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## Seasonal variation in bacterial community composition and $\beta$ – glucosidase expression in a tropical monsoon-influenced estuary

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### **ABSTRACT**Abstract:

Tidal estuaries receive carbohydrate-rich organic matter from various sources, which are mainly broken down by enzymatic processes and  $\beta$  – glucosidase ( $\beta$ -Glu) is one of the ectoenzymes produced by heterotrophic bacteria. An investigation was carried out along a tropical monsoon-influenced Zuari estuary to examine the seasonal variability in major bacterial taxa,  $\beta$ -Glu gene

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abundance, and number of cells expressing  $\beta$ -Glu. The results revealed a higher number of  $\beta$ -Glu-expressing cells,  $\beta$ -Glu gene, and bacterial abundance at the estuarine mouth when compared to the upstream and was coupled with high phytoplankton biomass and low transparent exopolysaccharides. A clear distinction in bacterial assemblages along with  $\beta$ -Glu expression levels was evident during different seasons. Likewise, a spatial pattern in major bacterial taxa was observed along the salinity gradient, with high proportions of  $\alpha$  and  $\gamma$ -*proteobacteria* at the saline mouth and  $\beta$ -*proteobacteria* at the upstream freshwater region. During the southwest monsoon which brings high freshwater influx, *Actinobacteria* and *Firmicutes* were high at the mid-estuarine region. Bacterial production at this time was mainly fueled by allochthonous riverine sources with a significant decrease in the number of cells expressing  $\beta$ -Glu to gene ratio. The  $\beta$ -Glu gene abundance was related to diverse groups ( $\alpha$ ,  $\gamma$ -*proteobacteria*, *Actinobacteria*, and *Firmicutes*). The number of cells expressing  $\beta$ -Glu covaried with  $\gamma$ -*proteobacteria* abundance and was influenced by suspended load, salinity, chlorophyll *a* and organic pool. Future studies should assess response of marine, brackish, and freshwater bacteria to different environmental conditions to ascertain their specific role in organic matter processing.

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**Key Words** **KEY WORDS:**  $\beta$  – Glucosidase; Estuary; Q-PCR; Ectoenzymes; Tides; Flow cytometry; Bacterial community

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## INTRODUCTION

Estuaries are considered as a resource pool of diverse microorganisms (Hu et al. 2014). The mixing between fresh and marine waters results in significant changes in structure and growth rates of the bacterial communities in an estuarine system (Henriques et al. 2006, Campbell & Kirchman 2013). These biological communities often respond to changes in the input of different substrates due to riverine influences, land run-off and thereby an alteration in organic matter (OM) quality and composition.

Carbohydrates, which form a significant portion of estuarine and marine OM, are released as simple sugars or complex polymeric forms (Hedges et al. 1988; Decho 1990). Transparent exopolysaccharides (TEP) are such polymers abundant in estuarine and oceanic waters (Passow 2002). In estuaries, they play a prominent role in OM cycling either through degradation, aggregates formation or sedimentation processes (Simon et al. 2002, Barrera-Alba et al. 2009, Mari et al. 2012). Microbial degradation of TEP and other polymeric forms is mainly mediated by a group of hydrolytic ecto or extracellular enzymes; the hydrolysis is a rate-limiting step in the aquatic environment (Hoppe 1983, Chrost 1990).  $\beta$ -Glucosidases ( $\beta$ -Glu; EC 3.2.1.21) are hydrolytic enzymes produced by bacteria to cleave beta(1-4) glycosidic linkages of complex carbohydrates. The production of these ectoenzymes is regulated by composition, concentration, and the availability of OM (Chrost 1991). The other environmental factors which can alter the ectoenzyme activity are temperature, salinity, dissolved oxygen, nutrients, and pH (Hoppe et al. 1996, Riemann et al. 2000, Cunha et al. 2001, Arrieta & Herndl 2002). Few researchers have observed a direct relation between  $\beta$ -Glu activity, bacterial abundance, and primary production (Chrost 1989, Romani & Sabater 1999, Cunha et al. 2000), but they focused on total bacterial population and their activity with not much emphasis on the bacterial community structure.

The recent developments in molecular methods have revealed an enormous bacterial diversity which is capable of producing an array of hydrolytic enzymes and established a link between bacterial community composition (BCC) and their metabolic activity (Lammle et al. 2007, Kielak et al. 2013). Many studies are carried out in estuarine and coastal waters relating BCC and hydrolytic activity, but all are restricted to temperate and sub-tropical regions (Kirchman et al. 2004, Haynes et al. 2007, D'ambrosio et al. 2014, Li et al. 2015), and not much have been explored in tropical monsoonal estuaries. Quantitative PCR (Q-PCR) and flow cytometry (FCM) are promising and cost-effective techniques in such community expression studies and relate to functional diversity (Deepak et al. 2007; Steenbergh et al. 2011). Few studies have also used FCM in differentiating cells, which are expressing different hydrolytic enzymes (e.g., chitinase, phosphatase) from total bacterial populations and linked to environmental variables (Steenbergh et al. 2011, Beier et al. 2012).

However, the studies involving the use of these tools in elucidating the relation between the number of cells expressing  $\beta$ -Glu and BCC are limited and was addressed in the present study.

The present study area, Zuari is a monsoonal estuary influenced by high runoff periods and shows a non-steady state (Vijith et al. 2009). Freshwater discharge occurs in high magnitude during southwest monsoon season, which regulates the hydrodynamics of this system on an annual scale. Hence it is expected that along with the changes in the biogeochemical processes; major bacterial taxa would significantly differ from those in dry condition. We hypothesized that high allochthonous inputs during the monsoon would influence and alter the bacterial populations and trigger or decline the  $\beta$ -Glu gene expression. The aim of the present work was to assess the influence of seasons on major bacterial taxa with an emphasis on  $\beta$ -Glu expressing populations and relate to environmental variables which trigger their expression.

## MATERIALS AND METHODS

### Sampling Sites and collection

Surface water samples were collected along the Zuari estuary during premonsoon (PreM; 20<sup>th</sup> February 2015 & 21<sup>st</sup> April 2015), southwest monsoon (SW-Mon; 24<sup>th</sup> August 2015 & 3<sup>rd</sup> September 2015), and post monsoon seasons (PostM; 16<sup>th</sup> October 2015 & 23<sup>rd</sup> October 2015) during spring – neap tidal cycles (Fig.1). The Zuari estuarine system represents large salinity variations ranging from 34 – 0 psu. Based on the salinity, sampling stations were divided into four parts representing mouth (S1, S2), lower middle (S3, S4), upper middle (S5, S6), and upstream (S7, S8) (Fig. 1). Samples from eight different stations were collected using 5 L Niskin sampler and transferred to acid washed carboys and stored on ice until brought to the laboratory.

### Physico – Chemical parameters

Water temperature and salinity were recorded in situ using a portable conductivity – temperature – depth (CTD) probe (Seabird 19plus, USA) with the accuracy of 0.005 °C in temperature and 0.005 psu in salinity. Dissolved oxygen (D.O) was measured using Winkler's method (Parsons et al. 1984). Dissolved nutrients concentration such as nitrite, nitrate, phosphate,

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silicate, and ammonia were measured using an automated nutrient analyzer (Skalar SAN PLUS 8505 Interface v3.31, Netherland).

Total phytoplankton biomass was estimated as total chlorophyll *a* (Chl *a*). Briefly, 500 ml of water samples were filtered through GF/F filter papers and subsequently overnight extraction was carried out using 90 % acetone and the raw fluorescent units (RFU) were measured using fluorometer (Turner, USA). Similarly, surface water samples were filtered on pre-ashed and pre-weighed filter papers (GF/F) in triplicate for measuring suspended particulate matter (SPM). To analyze dissolved organic carbon (DOC) content pre-ashed GF/F filtrates were also collected in pre-combusted glass vials (450 °C for 3 hrs) and acidified using 2 % H<sub>2</sub>SO<sub>4</sub>. Filter papers with the particulate materials were dried in the oven at 60 °C and reweighed on a weighing balance (Mettler Toledo). Total and dissolved organic carbon was measured using total organic carbon analyzer (Shimazu; Model: TOC – L). Particulate organic carbon (POC) values were calculated from the mean difference between the total and DOC and expressed as mg L<sup>-1</sup>. For TEP analysis, samples were filtered on 0.4 µm filter papers (HTTP; Millipore, USA) and analyzed using modified dye binding assay protocol as described by Klein et al. (2011). Gum Xanthan was used as the standard, and TEP values are expressed as µg equivalent Xanthan Gum litre<sup>-1</sup> (µg. equiv. X. L<sup>-1</sup>).

#### Flow cytometric analysis

##### Enumeration of number of cells expressing β-Glucosidase and total bacterial count (TBC)

The number of cells expressing β-Glu were enumerated using Fluorescein di-β-D-glucopyranoside (FDGlu; Molecular Probes, USA) coupled with flow cytometry. Before sample analysis, this method has been validated using β-Glu positive pure strain (*Exiguobacterium indicum*; NCBI accession number: KR047884), and details are provided in the supplementary material. Briefly, non-fluorescent FDGlu substrate is hydrolyzed by β-Glu via two-step process; first to fluorescein mono glucoside (FMG) and later to highly fluorescent fluorescein. A few studies have used FDGlu in measuring exo-β-Glu activity of yeast and recombinant bacterial strains (Kohen et al. 1993, Cid et al. 1994). The surface water samples were passed through the cell strainer cap (40 µm mesh size, BD Biosciences) to remove larger particles. For β-Glu activity, one set of samples (n = 3) were incubated with FDGlu (2mM final concentration, Molecular Probes, USA). The FDGlu working stock solutions were always stored on ice to prevent hydrolysis. Control samples (n=3) were also prepared without addition of FDGlu. Subsequently, the stained, as well as the control samples, were incubated for 30

mins at 30° C in the dark. After incubation, the samples were analyzed using a BD FACSAria™ II flow cytometer (BD-Biosciences, USA) equipped with a 488nm nuclear blue laser. The cells with exo β-Glu activity hydrolyzed FDGlu, and the emitted light signal was collected through different filter sets: (1) 488/10 band pass for right angle light scatter (RALS), (2) 530/30 band pass filter for green fluorescence signal from the fluorescein hydrolysis product of FDGlu (Suppl. Fig. 1A-E). Fluorescent beads (1µm, Polysciences) were used as internal standards for calibrating the above parameters. Gating was done against green versus RALS signals to determine the number of cells expressing β-Glu (Suppl. Fig. 1D).

The TBC Samples were preserved using 1 % paraformaldehyde (final concentration) at -20 °C were thawed to room temperature (n=3), stained with SYBR Green I and incubated in the dark for 15 mins (1:10000 final concentration; Molecular Probes, USA). After incubation, the stained samples were analyzed using a BD FACSAria™ II flow cytometer equipped with 530/30 bandpass green filter and calibrated using 1 µm fluorescent beads. The collected data sets were processed using BD FACS Diva software (Version 6.2).

#### Bacterial production (BP)

In the present study, 5-Bromo-2-Deoxyuridine (BrdU) was used to measure the BP in estuarine samples. BrdU is a thymidine analog and can be incorporated specifically into actively synthesizing DNA in place of thymidine. Many studies warrant that BrdU is a suitable substrate for measuring BP in pelagic environments (Pernthaler et al. 2002, Hamasaki 2006). BrdU was added to the surface water samples (1µM final concentration; BD-Biosciences, USA) and incubated for 3 hrs at in situ temperature. After incubation, the samples were fixed with paraformaldehyde (1% final concentration) and stored at -20° C until BrdU immunofluorescence detection (IFD). IFD was performed using FITC-labeled anti-BrdU. In brief, samples stained with FITC labeled anti – BrdU with DNase I (BD – Biosciences) were incubated for 30 mins in the dark and analyzed using a BD FACSAria™ II flow cytometer equipped with a 488nm nuclear blue laser. The samples without BrdU were used as controls. For calculating the bacterial carbon production, 11 fg C per bacterium as a cell-to-carbon conversion factor was used (Garrison et al. 2000).

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#### Enumeration of protists abundance

Protists abundance was determined using the modified protocol described by Christaki et al. (2011). Briefly, paraformaldehyde fixed (1% final conc) frozen samples were thawed, stained with SYBR Green I (1:10000 final conc Molecular Probes, USA) and incubated in the dark for 15 mins before measurement. Analysis of stained samples was carried out using BD FACS Aria II instrument. Emitted light was collected through 488/10 band pass filter for right angle light scatter (SSC), 530/30 band pass filter for green fluorescence and 695/40 for red fluorescence. The detector voltage was reduced based on SSC vs. green signal plot to cut off the intrusion of bacterial populations with protists abundance. 1µm yellow – green fluorescent beads (Polysciences) were used as an internal standard. The data sets were processed using BD FACS Diva (Version 6.2) software-

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#### Extraction of DNA

Water samples collected in the field were brought to the laboratory under cold condition. Subsequently, 2 L of samples were filtered onto 0.22 µm pore size filters (GSWP04700; Millipore, USA) under low vacuum. The filters were then stored at -20 °C until the extraction procedure was performed. DNA extraction was performed using power water DNA isolation kit (MOBio, USA) as per manufacturer's instructions.

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#### Quantitative-PCR (qPCR) analysis

##### Bacterial community composition (BCC)

Q - PCR analyses were performed to evaluate BCC using a set of primers which are specific to 16S rRNA genes of *Proteobacteria* subclass ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Table. 1). The reaction was carried out in a real-time PCR system (Rotor-Gene Q 5X HRM, Qiagen, Germany) using Quantitech SYBR Green PCR master mix (Qiagen, Germany). Each 20 µl reaction mix contained 10 µl of 2 × SYBR Green PCR Mix, 0.5 µl of each primer, 1 µl of a template and 8 µl of RNase – free water. Primer sets and thermal cycler conditions are described in

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Table 1 and Suppl. Table. 1 respectively. Melt curve analyses were performed to test the specificity of each primer sets at the end of the cycle. In brief, melt curves were obtained by adding a high-resolution melting (HRM) step at the end of the final cycle. The temperature was raised from 60 to 90 °C with 0.5 °C interval. A clear single peak was obtained between 84 – 86 °C indicating the presence of site-specific PCR amplicon (Suppl. Fig. 2). A tenfold serially diluted purified PCR product containing target DNA was used to generate the standard curve for quantification of unknown target samples. The curves were attained by plotting the cyclic threshold (Ct) values against the logarithm of the concentration of each 10-fold dilution series of PCR fragments. A known series of standards in triplicate were included during each run to correct the potential error for unknown samples. The reaction efficiency was always noted to be 0.98 to 1.0. Data sets were processed using Rotor-Gene Q Software 2.3.1.49 (Qiagen).

#### Quantification of $\beta$ -Glucosidase gene

In the present study, the gene encoding  $\beta$ -Glu was quantified by Q-PCR using a set of degenerated primers (BgluF2/BgluR4; Table. 1) as described by Canizares et al. (2011). The standard curve and Ct was calculated by serially diluting a purified PCR amplicon of a  $\beta$ -Glucogene from *Exiguobacterium indicum*. Further, the specificity of the primer set was evaluated using a melt curve analysis, and obtained data were processed using Rotor-Gene Q Software 2.3.1.49 (Qiagen).

#### Statistical analysis

Before statistical analysis, data were  $\log(x+1)$  transformed to meet assumptions of parametric tests. Shapiro-Wilk test proved normality of log-transformed data. Three-way analysis of variance (Three-way ANOVA) followed by post hoc Tukey's HSD test was performed to assess the significant temporal and spatial variation in bacterial components and environmental variables along the estuary with tides, seasons, and stations as factors (IBM SPSS Statistics 22). Further, a similarity (SIMPROF) test was performed to identify the spatial variation in BCC using Primer 6 software (Clarke & Gorley 2006).

The relations between biotic (TBC, BP, protists abundance,  $\beta$ -Glu gene abundance, number of cells expressing  $\beta$ -Glu, and BCC) and environmental variables were evaluated using multivariate

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redundancy analysis (RDA). The analyses were performed using CANOCO 4.5 for Windows software package (ter Braak & Smilauer 2002). Before RDA analyses, the data were checked for linear characteristics and standardized (length of first detrended correspondence analysis (DCA) axes  $< 2$  standard deviation (SD) units), and the Monte Carlo test was used to assess the significance of the RDA method ( $p < 0.05$ ; 999 permutations). Meanwhile, generalized linear models (GLM) in a stepwise manner followed by F-statistics were performed to predict the species response to environmental ordination axes in RDA.

## RESULTS

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## Environmental parameters, dissolved nutrients, Chl *a* and TEP

The variations in the environmental parameters along the Zuari estuary during the sampling periods are provided in Fig. 2 and 3 respectively. Briefly, the average surface water temperature was high during PreM spring tide from mouth to the upstream of the estuary (31.36 – 32.48 °C). Salinity ranged from 34 – 0 psu, was high in the mouth, decreased from upper mid to upstream during non-monsoon, with a significant reduction during SW-Mon throughout the estuary (df = 3; F=24.30; p = 0.001; Fig. 2A, B). A similar decreasing trend from mouth to upstream was evident in nitrite (df = 3; F = 17.93; p = 0.001) and nitrate (df = 3; F = 2.28; p = 0.01) concentration during spring tide (Fig. 2C, 3C). Whereas, phosphate and silicate were higher towards the upstream, especially in the SW-Mon (2.90 & 53.76  $\mu\text{mol L}^{-1}$ ) and PostM (4.62 & 95.69  $\mu\text{mol L}^{-1}$ ) irrespective of the tides (Fig. 2G, H, K, & L).

During all the seasons, total Chl *a*, and SPM increased with increasing salinity (Fig. 3A-D). TEP values were high towards the upstream and peaked during spring tide of PostM ( $497.47 \pm 153.81 \mu\text{g equiv. X. L}^{-1}$ ) and SW-Mon ( $345.10 \pm 29.47 \mu\text{g equiv. X. L}^{-1}$ ) respectively (Fig. 3E - F). Overall, organic carbon showed a significant seasonal variability (ANOVA; df = 2; F = 65.20; p = 0.001) with high POC during PostM, and DOC during PreM especially during spring tide (Fig. 3G-L).

## Variation in TBC, BP, and protists abundance

Overall, TBC and BP showed a significant temporal variability (ANOVA; df = 2; F = 13.14; p = 0.0001 & df = 2; F = 12.50; p = 0.0001 respectively) when compared to sites and tide (Fig. 4A-D). The TBC was high during PreM at the mouth ( $1.01 \pm 0.4 \times 10^7 \text{ cells mL}^{-1}$ ), lower mid ( $1.74 \pm 0.7 \times 10^7 \text{ cells mL}^{-1}$ ), upper mid ( $2.44 \pm 0.7 \times 10^7 \text{ cells mL}^{-1}$ ) with a clear decrease during SW-Mon from mouth to upstream ( $2.28 \pm 0.2 \times 10^6$  to  $4.70 \pm 0.9 \times 10^6 \text{ cells mL}^{-1}$ ) irrespective of the tides (Fig. 4A, B). The TBC was positively related to temperature, salinity, Chl *a*, and negatively with TEP (Fig. 5A, C, D, F). Unlike TBC, BP was high towards upstream during SW-Mon ( $7.61 \pm 1.46 \mu\text{g C L}^{-1} \text{ h}^{-1}$ ) and PostM ( $7.31 \pm 1.46 \mu\text{g C L}^{-1} \text{ h}^{-1}$ ), and negatively related to salinity, SPM, Chl *a* and positively with TEP especially during spring tide (Fig. 4C, D and Fig. 5A).

In the case of protists abundance, a significant temporal (ANOVA; df = 2; F=31.00; p=0.0001), spatial (ANOVA; df = 3; F=8.13; p=0.0001), and tidal variation was observed (ANOVA; df = 1; F=32.16; p=0.0001) with higher abundance during neap tide of SW-Mon ( $3.76 \pm 0.5 \times 10^5$

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cells mL<sup>-1</sup>) and PostM ( $4.31 \pm 0.4 \times 10^5$  cells mL<sup>-1</sup>) (Fig. 4E, F). Their abundance increased with decreasing salinity during SW-Mon (mouth<lower mid< upper mid<upstream) and reversed during PreM (mouth>lower mid>upper mid> upstream)(Fig. 4F). The protists numbers were positively related to temperature, SPM, TOC and negatively with Chl *a* and POC especially during neap tide (F= 15.1; p=0.0008; Fig. 5B, F).

#### Variation in $\beta$ -Glu gene abundance and number of cells expressing $\beta$ -Glu

In general,  $\beta$ -Glugene abundance and number of cells expressing  $\beta$ -Glu varied significantly with the sites (df = 3; F=10.85; p=0.0001 & df = 3; F=19.57; p=0.0001 respectively) as well as the seasons (df = 2; F=24.86; p=0.0001 & df = 2; F=43.54; p=0.0001 respectively) (Fig. 4G, H, K, L). The tidal variation was not significant. The number of cells expressing  $\beta$ -Glu increased during PreM ( $4.05 \pm 1.5 \times 10^6$  cells mL<sup>-1</sup>&49% with respect to TBC), and a clear decrease was evident during SW-Mon ( $3.65 \pm 1.3 \times 10^5$  cells mL<sup>-1</sup>&16% with respect to TBC) and PostM ( $1.62 \pm 0.9 \times 10^5$  cells mL<sup>-1</sup>&4% with respect to TBC) from mouth to upstream(Fig. 4G, H& Suppl. Fig. 3A, B).Whereas, gene abundance showed a significant increase from PreM ( $3.18 \pm 0.13 \times 10^6$  copies  $\mu$ L<sup>-1</sup>) to PostM ( $1.51 \pm 0.16 \times 10^8$  copies  $\mu$ L<sup>-1</sup>) (Fig. 4k, L). The gene abundance and number of cells expressing  $\beta$ -Glu were high at the mouth and decreased towards upstream, but vice versa was observed in the case of BP. The uncoupling between BP,  $\beta$ -Glugene and the number of cells expressing  $\beta$ -Glu was more pronounced during SW-Mon (Fig. 5A, D; Suppl. Table. 2C). The gene abundance was positively related to salinity, TOC, and POC (Fig. 5A, C, D,& F). While, number of cells expressing  $\beta$ -Glu were positively related to temperature, salinity, SPM, Chl *a*, DOC and negatively to TEP (Fig. 5A, C, D, F,& Suppl. Table. 2A-C).

#### Variation in BCC and their relation with $\beta$ -Glu gene abundance and number of cells expressing $\beta$ -Glu

Overall, a clear shift in BCC was evident between the seasons and sites (post hoc Tukey's HSD  $p < 0.05$ ; Fig. 6A-L; Fig. 5B, E). Likewise, the total community recovery also varied with the seasons (Suppl. Fig. 3C, D). A higher percentage of populations recovered during PostM (45 – 68%) as compared to other seasons and high recovery was mainly related to  *$\alpha$ -proteobacteria* abundance

(Suppl. Fig. 3C, D). The mouth and lower mid-estuary were dominated by  $\alpha$  and  $\gamma$ -*proteobacteria* which seems to be marine as evidenced by their significant positive relation to salinity (Fig. 5A, D;  $p < 0.05$  and Fig. 6A,B, E, & F). Both the groups were abundant during non-monsoon seasons. The  $\beta$ -*proteobacteria* dominated upper mid and upstream region of the estuary and were negatively related to salinity during SW-Mon (Fig. 6C, D). Similarly, the proportions of *Actinobacteria* and *Firmicutes* were high at the upper-mid estuary, and their abundance increased during SW-Mon and PostM seasons (Fig. 6I - L). The *Bacteroidetes* was observed in significant numbers along the estuary irrespective of the seasons (Fig. 6G, H). The RDA analysis showed a strong coupling between TBC, Chl *a*, SPM, DOC, number of cells expressing  $\beta$ -Glu, and  $\gamma$ -*proteobacteria* abundance during PreM (Fig. 5A-E;  $p < 0.001$ ; Table. 2A-C). Further, a decrease in  $\gamma$ -*proteobacteria* abundance and number of cells expressing  $\beta$ -Glu during SW-Mon was significantly related to protists abundance and salinity change. The  $\beta$ -Glucose abundance was associated with diverse groups ( $\alpha$ ,  $\gamma$ -*proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*) in which  $\alpha$ -*proteobacteria* appeared to be most significant especially during PostM (Fig. 5A, D, Table. 2C,  $p < 0.01$ ). However, non-expression of the gene in these communities could be related to the environmental factors mainly SPM, salinity, and TOC (Fig. 5A, D; Monte Carlo test;  $p < 0.05$ ). The BP was strongly influenced by  $\beta$ -*proteobacteria* abundance, which largely uncoupled with  $\beta$ -Glucose abundance and the number of cells expressing  $\beta$ -Glu during SW-Mon (Suppl. Table. 2C).

## DISCUSSION

In the present study, total bacterial abundance was high during PreM and was mainly driven by temperature, salinity, and TOC. Whereas, bacterial production was high during SW-Mon and PostM towards upstream. A recent study by Kaartokallio et al. (2016) reported similar results in the inner estuarine regions of west and south coast of Finland, and this was related to the strong influence of riverine water mixing with an estuarine gradient. The number of cells expressing  $\beta$ -Glu were high at the mouth (40 to 80% of the total abundance) and lower-mid estuary (10 to 50% of the total abundance) with a seasonal pattern similar as that of total bacterial abundance. Both, the number of cells expressing  $\beta$ -Glu and total abundance was positively related to Chl *a* and negatively with TEP. The high Chl *a* ( $3.44 \text{ mg L}^{-1}$ ) at the mouth indicated high phytoplankton abundance. Most of the estuaries are highly productive, and both autochthonous (in situ production) and allochthonous (external input) substrates influence the bacterioplankton abundance and their production (Coffin &

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Sharp 1987). Heterotrophic bacteria are known to actively consume DOM produced by the phytoplankton cells (Cole et al. 1982, Petit et al. 1999). The phytoplankton produces carbohydrate-rich polymers either as a low molecular weight or polymeric forms that are degraded during enzymatic degradation (Hama & Yanagi, 2001, Steen et al. 2008). TEP's are one of the organic polymers and activity/expression of  $\beta$ -Glu is frequently used to describe the degradation of these carbohydrate-rich polymers (Hoppe et al. 1998). Thus, low levels of TEP at the mouth in the present study indicated their active breakdown by high numbers of  $\beta$ -Glu expressing bacteria.

However, the TEP were high in numbers at the upstream especially during the onset of monsoon, and can be related to a decline in the number of cells expressing  $\beta$ -Glu from mouth to upstream. This relation suggests the influence of large salinity variations in our study area between monsoon and non-monsoon seasons (i.e., < 5 psu at the mouth, and >15 psu towards upstream). A study by Cunha et al. (2000) also pointed out that exo enzymatic activities are significantly related to temperature, salinity and Chl *a*. Similarly, a study at Baltic Sea region showed that the alteration in organic matter composition and salinity via high river loads resulted in a significant change in the enzymatic activity (Daniela et al. 2016). It is known that microbial processes in estuaries are also controlled by the nature, source, and composition of particles in SPM. In the present study, a significant positive relationship was observed between SPM and the number of cells expressing  $\beta$ -Glu. Observation at Elbe turbid estuary also reported a similar correlation between enzymatic activity and SPM and noted to be a controlling factor for the distribution of organisms and nutrients (Bernat et al. 1994). A recent study by Boras et al. (2015) at Blanes Bay too reported a similar relation with  $\beta$ -glucosidase activity. The suspended load in the present study area is influenced by an annual seasonal cycle with high inorganic mineral grains and allochthonous materials during SW-Mon as compared to dry seasons (Gonsalves et al. 2009). Thus, other than salinity, the decrease in the number of cells expressing  $\beta$ -Glu during SW-Mon could be due to change in the SPM composition.

A clear seasonal and spatial shifts in the bacterial community structure were evident. Among the sites, proportions of  *$\alpha$*  and  *$\gamma$ -proteobacteria* were higher at the marine mouth, lower mid region and decreased gradually towards the upstream. A similar finding was reported in other estuarine regions such as Gulf of Delaware (Cottrell & Kirchman 2004, Elifantz et al. 2005), Chesapeake Bay (Bouvier & del Giorgio 2002), Columbia River estuary (Crump et al. 1999), Baltic Sea (Herlemann et al. 2011), and River Pearl estuary (Zhang et al. 2006). The mouth and lower-mid stations showed similar bacterial community composition, and physical characteristics, i.e., salinity. On the other hand, the upstream region was distinctly different and was dominated by  *$\beta$ -proteobacteria*

irrespective of the seasons. Earlier studies have also reported the dominance of  $\beta$ -proteobacteria in freshwater and  $\alpha$ -proteobacteria in saltwater condition (Bouvier & del Giorgio 2002, Kan et al. 2006). The TOC and POC were linearly related to  $\alpha$ -proteobacteria abundance. The temporal variations in bacterial communities are linked to the changes in Chl *a*, temperature, salinity and availability of organic matter pool (Murray et al. 1998, Crump et al. 1999, Bouvier & del Giorgio 2002, Pinhassi et al. 2004, Kan et al. 2006).

Other than environmental factors, grazers can also play a significant role in shaping the bacterial communities (Pernthaler et al. 2001). In the present study, an increase in the protists abundance resulted in a significant decrease in the relative abundance of  $\gamma$ -proteobacteria towards the upstream and during SW-Mon. Beardsley et al. (2003) also reported that selective grazing by heterotrophic nanoflagellates controlled the  $\gamma$ -proteobacteria genera at North Sea region. While the upper mid-estuary showed higher proportions of *Actinobacteria* and *Firmicutes*. Members of these have been detected in the mesohaline condition (<20 psu) and related to freshwater taxa (Newton et al. 2011). Further, members of this clade are sensitive to salinity change and depend on a specific range of organic substrates (Sharp et al. 2008, Campbell & Kirchman 2013). A study by Harji (2011) in the study region showed that terrestrial-derived organic matter mainly dominated the lower-mid estuary due to riverine influence. Thus, it seems that low saline condition during SW-Mon and high input of riverine sources favored the proliferation of freshwater taxa with low  $\beta$ -glucosidase activity at the upper-mid estuary. However, *Bacteroidetes* were fairly persistent along the estuary as observed in other estuaries and their abundance was related to SPM and TEP (DeLong et al. 1993, Bouvier & del Giorgio 2002; Crump et al. 2004). Members of *Bacteroidetes* are known to be dominant in organic-rich particles and possess large degree of hydrolytic enzymes to degrade complex polysaccharides (Azam & Malfatti 2007, Dang et al. 2009, Edwards et al. 2010).

Microbial degradation via ectoenzyme process is one of the major sources of substrate addition in aquatic systems for bacterial utilization processes (Hoppe et al. 1988). Although previous studies had hypothesized that the source of OM and microbial community can significantly influence the seasonality of  $\beta$ -Glu compared to hydrography condition (Bhaskar & Bhosle 2008, Boucher & Debroas 2009), such links were not evaluated in this estuary. During this study,  $\beta$ -Glu gene abundance and the number of cells expressing  $\beta$ -Glu showed a strong seasonality along with the changes in BCC. The number of cells expressing  $\beta$ -Glu to gene abundance ratio were high during PreM and apparently decreased during SW-Mon and PostM as well as towards the upstream. The  $\beta$ -Glu gene copies covaried with the relative abundance of  $\alpha$ ,  $\gamma$ -proteobacteria, *Firmicutes*, and *Actinobacteria* irrespective of the tides. The number of cells expressing  $\beta$ -Glu and the relative

abundance of  $\gamma$ -*proteobacteria* were related and influenced by salinity, Chl *a*, TOC, and SPM. Most of the factors showed a positive loading on the number of cells expressing  $\beta$ -Glu and  $\gamma$ -*proteobacteria* abundance, and negative with  $\alpha$ -*proteobacteria*, *Firmicutes*, and *Actinobacteria*. In estuaries,  $\gamma$ ,  $\alpha$  – *proteobacteria* and *Bacteroidetes* play a significant role in the degradation of DOC and exopolymers and have shown association with microalgae (Hold et al. 2001, Grossart et al. 2005, Haynes et al. 2007). Specifically, members of  $\gamma$  – *proteobacteria* benefit from algae and their derived organic matter (Puddu et al. 2003, Teeling et al. 2012). Further, they contribute significantly in the decomposition of organic matter which is produced by algal communities suggesting  $\gamma$  – *proteobacteria* to be the potential group expressing the  $\beta$ -glucosidase enzyme. A few exopolymers enrichment studies have revealed that members of  $\gamma$  – *proteobacteria* mainly genus *Acinetobacter* and *Pantoea* possess genes for  $\beta$ -Glu production and degrade polysaccharides (Tajima et al. 2001, Arora et al. 2012). Zoppini et al. (2005) also pointed out that organic matter enrichment favored  $\gamma$  – *proteobacteria* growth in Northern Adriatic Sea. A previous study by Khandeparker et al. (2011) in this estuary showed that 65% of the bacterial isolates could produce complex carbohydrate degrading enzymes and majority of the strains included *Vibrio*, *Alteromonas*, *Enterobacter*, *Marinobacter*, *Aeromonas* and *Exiguobacterium* indicating major contribution of  $\gamma$  – *Proteobacteria* taxa in the degradation of carbohydrate derived organic matter. Similarly, a study by Yu et al. (2011) evaluated degradation capability of glucosidase substrates and reported that it was mainly distributed in  $\gamma$  – *Proteobacteria* and *Bacteroidetes* groups when compared to  $\alpha$ -*proteobacteria* and *Actinobacteria*. A study by Moreno et al. (2012) also observed higher genetic diversity of  $\beta$ -Glu encoding genes in *Proteobacteria* followed by the representative members of *Chloroflexi*, *Deionococci*, *Actinobacteria*, *Thermotogae*, and *Firmicutes*. Likewise, Berlemont and Martiny (2013) analysed 5,123 bacterial genome sequences and reported that 79 % of the analysed bacterial genome contained  $\beta$ -Glu gene which included phylum's such as *Proteobacteria* (2200), *Cyanobacteria* (70), *Actinobacteria* (514), *Fusobacteria* (33), *Spirochetes* (236), *Aquificae* (7), *Chloroflexi* (17), *Planctomycetes* (13) and *Acidobacteria* (9). Further, a transplant experiment by Lindh et al. (2015) at Baltic sea basin waters revealed a significant correlation between  $\beta$ -glucosidase activity and taxon such as *Flavobacteriaceae*, *Chromatiaceae*, and *Verrucomicrobia*.

The proportions of  $\gamma$  – *Proteobacteria* decreased during SW-Mon along with a decrease in the number of cells expressing  $\beta$ -Glu throughout the estuary along with the changes in DOM. It is known that in this estuary along the salinity gradient, an upper freshwater region is dominated by terrestrial, and phytoplankton derived DOC dominated the saline mouth region (Mannino & Harvey 2000, Harji 2011). Although the present study didn't measure DOM chemistry, the decoupling between BP,  $\beta$ -Glu

gene and the number of cells expressing  $\beta$ -Glu could be related to changes in organic matter composition which influences the bacterial community. An increase in BP can be related to  $\beta$ -*proteobacteria* and decrease in salinity, and this increase neither influenced the  $\beta$ -Glu gene abundance nor the number of cells expressing  $\beta$ -Glu. An earlier study carried out in Zuari estuary also indicated that BP is fueled by the allochthonous sources and behave as a net heterotrophy system during the monsoon months (Ram et al. 2007). A few studies also stated that decrease in glucosidase activity could be explained by substrate composition or by a change in the bacterial community (Martinez et al. 1996, Jost & Pollehne 1998). It has been reported that the physical and chemical factors partially control the ectoenzymatic patterns by indirectly controlling the bacterial community structure (Langenheder et al. 2005, Boucher & Debroas 2009).

Overall, the results of the present study indicated a clear seasonal shifts in BCC with high proportions of *Proteobacteria* ( $\gamma$ , and  $\alpha$ ) groups during PreM to *Actinobacteria* –  $\beta$  – *Proteobacteria* – *Firmicutes* groups during SW-Mon, a period of high riverine influx, which reversed to  $\alpha$  - *Proteobacteria* group during recovery PostM season. Although  $\beta$ -Glu gene abundance was related to the relative abundance of diverse phylogenetic groups, the number of cells expressing  $\beta$ -Glu were mainly related to the relative abundance of  $\gamma$ -*proteobacteria* taxa. A higher ratio of number of cells expressing  $\beta$ -Glu observed at saltwater and decline towards the freshwater region could be related to change in the spectrum of DOM input in this estuary. In conclusion, the number of cells expressing  $\beta$ -Glu was influenced by the shifts in BCC that seems to be regulated by different environmental drivers. Further studies should focus on translocation of marine, brackish and freshwater bacterial communities into different environmental conditions and evaluate their potential influence on the processing of the organic matter.

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### Legends to figures

**Fig. 1:** Map showing sampling stations along the Zuari estuary. Mouth (S1 & S2); Lower middle (LM; S3 & S4); Upper middle (UM; S5 & S6); and Upstream (S7 & S8).

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**Fig. 2:** The average values of salinity and dissolved nutrients concentration in surface water from mouth to upstream of the Zuari estuary during the spring and neap tides. values are mean  $\pm$  standard deviation; Lower Mid - Lower Middle; Upper Mid - Upper Middle; PreM - Premonsoon; SW-Mon - Southwest monsoon; PostM - Post monsoon; significant differences among the seasons and stations are evaluated using three-way ANOVA followed by post hoc Tukey's HSD test at the significance level of  $p < 0.05$ ; different letters (a-c) and numbers (1-7) on the bars denote significant differences among the seasons and stations respectively.

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**Fig. 3** The average values of transparent exopolysaccharides (TEP), suspended particulate matter (SPM), total organic carbon (TOC), dissolved organic carbon (DOC), particulate organic carbon (POC), and chlorophyll a (Chl a) concentration in surface water from mouth to upstream of the Zuari estuary during the spring and neap tides. values are mean  $\pm$  standard deviation; Lower Mid - Lower Middle; Upper Mid - Upper Middle; PreM - Premonsoon; SW-Mon - Southwest monsoon; PostM - Post monsoon; significant differences among the seasons and stations are evaluated using three-way ANOVA followed by post hoc Tukey's HSD test at the significance level of  $p < 0.05$ ; different letters (a-c) and numbers (1-6) on the bars denote significant differences among the seasons and stations respectively.

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**Fig. 4:** The average values of total bacterial count (TBC), bacterial production (BP), protists abundance, number of cells expressing  $\beta$ -glucosidase (no. of cells exp  $\beta$ -glu), 16S ribosomal RNA (16S rRNA),  $\beta$ -glucosidase ( $\beta$ -glu) gene abundance in surface water from mouth to upstream of Zuari estuary during the spring and neap tides, values are mean  $\pm$  standard deviation; Lower Mid - Lower Middle; Upper Mid - Upper Middle; PreM - Premonsoon; SW-Mon - Southwest monsoon; PostM - Post monsoon; significant differences among the seasons and stations are evaluated using

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three-way ANOVA followed by post hoc Tukey's HSD test at the significance level of  $p < 0.05$ ; different letters (a-c) and numbers (1-7) on the bars denote significant differences among the seasons and stations respectively.

**Fig. 5.** Redundancy analysis (RDA) showing the relation between biotic and abiotic variables during the spring and neap tides; A and D) RDA triplots; Red arrows indicate environmental variables; Dotted red lines are highly significant ( $p < 0.05$ ; Monte Carlo test); Blue arrows indicates species variables; B and E) RDA sample ordination plots; individual colored dots denote all biotic data set of a particular station and season; C and F) Eigenvalues and intersect correlation scores of RDA axes 1 and 2 respectively.

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**Fig. 6** The average copy numbers of different bacterial groups in surface water from mouth to upstream of the Zuari estuary during the spring and neap tides. values are mean  $\pm$  standard deviation; Lower Mid - Lower Middle; Upper Mid - Upper Middle; PreM - Premonsoon; SW-Mon - Southwest monsoon; PostM - Post monsoon; significant differences among the seasons and stations are evaluated using three-way ANOVA followed by post hoc Tukey's HSD test at the significance level of  $p < 0.05$ ; different letters (a-c) and numbers (1-7) on the bars denote significant differences among the seasons and stations respectively.

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**Suppl. Fig. 1:** Validation of flow cytometric analysis A)  $\beta$  - glucosidase positive pure culture *Exiguobacterium indicum* isolated from Zuari estuary; B) Dot plot showing control (I)- sample without addition of FDGlu; C) Dot plot showing control (II) – addition of FDGlu without bacteria; D) Dot plot showing positive sample; E) Histogram plot.

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**Suppl. Fig. 2:** Melt curve analysis.

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**Suppl. Fig. 3:** A-B) The number of cells expressing  $\beta$  - Glucosidase in percentage with respect to total bacterial count from mouth to upstream of Zuari estuary during the spring and neap tides; C-D) The percentage of bacterial community recovered by Q-PCR with respect to total community from mouth to upstream of Zuari estuary during the spring and neap tides; Lower Mid - Lower Middle;

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### Legends to tables

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**Suppl. Table 1:** PCR cycling conditions.

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**Suppl. Table 2A-C:** RDA associated statistics; A) Monte Carlo permutation test-spring tide; B) Monte Carlo permutation test-neap tide; C) Species response on RDA environmental axes 1 and 2 using generalized linear model and F statistics; GLM – generalized linear model; no. of cells exp.  $\beta$ -Glu number of cells expressing  $\beta$ -Glucosidase;  $\beta$ -Glu gene -  $\beta$ -Glucosidase gene; NS – Not Significant.

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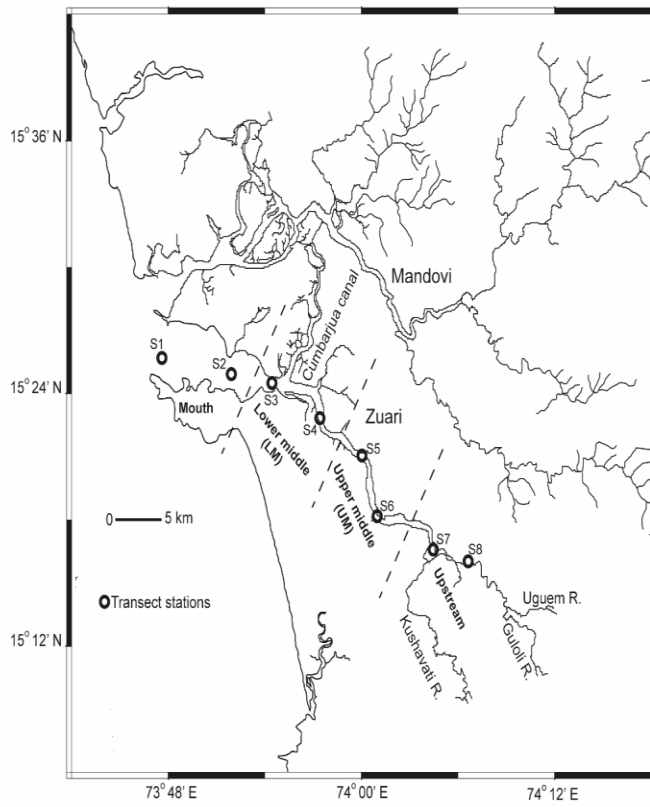


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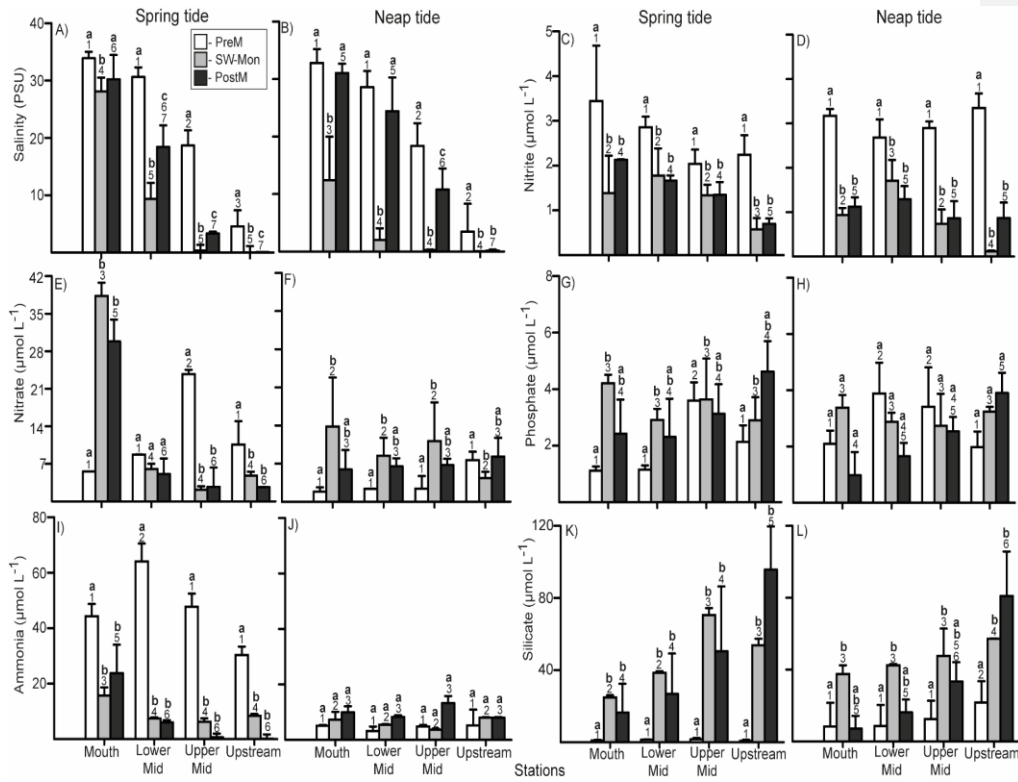


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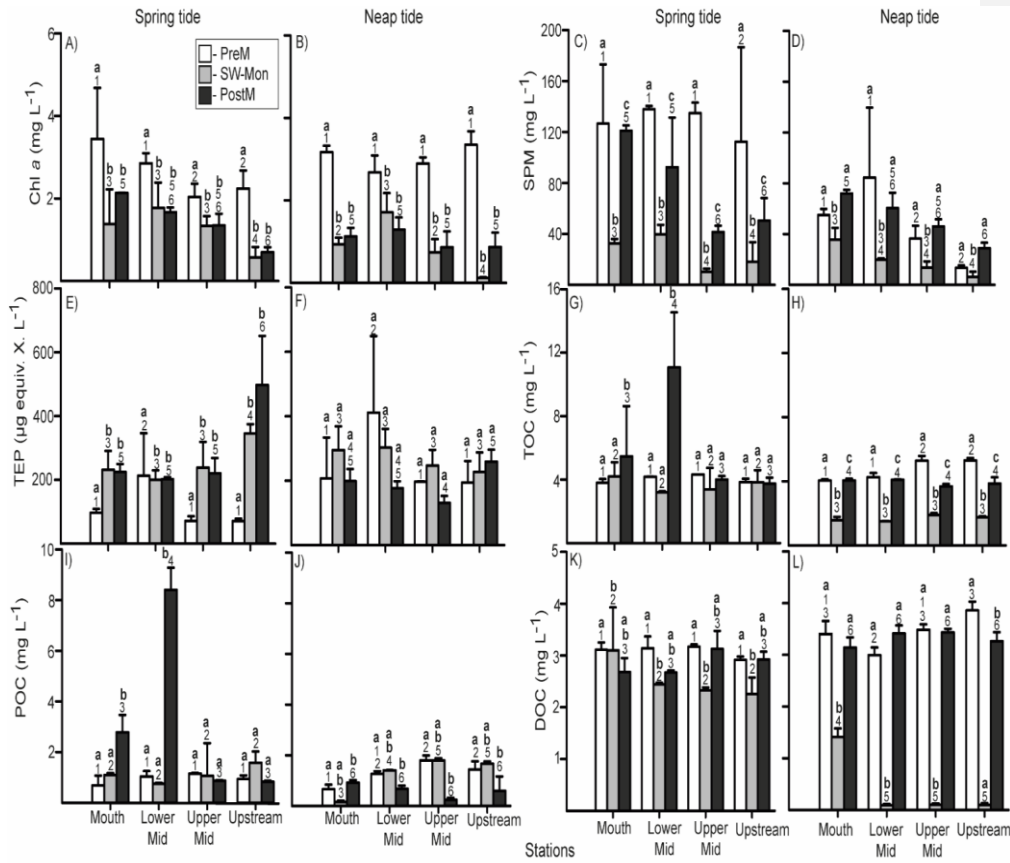


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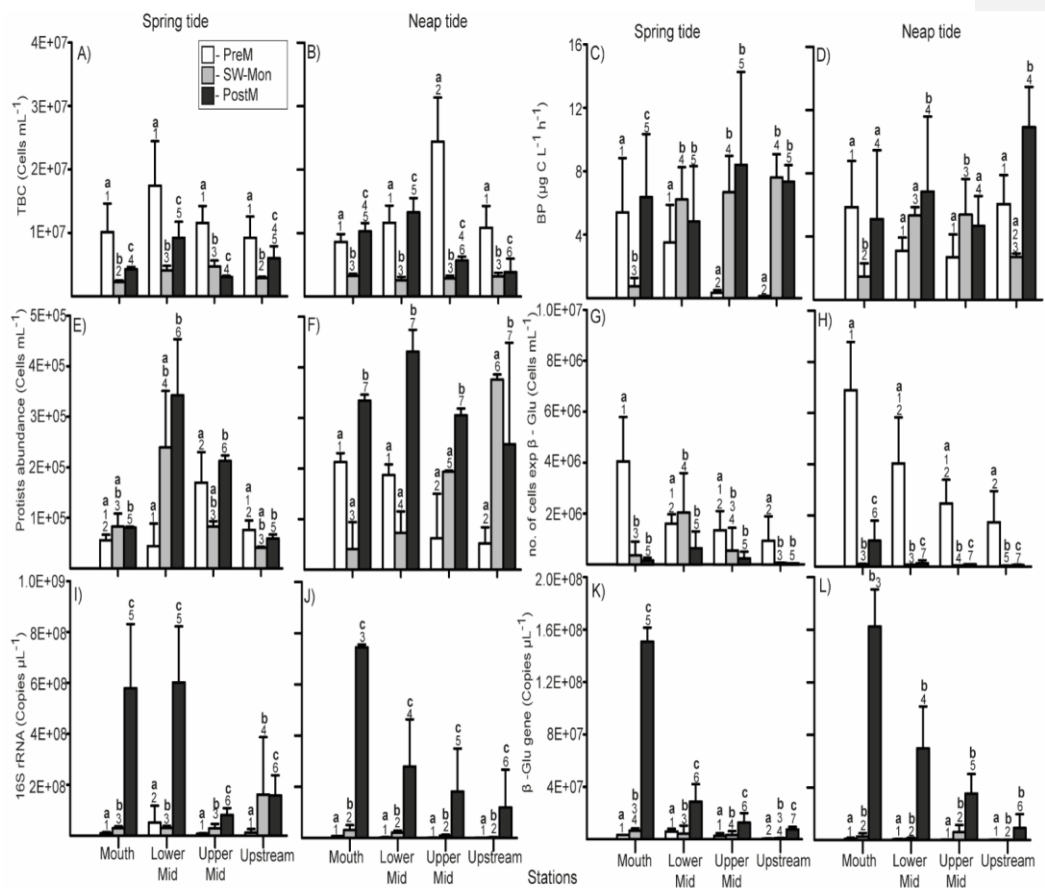


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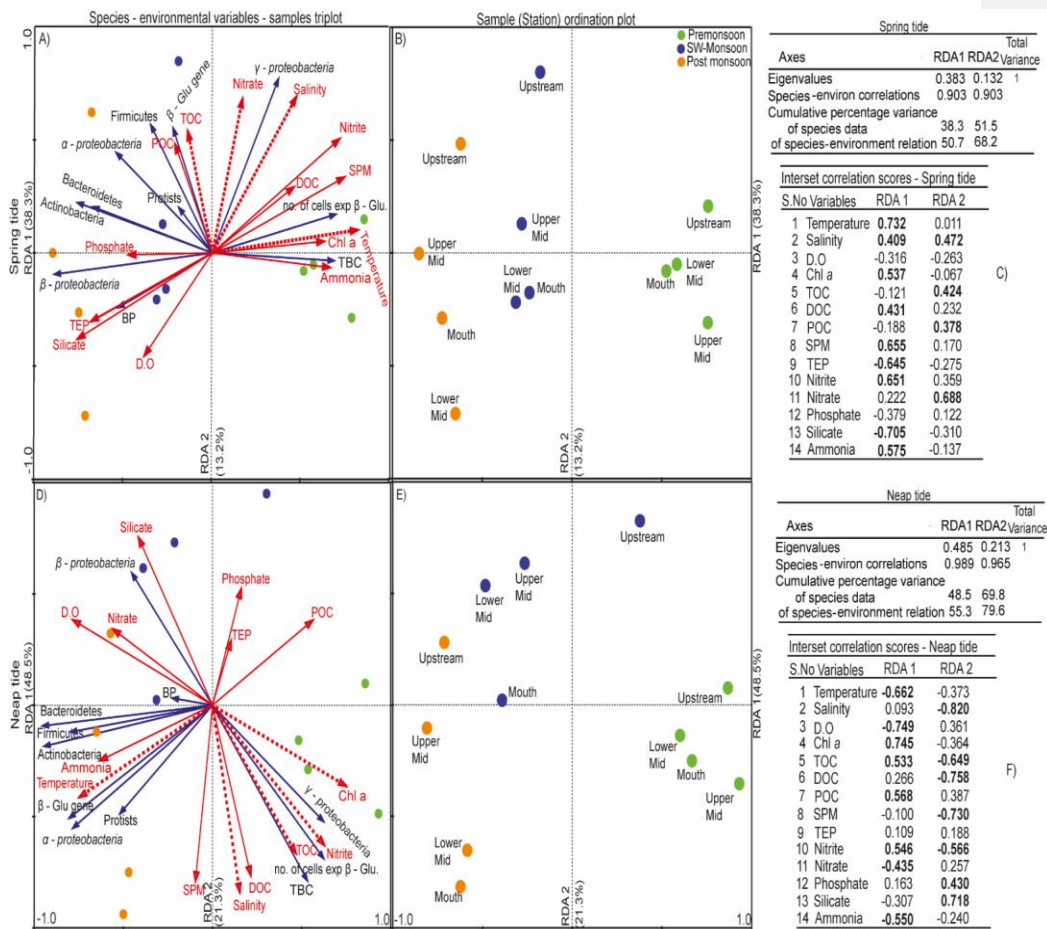


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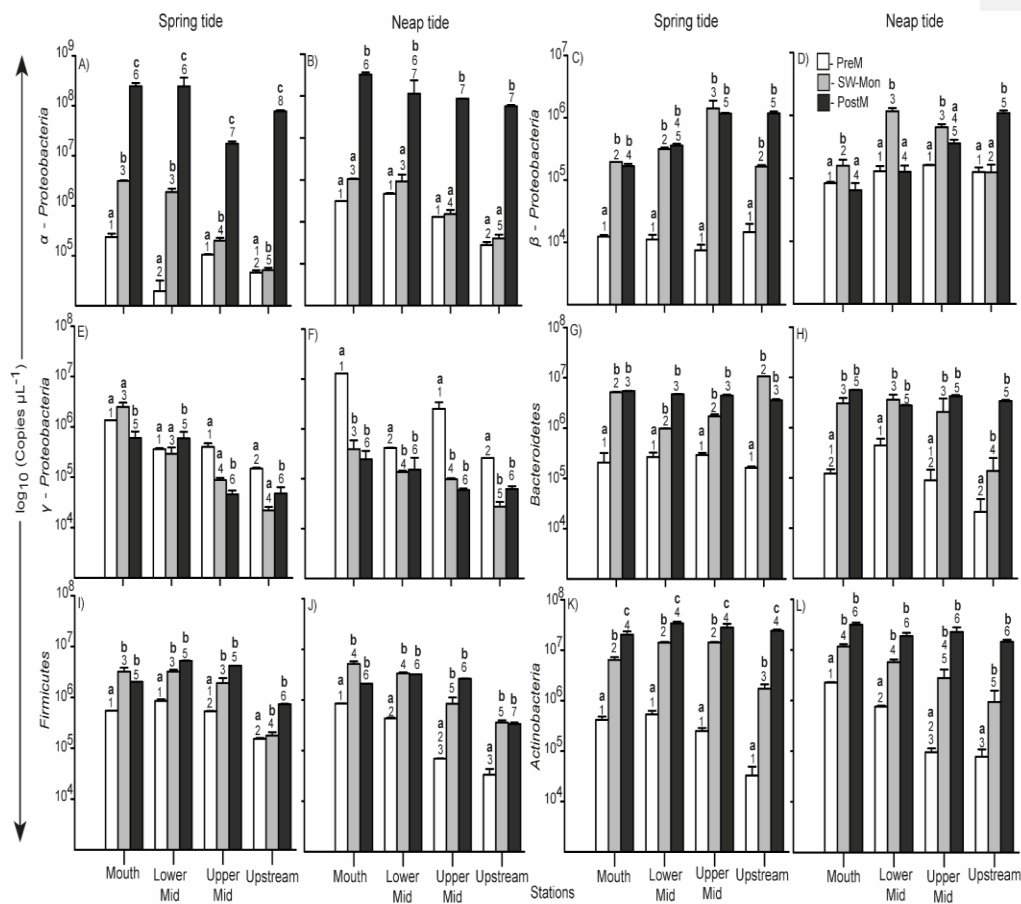


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Target group	Target gene	Name	Sequence	References
Universal	16S rRNA	8F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTACGACTT	Lane, 1991
<i>α-Proteobacteria</i>	16S rRNA	926F 1062R	AAACTCAAAGAATTGACGG CTCACRRCACGAGCTGAC	De Gregoris et al. 2011
<i>β-proteobacteria</i>	16S rRNA	Beta359f Beta682r	GGGGAATTTTGGACAATGGG ACGCATTCACTGCTACACG	Ashelford et al. 2002
<i>γ-proteobacteria</i>	16S rRNA	1080yF Y1202R	TCGTCAGCTCGTGTGTGA CGTAAGGGCCATGATG	De Gregoris et al. 2011
<i>Bacteroidetes</i>	16S rRNA	798cfbF cfb967R	CRAACAGGATTAGATACCCT GGTAAGGTTCTCGCGTAT	De Gregoris et al. 2011
<i>Firmicutes</i>	16S rRNA	928f-Firm 1040FirmR	TGAAACTYAAAGGAATTGACG ACCATGCACCACCTGTC	De Gregoris et al. 2011
<i>Actinobacteria</i>	16S rRNA	Act920F3 Act1200R	TACGGCCGCAAGGGCTA TCRTCCCCACCTTCCTCCG	De Gregoris et al. 2011
<i>β-Glucosidase gene</i>	<i>β-Glu</i>	bgluF2 bgluR4	TTCYTBGGYRTCAACTACTA CCGTTYTCGGTBAYSWAGA	Cañizares et al. 2011

**Table 1.** Q-PCR Primer sets used in this study

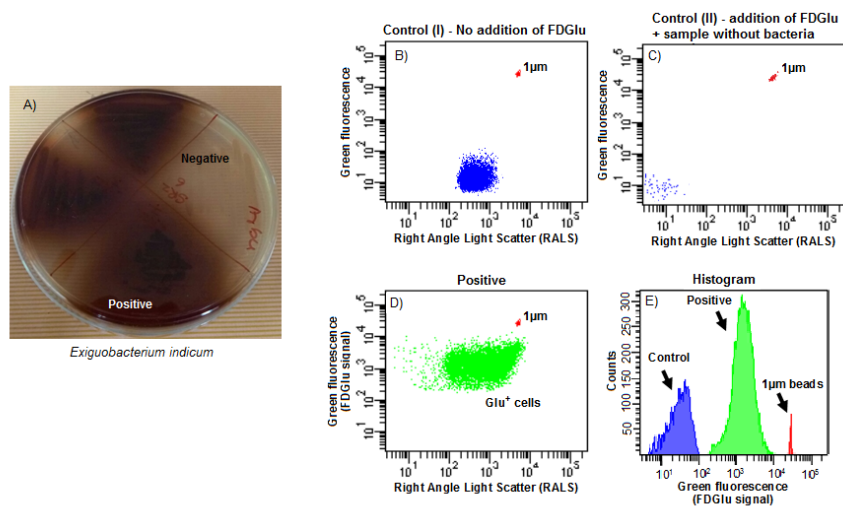
#### Isolation and identification $\beta$ - Glu producing bacteria

Water samples were collected from eight different stations located along the Zuari estuary (Fig. 1), West Coast of India. One ml of sub-sample was serially diluted and spread plated on Zobell Marine Agar 2216 (ZMA 2216) and incubated for 24 hours. After incubation, pure cultures were isolated from agar medium and screened for  $\beta$  - Glu activity. Briefly, isolates were streaked on an agar plate containing 0.5% arbutin and 0.02 % Ammonium ferric (II) citrate (which was filter sterilized and added to the autoclaved medium) and incubated at 30<sup>0</sup> C for 24 hrs.  $\beta$  - Glu positive isolates hydrolyse the arbutin substrate, and a dark brown colour zone develops in the agar plates (Suppl. Fig. 1A). Based on the biochemical characterization and protein profile using MALDI-TOF/TOF (Bruker) with biotyper software, the strain identified as *Exiguobacterium indicum*. Further, bacterial genomic DNA was extracted using pure link genomic DNA extraction kit (Invitrogen, USA) and 16S rRNA gene amplification was carried out by PCR using the universal primer set 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTGTTACGACTT-30). The sequence obtained by analyzing with an automated DNA sequencer (Applied Biosystems) was aligned with Clustal X, and MEGA 4 software was used to built the phylogenetic tree (NCBI accession number: KR047884). The analysis of the isolated strain's 16S rRNA confirms the taxonomy obtained from biochemical and protein profiling.

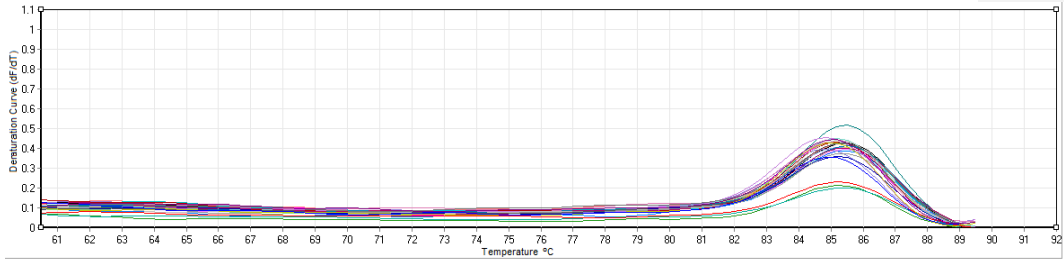
#### Standardization of flow cytometric (FCM) analysis

For FCM analysis, *E. indicum* inoculated in the broth containing 0.5% of arbutin and incubated for 24 hrs at 30<sup>0</sup> C. After incubation, cells were washed and diluted with 0.05M sodium acetate buffer (pH 5.5), and subsequently stained with FDGlu for 30 mins in the dark (2mM final concentration; Molecular Probes, USA). Control samples also prepared without FDGlu and FDGlu-sample without bacteria to cut off the background noise. After incubation, the samples from both the

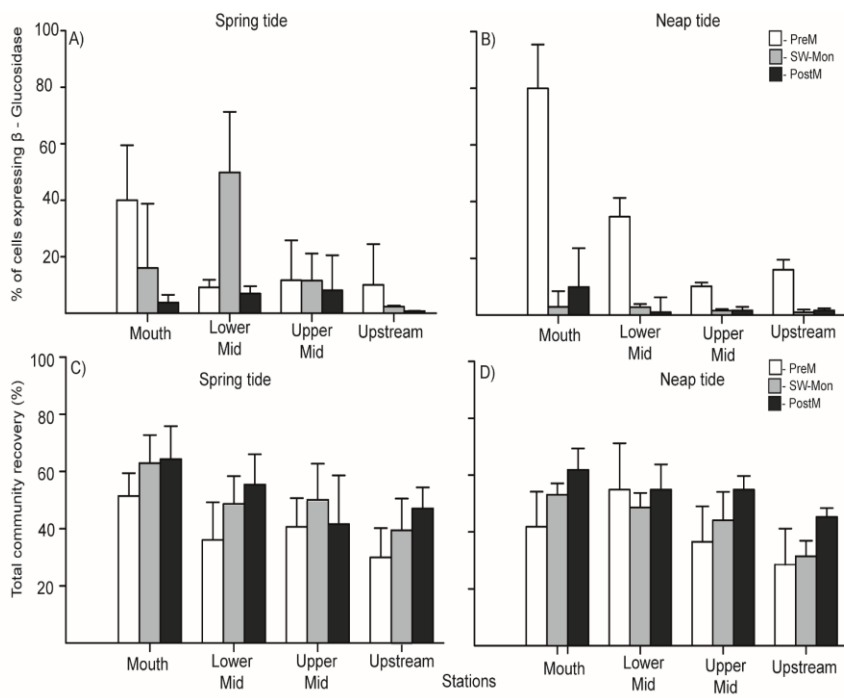
sets were analyzed using a BD FACSAria™ II flow cytometer equipped with a 488nm nuclear blue laser using following detector settings (FSC – 286; SSC – 310; FL1 – 464) at a threshold rate of 200 (FITC). Emitted light collected through different filter sets: (1) 488/10 band pass for right angle light scatter (RALS), (2) 530/30 band pass filter for green fluorescence signal from the fluorescein hydrolysis product of FDGlu (Suppl. Fig. 1B-E).



**Supplementary Fig. 1:** Validation of flow cytometric analysis; A)  $\beta$ -glucosidase positive pure culture *Exiguobacterium indicum* (NCBI accession number: KR047884) isolated from Zuari estuary; B) Dot plot showing control (I) sample without addition of FDGlu; C) Dot plot showing control (II) addition of FDGlu without bacteria; D) Dot plot showing positive sample; E) Histogram plot



Supplementary Figure. 2: Melt curve analysis



**Supplementary Fig. 3:** A-B) The number of cells expressing  $\beta$  - Glucosidase in percentage with respect to total bacterial count from mouth to upstream of Zuari estuary during the spring and neap tides; C-D) The percentage of bacterial community recovered by Q-PCR with respect to total community from mouth to upstream of Zuari estuary during the spring and neap tides; Lower Mid - Lower Middle; Upper Mid - Upper Middle; PreM - Premonsoon; SW-Mon - Southwest Monsoon; PostM - Post monsoon.

**Supplementary Table 1.** Q-PCR cycling conditions (' – Min; " – Seconds).

<b>PCR REACTION COMPONENTS</b>					
2X SYBR green buffer containing taq polymerase					
0.5 µl of each primer					
DNA template					
Water to 20 µl					
<b>PCR THERMOCYCLE</b>					
Universal	Initial denaturation	95° C 5'			
	40 cycles	<table border="0"> <tr> <td rowspan="4" style="font-size: 3em; vertical-align: middle;">{</td> <td>94° C 60"</td> </tr> <tr> <td>55° C 60"</td> </tr> <tr> <td>72° C 60"</td> </tr> </table>	{	94° C 60"	55° C 60"
{	94° C 60"				
	55° C 60"				
	72° C 60"				
	Group specific	Initial denaturation	95° C 5'		
40 cycles		<table border="0"> <tr> <td rowspan="4" style="font-size: 3em; vertical-align: middle;">{</td> <td>95° C 15"</td> </tr> <tr> <td>53° C 15"</td> </tr> <tr> <td>72° C 15"</td> </tr> </table>	{	95° C 15"	53° C 15"
{	95° C 15"				
	53° C 15"				
	72° C 15"				
	β - Glucosidase gene	Initial denaturation	95° C 5'		
40 cycles		<table border="0"> <tr> <td rowspan="4" style="font-size: 3em; vertical-align: middle;">{</td> <td>95° C 60"</td> </tr> <tr> <td>53° C 60"</td> </tr> <tr> <td>72° C 45"</td> </tr> </table>	{	95° C 60"	53° C 60"
{	95° C 60"				
	53° C 60"				
	72° C 45"				

**Supplementary Table. 2** Associated statistics of RDA analyses

A) Monte Carlo permutation test - Spring tide

Marginal Effects			Conditional Effects				
Variables	Var.N	$\lambda_1$	Variables	Var. N	$\lambda_A$	P	F
Temperature	1	0.27	<b>Temperature</b>	<b>1</b>	<b>0.27</b>	<b>0.001</b>	<b>8.04</b>
Silicate	13	0.25	<b>Nitrate</b>	<b>11</b>	<b>0.11</b>	<b>0.002</b>	<b>3.75</b>
SPM	8	0.23	<b>TOC</b>	<b>5</b>	<b>0.07</b>	<b>0.012</b>	<b>2.65</b>
Nitrite	10	0.23	<b>Salinity</b>	<b>2</b>	<b>0.07</b>	<b>0.034</b>	<b>2.69</b>
TEP	9	0.22	Silicate	13	0.05	0.098	1.94
Ammonia	14	0.17	TEP	9	0.04	0.103	1.87
Salinity	2	0.15	D.O	3	0.03	0.267	1.29
Chl <i>a</i>	4	0.15	POC	7	0.06	0.254	1.14
Nitrate	11	0.11	Phosphate	12	0.02	0.296	1.14
DOC	6	0.1	Nitrite	10	0.04	0.201	1.47
D.O	3	0.1	Ammonia	14	0.01	0.52	0.85
Phosphate	12	0.09	DOC	6	0.02	0.639	0.7
POC	7	0.06	Chl <i>a</i>	4	0.01	0.753	0.54
TOC	5	0.05	SPM	8	0.02	0.756	0.5

B) Monte Carlo permutation test - Neap tide

Marginal Effects			Conditional Effects				
Variables	Var.N	$\lambda_1$	Variables	Var.N	$\lambda_A$	P	F

Chl <i>a</i>	4	0.31	<b>Chl <i>a</i></b>	<b>4</b>	<b>0.31</b>	<b>0.001</b>	<b>9.93</b>
D.O	3	0.31	<b>Temperature</b>	<b>1</b>	<b>0.17</b>	<b>0.001</b>	<b>7</b>
Temperature	1	0.27	<b>TOC</b>	<b>5</b>	<b>0.13</b>	<b>0.001</b>	<b>6.34</b>
TOC	5	0.25	<b>Salinity</b>	<b>2</b>	<b>0.12</b>	<b>0.001</b>	<b>8.28</b>
Nitrite	10	0.23	<b>Nitrite</b>	<b>10</b>	<b>0.03</b>	<b>0.035</b>	<b>2.49</b>
POC	7	0.2	DOC	6	0.02	0.132	1.65
DOC	6	0.18	D.O	3	0.02	0.11	1.76
Silicate	13	0.17	Silicate	13	0.02	0.074	1.9
Salinity	2	0.17	TEP	9	0.01	0.655	0.75
Ammonia	14	0.17	POC	7	0.02	0.621	0.712
SPM	8	0.13	Phosphate	12	0.01	0.504	0.9
Nitrate	11	0.12	SPM	8	0.02	0.318	1.13
Phosphate	12	0.06	Nitrate	11	0.01	0.625	0.77
TEP	9	0.02	Ammonia	14	0.01	0.537	0.81



C) Species response on RDA environmental axes 1 and 2 using generalized linear model and F statistics; GLM – generalized linear model; NS – Not Significant; No. of cells exp  $\beta$ -Glu - number of cells expressing  $\beta$ -Glucosidase;  $\beta$ -Glu gene -  $\beta$ -Glucosidase gene

C)		Surface water							
		Spring tide				Neap tide			
		GLM		Score		GLM		Score	
S. No	Dependent	F	P	RDA 1	RDA 2	F	P	RDA 1	RDA 2
1	<i>a-proteobacteria</i>	5.70	<0.01	<b>-0.52</b>	<b>0.42</b>	24.97	<0.0001	<b>-0.72</b>	<b>-0.54</b>
2	<i><math>\beta</math>-proteobacteria</i>	16.17	<0.0005	<b>-0.88</b>	-0.02	5.5	<0.02	-0.34	<b>0.54</b>
3	<i><math>\gamma</math>-proteobacteria</i>	23.0	<0.0001	<b>0.40</b>	<b>0.71</b>	19.9	<0.0001	0.37	-0.39
4	<i>Bacteroidetes</i>	5.50	<0.02	<b>-0.64</b>	0.28	57.9	<0.00001	<b>-0.94</b>	-0.07
5	<i>Firmicutes</i>	4.9	<0.01	<b>-0.34</b>	<b>0.52</b>	36.9	<0.0001	<b>-0.84</b>	-0.09
6	<i>Actinobacteria</i>	16.95	<0.0001	<b>-0.76</b>	0.21	26.8	<0.0001	<b>-0.89</b>	-0.20
7	Total Bacterial Count	16.20	<0.001	<b>0.64</b>	-0.18	11.3	<0.01	<b>0.52</b>	<b>-0.77</b>
8	Protists abundance	NS	NS	-0.13	0.31	15.1	<0.0005	<b>-0.43</b>	<b>-0.43</b>
9	No. of cells exp $\beta$ -Glu	15.50	<0.001	<b>0.66</b>	0.03	12.3	<0.001	<b>0.64</b>	-0.66
10	Bacterial Production	7.1	<0.01	<b>-0.61</b>	-0.37	NS	NS	-0.06	0.07
11	$\beta$ -Glu gene	4.10	<0.03	-0.22	<b>0.45</b>	47.52	<0.00001	<b>-0.65</b>	<b>-0.47</b>