showed > 99% similarities with *Exiguobacterium* spp. (MH429958.1 and MH429959.1), *Bacillus subtilis* (MH429960.1) and *B. cereus* (MH429960.1) while marine biofilm-forming bacteria showed > 99% similarity with *S. lentus* (MH429957.1).

Experiment II

Characterization of CB-EPS extracts from selected biofilm-forming isolates with high biofilm-forming potential using FT-IR

The Fourier-transform infrared spectra of cell-bound EPS extracted from the biofilm bacteria are illustrated in Fig. 3. The possible assignments of the absorption bands and their references are tabulated in Table 1. The spectral patterns of freshwater and marine bacterial EPS differed from the estuarine bacterial EPS (Fig. 3a–c). The pattern was similar for the rest of the freshwater and estuarine biofilm-forming bacteria (ESM_3a–b), except for one of the estuarine bacteria (*S. lentus*), which showed a spectral pattern similar to the freshwater bacteria (ESM_3c). The spectra of the freshwater, estuarine and marine bacterial CB-EPS displayed a broad peak at around 3,600–3,200 cm^{-1} indicating the presence of hydroxyl groups, followed by an asymmetrical stretching peak in the range of 1,593–1,629 cm^{-1} corresponding to ring stretching of mannose or galactose (Fig. 3a–c; Table 1). Moreover, the absorption peaks in the range from 1,000 cm^{-1} to 1,200 cm^{-1} were also linked to the presence of C–O–H side groups and C–O–C glycosidic bond vibrations which are characteristics of all sugar derivatives (Fig. 3a–c; Table 1). The presence of carboxylic acids COO– group (strong peak at 1,402–1,408 cm^{-1}), uronic acids (1,111–1,112 cm^{-1}) and ester sulphate groups (810–816 cm^{-1}) were also evident in the spectra (Fig. 3a–c; Table 1). The freshwater and marine bacterial EPS spectrum contained, in addition to spectral bands noted above, the presence of terpenoids and steroids which were evident from peaks at 1,475/1,473 and 1,363/1,365 cm^{-1}, respectively (Fig. 3a, c; Table 1). Overall, the spectroscopic results were suggestive of carbohydrate nature of CB-EPS in all the biofilm bacteria, irrespective of types. Therefore, it was further characterized for its sugar content by using HPLC.

Sugar characterization (%) of CB-EPS from biofilm bacteria

The sugar composition (%) of the bacterial cell-bound EPS belonging to different biofilm types is given in Table 2. Furthermore, these sugar compositions were subjected to NMDS to cluster different biofilm bacterial types on the basis of their sugar composition (ESM_4). Nearly all of the freshwater bacterial cell-bound EPS showed the dominance of galactose with a minor contribution of mannose, xylose and others (Table 2, ESM_4). On the other hand, the estuarine bacterial cell-bound EPS were distinct, wherein mannose was dominant along with galactose, rhamnose, ribose, arabinose and others (Table 2, ESM_4). On the contrary, the marine bacterial cell-bound EPS composition differed, wherein fructose and sorbitol were dominant sugars, along with ribose and galactose (Table 2, ESM_4). The components of the sugars varied with the biofilm bacterial types; moreover, the relative proportions of the individual saccharides differed, suggesting that the exopolymer is a heteropolysaccharide (ESM_4). Analysis of Similarity (ANOSIM) also revealed these differences ($R = 0.56$, $P < 0.05$). The results of SIMPER analysis showing overall dissimilarities in the sugar composition and

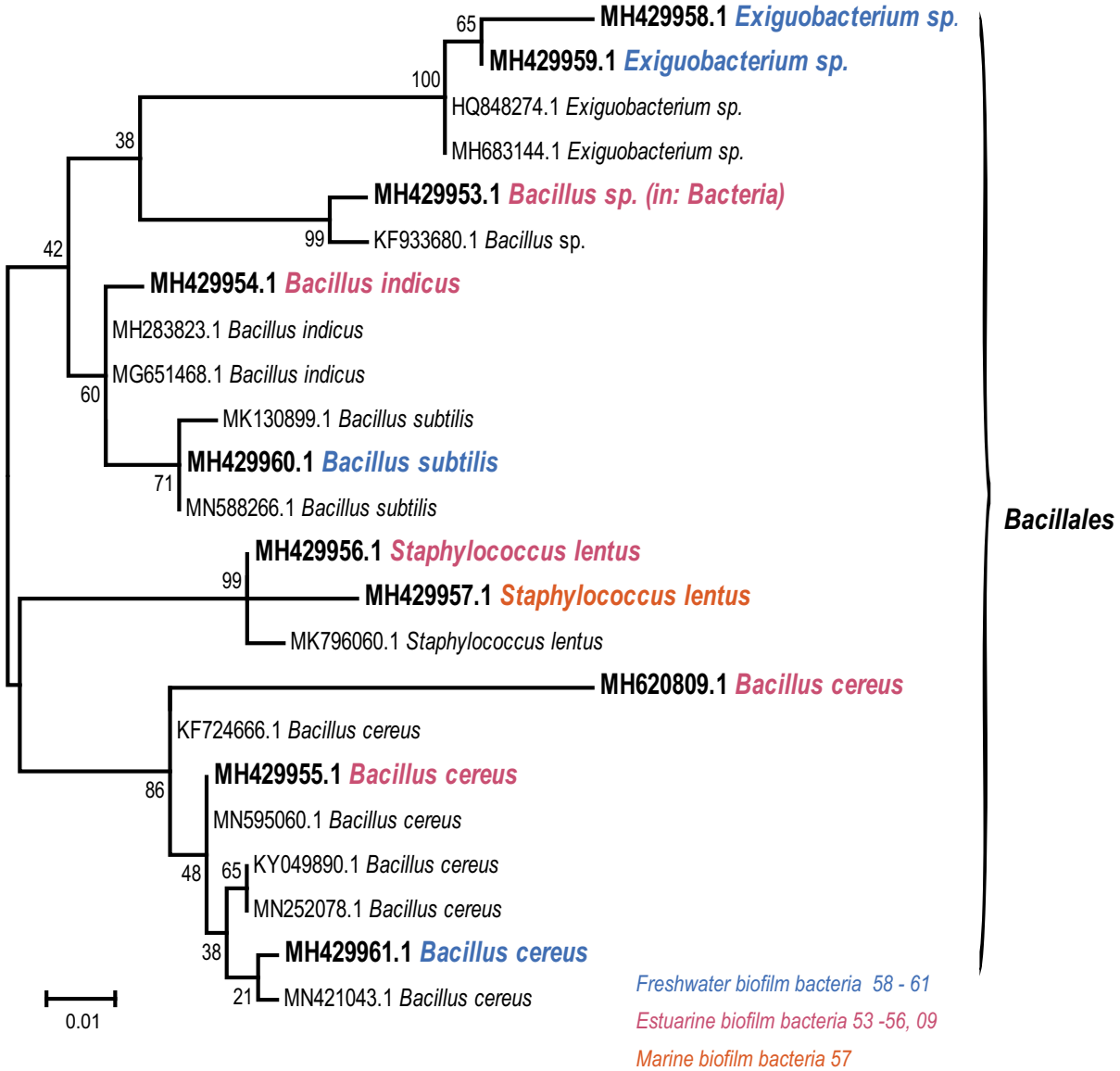
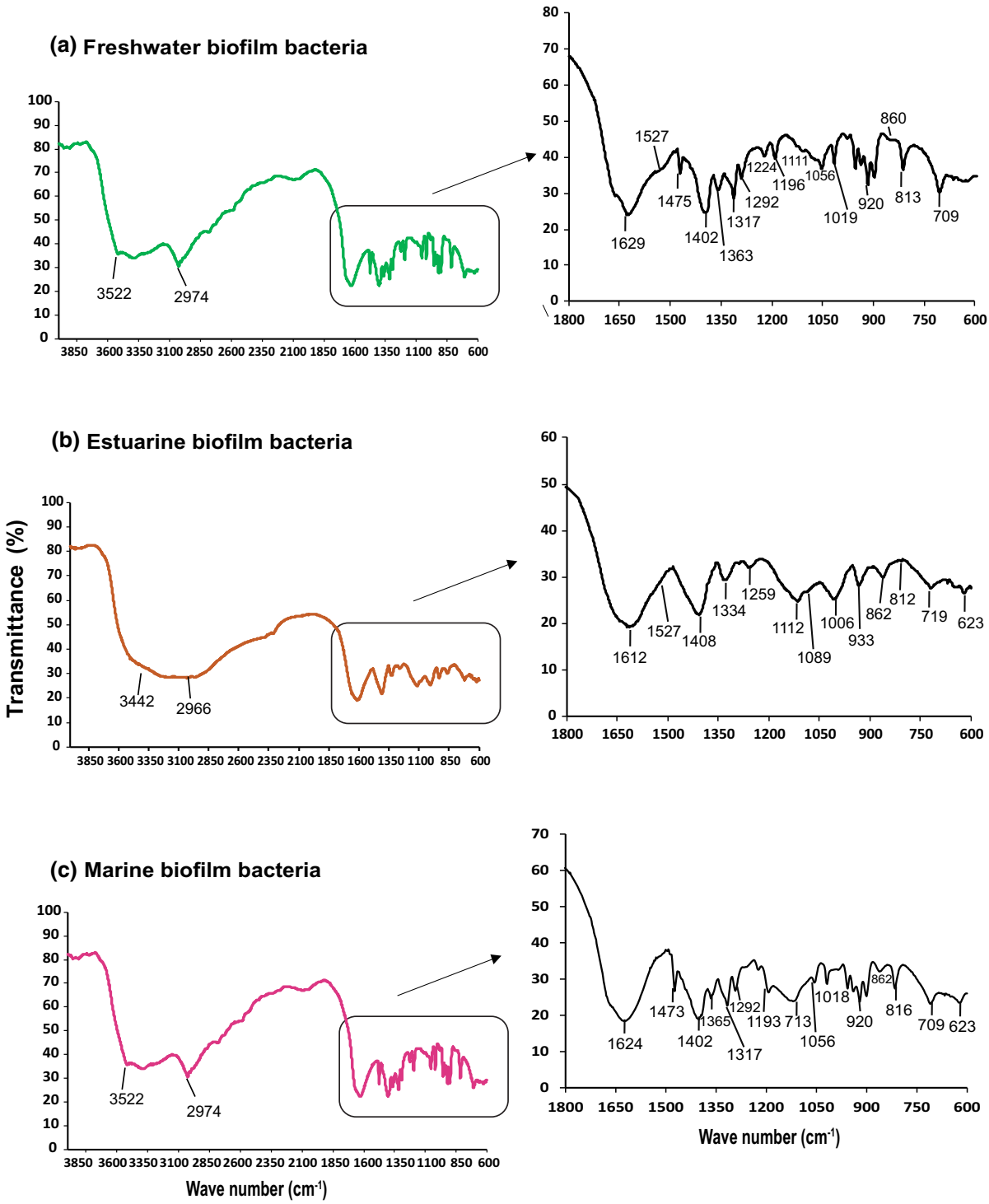


Fig. 2 Phylogenetic relationship of the chosen biofilm isolates from the freshwater, estuarine and marine biofilms based on 16S rDNA gene sequence analysis. The sequences obtained from the present study are highlighted in blue for freshwater, pink for estuarine and orange colour for marine. Scale bar, 0.01 substitutions per nucleotide position. *sp*. Species

sugars that significantly contributed to differences between biofilm types are tabulated in Table 3. The average dissimilarity was comparable between freshwater and estuarine (43.63%) and freshwater and marine (43.84%), while it was 52.84% between the estuarine and marine group. The mannose, melibiose, galactose, fructose and D-sorbitol significantly contributed to the dissimilarity between the biofilm types (refer contribution % in Table 3a–c).

Role of CB-EPS in biofilm formation

Most of the biofilm bacteria, including marine, lost their ability to coaggregate in the absence of CB-EPS, which was evident from turbid supernatant after mixing the bacterial suspensions (data not shown). For those bacterial pairs, i.e. *Exiguobacterium* spp., (MH429959) and *B. cereus* (MH429961) from freshwater, and *B. indicus* (MH429954) and *B. cereus*


(a) Freshwater biofilm bacteria
3522
2974
1629
1527
1475
1402
1363
1317
1292
1224
1196
1111
1056
1019
920
860
813
709
(b) Estuarine biofilm bacteria
Transmittance (%)
3442
2966
1612
1527
1408
1334
1259
1112
1089
1006
933
862
812
719
623
(c) Marine biofilm bacteria
3522
2974
1624
1473
1402
1365
1317
1292
1193
1056
1018
920
862
816
713
709
623
Wave number (cm-1)
Wave number (cm-1)

◀**Fig. 3** Fourier-transform infrared (FT-IR) spectra's of cell-bound bacterial EPS from representative **a** freshwater, **b** estuarine and **c** marine biofilm-forming bacteria. The freshwater bacterial EPS spectra are represented in green colour, estuarine bacterial EPS spectra in orange colour and marine bacterial EPS spectra in pink colour. The zoomed infrared (1,800–600 cm^{-1}) spectral section is shown in black colour. The spectral pattern for the other freshwater and estuarine biofilm-forming bacteria was similar, except for one of the estuarine (MH429956.1), which showed a spectral pattern similar to the freshwater bacteria

(MH620809) from the estuarine biofilms, which were able to coaggregate, even in the absence of CB-EPS, were chosen for further characterization.

The visualization of the selected freshwater and estuarine biofilm bacterial coaggregates was done by using epifluorescence microscopy, which showed that two different species were closely associated with one another (Fig. 4a, b). Moreover, this distribution was confirmed by scanning electron microscopy, which revealed the presence of highly dense coaggregates covered by an extracellular EPS, which helped in holding the bacteria together (Fig. 4c, d).

Comparative characterization of freshwater and estuarine bacterial pairs and their coaggregates before (control) and after the extraction of CB-EPS

Characterization of glycoconjugates on coaggregate surfaces using fluorescent markers The selected coaggregation pairs showed high levels of specific binding to D-mannose and D-glucose residues (alpha polysaccharides as detected by Concanavalin A) as well as *N*-acetyl-D-glucosamine and sialic acids (as detected by wheat germ agglutinin) (Fig. 5). On the other hand, very low levels of *N*-acetyl-D-galactosamine residues (as detected by *G. max*) were associated with coaggregation pairs during the study period. A similar type of expression of these glycoconjugates was observed by these aggregates even after extraction of CB-EPS (Fig. 5).

Table 1 Interpretation of infrared (IR) spectra and peak assignments of the cell-bound EPS

Wave number (cm^{-1})	Molecular vibrations of functional groups and biomolecule contributor	References
3,600–3,200	Broad O–H stretching absorption peak indicating the presence of hydroxyl groups, which are characteristics for carbohydrates	Khandeparker et al. (2002), Seedevi et al. (2013), Zeng et al. (2016) and Elnahas et al. (2017)
3,000–2,800	C–H stretching vibration band of the aliphatic CH_2 group by fatty acids and lipids	Cheng et al. (2013) and Fang et al. (2014)
1,593–1,629	Presence of an asymmetrical stretching peak corresponding to ring stretching of mannose or galactose	Freitas et al. (2009) and Kavita et al. (2011, 2013, 2014)
Peaks at 1,527 and 1,533 cm^{-1}	C–N stretching vibrations in combination with N–H bending of proteins (amide I and II bands)	Lorite et al. (2011) and Fang et al. (2014)
1,402–1,408	Symmetric stretching of the carboxylic acids COO– group	Zhao et al. (2007) and Lorite et al. (2011)
Peaks at 1,475 and 1,363 cm^{-1}	Presence of terpenoids and steroids	Khandeparker et al. (2002)
1,000–1,125	*O*-acetyl ester linkage bonds indicating the presence of uronic acids	Bramhachari & Dubey (2006) and Kavita et al. (2014)
1,000–1,200	C–O–H side groups and C–O–C glycosidic bond vibrations which are characteristics of all sugar derivatives	Suh et al. (1997), Sheng et al. (2005), Bramhachari & Dubey (2006), Cai et al. (2013) and Kavita et al. (2013)
Peaks at 810–816 cm^{-1}	Presence of ester sulphate groups	Lloyd et al. (1961); D'souza (2004), Seedevi et al. (2013) and Guezennec et al. (1998)
858–862	Presence of α-glycosidic linkages between individual glycosyl residues	Kodali et al. (2009)

Table 2 Composition (%) of sugars in the cell bound EPS extracted from biofilm bacteria from different aquatic environments

	Rha	Rib	Xyl	Arab	GlcNAc	Fruc	Man	Glu	Sorbitol
Freshwater									
F1 (*Exiguobacterium* spp.)	0.00	3.73	10.23	3.22	0.00	0.00	22.40	0.00	12.04
F2 (*Exiguobacterium* spp.)	0.00	2.70	6.90	0.00	0.00	0.00	24.50	0.00	3.10
F3 (*Bacillus subtilis*)	0.00	6.04	9.82	2.97	0.00	4.23	14.17	0.00	7.36
F4 (*Bacillus cereus*)	0.16	0.89	5.75	7.36	0.00	12.11	14.37	0.00	8.06
Estuarine									
E1 (*Bacillus* spp.)	0.00	0.72	0.00	0.35	0.07	0.00	61.43	0.00	4.49
E2 (*Bacillus cereus*)	3.29	4.70	0.00	0.00	0.00	0.00	0.00	0.61	5.39
E3 (*Bacillus indicus*)	1.76	5.45	4.11	6.13	0.00	2.79	19.08	0.00	4.42
E4 (*Bacillus cereus*)	0.00	2.39	5.88	2.95	1.38	0.00	59.37	0.00	0.00
E5 (*Staphylococcus lentus*)	0.00	10.50	0.00	7.60	1.00	5.70	0.00	0.00	0.00
Marine									
M1 (*Staphylococcus lentus*)	0.00	10.32	0.00	0.00	0.00	19.76	0.00	0.00	22.54

	Mannitol	Gal	Sucrose	Inositol	Cellobiose	Maltose	Lactose	Melibiose	Trehalose
Freshwater									
F1 (*Exiguobacterium* spp.)	0.00	18.02	1.80	2.40	2.20	7.00	3.92	9.10	3.94
F2 (*Exiguobacterium* spp.)	0.60	47.00	1.60	0.40	1.70	3.70	2.30	3.30	2.20
F3 (*Bacillus subtilis*)	0.00	24.37	0.44	3.20	2.60	4.50	4.00	9.03	7.27
F4 (*Bacillus cereus*)	1.70	20.46	2.88	1.73	4.65	7.00	4.27	5.36	3.25
Estuarine									
E1 (*Bacillus* spp.)	0.37	5.36	1.15	2.17	3.14	3.88	5.95	7.92	3.00
E2 (*Bacillus cereus*)	0.00	19.58	3.29	3.28	4.08	5.88	8.02	37.65	4.23
E3 (*Bacillus indicus*)	0.85	12.90	1.54	2.63	3.29	4.37	6.87	20.09	3.72
E4 (*Bacillus cereus*)	0.34	6.05	1.61	1.23	1.47	1.99	3.95	9.00	2.39
E5 (*Staphylococcus lentus*)	0.40	14.60	2.50	6.30	5.60	8.50	11.30	21.50	4.50
Marine									
M1 (*Staphylococcus lentus*)	1.75	20.61	1.36	1.25	2.63	4.59	5.30	7.65	2.24

F freshwater, *E* estuarine, *M* marine, *spp.* species, *Rha* rhamnose, *Rib* ribose, *Xyl* xylose, *Arab* arabinose, *GlcNAc* *N*-acetyl-D-glucosamine, *Fruc* fructose, *Man* mannose, *Gal* galactose

Table 3 SIMPER analysis showing sugars that contributed to the differences among the biofilm types

(a) Average dissimilarity = 43.63

Group				Freshwater	Estuarine	
Sugars	Av. Diss	Contrib%	Cum.%	Av. Abund	Av. Abund	Diss/SD
Mannose	11.15	25.55	25.55	18.88	27.98	1.79
Melibiose	7.65	17.53	43.09	6.7	22.84	1.71
Galactose	6.25	14.32	57.4	27.48	14.16	1.1
Xylose	3.25	7.45	64.85	8.15	3.72	2.56
Lactose	2.54	5.83	70.68	3.63	9.12	3.02
D-sorbitol	2.3	5.28	75.96	7.65	3.42	1.47
Fructose	1.97	4.53	80.49	4.08	1.7	1.01
Arabinose	1.68	3.84	84.33	3.4	4.32	1.43
Ribose	1.57	3.59	87.92	3.33	5.56	1.22
Inositol	0.91	2.09	90.01	1.93	3.76	1.13

(b) Average dissimilarity = 43.84

Group				Freshwater	Marine	
Sugars	Av. Diss	Contrib%	Cum.%	Av. Abund	Av. Abund	Diss/SD
Mannose	9.44	21.54	21.54	18.88	0	9.44
Fructose	7.87	17.95	39.49	4.08	19.8	7.87
D-sorbitol	7.43	16.95	56.44	7.65	22.5	7.43
Galactose	4.11	9.39	65.82	27.48	20.6	4.11
Xylose	4.08	9.3	75.12	8.15	0	4.08
Ribose	3.49	7.96	83.08	3.33	10.3	3.49
Arabinose	1.7	3.88	86.96	3.4	0	1.7
Melibiose	1.18	2.68	89.64	6.7	7.6	1.18
Trehalose	0.98	2.23	91.87	4.15	2.2	0.98

(c) Average dissimilarity = 52.84

Group				Estuarine	Marine	
Sugars	Av. Diss	Contrib%	Cum.%	Av. Abund	Av. Abund	Diss/SD
Mannose	11.97	22.65	22.65	27.98	0	0.94
D-sorbitol	8.86	16.77	39.42	3.42	22.5	5.54
Fructose	8.38	15.87	55.28	1.7	19.8	7.61
Melibiose	7.23	13.69	68.97	22.84	7.6	1.54
Galactose	2.96	5.61	74.58	14.16	20.6	1.66
Ribose	2.21	4.18	78.76	5.56	10.3	1.52
Arabinose	1.99	3.76	82.52	4.32	0	1.15
Lactose	1.76	3.33	85.86	9.12	5.3	2.15
Xylose	1.53	2.89	88.75	3.72	0	0.63
Inositol	1.21	2.3	91.04	3.76	1.2	1.58

(a) Freshwater and estuarine, (b) freshwater and marine and (c) estuarine and marine

Av. Diss average dissimilarity, *Contrib%* contribution, *Cum. %* Cumulative %, *Av. Abund* average abundance

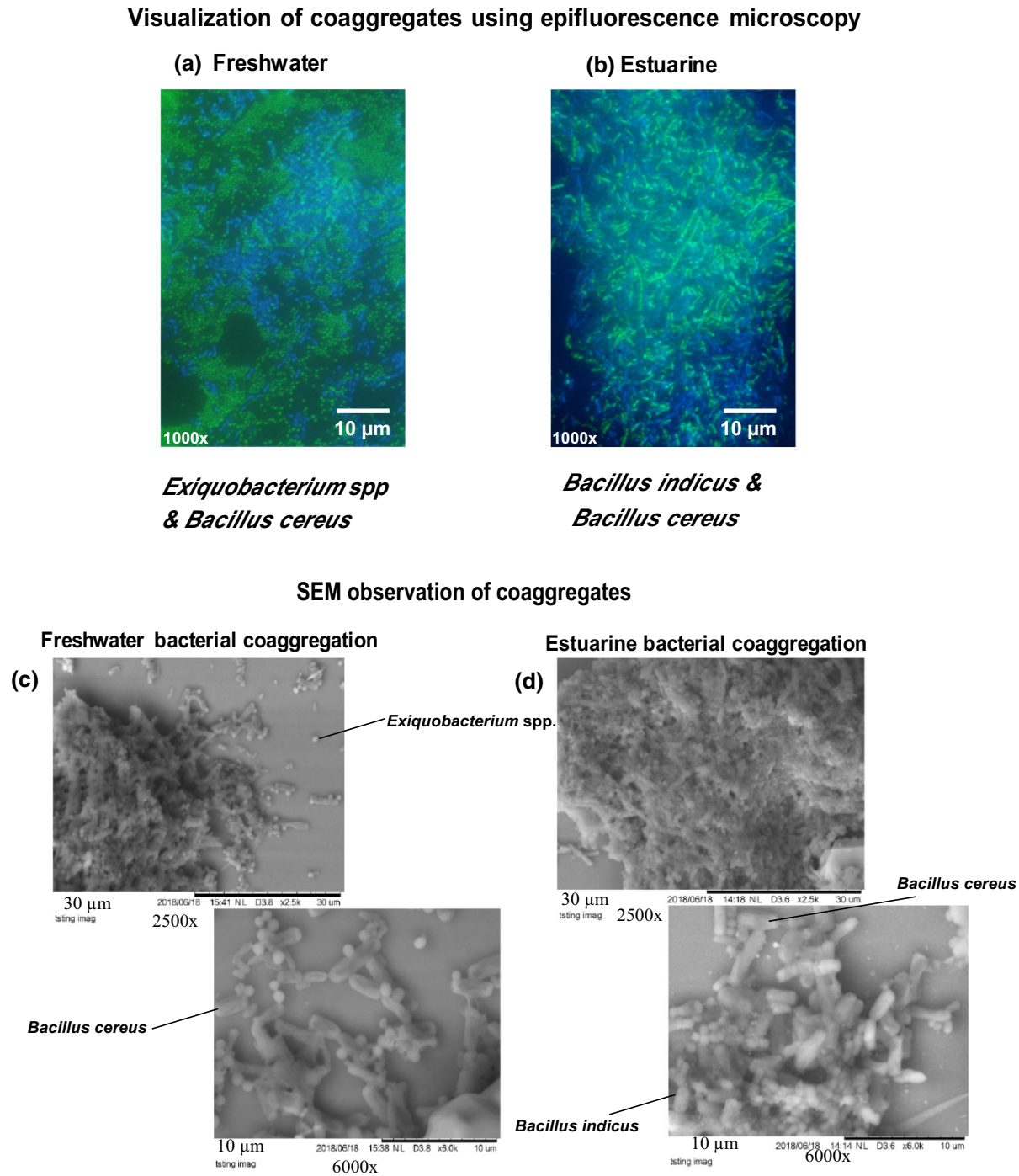


Fig. 4 Visualization of coaggregates using epifluorescence microscopy (× 1,000 magnification) from **a** freshwater, and **b** estuarine biofilm bacterial pairs (cultures grown till stationary phase), stained either with DAPI (blue) or SYBR green nucleic acid (green) nucleic acid stain, and scanning electron microscopy (SEM) images of coaggregates (× 2,500 and × 6,000 magnification) formed by **c** freshwater and **d** estuarine biofilm bacterial pairs (cultures grown till stationary phase)

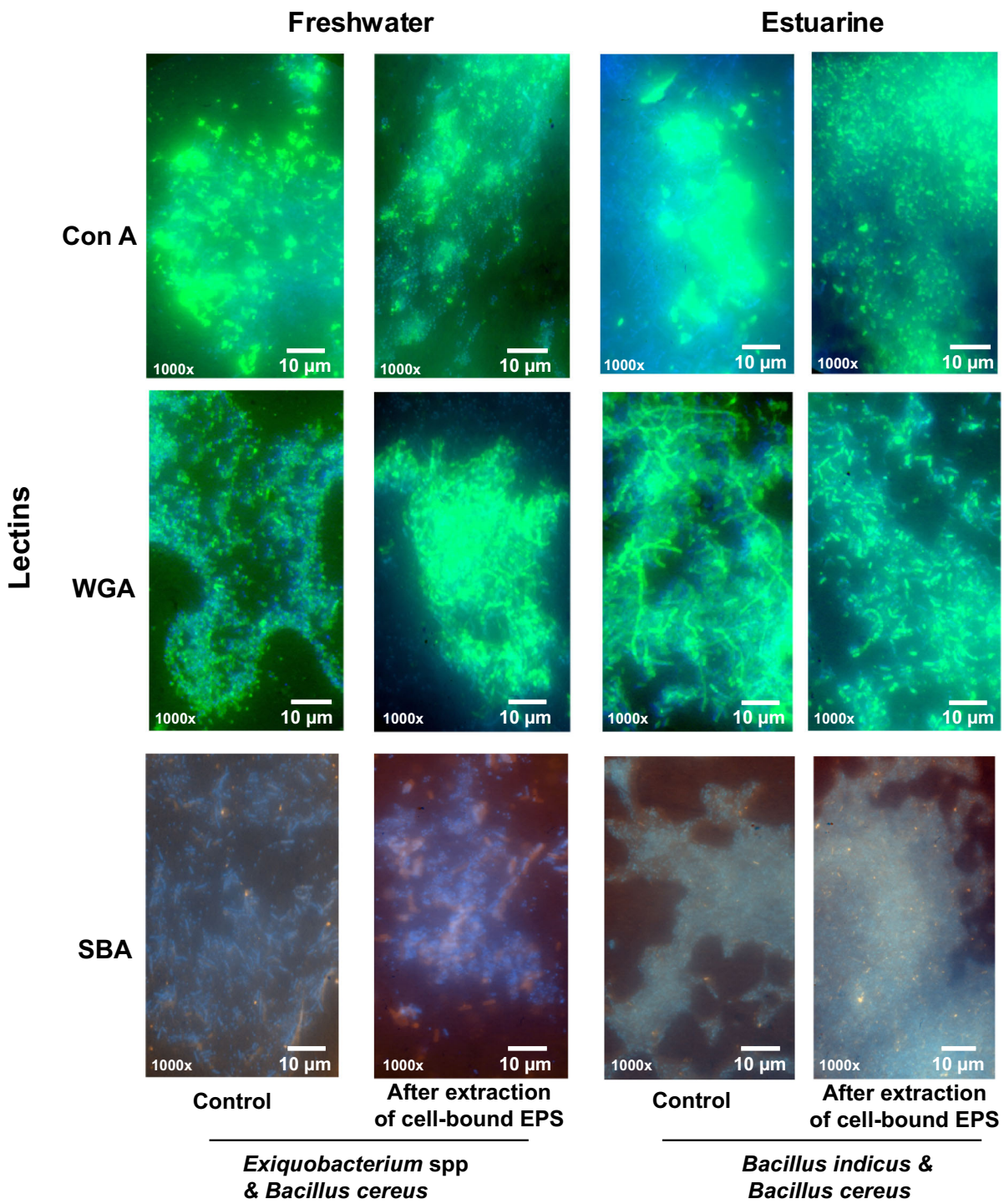


Fig. 5 Fluorescent labelling of glycoconjugates associated with coaggregates using epifluorescence microscopy (× 1,000 magnification). *Con A* Concanavalin A, *WGA* wheat germ agglutinin and *Glycine max* (soya bean agglutinin SBA). Bacterial coaggregation pairs are visible as blue while D-mannose/D-glucose residues (as detected by Con A) and *N*-acetyl-D-glucosamine and sialic acids (as detected by WGA) are visible as green. In the case of *Glycine max*, bacterial coaggregation pairs are visible as blue and *N*-acetyl-D-galactosamine as orange

Table 4 Interpretation of IR spectra of the bacterial cell pellets

Wave number (cm^{-1})	Molecular vibrations of functional groups and biomolecule contributor	References
3,000–2,800	Membrane lipids and fatty acids	Naumann (2000)
1,739–1,725	Stretching C=O of ester functional groups from the membrane lipids and fatty acids	Ojeda et al. (2009), Deepika et al. (2012)
1,700–1,500	Proteins and peptides containing amide I and II bonds	Naumann (2000)
1,500–1,200	Mixed region which includes	Naumann (2000)
	(1) Fatty acids around 1468, 1455	
	(2) P=0 stretching of PO_4^- (phosphodiesters, backbone of nucleic acids) around 1,250–1,200	
	(3) Amide III band of proteins around 1,350–1,240	
1,200–900	Carbohydrate region	Naumann (2000)
900–600	Fingerprint region	Naumann (2000)

Refer to the Electronic Supplementary Material (ESM_5 and ESM_6) for the Fourier-transformed infrared (FT-IR) spectra's of the freshwater and estuarine bacterial cell pellets and coaggregates

FT-IR analysis of freshwater and estuarine bacterial strains and their coaggregates The infrared spectra of the freshwater and estuarine bacterial strains and coaggregates exhibited marked changes in the spectral regions (as tabulated in Table 4) before and after the extraction of cell-bound EPS (ESM_5a–b, ESM_6a–b). The FT-IR of freshwater coaggregates showed distinct peaks at 2957, 1658 and 1537 cm^{-1} attributed to –CH asymmetric stretching of CH_3 in the fatty acids, amide I and II regions of proteins, respectively (Table 4, ESM_5a). On the other hand, a weak peak pattern was observed in the case of the estuarine coaggregated bacteria (ESM_6a). One common trend observed in both were notable changes in the peaks 1,058–1,072 cm^{-1}, which were attributed to alterations in the carbohydrate region (Table 4, ESM_5a, ESM_6a). However, the opposite trend was observed in the expression pattern by these coaggregates after extraction of cell-bound EPS. In the case of estuarine bacteria, the coaggregates showed the involvement of lipids and proteins which was evident from the emergence of peaks at 2956, 1664 (amide I) and at 1535 cm^{-1} (amide II), respectively, as observed in the spectra (Table 4, ESM_6b). On the other hand, a weak peak pattern was observed in the case of the freshwater coaggregated bacteria (ESM_5b), except for the appearance of a peak at 1,724 cm^{-1} corresponding to stretching C=O of ester functional groups from the membrane lipids and fatty acids (ESM_5b). Moreover, notable alterations were also observed in the carbohydrate spectral region (1,200 to 900 cm^{-1}) of both the biofilm bacteria, which could be attributed to compositional alterations in the cell wall or cell membrane (Table 4, ESM_5b, ESM_6b). A similar type of spectral expression pattern was observed by these coaggregates, formed after exposure of these bacterial pairs to salt stress. The spectra observed were indicative of changes that occurred in the surfaces of the individual and coaggregated bacteria when subjected to environmental changes (ESM_5c, ESM_6c). The estuarine biofilm bacteria expressed lipids and proteins, which was evident from the emergence of peaks at 2957, 1664 (amide I) and 1535 cm^{-1} (amide II), respectively, different from the freshwater spectra (Table 4, ESM_5c, ESM_6c). The most remarkable modifications were observed in the carbohydrate spectral region of both the bacterial spectra (1,200 to 900 cm^{-1}) due to alterations in the cell membrane along with the appearance of a peak at 1,722 cm^{-1} corresponding to the membrane lipids and fatty acids (Table 4, ESM_5c, ESM_6c).

Discussion

In the present study, the estuarine and freshwater biofilm bacteria showed higher autoaggregation and coaggregation potential as indicated by strong auto- and coaggregation scores, compared to marine biofilm

bacteria. This coaggregation potential was growth phase-dependent, i.e. aggregation was observed at the stationary phase. This finding corroborates well with earlier studies which reported growth phase-dependent aggregation in the biofilm bacteria from freshwater and marine environments (Rickard et al., 2000, 2002; Saravanan et al., 2014). The bacteria possessing the aggregation capabilities, i.e. autoaggregation and coaggregation, impart selective advantage over non-coaggregating ones, thereby playing an important role in the development of enhanced biofilm formation via interbacterial interactions (Rickard et al., 2004). On the other hand, weak coaggregation was observed among most of the marine biofilm bacterial strains isolated in this study, except for one marine biofilm-forming bacteria, which showed the highest coaggregation scores. An earlier study by Buswell et al. (1997) reported that low coaggregation scores do not imply weak interactions. Moreover, these coaggregation properties may depend on the relative size or morphologies of bacteria and the density of interacting ligands present on the cell surface (Buswell et al., 1997). Overall, these variations observed in the coaggregation potential among biofilm bacteria within different aquatic environments based on their salinity differences could be attributed to the diverse bacterial species and differences in the mechanisms mediating this process. Besides, the role of pH in influencing this coaggregation process cannot be ruled out for the reason that the pH profiles in the aquatic environments differ, and such changes in physicochemical factors can have a profound effect on the composition and viscosity of the bacterial adhesive exopolymers as well as cell surface molecules, which in turn may influence the coaggregation process but this needs further validation.

Although the bacterial strains used in the present study represented only a small proportion of total biofilm bacteria, the coaggregation appeared to be a significant phenomenon within these culturable biofilm-associated bacteria. The 16S rDNA sequencing data of the selected biofilm-forming bacterial isolates and their comparison to the gene bank database indicated that most of the isolates were identified as *Bacillus* genera followed by *Exiguobacterium* and *Staphylococcus*, which are Gram-positive bacteria belonging to Firmicutes. These genera are predominantly found in the oral infections and dental caries as well (Helgason et al., 2000; Raju & Anitha, 2015; Rani et al., 2016; Sakthivel et al., 2016). Moreover, members of Firmicutes have been reported as the most frequent isolates identified from the culturable bacterial diversity in the saline conditions (Kalwasinska et al., 2017; Remonsellez et al., 2018). Phylogenetic analysis revealed that all the *Bacillus* species clustered together differed in the rDNA sequence compositions. Members of genus *Bacillus* are ubiquitous in the terrestrial, freshwater as well as in the seawater habitats (Ruger, 1989). This genus includes several species which can colonize different specialized niches and adapt to changing environmental conditions such as salinity (Khandeparker et al., 2011; Sravankumar et al., 2014). This suggests that *Bacillus* spp. may be one of the many bridging organisms which facilitate the coaggregation with other bacteria, which are incapable of aggregating but needs further validation. Likewise, in the case of oral biofilms, it is known that the pathogenic bacteria will attach only to biofilms formed by initial colonizers, which are non-pathogenic ones (mostly Gram-positive bacteria) that contribute to coaggregation process and form a suitable favourable environment by producing metabolic end products such as lactate, *N*-acetyl muramic acid, *p*-amino benzoic acid, etc., for pathogenic ones (mostly Gram-negative bacteria) to adhere (Bowden et al., 1979; Vasudevan, 2017). It is thought-provoking whether such a scenario exists in aquatic biofilm bacteria with *Bacillus* spp., as one of the many other species involved in creating a favourable environment for pathogens to adhere.

As the coaggregation process includes the interaction of different bacteria at the cell surface and involves cell–cell interactions, we focused on characterizing cell-bound EPS and identifying their role in the coaggregation. The nature of cell-bound EPS was characterized more specifically in terms of chemical functional groups using FT-IR. The cell-bound EPS were rich in carbohydrates showing the presence of hydroxyl, carbonyl groups, mannose and uronic acids, which have great relevance in the adhesion, aggregation and cohesion processes (Decho, 1990; Jain & Bhosle, 2008; Casillo et al., 2018). The HPLC analysis revealed the freshwater cell-bound EPS to be a sulphated heteropolysaccharide, wherein galactose was dominant sugar along with terpenoids and steroids as evident from spectra (Fig. 3a; Tables 1, 2). These terpenoids are known as the largest class of natural products, which serve as a medium of communication

among species, thereby playing a significant role in antagonistic and beneficial interactions among organisms (Gershenzon & Dudareva, 2007). Whereas steroids are mostly chemical signalling compounds which trigger phenotypic changes in microbes via quorum sensing and enhance their adherence to surfaces (Patt et al., 2018). This suggests that these compounds might play an important role in the biofilm formation of these freshwater strains.

On the other hand, the estuarine bacterial EPS, also a sulphated heteropolysaccharide, were dominated by mannose. Although the relative proportions of the other individual sugars differed between the estuarine bacterial strains, it was not significant. This result suggests that diversity in sugar composition could further possibly result in variation in functionality among the estuarine strains. It has been reported that the sugars found in the bacterial EPS facilitate and enhance the bacterial adhesion (van Loosdrecht et al., 1990; Azeredo & Oliveira, 2000; Tsuneda et al., 2003; Cavalcante et al., 2014). The presence of the sulphate moieties in the bacterial EPS provides flexibility to EPS, imparts gel-like consistency and thus help in stabilizing the polymer, and might also be useful in the aggregation of cells (Hoagland et al., 1993; Bhasker, 2003). Whereas the cell-bound EPS extract of marine strain showed fructose and sorbitol as dominant sugars along with terpenoids and steroids. Overall, the sugar composition of estuarine bacterial EPS was more diverse, followed by freshwater and least was observed in the marine. Hence, the sugar composition of the CB-EPS seems to be crucial for strong coaggregation capabilities. However, since the present study included only one marine isolate, it makes the comparison across different aquatic systems rather difficult and requires further validation.

Most of the biofilm bacteria, including marine bacteria used in the present study, lost their ability to coaggregate after extraction of the cell-bound EPS, suggesting the importance of cell-bound EPS in the aggregation process. For those bacterial pairs, i.e. *Exiguobacterium* spp. and *B. cereus* from freshwater, and *B. indicus* and *B. cereus* from the estuarine biofilms, which still retained their ability to coaggregate even after extraction of the cell-bound EPS, were chosen for further studies. The scanning electron micrographs of these coaggregated bacterial pairs revealed that highly dense bacterial aggregates were embedded in an abundant extracellular material. Hence, it was evident that the presence of some specific molecules produced by the coaggregates helped in holding the bacteria together. Earlier studies have investigated the nature of bacterial cell surface molecules using the sugar reversal tests, i.e. by comparing the coaggregation capability in the presence of sugars (Kolenbrander et al., 1993; Rickard et al., 2000; Kolenbrander et al., 2006; Stevens et al., 2015). Lactose or *N*-acetyl-D-galactosamine were major sugars reported in blocking coaggregation between bacteria from human oral biofilms (Kathariosos-Lanwermeyer et al., 2014 and references with). However, this does not provide information on the type of the surface molecules associated with the coaggregates. Hence, these coaggregated pairs were characterized for the presence of glycoconjugates using specific fluorescent lectins.

Lectin molecules specifically bind to carbohydrates and thus are powerful tools used for analysing glycidic structures of microbial origin aggregates (Cavalcante et al., 2014). In the present study, both the estuarine (*B. indicus* and *B. cereus*) and freshwater (*Exiguobacterium* spp., and *B. cereus*) coaggregates showed high levels of specific binding to D-mannose, D-glucose residues and *N*-acetyl-D-glucosamine/sialic acids and these molecules were expressed even after the extraction of the cell-bound EPS. The mannose is a hexose sugar with more hydroxyl groups than a pentose sugar and is considered as a promotory sugar (Khandeparker & Anil, 2011; Sahoo & Khandeparker, 2018). Both D-glucose and D-mannose serve as important cues for the settlement of *Balanus amphitrite* cyprids (Khandeparker et al., 2002, 2003; Khandeparker & Anil, 2011). The interactions between these sugars and cypris temporary adhesive are most likely to be affected via polar groups. The higher number of hydroxyl groups influence stronger attachment with polar groups of cyprid antennules (Neal & Yule, 1996; Sahoo & Khandeparker, 2018). On the other hand, sialic acids are sugars, which play an important role in cellular recognition, cell–cell attachment and signalling (Sigma-Aldrich, 2009). It is well known that several pathogenic bacteria or parasites use surface sialic acids for attachment to the host cell (Sigma-Aldrich, 2009). Moreover, relatively high amounts of these molecules (*N*-acetyl-D-glucosamine, D-glucose and D-mannose) have been detected in the algal cell wall surfaces as well (Tien et al., 2005). Earlier studies have also reported that *N*-acetyl-D-

glucosamine also plays a significant role in the attachment of fouling diatoms (Bahulikar & Kroth, 2008; Khodse & Bhosle, 2010). A recent study demonstrated the importance of *N*-acetyl-D-glucosamine expressed by diatoms and its interaction with barnacle shell proteins in facilitating cyprid metamorphosis (Sahoo & Khandeparker, 2018). *N*-acetyl-D-galactosamine along with D-glucose and D-mannose produced by haemocytes have been reported as cues for the settlement of *B. amphitrite* (Khandeparker et al., 2019). Thus, the results from the present study indicate the involvement of *N*-acetyl-D-glucosamine/sialic acid, D-mannose and D-glucose in the bacterial cell–cell interactions, which are similar to the cues reported for the attachment of fouling diatoms and recruitment of larger macrofouling organisms.

FT-IR analysis has been recognized as a powerful tool to understand the structural changes occurring in the bacterial cell surfaces in response to environmental changes (Kamnev, 2008). The biomolecules, mainly sugars, proteins and lipids in the bacterial membrane have distinct infrared (IR) vibrations that indicate their conformation and physical state (Pan et al., 2017). The results revealed that before the extraction of cell-bound CB-EPS, some peculiar proteins and lipids were expressed by the freshwater coaggregates, as evident from the spectra, probably to carry out cell–cell interactions. On the other hand, after EPS extraction, the proteins and lipids were expressed by the estuarine coaggregates, which possibly helped them in retaining the biofilm-forming capability. Alternatively, the same was not the case with the freshwater bacteria, which coaggregated by expression of lipids in the absence of CB-EPS. An earlier study by Andrews et al. (2010), confirmed the importance of lipids, which can influence and facilitate the attachment of different bacteria. Moreover, in this study, the alterations in the carbohydrate region of the cell membrane were also observed, irrespective of the biofilm types, thus highlighting the importance of carbohydrates in the cell–cell attachment (coaggregation) by biofilm bacterial cells over individual bacterial cells. This result is in accordance with the previous studies by Bengtsson (1991), Vandervivere & Kirchman (1993) and Cheung et al. (2000).

Formation of biofilms is a quorum-sensing controlled process (involving cell–cell communication and gene regulation), which involves various bacterial physiological activities, including EPS production (Dobretsov et al., 2009; Hmelo, 2017; Jemielita et al., 2018). Moreover, the secretion of EPS depends on the type of bacteria and environmental factors, which further influences their chemical composition (Sonak, 1998; Khandeparker et al., 2002, 2003; Camilli & Bassler, 2006; Decho & Gutierrez, 2017). As environmental conditions change rapidly, biofilm-forming bacteria possess different mechanisms for sensing and adapting to these changes. One of the important environmental stressors for bacteria is salt stress, i.e. exposure to either high or low salt concentration, which can cause physiological changes in the bacteria and subsequently influence the biofilm formation. Earlier studies have reported that enhanced production of carbohydrates and proteins in the EPS was used as a protective adaptation by bacteria to cope up with salinity stress (Zhao et al., 2016; Kim & Chong, 2017). The present study revealed that the exposure of biofilm-forming bacterial cells (*B. indicus*, *B. cereus* from estuarine and *Exiguobacterium* spp., *B. cereus* from freshwater) to abrupt changes in the salt concentrations (salinity stress) resulted in different physiological response among these bacteria and the subsequent alterations in the surface chemistry of the coaggregates were detected using FT-IR. The estuarine biofilm bacteria could retain their biofilm-forming capability, i.e. by forming aggregates through the expression of proteins and lipids in response to salt stress. Whereas, the freshwater biofilm bacteria also coped up with the salinity stress and formed aggregates through the expression of lipids. Moreover, this type of expression pattern was also observed by the estuarine and freshwater aggregates in the absence of CB-EPS, and probably this is the mechanism by which these bacteria still retained their biofilm-forming ability under unfavourable conditions. The response and mechanism of *Exiguobacterium* spp., under varying salinities, have been reported earlier, wherein the salt-dependent alterations in the cell membranes were reflected in the concentration of phospholipids that dominated it (Remonsellez et al., 2018). Whereas, the involvement of fatty acids and their composition in the *Bacillus* spp. has been reported in response to a wide variety of adaptations (Diomandé et al., 2015 and references within). Unlike *Bacillus* spp., the exposure of salt stress had an influence on the intensity of orange colour pigmentation of *Exiguobacterium* strains during the study period (data not shown), indicating some relation between salt tolerance and pigmentation. One

common trend observed in both the biofilm types was notable changes in the carbohydrate region indicating the significance of carbohydrates in the coaggregation process. Thus, it seems like the salt stress conditions effectively induced quorum sensing in the *Exiguobacterium* spp., and *Bacillus* spp., irrespective of biofilm types, resulting in the alterations in their cell surface chemistry in response to environmental changes, thus retaining their coaggregation potential and these modifications aided in their survival.

Conclusions

The present study is a first report to demonstrate the coaggregation potential of biofilm-forming bacteria from different aquatic environments (freshwater, estuarine and marine), and identify the molecules involved in their aggregation. The biofilm-forming potential via cell–cell interactions of estuarine and freshwater biofilm bacteria was higher, attributed to cell-bound EPS rich in carbohydrates with distinct sugar composition, compared to marine. The carbohydrate content of the cell-bound EPS was influenced by the environment and varied among the biofilm bacteria. Most of the biofilm bacteria, including marine, lost their ability to coaggregate in the absence of cell-bound EPS suggesting its importance in the aggregation process. The estuarine (*B. indicus* and *B. cereus*) and freshwater (*Exiguobacterium* spp. and *B. cereus*) biofilm bacterial pairs, which retained their ability to coaggregate even in the absence of cell-bound EPS, produced specific biomolecules (D-mannose, D-glucose, *N*-acetyl-D-glucosamine on the cell surface) in common. However, the underlying mechanism of the formation of estuarine coaggregates differed from freshwater ones, by expression of proteins and lipids. A similar expression pattern was also observed by the estuarine coaggregates after subjecting to salt-stress conditions, and probably this is the mechanism by which estuarine bacteria switch over and still retain their biofilm-forming capability under changing environmental conditions. Thus, it seems that the capability to coaggregate is influenced by the interplay of the environmental factors, biofilm bacterial species and molecules expressed at the cell surface. As biofilms serve as important settlement cues for the recruitment of macrofouling organisms, understanding the role of the molecules expressed by these biofilms via cell–cell interactions and their influence on the larval settlement and recruitment of macrofoulers under different environmental settings is an important topic for future investigation.

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