

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

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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.

Keywords: Biofilms; Periphyton; Bacteria; Microcosms; *Vibrio* spp; Estuary

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 (Elliot and Quintino, 2007; Elliott and Whitefield, 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 estuary 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., 2010, Malham et al., 2012). The bacterial load, especially potential pathogens in the coastal waters are generally higher than the open sea (Brettar et al., 2007). Monitoring tropical 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, Kastner 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 ~ 5m. 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 2-32 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 x 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 hours. 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=1x3) and each one was scraped separately into 10 ml of 0.22µm autoclaved filtered seawater for microbial counts. 1ml of subsample was used for the analysis of viable and pathogenic bacterial abundance while 5ml of subsample was fixed with paraformaldehyde for enumeration of total bacterial counts. For pigment analysis, twenty seven slides were sampled (n=9x3) and nine slides were scraped separately in 25ml 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 (NO₃), nitrite (NO₂), phosphate (PO₄) and silicate (SiO₄) were analysed using autoanalyser (Skalar SAN PLUS 8505 Interface v3.31, Netherland). The nutrient samples were analyzed 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 hours in the field. After this, one set of slides (n=30) from each station was kept as control as described above in section 2.3 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 (0.22µ 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 x 74 x 138 mm, 450ml, Himedia). All the jars (or microcosms) were closed and static during the incubation. The experimental units were incubated for 5 days under 12h light: 12h dark cycle at room temperature. At the end of the incubation, 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, 5ml 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 analyzed within 30 days of sampling date. For the flow cytometric analysis, 1ml of subsample was sonicated (30W for 60secs) and subsequently passed through BD cell strainer cap (Cat no: 352235) to remove larger particles. The samples were then stained with DAPI (Sigma) at 1µg/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 (1µm, 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 Count (TBC) is expressed as cells/cm² of the slide surface area sampled.

2.6. Enumeration of viable and pathogenic bacteria in biofilms

To enumerate Total Viable Count (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 hours, viable colonies were counted and expressed as CFU/cm² 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=9x3) were scraped and pooled in 25 ml of 0.22 µm filtered autoclaved seawater. Subsequently, the pooled samples were filtered on GF/F 25mm Whatman filters in dark condition and kept in -20°C until further analysis. The samples were analyzed within 30 days of sampling date. For pigment analysis, filters were extracted using 90 % HPLC grade acetone and extracts were filtered through 0.22µm 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 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 *c*2), 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.

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 & 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 6.0 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 25.2-31.2 (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 NO₃ (PostM (50.6 μmol) > PreM (12.5 μmol) > MoN (6.7 μmol) and PO₄ (PostM (12.3 μmol) > MoN (6.7 μmol) > PreM (4.2 μmol) concentrations were higher at the upstream end and decreased towards the mouth of the estuary during spring tide. SiO₄ 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' Lambda: $p < 0.001$). 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. 3A3-3B3).

Total coliforms were abundant in marine and brackish biofilms during MoN, irrespective of tides (Fig 2C). They were influenced by SiO₄ during spring tide and NO₂ 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, 2F). Other than salinity, *V. cholerae* were also related to SiO₄ 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 ($r = 0.68$, $p \leq 0.05$) (Fig. 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 (Fig. 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, $p \leq 0.001$).

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 and brackish water showed Vio and Allo pigments with 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 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 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, 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 silicates 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., 2008, 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 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 Jurgens, 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 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 allow them to pass through 0.22µm membrane filters (Li and Dickie, 1985). They have developed starvation-survival strategies to survive prolonged 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 favourable, 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 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 5day aged biofilms during PreM spring tide. Pheophytin is an indicative of the physiological status of phytoplankton biomass and its transformation (Ahel et al., 1996). 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 et al., 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 lead 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.

Acknowledgements

We are grateful to the Director, National Institute of Oceanography, for his support and encouragement. We gratefully acknowledge Dr Dattesh Desai, Senior Scientist, NIO, for his support during the course of this work. This work was supported by Ballast Water Management Program, India (Ministry of Shipping and DG shipping) and CSIR funded Ocean Finder Program (PSC0105). We also thank colleagues of the BBD department for their help. This is an NIO contribution No #####

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Legends to Figures

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

Fig. 2 Spatio-temporal and tidal variations of (A) Total bacterial count, (B) Total viable count, (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 mean based on three replicate samples. The asterisk (*) above bars indicate significant differences within the samples. Different letters indicate significant seasonal difference between the groups (Tukey's HSD, $p < 0.05$).

Fig. 3 Redundancy analysis (RDA) of one-day old biofilms showing 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 – RDA triplot with viable bacteria; A2 – 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 surface and near bottom waters respectively. $P \leq 0.05$ are highlighted in bold.

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 abundance, TVC- total viable abundance, TC- total coliforms. Shaded grey area represents prominent response of bacteria after translocation. The asterisk (*) represent significant response (Tukey's HSD, $p < 0.05$) and arrows represent seasonal response of biofilm bacteria to environmental changes.

Fig. 5 Response of total bacteria count, total viable count 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 mean based on three replicate samples. Different letters indicate significant difference between control and treatments (Tukey's HSD, $p < 0.05$).

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 mean based on three replicate samples. Different letters indicate significant differences between the control and treatments (Tukey's HSD, $p < 0.05$).

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 ($\mu\text{g. cm}^{-2}$) are indicated in the figures by the side of the pie charts).

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 ($\mu\text{g. cm}^{-2}$) 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 ($\mu\text{g. cm}^{-2}$) are indicated in the figures by the side of the pie charts).

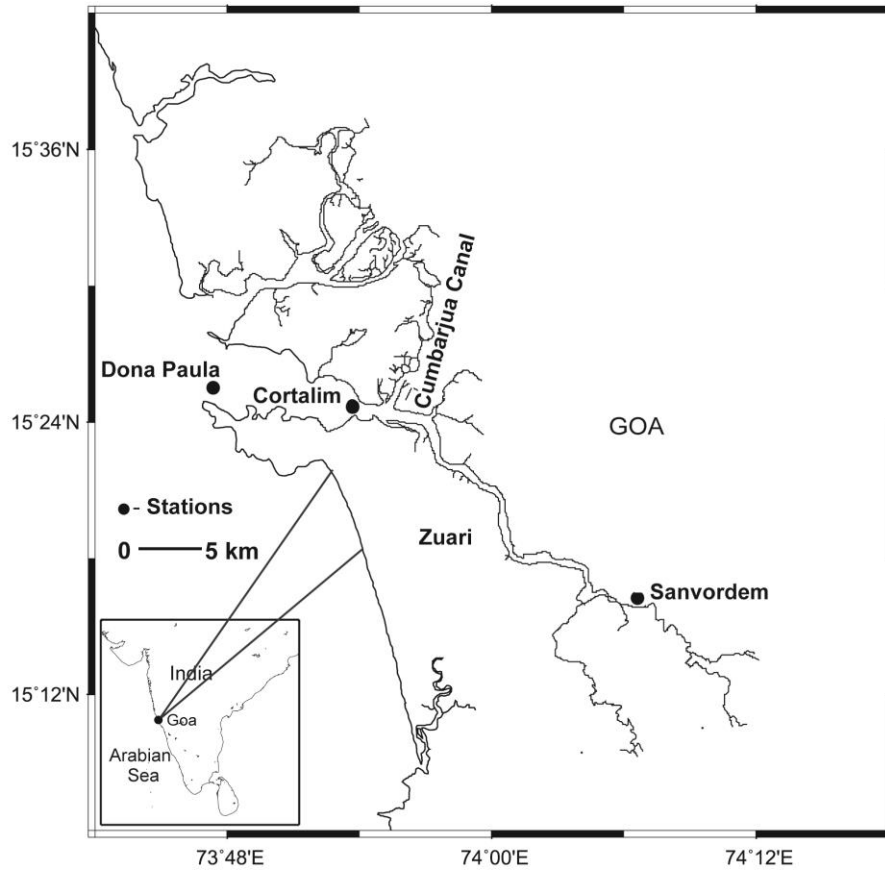
Fig. 10 Response of periphyton pigment composition in one-day old freshwater biofilm communities developed during neap tide, after five day incubation in the laboratory using microcosm experiments. MBF – Marine biofilms, BBF – Brackish biofilms, FBF – Freshwater biofilms, MW – Marine water, BW – Brackish 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 ($\mu\text{g. cm}^{-2}$) are indicated in the figures by the side of the pie charts).

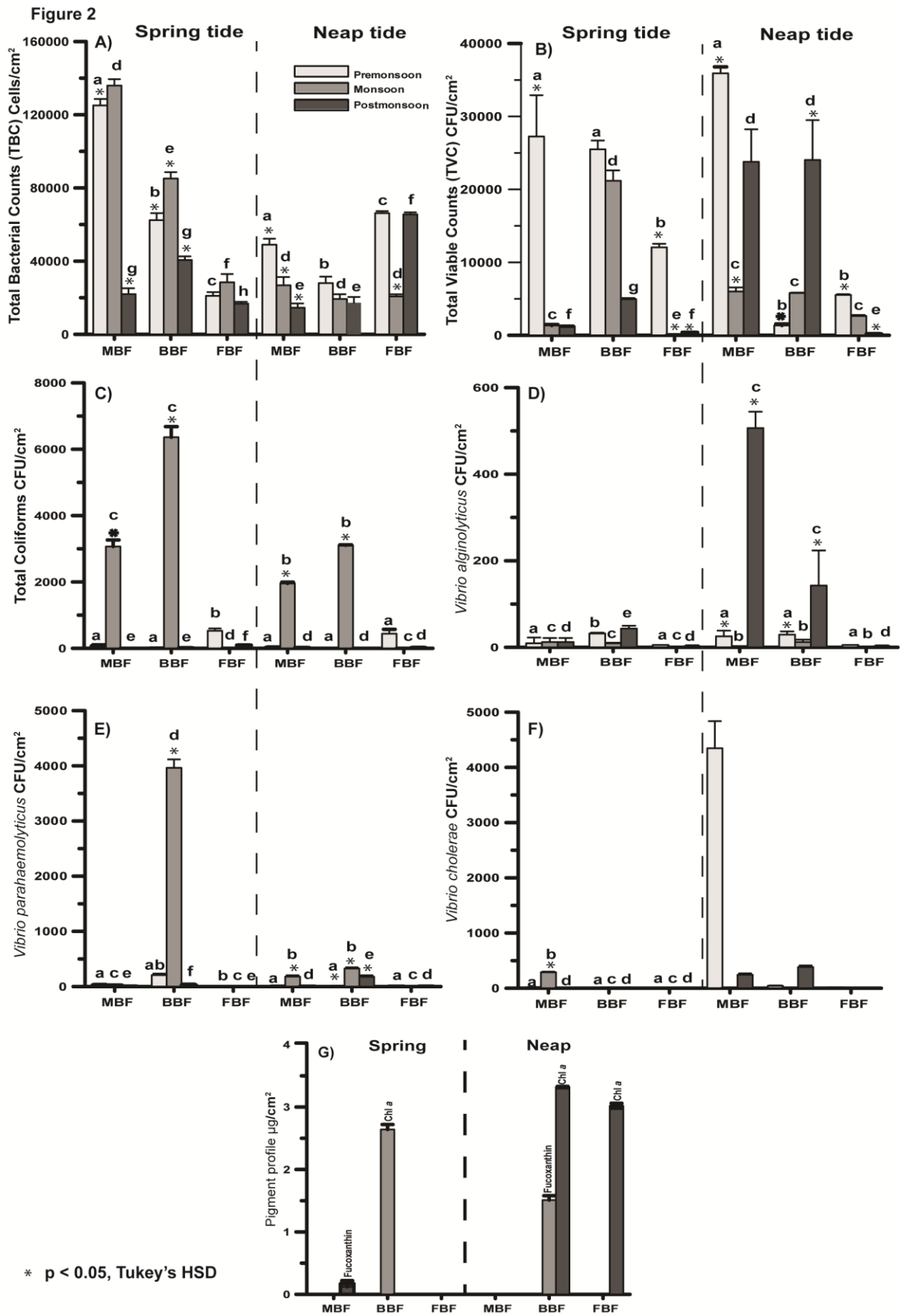
Legends to Supplementary tables

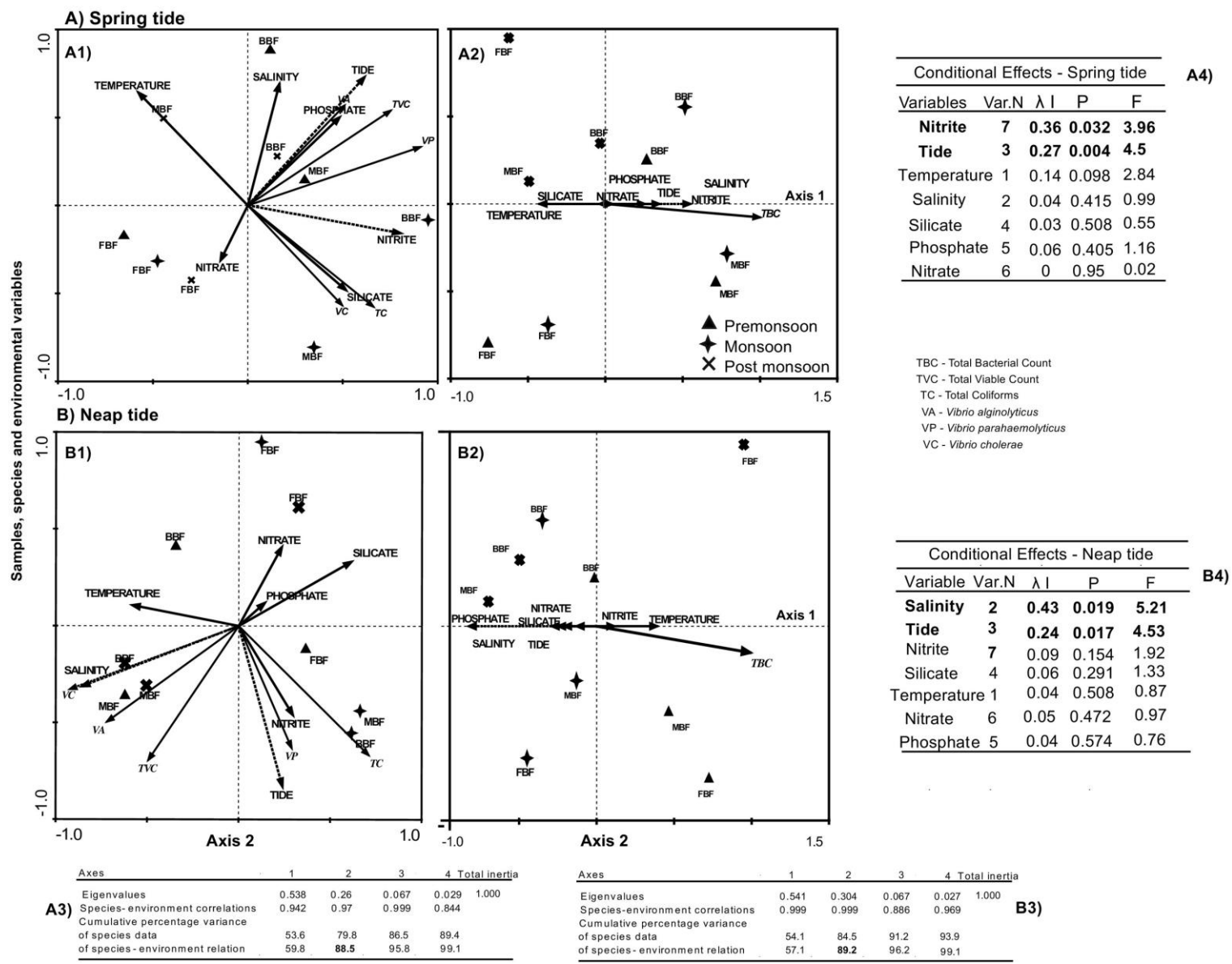
Supp. Table 1 Mean variation of physicochemical factors from marine to freshwater end of Zuari estuary during spring and neap tide

Supp. Table 2 Pigment signatures used in the phytoplankton taxonomy.

Figure 1







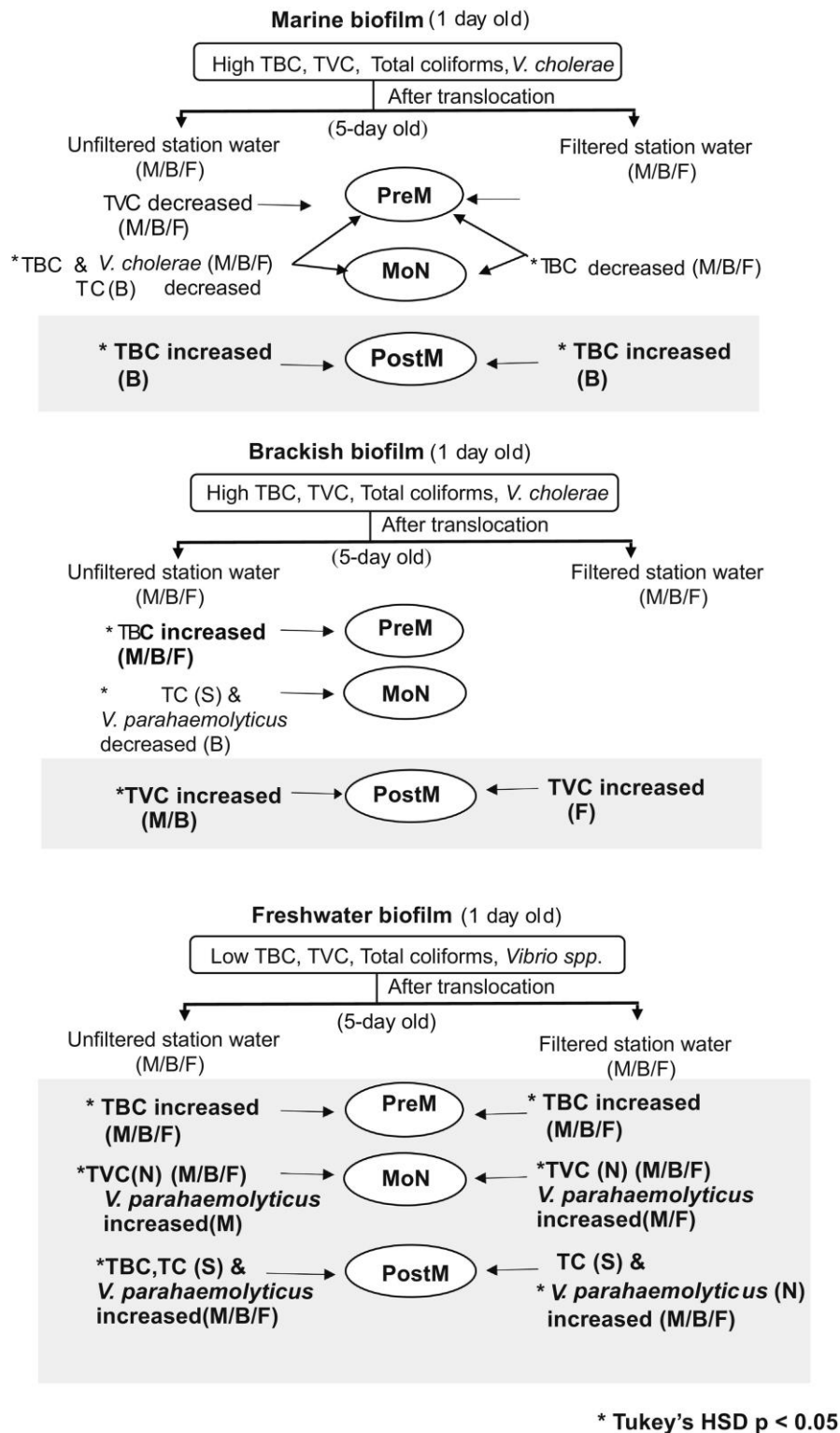


Figure 4

Figure 5

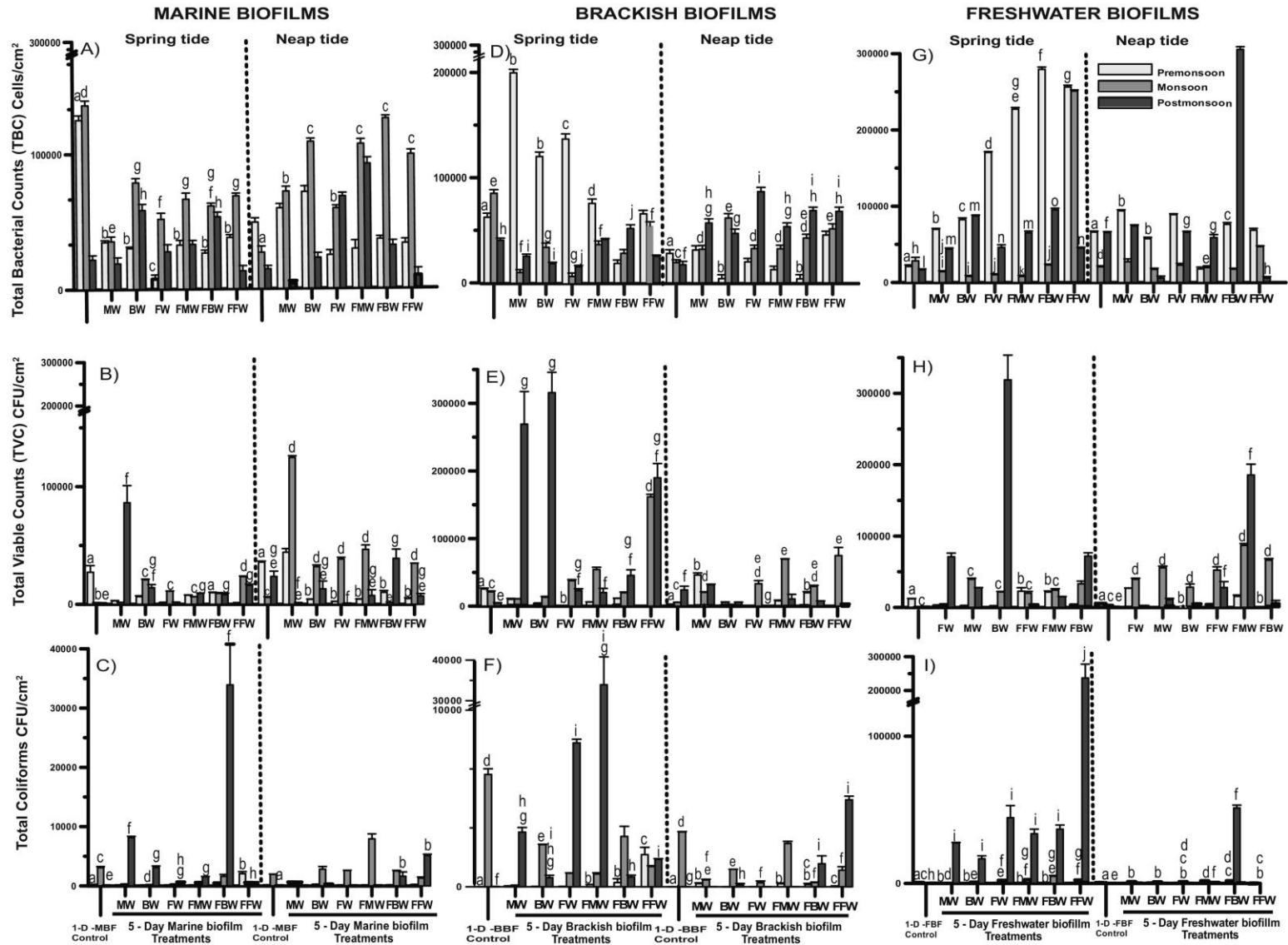


Figure 6

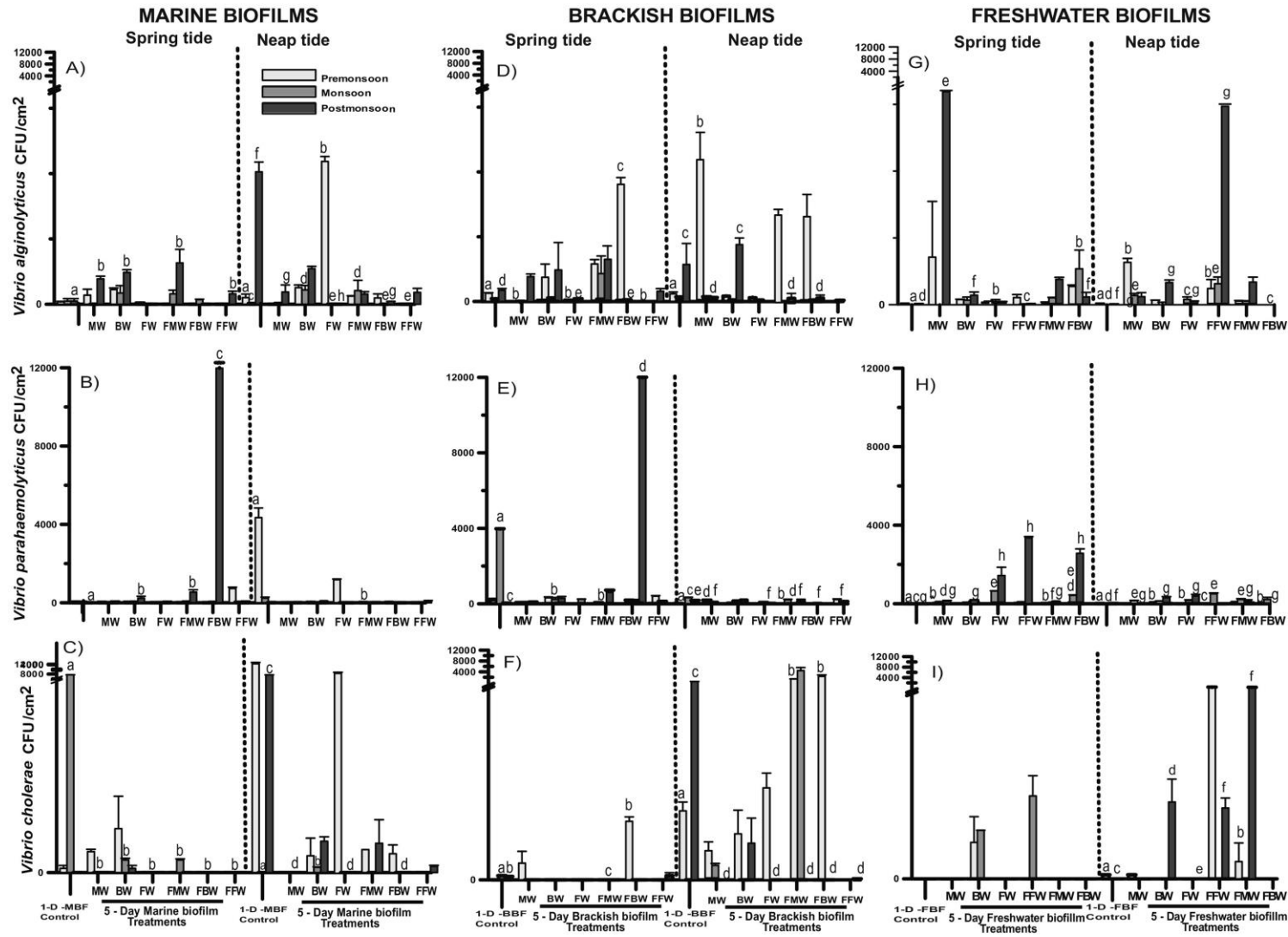
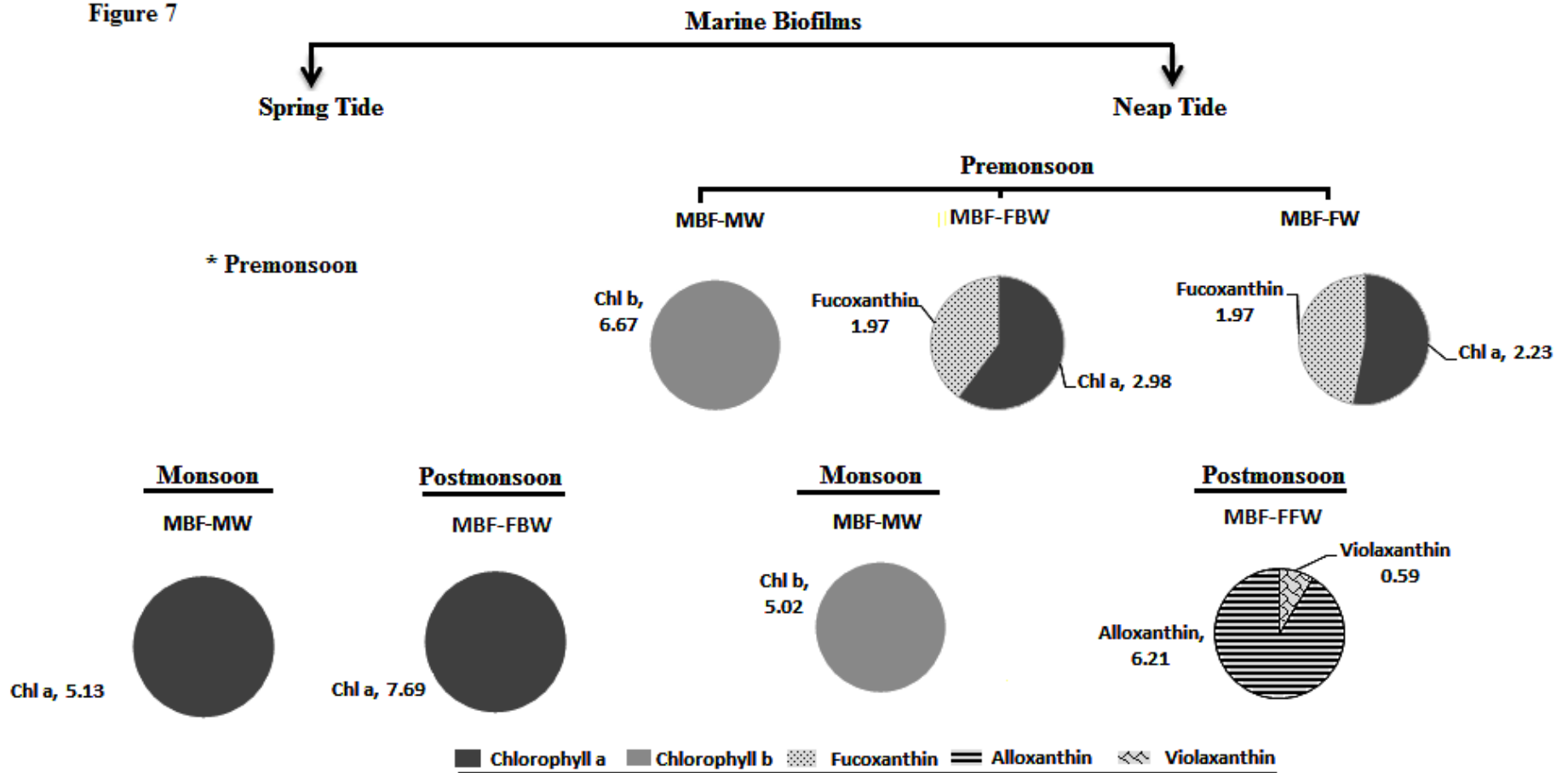


Figure 7



* Not Detected

Figure 8

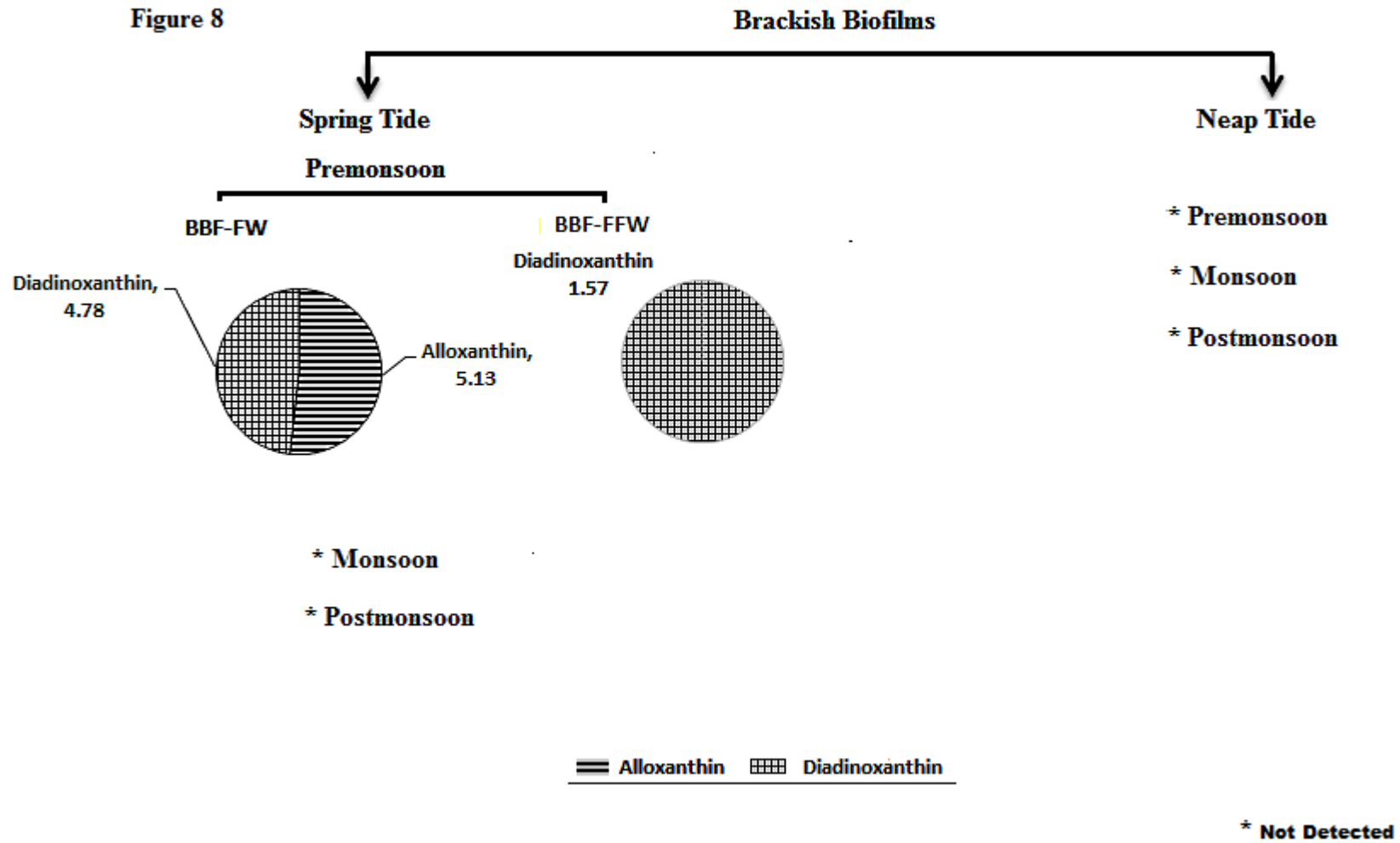


Figure 9

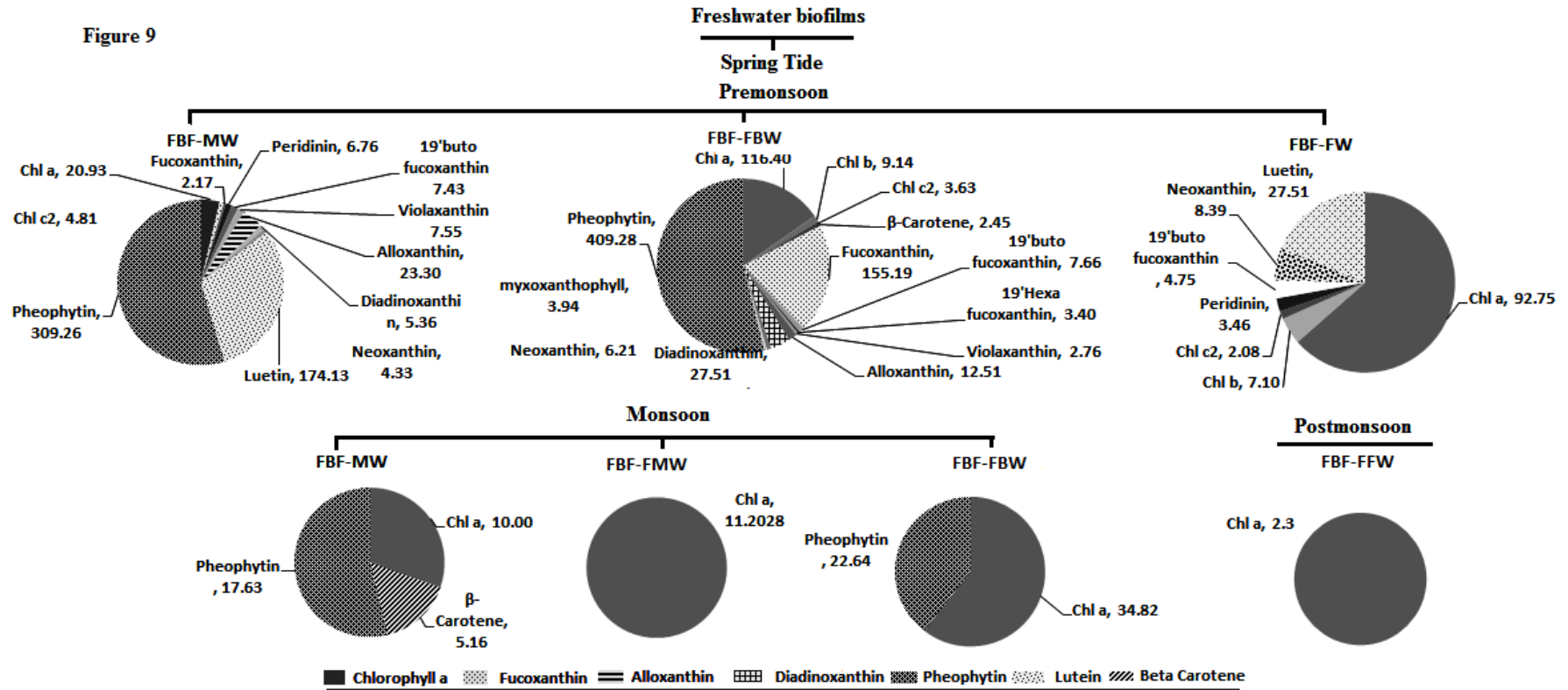
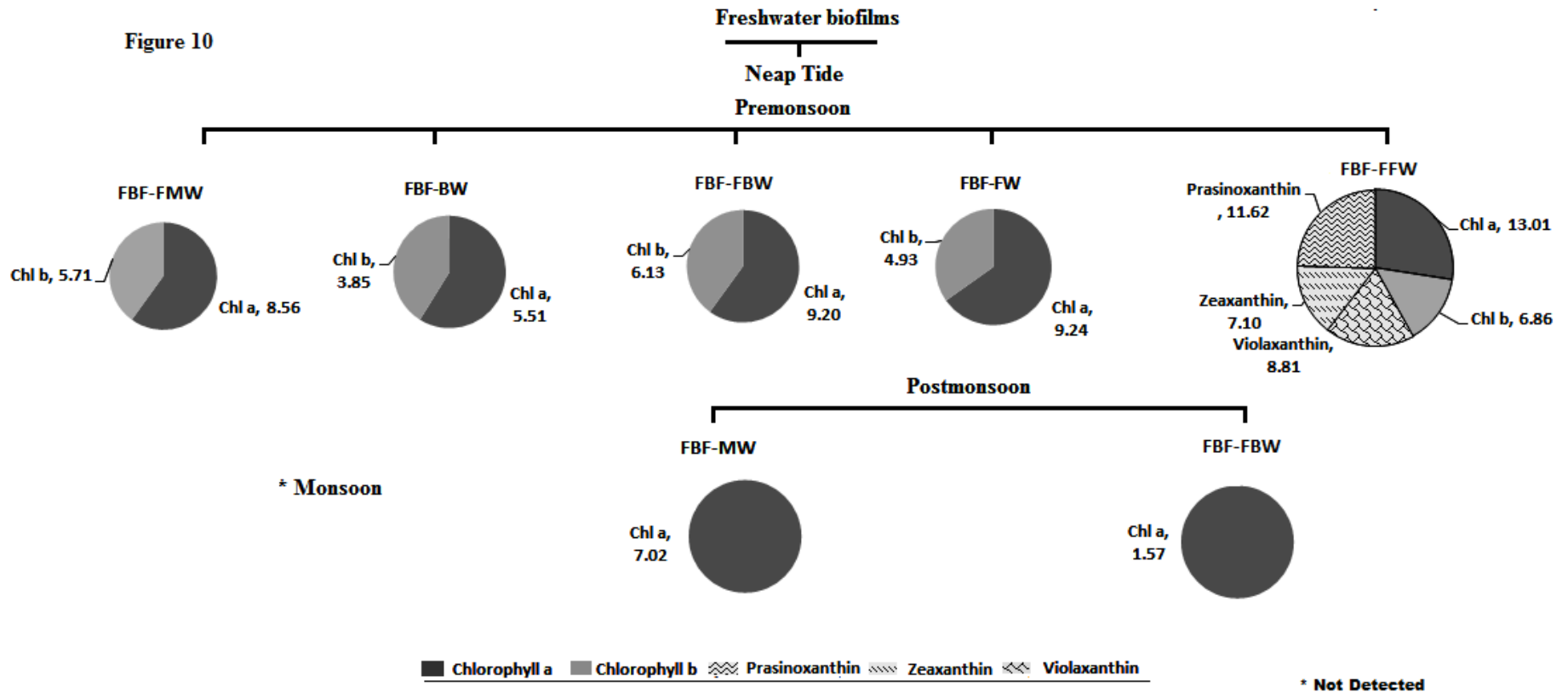


Figure 10



Supplementary Table 1

Mean variation of physicochemical factors from marine to freshwater end of Zuari estuary during spring and neap tide

Parameters	Premonsoon (PreM)						Monsoon (MoN)						Postmonsoon (PostM)					
	Marine		Brackish		Freshwater		Marine		Brackish		Freshwater		Marine		Brackish		Freshwater	
	Sp	Np	Sp	Np	Sp	Np	Sp	Np	Sp	Np	Sp	Np	Sp	Np	Sp	Np	Sp	Np
Temperature (°C)	30.2	30.79	31.11	31.85	31.23	32.31	25.6	25.8	27.0	27.76	29.46	27.76	29.65	30.00	30.19	30.82	29.46	29.96
Salinity (psu)	35.1	34.85	31.21	28.68	0.37	0.128	15	12	2.13	0.062	0.026	0.648	32.83	31.96	25.21	24.37	0.026	0.036
Tide (cm)	195	189.4	214.4	167.1	175.9	199.8	185	198	205	212.4	174.8	173.5	194.3	189.9	211.5	204.6	176.2	175.9
SiO ₄ (μM)	26.2	8.74	67.3	31.93	69.96	68.94	167	173	193	146.9	131.4	222.9	27.82	36.88	42.52	125.4	26.04	131.2
NO ₃ (μM)	12.2	8.93	11.65	ND	12.48	0.83	8.02	4.41	9.23	13.78	6.69	21.25	0.07	ND	8.67	8.33	50.58	10.61
NO ₂ (μM)	6.06	2.31	2.8	ND	0.83	0.27	3.95	4.68	7.88	0.94	2.23	ND	0.75	1.27	0.12	0.68	1.76	1.76
PO ₄ (μM)	1.44	0.55	20.18	1.61	4.23	0.38	2.48	2.8	26.7	4.99	3.21	6.21	13.03	4.03	20.18	3.16	12.33	0.9

Sp – Spring tide

Np- Neap tide

ND- Not determined

Supplementary Table 2

Pigment signatures used in the phytoplankton taxonomy (Jeffrey et al., 1997; Ston and Kosakowska, 2000; Barlow et al., 2012; Moreno et al., 2012)

Biomarker	Phytoplankton Groups
Chlorophyll a Chlorophyll b Chlorophyll c1c2	Total algal biomass Green Flagellates Diatoms, Prymnesiophytes, Cryophytes, Dinoflagellates
Fucoxanthin Peridinin 19'BF +19'HF	Diatoms Dinoflagellates Nanoflagellates Haptophytes Chrysophytes
19' - (Butanoyloxyfucoxanthin) + 19' - (Hexanoyloxyfucoxanthin)	Prymnesiophytes
Alloxanthin Zeaxanthin Myxoxanthophyll Neoxanthin	Cryptophytes Cyanobacteria Green Algae and Land plants
Violaxanthin Lutein Diadinoxanthin	Prasinophyta Chlorophytes Euglenophyta Bacillariophyta Bolidophyta Chrysophyta Raphidophyta Haptophyta Dinophyta Prasinophyta
Prasinoxanthin	
Photosynthetic pigments (PSC)	Peridinin, Fucoxanthin, α -carotene
Photo protecting carotenoids (PPC)	Diadinoxanthin, Alloxanthin, Zeaxanthin, Lutein, Neoxanthin, Violaxanthin and β -carotene.

Reference

Ston, J., Kosakowska, A., 2000. Qualitative and quantitative analysis of Baltic phytoplankton pigments. *Oceanologia* 42, 449–471.