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Seasonal variation in bacterial community composition and β-glucosidase expression in a tropical monsoon-influenced estuary

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ABSTRACT: Tidal estuaries receive carbohydrate-rich organic matter from various sources, which are mainly broken down by enzymatic processes. β-glucosidase (β-Glu) is one of the ectoenzymes produced by heterotrophic bacteria. An investigation was carried out along the tropical monsoon-influenced Zuari estuary to examine seasonal variability in major bacterial taxa, β-Glu gene abundance, and number of cells expressing β-Glu. The results revealed a higher number of β-Glu expressing cells, and higher β-Glu gene and bacterial abundance at the estuarine mouth compared to the upstream area, and this was coupled with high phytoplankton biomass and low transparent exopolysaccharides. A clear distinction in bacterial assemblages along with β-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 *Gammaproteobacteria* at the saline mouth and *Betaproteobacteria* at the upstream, freshwater region. During the southwest monsoon, which brings high freshwater influx, *Actinobacteria* and *Firmicutes* were abundant in 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 the β-Glu to gene ratio. β-Glu gene abundance was related to diverse groups (*Alpha-* and *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes*). The number of cells expressing β-Glu covaried with *Gammaproteobacteria* 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.

KEY WORDS: β-glucosidase · Estuary · qPCR · Ectoenzymes · Tides · Flow cytometry · Bacterial community

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 and 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 and are abundant in estuarine and oceanic waters (Passow 2002). In estuaries, they play a prominent role in OM cycling either through degradation, aggregate formation, or sedimentation processes (Simon et al. 2002, Barrera-Alba

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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, Chróst 1990). β-glucosidases (β-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 the composition, concentration, and availability of OM (Chróst 1991). Other environmental factors that can alter 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 relationship between β-Glu activity, bacterial abundance, and primary production (Chróst 1989, Romaní & Sabater 1999, Cunha et al. 2000); rather, the focus is mainly on total bacterial population and its activity with less emphasis on bacterial community structure.

Recent developments in molecular methods have revealed enormous bacterial diversity which is capable of producing an array of hydrolytic enzymes, and have established a link between bacterial community composition (BCC) and their metabolic activity (Lämmle et al. 2007, Kielak et al. 2013). Many studies relating BCC and hydrolytic activity have been carried out in estuarine and coastal waters, 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 few have been explored in tropical monsoonal estuaries. Quantitative PCR (qPCR) 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 used FCM in differentiating cells, which express 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 relationship between the number of cells expressing β-Glu and BCC are limited, and this was addressed in the present study.

Our 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 the southwest monsoon season, which regulates the hydrodynamics of this system on an annual scale. Hence, it is expected that along with changes in biogeochemical processes, major bacterial taxa would significantly differ from those present in the dry season. We hypothesized that high allochthonous inputs during the monsoon would influence and alter bacterial populations and trigger an increase or decrease in β-Glu gene expression. The aim of the present work was to assess the influence of seasons on major bacterial taxa with an emphasis on β-Glu expressing populations and relate this 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 February 2015 and 21 April 2015), southwest monsoon (SW-Mon; 24 August 2015 and 3 September 2015), and post monsoon seasons (PostM; 16 October 2015 and 23 October 2015) during spring–neap tidal cycles (Fig. 1). The Zuari estuarine system exhibits large salinity variations ranging from 34 to 0 psu. Based on salinity, sampling stations were divided into 4 areas representing the mouth (Stns S1, S2), lower middle (Stns S3, S4), upper middle (Stns S5, S6), and upstream (Stns S7, S8) (Fig. 1). Samples from 8 different stations were collected using 5 l Niskin

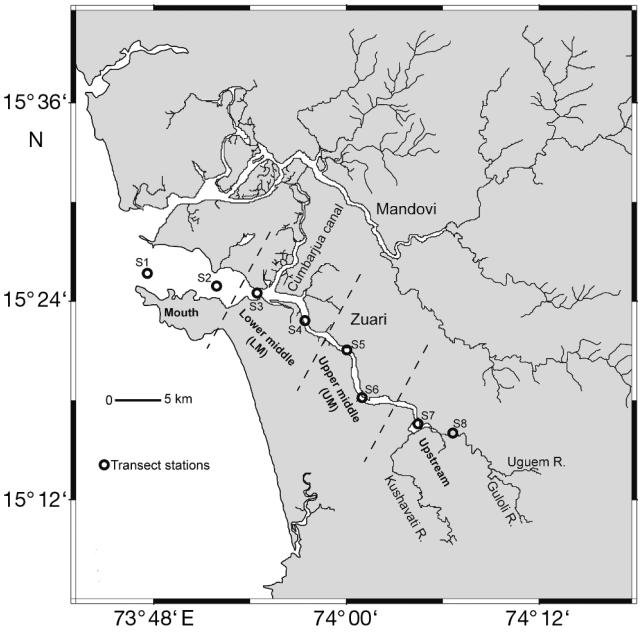

Fig. 1. Sampling station locations along the Zuari estuary: mouth (Stns S1 and S2); lower middle (LM; Stns S3 and S4); upper middle (UM; Stns S5 and S6); and upstream (Stns S7 and S8)

bottles, 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) with an accuracy of 0.005°C and 0.005 psu. Dissolved oxygen (DO) was measured using Winkler's method (Parsons et al. 1984). Concentrations of dissolved nutrients such as nitrite, nitrate, phosphate, silicate, and ammonia were measured using an automated nutrient analyzer (Skalar SAN PLUS 8505 Interface v.3.31).

Total phytoplankton biomass was estimated as total chlorophyll *a* (chl *a*). Briefly, 500 ml of water samples were filtered through GF/F filter papers; subsequently, overnight extraction was carried out using 90% acetone and the raw fluorescent units (RFU) were measured using a fluorometer (Turner). 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 h) and acidified using 2% H_2SO_4. Filter papers with the particulate materials were dried in an oven at 60°C and reweighed on a weighing balance (Mettler Toledo). Total and DOC was measured using a 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 are expressed as mg l^{-1}. For TEP analysis, samples were filtered on 0.4 µm filter papers (HTTP; Millipore) and analyzed using a modified dye binding assay protocol as described by Klein et al. (2011). Xanthan gum was used as a standard, and TEP values are expressed as µg xanthan gum equivalent l^{-1} (µg Xeq. l^{-1}).

Flow cytometry analysis

Enumeration of cells expressing β-Glu

The number of cells expressing β-Glu were enumerated using Fluorescein di-β-D-glucopyranoside (FDGlu; Molecular Probes) coupled with flow cytometry. Before sample analysis, this method was validated using a β-Glu-positive pure strain (*Exiguobacterium indicum*; NCBI accession number KR047884); details are provided in the Supplement at www.int-res.com/articles/suppl/a080p273_supp.pdf. Briefly, non-fluorescent FDGlu substrate was hydrolyzed by β-Glu via a 2-step process: first to fluorescein mono glucoside (FMG), and then to highly fluorescent fluorescein. Previous 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 a 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 (2 mM final concentration; Molecular Probes). 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 and control samples were incubated for 30 min at 30°C in the dark. After incubation, the samples were analyzed using a BD FACSAria™ II flow cytometer (BD Biosciences) equipped with a 488 nm 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 (see Fig. S1 in the Supplement). Fluorescent beads (1 µm; 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 (Fig. S1D).

Total bacterial count

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

Bacterial production

In the present study, 5-bromo-2-deoxyuridine (BrdU) was used to measure bacterial production (BP) in estuarine samples. BrdU is a thymidine ana-

log 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 (1 µM final conc.; BD Biosciences) and incubated for 3 h at *in situ* temperature. After incubation, the samples were fixed with paraformaldehyde (1% final conc.) 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 min in the dark and analyzed using a BD FACSAria™ II flow cytometer equipped with a 488 nm nuclear blue laser. The samples without BrdU were used as controls. For calculating bacterial carbon production, 11 fg C per bacterium as a cell-to-carbon conversion factor was used (Garrison et al. 2000).

Enumeration of protist abundance

Protist abundance was determined using a 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) and incubated in the dark for 15 min before measurement. Analysis of stained samples was carried out using an 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 versus green signal plot to cut off the intrusion of bacterial populations with protist abundance. Yellow-green fluorescent beads (1 µm; Polysciences) were used as an internal standard. The data sets were processed using BD FACS Diva (v.6.2) software.

Extraction of DNA

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

qPCR analysis

Bacterial community composition

The qPCR analyses were performed to evaluate BCC using a set of primers which are specific to 16S rRNA genes of the *Proteobacteria* subclasses (*Alpha*, *Beta*, and *Gamma*), *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Table 1). The reaction was carried out in a real-time PCR system (Rotor-Gene Q 5X HRM; Qiagen) using Quantitech SYBR Green PCR master mix (Qiagen). Each 20 µl reaction mix contained 10 µl of 2× SYBR Green PCR Mix, 0.5 µl of

Table 1. Quantitative PCR (qPCR) primer sets used in this study

Target group	Target gene	Name	Sequence	Reference
Universal	16S rRNA	8F	AGA GTT TGA TCC TGG CTC AG	Lane (1991)
		1492R	GGT TAC CTT GTT ACG ACT T	
Alphaproteobacteria	16S rRNA	926F	AAA CTC AAA KGA ATT GAC GG	Bacchetti De Gregoris et al. (2011)
		1062R	CTC ACR RCA CGA GCT GAC	
Betaproteobacteria	16S rRNA	Beta359f	GGG GAA TTT TGG ACA ATG GG	Ashelford et al. (2002)
		Beta682r	ACG CAT TTC ACT GCT ACA CG	
Gammaproteobacteria	16S rRNA	1080 F	TCG TCA GCT CGT GTY GTG A	Bacchetti De Gregoris et al. (2011)
		1202R	CGT AAG GGC CAT GAT G	
Bacteriodetes	16S rRNA	798cfbF	CRA ACA GGA TTA GAT ACC CT	Bacchetti De Gregoris et al. (2011)
		cfb967R	GGT AAG GTT CCT CGC GTA T	
Firmicutes	16S rRNA	928f-Firm	TGA AAC TYA AAG GAA TTG ACG	Bacchetti De Gregoris et al. (2011)
		1040FirmR	ACC ATG CAC CAC CTG TC	
Actinobacteria	16S rRNA	Act920F3	TAC GGC CGC AAG GGC TA	Bacchetti De Gregoris et al. (2011)
		Act1200R	TCR TCC CCA CCT TCC TCC G	
β-glucosidase gene	β-Glu	bgluF2	TTC YTB GGY RTC AAC TAC TA	Cañizares et al. (2011)
		bgluR4	CCG TTY TCG GTB AYS WAG A	

each primer, 1 µl of a template, and 8 µl of RNase-free water. Primer sets and thermal cycler conditions are described in Tables 1 & S1, respectively. Melt curve analyses were performed to test the specificity of each primer set 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 at 0.5°C intervals. A clear single peak was obtained between 84 and 86°C, indicating the presence of site-specific PCR amplicon (see Fig. S2 in the Supplement). A 10-fold 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 (C_T) values against the logarithm of the concentration of each 10-fold dilution series of PCR fragments. A known series of standards were included in triplicate during each run to correct the potential error for unknown samples. The reaction efficiency was always between 0.98 and 1.0. Datasets were processed using Rotor-Gene Q Software v.2.3.1.49 (Qiagen).

Quantification of the β-Glu gene

In the present study, the gene encoding β-Glu was quantified by qPCR using a set of degenerate primers (BgluF2/BgulR4; Table 1) as described by Cañizares et al. (2011). The standard curve and C_T were calculated by serially diluting a purified PCR amplicon of a β-Glu gene from *E. 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 v.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. A 3-way analysis of variance (3-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 (SPSS Statistics v.22; IBM). Further, a similarity (SIMPROF) test was performed to identify the spatial variation in BCC using Primer v.6 software (Clarke & Gorley 2006).

The relationship between biotic (TBC, BP, protist abundance, β-Glu gene abundance, number of cells expressing β-Glu, and BCC) and environmental variables were evaluated using multivariate redundancy analysis (RDA). The analyses were performed using CANOCO v.4.5 for Windows software package (ter Braak & Smilauer 2002). Before RDA analyses, data were checked for linear characteristics and standardized (length of first detrended correspondence analysis axes < 2 SD units), and a 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

Environmental parameters, dissolved nutrients, chl *a* and TEP

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

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

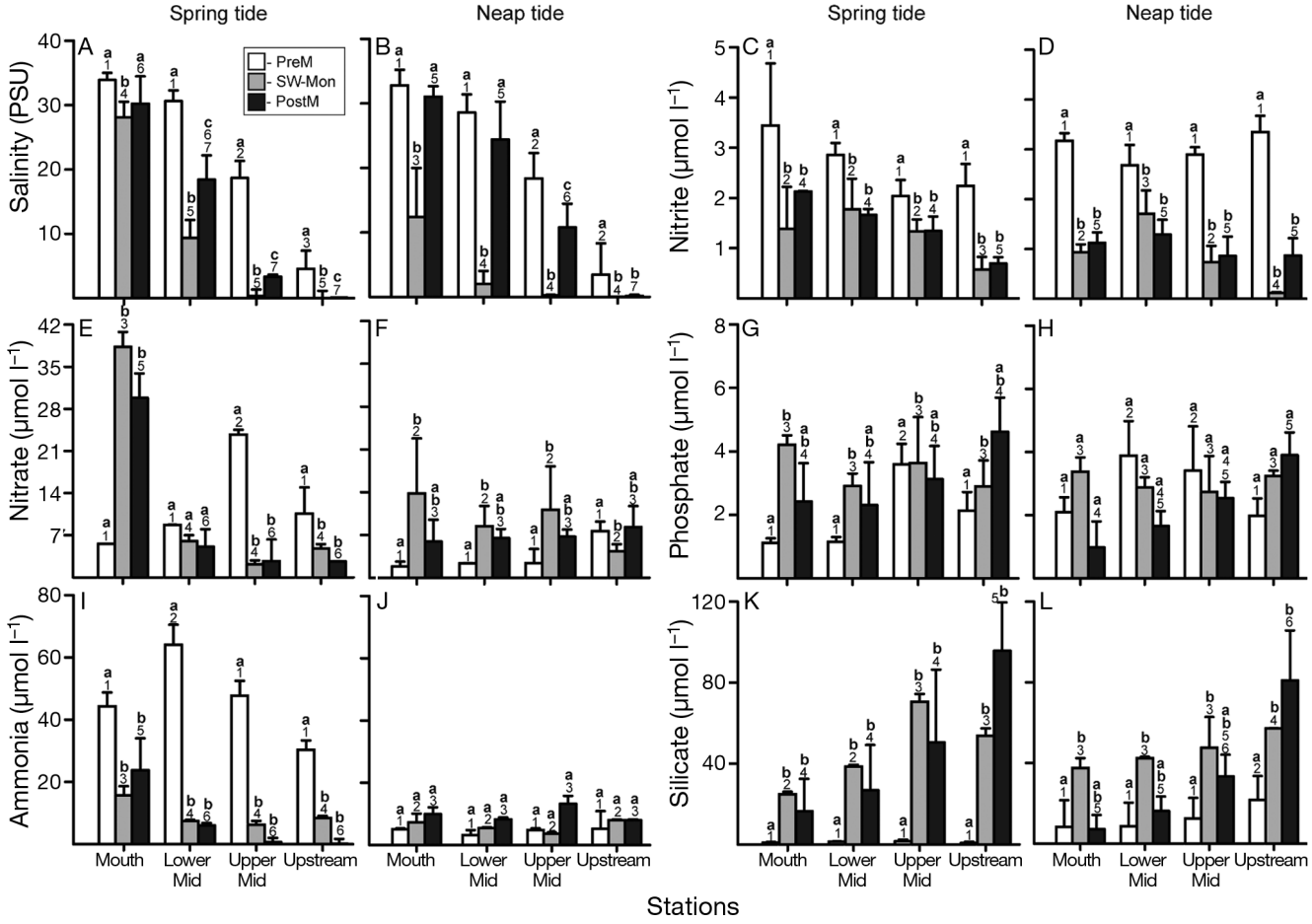

Fig. 2. Mean (±SD) values of salinity and dissolved nutrient concentrations in surface waters from the mouth to upstream regions of the Zuari estuary during spring and neap tides. Lower mid: lower middle; upper mid: upper middle; PreM: premonsoon; SW-Mon: southwest monsoon; PostM: post monsoon. Significant differences among the seasons and stations were evaluated using 3-way ANOVA followed by post hoc Tukey's HSD test at a significance level of $\alpha = 0.05$. Different letters (a–c) and numbers (1–7) above the bars denote significant differences among seasons and stations, respectively

Variation in TBC, BP, and protist abundance

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

In the case of protist abundance, a significant temporal (ANOVA; $F = 31.00$, df = 2, p = 0.0001), spatial (ANOVA; $F = 8.13$; df = 3, p = 0.0001), and tidal variation was observed (ANOVA; $F = 32.16$; df = 1, p = 0.0001) with higher abundance during neap tide of SW-Mon ($3.76 \pm 0.5 \times 10^5$ cells ml^{-1}) and PostM ($4.31 \pm 0.4 \times 10^5$ cells ml^{-1}) (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 number of protists was positively related to temperature, SPM, and TOC, and negatively related to chl *a* and POC, especially during neap tide ($F = 15.1$, p = 0.0008; Fig. 5D,F).

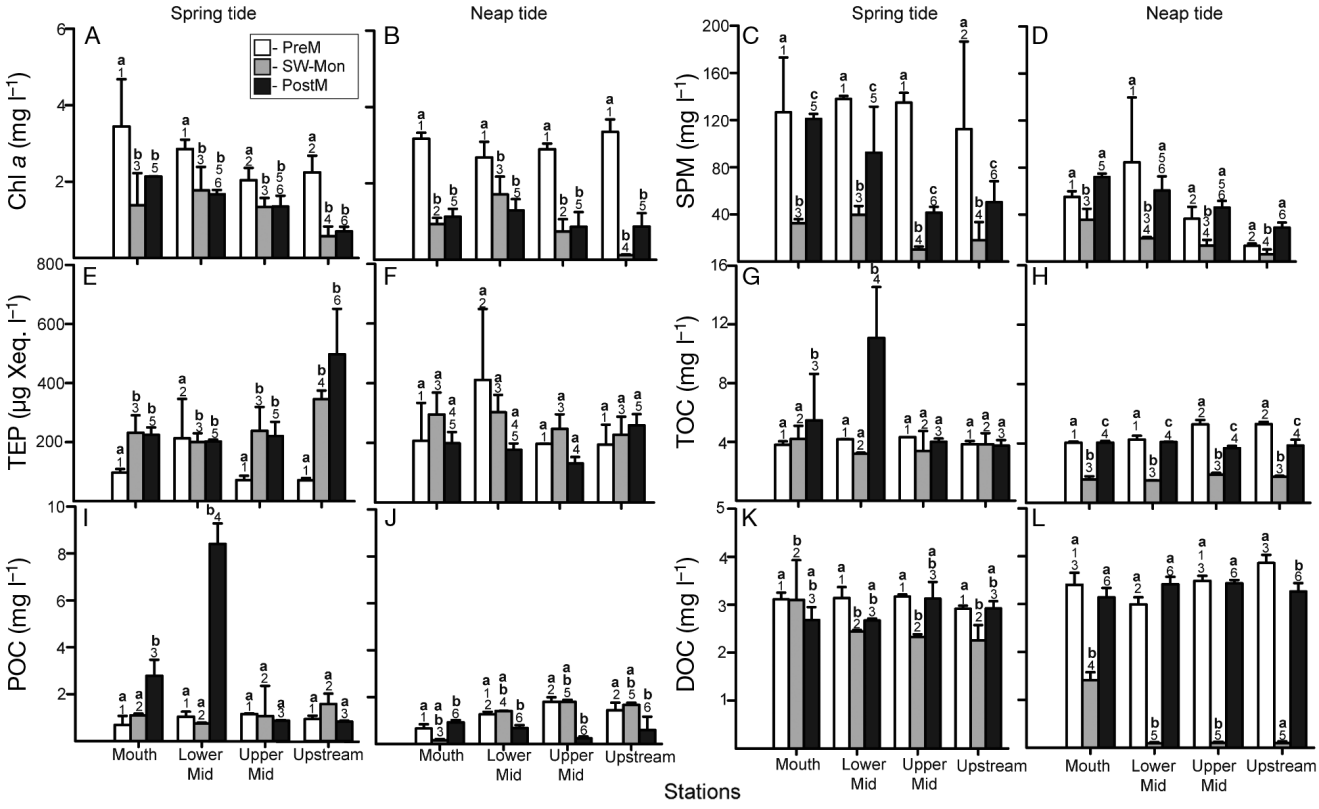

Fig. 3. Mean (±SD) values of chlorophyll *a* (chl *a*), suspended particulate matter (SPM), transparent exopolysaccharides (TEP, as Xanthan gum equivalent [Xeq.]), total organic carbon (TOC), particulate organic carbon (POC) and dissolved organic carbon (DOC) concentrations in surface waters from the mouth to upstream regions of the Zuari estuary during spring and neap tides. See Fig. 2 for abbreviations

Variation in β-Glu gene abundance and number of cells expressing β-Glu

In general, β-Glu gene abundance and number of cells expressing β-Glu varied significantly among the sites ($F = 10.85$, df = 3, p = 0.0001 and $F = 19.57$, df = 3; p = 0.0001, respectively) as well as seasons ($F = 24.86$, df = 2, p = 0.0001 and $F = 43.54$, df = 2, p = 0.0001, respectively) (Fig. 4G,H,K,L). Tidal variation was not significant. The number of cells expressing β-Glu increased during PreM ($4.05 \pm 1.5 \times 10^6$ cells ml^{-1} and 49% with respect to TBC), and a clear decrease was evident during SW-Mon ($3.65 \pm 1.3 \times 10^5$ cells ml^{-1} and 16% with respect to TBC) and PostM ($1.62 \pm 0.9 \times 10^5$ cells ml^{-1} and 4% with respect to TBC) from the mouth to upstream region (Fig. 4G,H & Fig. S3A,B in the Supplement at www.int-res.com/articles/suppl/a080p273_supp.pdf). Whereas gene abundance showed a significant increase from PreM ($3.18 \pm 0.13 \times 10^6$ copies µl^{-1}) to PostM ($1.51 \pm 0.16 \times 10^8$ copies µl^{-1}) (Fig. 4K,L). Gene abundance and the number of cells expressing β-Glu were high at the mouth and decreased towards upstream, but the opposite was observed in the case of BP. The uncoupling between BP, β-Glu gene abundance, and the number of cells expressing β-Glu was more pronounced during SW-Mon (Fig. 5A,D, Table S2C in the Supplement). Gene abundance was positively related to salinity, TOC, and POC (Fig. 5A,C,D,F), while the number of cells expressing β-Glu was positively related to temperature, salinity, SPM, chl *a*, and DOC, and negatively related to TEP (Fig. 5A,C,D,F Table S2A–C).

Variation in BCC and its relationship to β-Glu gene abundance and number of cells expressing β-Glu

Overall, a clear shift in BCC was evident between the seasons and sites (post hoc Tukey's HSD $p < 0.05$; Figs. 5B,E & 6). Likewise, total community recovery also varied with the seasons (Fig. S3C,D). A higher percentage of populations recovered during PostM (45 to 68%) compared to other seasons; high recovery was mainly related to *Alphaproteobacteria* abundance (Fig. S3C,D). The mouth and lower mid-

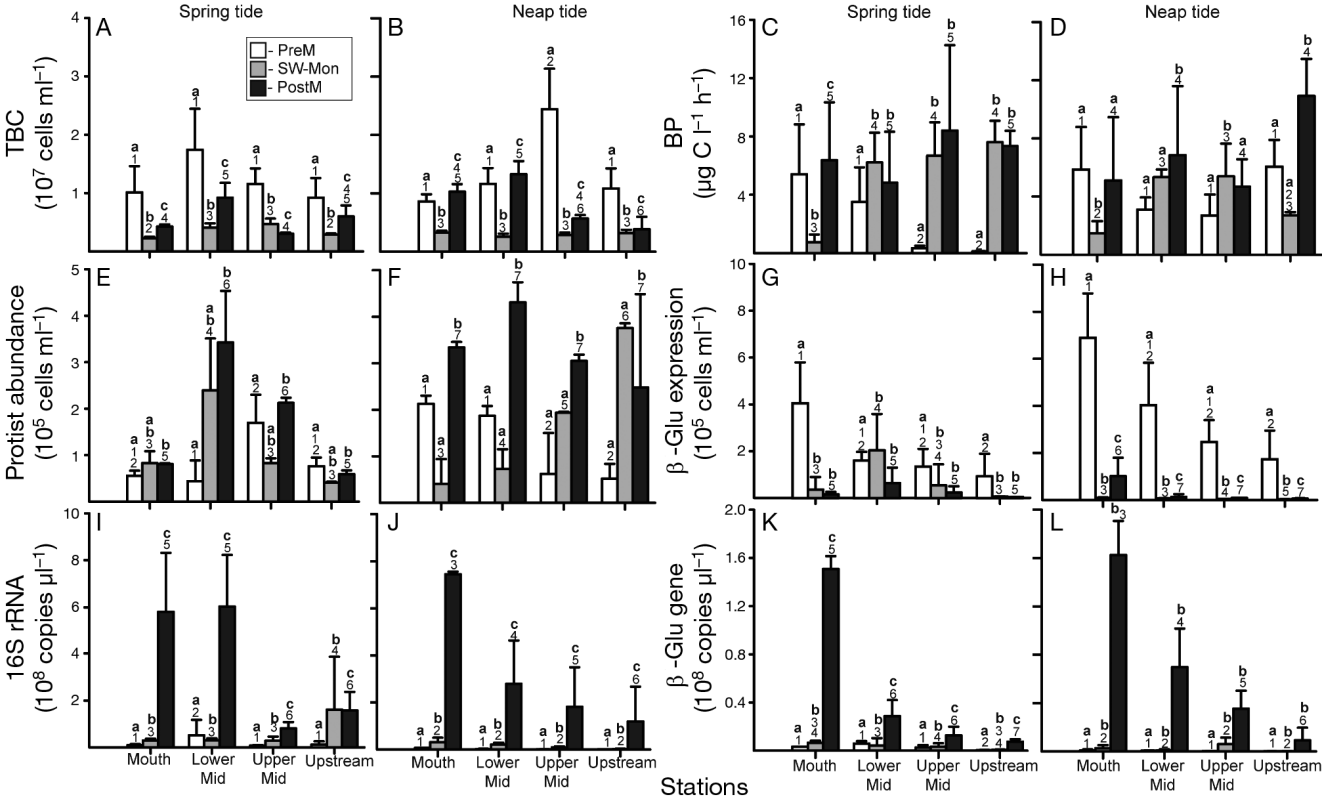

Fig. 4. Mean (±SD) values of total bacterial count (TBC), bacterial production (BP), protist abundance, number of cells expressing β-glucosidase (β-Glu expression), 16S ribosomal RNA (16S rRNA), and β-Glu gene abundance in surface waters from the mouth to upstream regions of Zuari estuary during spring and neap tides. See Fig. 2 for abbreviations

estuary were dominated by *Alpha-* and *Gammaproteobacteria*, which appear to be marine as evidenced by their significant positive relation to salinity (Figs. 5A,D & 6A,B,E,F; $p < 0.05$). Both groups were abundant during non-monsoon seasons. The *Betaproteobacteria* dominated the upper mid and upstream regions of the estuary and were negatively related to salinity during SW-Mon (Fig. 6C,D). Similarly, the proportions of *Actinobacteria* and *Firmicutes* were high in the upper-mid estuary, and their abundance increased during SW-Mon and PostM seasons (Fig. 6I–L). *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 β-Glu, and *Gammaproteobacteria* abundance during PreM (Fig. 5A–E, Table S2A–C; $p < 0.001$). Further, a decrease in *Gammaproteobacteria* abundance and number of cells expressing β-Glu during SW-Mon was significantly related to protist abundance and salinity change. β-Glu gene abundance was associated with diverse groups (*Alpha-* and *Gammaproteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*) in which *Alphaproteobacteria* appeared to be most significant, especially during PostM (Fig. 5A,D, Table S2C; $p < 0.01$). However, non-expression of the gene in these communities could be related to environmental factors, mainly SPM, salinity, and TOC (Fig. 5A,D; Monte Carlo test, $p < 0.05$). BP was strongly influenced by *Betaproteobacteria* abundance, which largely uncoupled with β-Glu gene abundance and the number of cells expressing β-Glu during SW-Mon (Table S2C).

DISCUSSION

In the present study, total bacterial abundance was high during the PreM season and was mainly driven by temperature, salinity, and TOC, whereas bacterial production was high during SW-Mon and PostM towards the upstream region of the Zuari estuary. A recent study by Kaartokallio et al. (2016) reported similar results in the inner estuarine regions of the west and south coast of Finland, which was related to

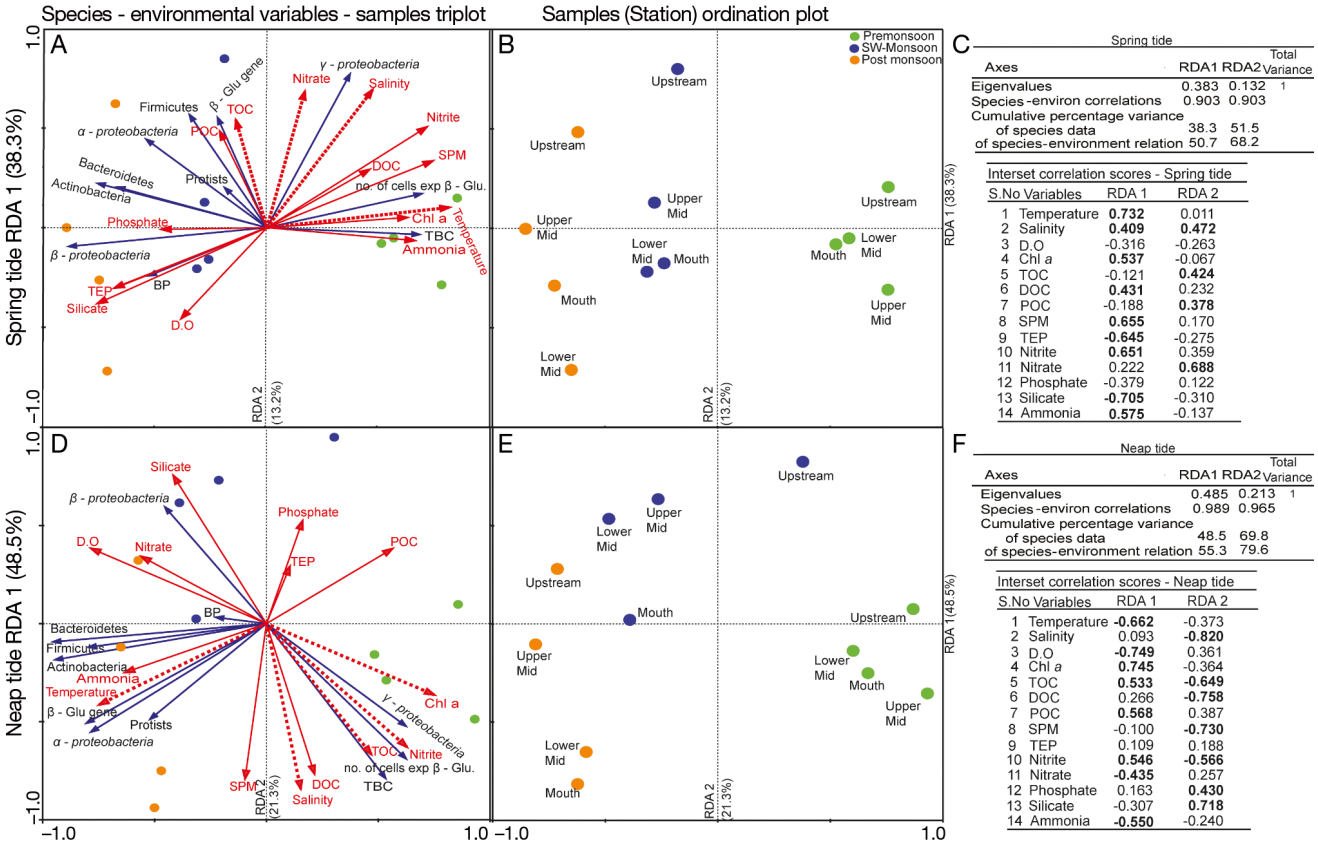

Fig. 5. Redundancy analysis (RDA) showing the relationship between biotic and abiotic variables during the spring and neap tides in Zuari estuary. (A,D) RDA triplots: red arrows indicate environmental variables; dotted red lines are highly significant (Monte Carlo test, $p < 0.05$); blue arrows indicate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

the strong influence of riverine water mixing with an estuarine gradient. In the present study, the number of cells expressing β-Glu were high at the mouth (40 to 80% of the total abundance) and lower-mid estuary (10 to 50% of total abundance) with a seasonal pattern similar to that of total bacterial abundance. Both the number of cells expressing β-Glu and total abundance was positively related to chl *a* and negatively related to TEP. The high chl *a* (3.44 mg l^{-1}) level at the mouth of the estuary indicated high phytoplankton abundance. Most estuaries are highly productive, and both autochthonous (*in situ* production) and allochthonous (external input) substrates influence bacterioplankton abundance and production (Coffin & Sharp 1987). Heterotrophic bacteria are known to actively consume DOM produced by the phytoplankton cells (Cole et al. 1982, Petit et al. 1999). Phytoplankton produce carbohydrate-rich polymers, either as low molecular weight or polymeric forms, that are degraded through enzymatic activity (Hama & Yanagi 2001, Steen et al. 2008). TEPs are one of these organic polymers, and activity/expression of β-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 of the estuary in the present study indicates their active breakdown by high numbers of β-Glu expressing bacteria.

However, TEPs were abundant in the upstream region, especially during the onset of the monsoon, and this can be related to a decline in the number of cells expressing β-Glu from the mouth to upstream. This relationship suggests an influence of the large salinity variations in our study area that occur between monsoon and non-monsoon seasons (i.e. <5 psu at the mouth and >15 psu upstream). A study by Cunha et al. (2000) also pointed out that exoenzymatic activities are significantly related to temperature, salinity and chl *a*. Similarly, a study in the Baltic Sea region showed that the alteration in OM composition and salinity via high river loads resulted in a significant change in enzymatic activity (Figueroa et

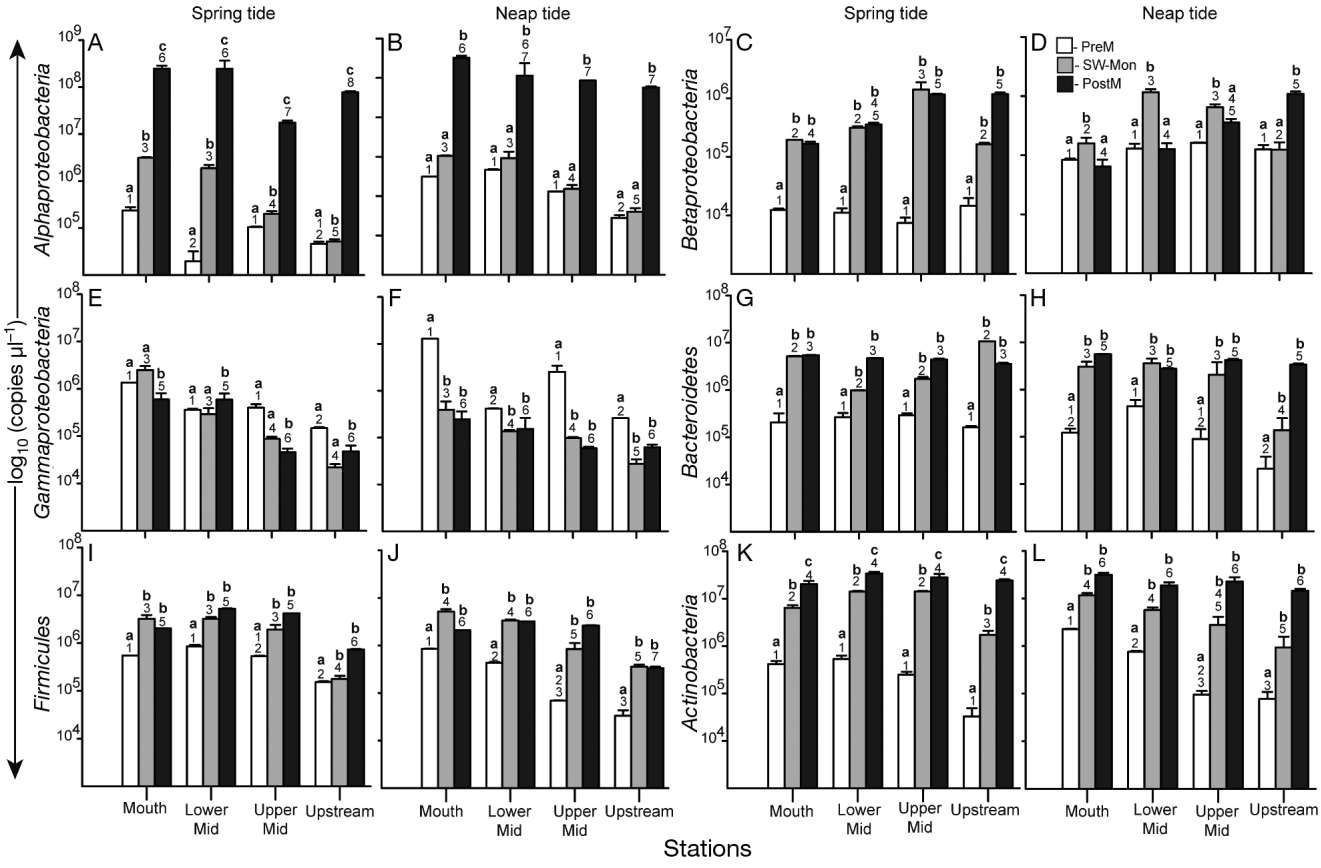

Fig. 6. Mean (±SD) copy numbers of different bacterial groups in surface waters from the mouth to upstream regions of the Zuari estuary during spring and neap tides. See Fig. 2 for abbreviations

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 β-Glu. Observation at the turbid Elbe estuary also reported a similar correlation between enzymatic activity and SPM and noted it to be a controlling factor for the distribution of organisms and nutrients (Bernát et al. 1994). A recent study by Boras et al. (2015) at Blanes Bay also reported a similar relationship with β-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 compared to the dry season (Gonsalves et al. 2009). Thus, other than salinity, the decrease in the number of cells expressing β-Glu during SW-Mon could be due to a change in the SPM composition.

Clear seasonal and spatial shifts in the bacterial community structure were evident. Among the sites, proportions of *Alpha-* and *Gammaproteobacteria* were higher at the marine mouth, lower in the mid region and decreased gradually towards the upstream area. Similar findings have been reported in other estuarine regions such as the 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). In our study, the mouth and lower-mid stations showed similar bacterial community composition and had similar physical characteristics, i.e. salinity. However, the upstream region was distinctly different and was dominated by *Betaproteobacteria* irrespective of the season. Earlier studies have also reported dominance of *Betaproteobacteria* in freshwater and *Alphaproteobacteria* in salt water areas (Bouvier & del Giorgio 2002, Kan et al. 2006). TOC and POC were linearly related to *Alphaproteobacteria* abundance. The temporal variations in bacterial communities are linked to the changes in chl *a*, temperature, salinity, and availability of OM (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 bacterial communities (Pernthaler et al. 2001). In the present study, an increase in protist abundance resulted in a significant decrease in the relative abundance of *Gammaproteobacteria* towards the upstream region and during the SW-Mon. Beardsley et al. (2003) also reported that selective grazing by heterotrophic nanoflagellates controlled the *Gammaproteobacteria* genera in the North Sea. The upper mid-estuary showed higher proportions of *Actinobacteria* and *Firmicutes*. Members of these have been detected in the mesohaline waters (<20 psu) and related to freshwater taxa (Newton et al. 2011). Furthermore, members of this clade are sensitive to salinity changes and depend on a specific range of organic substrates (Sharp et al. 2009, Campbell & Kirchman 2013). A study by Harji (2011) in this region showed that terrestrially derived OM mainly dominated the lower-mid estuary due to riverine influence. Thus, it seems that low saline conditions during SW-Mon and high input of riverine sources favored the proliferation of freshwater taxa with low β-glucosidase activity at the upper-mid estuary. However, *Bacteroidetes* were fairly persistent along the estuary, as has been 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 amounts of hydrolytic enzymes to degrade complex polysaccharides (Azam & Malfatti 2007, Dang et al. 2009, Edwards et al. 2010).

Microbial degradation via ectoenzymes is one of the major sources of substrate addition in aquatic systems for bacterial utilization (Hoppe et al. 1988). Although previous studies have hypothesized that the source of OM and the microbial community can significantly influence the seasonality of β-Glu compared to hydrography (Bhaskar & Bhosle 2008, Boucher & Debroas 2009), such links were not evaluated in this estuary. During this study, β-Glu gene abundance and the number of cells expressing β-Glu showed strong seasonality along with the changes in BCC. The number of cells expressing β-Glu to gene abundance ratios were high during PreM and apparently decreased during SW-Mon and PostM, as well as towards the upstream region. The β-Glu gene copies covaried with the relative abundance of *Alpha* and *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria* irrespective of the tides. The number of cells expressing β-Glu and the relative abundance of *Gammaproteobacteria* 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 β-Glu and *Gammaproteobacteria* abundance, and negative with *Alphaproteobacteria*, *Firmicutes*, and *Actinobacteria*. In estuaries, *Gamma-* and *Alphaproteobacteria* 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 *Gammaproteobacteria* benefit from algae and their derived OM (Puddu et al. 2003, Teeling et al. 2012). Furthermore, they contribute significantly to the decomposition of the OM produced by algal communities, suggesting that *Gammaproteobacteria* are the potential group expressing the β-Glu enzyme. A few exopolymer enrichment studies have revealed that members of *Gammaproteobacteria* (mainly *Acinetobacter* and *Pantoea*) possess genes for β-Glu production and can degrade polysaccharides (Tajima et al. 2001, Arora et al. 2012). Zoppini et al. (2005) also pointed out that OM enrichment favored *Gammaproteobacteria* growth in the 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 the majority of the strains included *Vibrio*, *Aeromonas*, *Enterobacter*, *Marinobacter*, *Aeromonas*, and *Exiquinobacterium* — indicating a major contribution of *Gammaproteobacteria* taxa in the degradation of carbohydrate-derived OM. Similarly, a study by Yu et al. (2011) evaluated the degradation capability of glucosidase substrates and reported that it was mainly distributed in the *Gammaproteobacteria* and *Bacteroidetes* groups when compared to *Alphaproteobacteria* and *Actinobacteria*. A study by Moreno et al. (2013) also observed higher genetic diversity of β-Glu encoding genes in *Proteobacteria* followed by the representative members of *Chloroflexi*, *Deionococci*, *Actinobacteria*, *Thermotogae*, and *Firmicutes*. Likewise, Berlemont & Martiny (2013) analysed 5123 bacterial genomes sequences and reported that 79% of the genome contained the β-Glu gene, which included phyla 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) in Baltic Sea basin waters revealed a significant correlation between β-Glu activity and taxa such as *Flavobacteriaceae*, *Chromatiaceae*, and *Verrucomicrobia*.

The proportions of *Gammaproteobacteria* decreased during the SW-Mon, along with a decrease in the number of cells expressing β-Glu throughout the

estuary and changes in DOM. It is known that in this estuary along the salinity gradient, an upper freshwater region is dominated by terrestrial inputs, whereas phytoplankton-derived DOC dominates the saline mouth region (Mannino & Harvey 2000, Harji 2011). Although the present study did not measure DOM chemistry, the decoupling between BP, β-Glu gene abundance, and the number of cells expressing β-Glu could be related to changes in OM composition, which influences the bacterial community. An increase in BP can be related to *Betaproteobacteria* and a decrease in salinity, but this increase neither influenced the β-Glu gene abundance nor the number of cells expressing β-Glu. An earlier study carried out in the Zuari estuary also indicated that BP is fueled by allochthonous sources and behave as a net heterotrophy system during the monsoon months (Pradeep Ram et al. 2007). A few studies also stated that a 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 physical and chemical factors partially control ectoenzymatic patterns by indirectly controlling bacterial community structure (Langenheder et al. 2005, Boucher & Debroas 2009).

Overall, the results of the present study indicate clear seasonal shifts in BCC, with high proportions of *Proteobacteria* (*Gamma* and *Alpha*) groups during PreM shifting to *Actinobacteria*/*Betaproteobacteria*/*Firmicutes* groups during SW-Mon (a period of high riverine influx), which then reverse to *Alphaproteobacteria* during the PostM recovery season. Although β-Glu gene abundance was related to the relative abundance of diverse phylogenetic groups, the number of cells expressing β-Glu was mainly related to the relative abundance of *Gammaproteobacteria* taxa. The higher ratio in the number of cells expressing β-Glu observed in saltwater, declining towards the freshwater region could be related to changes in the spectrum of DOM input in this estuary. In conclusion, the number of cells expressing β-Glu was influenced by shifts in the BCC that appear to be regulated by different environmental drivers. Further studies should focus on the translocation of marine, brackish, and freshwater bacterial communities into different environmental conditions and evaluate their potential influence on the processing of OM.

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