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Environmental and hydroclimatic factors influencing *Vibrio* populations in the estuarine zone of the Bengal delta

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Abstract The objective of this study was to determine environmental parameters driving *Vibrio* populations in the estuarine zone of the Bengal delta. Spatio-temporal data were collected at river estuary, mangrove, beach, pond, and canal sites. Effects of salinity, tidal amplitude, and a cyclone and tsunami were included in the study. *Vibrio* population shifts were found to be correlated with tide-driven salinity and suspended particulate matter (SPM). Increased abundance of *Vibrio* spp. in surface water was observed after a cyclone, attributed to re-suspension of benthic particulate organic carbon (POC), and increased availability of chitin and dissolved organic carbon (DOC). Approximately a two $\log_{10}$ increase in the ($p < 0.05$) number of *Vibrio* spp. was observed in < 20 μm particulates, compared with microphytoplankton (20–60 μm) and zooplankton > 60 μm fractions. Benthic and suspended sediment comprised a major reservoir of *Vibrio* spp. Results of microcosm experiments showed enhanced growth of vibrios was related to concentration of organic matter in SPM. It is concluded that SPM, POC, chitin, and salinity significantly influence abundance and distribution of vibrios in the Bengal delta estuarine zone.

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Introduction

The ecology and hydrodynamics of low-lying coastal sectors of tropical areas, such as the Bengal delta, are predicted to be affected by sea-level rise, elevated temperatures, more frequent cyclone events, and increased rainfall, factors associated with climate change (Cruz et al. 2007). Historically, the Bay of Bengal estuaries endure cyclonic storms, monsoon rainfall, and floods each year. They also serve as a reservoir of *Vibrio cholerae*, the causative agent of cholera, and many other *Vibrio* spp. (Alam et al. 2009; Akther et al. 2016). Ecological factors that regulate the dynamics of toxigenic serogroups of *V. cholerae* and *Vibrio parahaemolyticus* in the Bengal delta estuaries have been explored, including salinity, temperature, rainfall, and plankton (Huq et al. 2005; Alam et al. 2006; Cruz et al. 2007; Constantin de Magny et al. 2008). However, micro-ecosystem parameters, such as physicochemical factors, nutritional substrates, and tidal amplitude related to *Vibrio* populations in the Bengal estuaries have received limited attention (Neogi et al. 2011; Batabyal et al. 2014).

In this context, it is important to note that *Vibrio* spp. uniformly possess the ability to degrade chitin, a polymer of *N*-acetylglucosamine, produced in the aquatic environment ($\sim 10^{14}$ kg year^{-1}) (Keyhani and Roseman 1999). Vibrios also secrete a variety of enzymes that degrade organic matter, e.g., proteases, lipases, and laminarinase (Oliver et al. 1986; Alderkamp et al. 2007). Thus, turnover of complex organic compounds by vibrios represents an important contribution to the aquatic food web, making available a large fraction of particulate organic carbon (POC) and nitrogen (PON), as well as dissolved organic carbon (DOC) and nitrogen (DON). In the planktonic stage, vibrios attach to both small (< 20 μm) particulate organic matter (POM) and larger living or dead and degrading plankton. This association with plankton provides an ecological advantage, including nutrition, enhanced survival, and escape from predators (Tamplin et al. 1990; Neogi et al. 2014). In the estuarine environment, *Vibrio* populations are affected by multi-factorial interactions of physicochemical conditions, plankton dynamics, and availability of substrates as nutrient. A stimulating influence of suspended particulate matter (SPM), comprising silt, clay, and very fine sand, collectively termed "suspended sediment" (SS), as well as colloids, organic detritus, and plankton, on the occurrence of *Vibrio* populations has been reported (Colwell et al. 1981; Julie et al. 2010; Johnson et al. 2012). Thus, the benthic sediment of a coastal zone provides shelter and nutrients for *Vibrio* populations (Vezzulli et al. 2009; Johnson et al. 2012).

The seventh cholera pandemic is reported to have emerged in the coastal region of Bangladesh near the Karnaphuli estuary, approximately 2 years after its initial appearance in Indonesia in 1961 (Rizvi et al. 1965). Also, the current pandemic of *V. parahaemolyticus*-related gastroenteritis was first detected in 1996 in the coastal zone of Sundarban in the Bengal delta which comprises one of the largest mangroves in the world (Nair et al. 2007). The Ganges-Brahmaputra river mouth constitutes one of the world's highest sediment discharge zones, depositing significant amounts (ca. 1100 million MT year^{-1}) of sediment into the coastal Bay of Bengal (Darby et al. 2015). The interplay between these and bentho-pelagic events occurring in the estuarine zone of the Bengal delta is poorly understood, as is the effect of a cyclones or tsunamis on SPM, POM, chitin, and DOC and, subsequently, *Vibrio* populations (Neogi et al. 2011; Batabyal et al. 2014).

In the present study, *Vibrio* populations in surface water and benthic samples collected over a multiple year period were analyzed to establish their dynamics in three estuarine zones of the Bengal delta, the Karnaphuli estuary, Kuakata coast (near the Ganges-Brahmaputra river mouth), and Sundarban mangrove of Bangladesh. Environmental conditions before and after a cyclone, a tsunami and monsoon, and during a tidal cycle were determined. *Vibrio* dynamics were correlated with specific factors, including particulates, chitin, and dissolved inorganic and organic nutrients. The study was designed to explore the aquatic reservoir of *Vibrio* spp. on a broader scale and identify key ecohydrological factors regulating their population dynamics in the estuarine ecosystem of the Bengal delta.

Materials and methods

Study sites and sampling

Surface water (W) and benthic sediment (S) samples were collected from the Karnaphuli River estuary, Sundarban mangrove, and Kuakata coast in the east, west, and central coastal regions of Bangladesh. Major

features of the sampling regime and sample types are provided in Table 1. In the Karnaphuli estuary, near the port city of Chittagong (Fig. 1), samples were collected monthly from December 2004 to February 2005 and at 2-week intervals in May 2007 to determine spatio-temporal variation under different hydroclimatic conditions. Sampling during May 2007 captured the following conditions: pre-monsoon and 3 days before and 2 days after a cyclone at 14 sites that included high-, medium-, and low-salinity regions ($n = 5$, 5, and 4, respectively). During 2004–2005, the sample collection was carried out at nine sites, three times at three sites in each salinity region, 12 days before and 12 and 42 days after the 26 December 2004 Asiatic tsunami. In 2004–2005 and 2007, surface water samples were collected 0.5 m along the longitudinal axis of the river, starting at the river mouth as the ebb tide began. Surface water samples (300–400 L) collected before the 2007 cyclone were filtered, using 60 and 20 μm plankton nets to obtain ~ 100 mL of > 60 μm zooplankton, 20–60 μm predominantly phytoplankton, and < 20 μm particulates. These fractions were examined for *Vibrio* spp., SPM, and chitin content. Tidal variation was examined by collecting water samples at 0.5 m depth ($n = 11$) hourly during the monsoon season in June 2007. The water samples were collected at low tide to high tide at a medium-salinity site located ca. 7 km inland from the river mouth. In the Sundarban mangrove, surface water samples were collected hourly ($n = 12$) and plankton samples at 2 h intervals ($n = 5$) at 0.5 m depth at a salinity-stable creek and at high tide over a full tidal cycle during December 2007 (post-monsoon). *Vibrio* populations in the surface water samples collected at 0.5 m depth and benthic sediment at 0.25 m depth were analyzed using synchronized collections in the high salinity region of the Karnaphuli estuary ($n = 3$) and the Sundarban mangrove creek ($n = 3$) in May 2007 and December 2007, respectively. Synchronized sampling was also carried out from September to November 2012, at ten sites of Kuakata, including canal, river, pond, and beach sites (Fig. 1). Five subsamples of water and sediment were randomly collected at each site and combined to provide a composite.

Physicochemical measurements

Physicochemical parameters of water samples were measured in situ as follows. Temperature and pH were measured using a portable pH meter (sensION, HACH, Loveland, CO, USA), salinity by conductivity (WTW 340i with TetraCon325, Weilheim, Germany), and turbidity by nephelometry (Oakton T-100, Vernon Hills, IL, USA).

Analysis of dissolved nutrients

Water samples were filtered through GF/F filters (Whatman), and the filtrates (50 mL) were poisoned with 150 μL of $HgCl_2$ (20 g L^{-1}) and stored at 4 °C for nutrient analyses. Dissolved inorganic nitrogen (nitrate, nitrite, and ammonium, μM N), and phosphate (μM PO_4^{-3}) were determined spectrophotometrically in a Skalar-SAN-plus autoanalyzer (Skalar Analytical BV, Breda, the Netherlands), according to standard methods for seawater (Kattner and Becker 1991). Each sample was measured four times and corrections for blank absorptions were included.

Dissolved organic matter was quantified by measuring the concentration of DOC. Samples were acidified with phosphoric acid (20%, *v*/*v*) to remove inorganic carbon, and DOC was measured after high-temperature (680 °C) oxidation in a TOC analyzer (Dohrmann DC-190, Rosemount Analytical Inc., Santa Clara, CA, USA), following the method of Skoog et al. (1997).

Analysis of particulate nutrients

Suspended particulate matter (SPM) in water (< 20 μm fraction) and plankton (20–60 μm and > 60 μm fractions) were quantified, as described by Neogi et al. (2011). For POC and PON determination, the inorganic content in SPM was removed by acidification with 1 M HCl prior to quantification in a Flash EA 1112 elemental analyzer (Thermo Fisher Sci GmbH, Bremen, Germany), according to Verado et al. (1990). Standard Reference Material 1515 (LECO Corp., St. Joseph, MI, USA) was used for calibration after every five samples had been analyzed.

Chitin content in SPM and in the phytoplankton and zooplankton concentrates was determined, based on the high affinity of wheat germ agglutinin (WGA) to *N*-acetyl glucosamine residues, following the WGA-FITC method of Montgomery et al. (1990) using a fluorometer (Turner 450) at excitation of 426 nm and emission of 520 nm. Prior to labeling with WGA-FITC (Vector Laboratories Ltd., Peterborough, UK), particulates collected on filters were re-suspended in 3.0 mL borate buffer (0.1 M, pH 7.4) by sonication and extensive

Table 1 Details of sampling and samples collected in the Bengal delta

Location	Ecosystem (Campaigns: sites)	Pollution level[a]	Date	Season/ climate	Sample type (*n*, sites/samples)			Purpose
					High salinity	Medium salinity	Low salinity	
Karnaphuli	Riverine estuary (2004–2005, 9 sites)	Upstream: low	14 Dec 2004	Post-monsoon	W (3)	W (3)	W (3)	Spatial biophysicochemical change
		Mid region: high	07 Jan 2005	Post-tsunami	W (3)	W (3)	W (3)	Tsunami impact
		Upstream: medium	08 Feb 2005	Pre-monsoon	W (3)	W (3)	W (3)	Spatio-temporal variation
	(2007 May, 14 sites; Jun, 4 sites, 1 for tidal variation)		12 May 2007	Pre-monsoon	W (5), P (5), Z (5)	W (5), P (5), Z (5)	W (4), P (4), Z (4)	*Vibrio* distribution, key correlating factors
			17 May 2007	Post-cyclone	W (5)	W (5)	W (4)	Cyclone impact
			12 Jun 2007	Monsoon		W (11)		Tidal variation, monsoon impact
			12 Jun 2007		W (3), BS (3)			Comparative *Vibrio* occurrence
Sundarban	Mangrove wetland (2007, 1 site)	Very low	17 Dec 2007	Post-monsoon	W (12), P (5), Z (5)			Tidal variation, *Vibrio* distribution
			17 Dec 2007		W (3), BS (3)			Comparative *Vibrio* occurrence
Kuakata coast	Beach (2012, 5 sites)	Low	14 Sep 2012	Monsoon	W (5), BS (5)			Comparative *Vibrio* occurrence
			16 Oct 2012	Post-monsoon		W (5), BS (5)		Seasonal variation in micro-ecosystem
			15 Nov 2012	Post-monsoon			W (5), BS (5)	
	Canal (2012, 1 site)	High	14 Sep 2012	Monsoon			W (1), BS (1)	Comparative *Vibrio* occurrence
			16 Oct 2012	Post-monsoon			W (1), BS (1)	Seasonal variation in micro-ecosystem
			15 Nov 2012	Post-monsoon		W (1), BS (1)		
	Pond (2012, 2 sites)	Medium	14 Sep 2012	Monsoon			W (2), BS (2)	Comparative *Vibrio* occurrence
			16 Oct 2012	Post-monsoon			W (2), BS (2)	Seasonal variation in micro-ecosystem
			15 Nov 2012	Post-monsoon			W (2), BS (2)	
	River (2012, 2 sites)	Medium	14 Sep 2012	Monsoon			W (2), BS (2)	Comparative *Vibrio* occurrence
			16 Oct 2012	Post-monsoon		W (2), BS (2)	W (2), BS (2)	Seasonal variation in micro-ecosystem
			15 Nov 2012	Post-monsoon	W (2), BS (2)		W (2), BS (2)	

W, surface water (0.5 m depth); *P*, phytoplankton (20–60 μm); *Z*, zooplankton (> 60 μm); *BS*, benthic sediment (0.25 m depth)

[a] High, medium, low, and very low levels of pollution were indicated by mean fecal coliform counts $>10^2$, 10^1–10^2, and 10^1–10^0, and < 1 CFU mL^{-1}, respectively

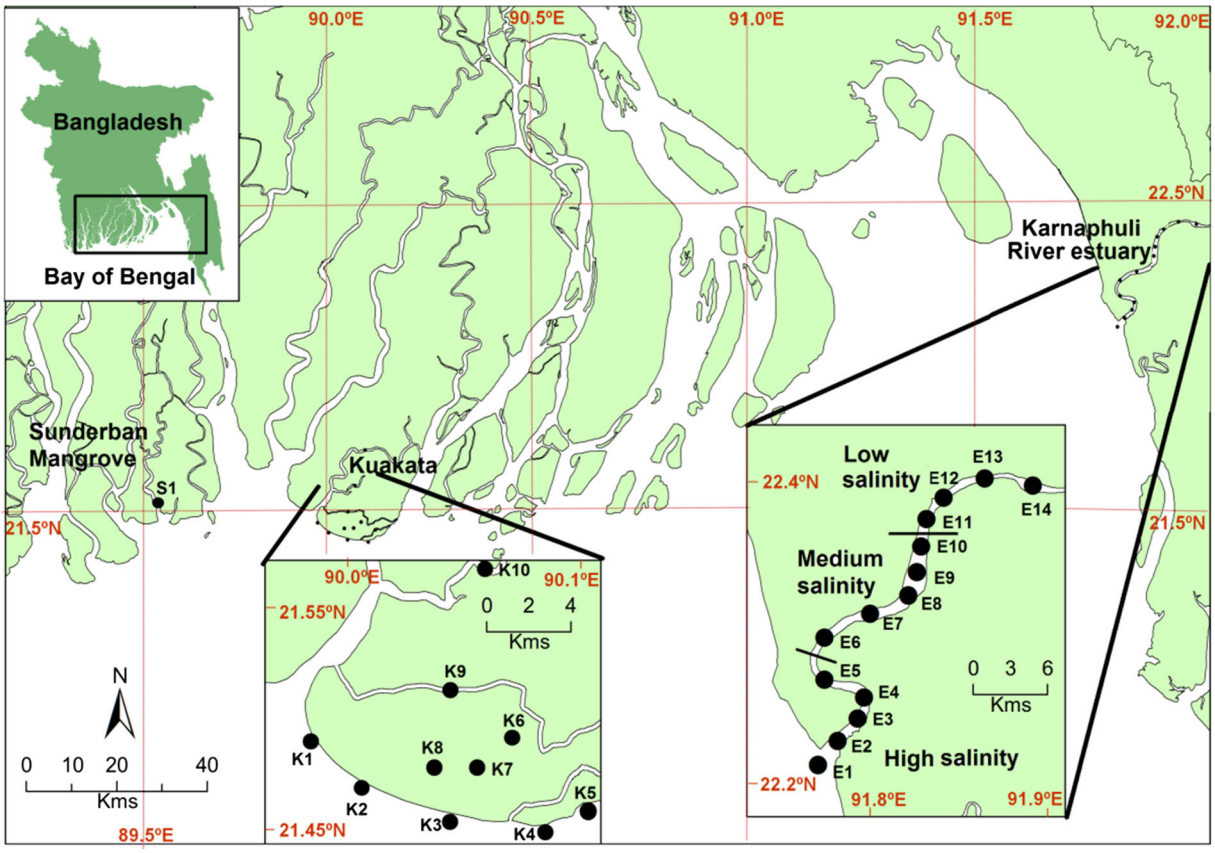

Fig. 1 Sampling sites in the eastern (Karnaphuli River estuary, during December 2004–February 2005 and May–June 2007), central (Kuakata, during October–December 2012), and western (Sundarban mangrove, during December 2007) coastal regions of Bangladesh. Closed circles indicate sites where samples were collected. The Karnaphuli estuarine sites were categorized based on salinity during high tide where the high-, medium-, and low-salinity zones ranged from 10 to 20, 2 to 9, and 0 to 1 practical salinity unit (PSU), respectively. Land-locked sites in Kuakata represent pond and canal ecosystems

pipetting. Purified crab chitin (Sigma-Aldrich, St. Louis, MO, USA) was used as standard for calibration. Background fluorescence of each sample without adding WGA-FITC was subtracted.

Culture and identification of *Vibrio* spp.

A portion of each water sample (100 μL, in triplicate), concentrated by filtration (0.2 μm filter, Millipore) with or without enrichment in alkaline peptone water (Difco, at 37 °C for 18 h), was plated on thiosulfate citrate bile salts sucrose (TCBS) agar (Difco, Detroit, MI, USA), and presumptive vibrios (colony forming units, CFU) were counted after 24 h incubation at 37 °C. For concentrated plankton fractions, 10 mL aliquots were homogenized with an Ultra-Turrax (T25, Ika-Werke, Staufen, Germany) and cultured for vibrios, as described for the water samples. The NaCl concentration in each medium was adjusted to the salinity of the original water sample. Representative *Vibrio* spp. (10 to 20 isolates from each of three replicates for each sample) were identified by species-specific multiplex PCR (Haldar et al. 2010; Neogi et al. 2010) and API 20E biochemical tests (bioMerieux, Marcy I'Etoile, France). Based on results of confirmatory tests, the proportion of positive isolates in the TCBS plate count and an average of three replicates of each sample were calculated to obtain the culturable *Vibrio* count.

Direct fluorescent antibody detection of *V. cholerae* O1

For detection of viable *V. cholerae* O1, yeast extract (0.002%) and nalidixic acid (0.025%) were added to the water and plankton homogenates (Kogure et al. 1979), incubated overnight, and preserved with formaldehyde (4%). Samples were labeled with monoclonal

fluorescein isothiocyanate (FITC)-conjugated anti-O1 antibody provided inthe Cholera direct fluorescent antibody (DFA) kit (New Horizon Diagnostics Corp., Baltimore, MD, USA), following manufacturer's instructions, and examined under an epifluorescence microscope (DM2500, Leica Microsystems).

Catalyzed reporter deposition fluorescence in situ hybridization

Five-milliliter portions of the water samples were filtered using 0.2 μm polycarbonate membranes (Type GTTP, diameter 47 mm, Millipore) which were subjected to catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) analysis, as described by Pernthaler et al. (2002), using probes for bacteria, gammaproteobacteria, betaproteobacteria, and *Vibrio* (Glöckner et al. 1999; Eilers et al. 2000). Filter sections were counterstained with DAPI (100 ng mL^{-1}), washed with sterile distilled water and 80% ethanol, air dried, mounted in 4:1 mixture of Citifluor (Citifluor Ltd., London, UK) and Vecta Shield (Vector Laboratories, CA, USA), and examined with an epifluorescence microscope. For each count, at least 1000 DAPI-stained cells at × 1000 magnification were counted to obtain specific probe-labeled counts.

Microcosm experiments

The SPM (< 20 μm) of the water samples was trapped on GF/F filters, and a portion (dried) was treated with H_2O_2 (15%) several times, to remove organic matter, and dried at 50 °C to constant weight. Both SPM fractions, with and without organic matter, were sterilized by UV for 15 min. Artificial sea water (ASW) was prepared by dissolving sea-salt (Sigma-Aldrich, 40 g L^{-1}) and the salinity adjusted to 15, 7.5, and 3.75 PSU and pH to 8.0. Fifty milliliters of autoclaved ASW was placed into 100 mL sterile glass bottles, and three types of microcosms were prepared: sediment with and without organic matter (1 mg mL^{-1}, the observed SPM load in original samples) and control without sediment, for each salinity. Ca. ~ 10^4 CFU mL^{-1} of a *V. parahaemolyticus* isolate from the Karnaphuli estuary was added to each bottle, which was tilted horizontally and incubated at 25 °C with shaking (50 rev min^{-1}). At regular intervals, 100 μL of each sample was plated on gelatin agar (Difco, pH 7.5) and culturable *V. parahaemolyticus* enumerated. The SPM in 100 mL from the microcosms and collected from the field sites were settled (12 h) by gravitation. Samples from the top layer, without sediment, and from the turbid bottom layer, with sediment, were analyzed for total *Vibrio* counts. Each microcosm and settling experiment was performed in triplicate and median ($n = 3$) counts of culturable *Vibrio* populations were compared.

Statistical analysis

Statistical analyses were done using "Xact" (ver. 7.21d, SciLab GmbH, St. Yrieix, France), Statistica (ver. 10.0, StatSoft Inc., USA), and SPSS (ver. 17.0, SPSS Inc., Chicago, USA). Differences among bacterial populations and environmental parameters were compared by site and hydroclimatic event, i.e., before and after a cyclone and tsunami using the student's t test (two-tailed). Regression fits were applied to explore correlations between variables. Influence of environmental factors on *Vibrio* dynamics during a tidal cycle was evaluated using Kendall Tau nonparametric correlation (τ_b, two-tailed; CI, 95%), obtained from transformed z values (Bonett and Wright 2000), since the sample size was relatively small ($n < 20$). A p value of < 0.05 was considered significant. Non-metric multi-dimensional scaling (MDS) was performed to elucidate relationships among bacteria and physicochemical variables for the water samples. Similarities among variables were computed by Euclidean distance.

Results

Tidal influence on biophysicochemical conditions and *Vibrio* populations

During June 2007 (monsoon), surface water salinity in the mid-saline zone of the Karnaphuli estuary (Fig. 1) increased gradually (0 to 8 practical salinity units (PSU), for practical purposes equivalent to ‰) with rising tide when temperature (30–31 °C) and pH (7.6–7.9) were relatively stable. Culturable *Vibrio* numbers were low (< 10^1 CFU mL^{-1}) at low tide, but increased significantly (> 10^2–10^4 CFU mL^{-1}, $p < 0.05$) as the tide came in, bringing higher salinity water (> 5 PSU) and turbidity (> 500 nephelometricturbibity units, NTU) (Fig. 2a). SPM was significantly correlated with culturable *Vibrio* counts ($\tau_b = 0.60$; CI, 0.30 to 0.79, $p < 0.01$, $n = 11$) (Online resource 1 (supplementary Table)). Dissolved

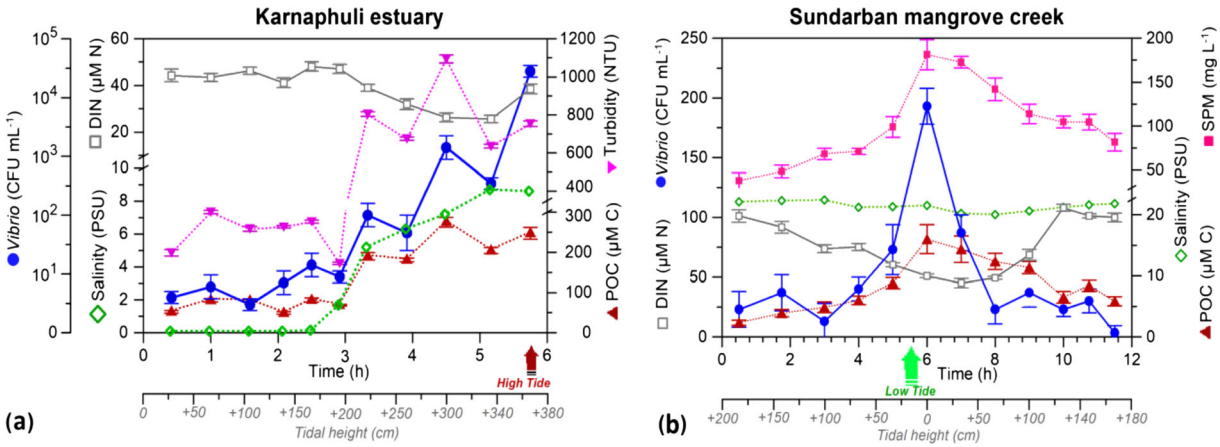

Fig. 2 Dynamics of *Vibrio* spp. and environmental factors, including biophysicohemical and nutritional, during tidal intervals at a stationary point of the Karnaphuli River estuary (**a**) and Sundarban mangrove creek (**b**) during June and December 2007, respectively. Changes in culturable *Vibrio* counts, salinity, and micro-ecosystem factors are shown in the *y*-axis, while tidal times, with changes in tidal height, are shown in the *x*-axis. Results are presented as mean ± SD ($n = 3$). Abbreviations: SPM, suspended particulate matter; DIN, dissolved inorganic nitrogen; POC, particulate organic carbon; PON, particulate organic nitrogen. Both POC and PON significantly (τ_b, two-tailed; CI, 95%, $p < 0.05$) correlated with *Vibrio* populations (Online resource 1 (supplementary Table 1))

inorganic nitrogen (DIN) concentration (74–95% nitrate, 0.5–7% nitrite, 3–20% ammonium) was low at high tide. Tidal influence on dissolved phosphate concentration (1.1–4.3 μM) was minimal. POC concentration was > 2 times higher at high tide (160–290 μM C) compared with low tide (Fig. 2a) and was significantly correlated ($\tau_b = 0.75$; CI, 0.52 to 0.87, $p < 0.005$, $n = 11$) with culturable *Vibrio* counts. POC was also significantly correlated ($\tau_b = 0.86$; CI, 0.71 to 0.93, $p < 0.001$, $n = 11$) with PON. Likewise, PON was significantly correlated ($\tau_b = 0.67$, CI: 0.40 to 0.84, $p < 0.005$, $n = 11$) with culturable *Vibrio* counts (Online resource 1 (supplementary Table)). Thus, during monsoon season, the occurrence of culturable *Vibrio* populations appeared to be dependent on tidal variation and associated changes in salinity, turbidity, SPM, POC, and PON.

Influence of factors other than salinity on *Vibrio* populations was assessed by collecting surface water samples during the changing tide at a fixed station in a creek located in the Sundarban mangrove during December (post-monsoon) 2007 (Fig. 1). Salinity was relatively constant (21–22 PSU) and *Vibrio* counts were low (< 50 CFU mL^{-1}) at high tide (0 and 12 h) and during inter-tidal periods. However, at low tide (7 h), the *Vibrio* counts increased significantly (~ 200 CFU mL^{-1}) (Fig. 2b). The increase in culturable *Vibrio* counts during low tide was accompanied with increase in SPM, POC, and PON. Temperature (28–30 °C), pH (6.8–7.7), and DIN (65–70% nitrate, 15–20% nitrite, 5–20% ammonium) had negligible impact on bacterial populations in the water samples. Phosphate concentrations ranged from 0.5 to 0.8 μM, without a discernible tidal influence (Fig. 2b). However, SPM concentration increased during low tide and was significantly correlated with particulate organic nutrient, e.g., POC ($\tau_b = 0.88$; CI, 0.76 to 0.94, $p < 0.001$, $n = 12$) and PON ($\tau_b = 0.76$; CI, 0.55 to 0.88, $p < 0.005$, $n = 12$). Significant positive correlations between *Vibrio* and POC ($\tau_b = 0.47$; CI, 0.14 to 0.71, $p < 0.05$, $n = 12$) and *Vibrio* and PON ($\tau_b = 0.53$; CI, 0.22 to 0.75, $p < 0.05$, $n = 12$) were observed (Online resource 1 (supplementary Table)).

Spatio-temporal variation and effect of the tsunami on *Vibrio* culturability

Culture-independent detection, employing CARD-FISH, revealed spatio-temporal abundance of bacterioplankton (bacteria, gammaproteobaceteria, betaproteobacteria, and *Vibrio* populations) in surface water of the Karnaphuli estuary. During December 2004 to February 2005, total bacterial counts for surface water were relatively stable, ~ 10^6 cells mL^{-1}, despite the salinity increase from 19.3 to 22.9 PSU, 7.6 to 10.4 PSU, and 0.1 to 1.3 PSU in the high-, medium-, and low-salinity regions (Fig. 3). During this time period, the water temperature was between 23.4 and 24.6 °C. The pH and dissolved oxygen (DO) varied from 7.5 to 8.0 and 5.7 to 6.5 mg L^{-1}, respectively. Culture independent *Vibrio* numbers (FISH counts) were

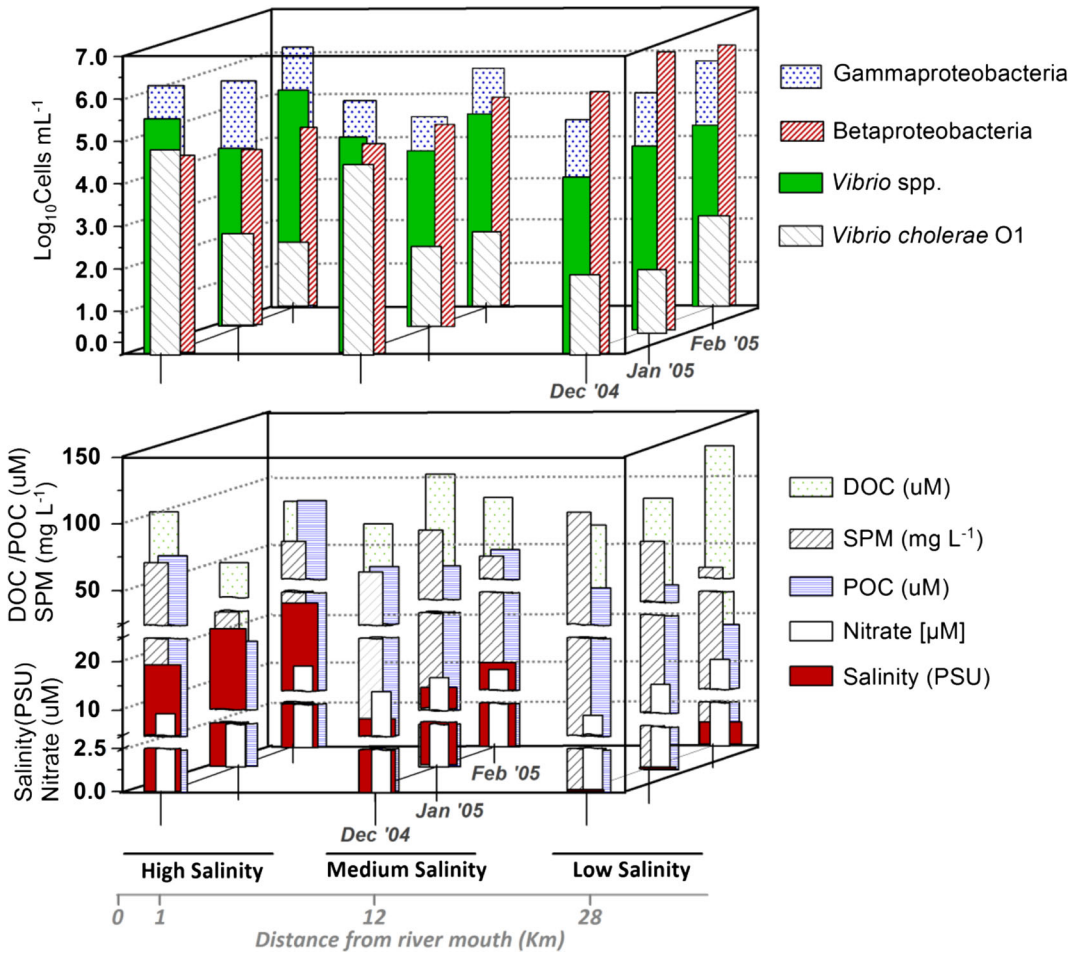

Fig. 3 Culture-independent occurrence of the major bacterial populations influenced by environmental factors of the Karnaphuli river estuary during December 2004 to February 2005. Values shown in the graphs represent means of the water samples collected from three closely located sites at each of the high-, medium-, and low-salinity environments. Comparative occurrence (mean values, $n = 3$) of the major bacterial populations, including *Vibrio* spp., and associated environmental factors of surface water, are shown in the top and bottom panels, respectively. The purpose was to check whether *Vibrio* and other major groups of bacteria are influenced similarly or in variable manner by the environmental changes. *Vibrio*, gammaproteobacteria (a major group of marine microbes) and betaproteobacteria (which are predominant in wastewater, plant, and soil) counts were obtained by CARD-FISH, and *Vibrio cholerae* O1 counts by DFA. Abbreviations: SPM, suspended particulate matter; POC, particulate organic carbon; DOC, dissolved organic carbon

$> 10^5$ cells mL^{-1} in the high-salinity zone and decreased to $\sim 10^4$ cells mL^{-1} in the upstream low-salinity samples. *Vibrio* counts represented 1.0–8.0, 1.7–4.8, and 0.3–0.6% of total bacteria in the high-, medium-, and low-salinity regions, respectively, of the Karnaphuli estuary. Gammaproteobacteria, which includes marine bacteria, followed a similar pattern, decreasing (from $\sim 10^6$ to $\sim 10^5$ cells mL^{-1}) toward freshwater, but the betaproteobacteria, predominantly of wastewater, plant, and soil origin, showed an opposite trend (Fig. 3). Culture-independent and culturable *Vibrio* counts displayed a positive and significant correlation ($r = 0.98$, $p < 0.001$, $n = 27$). Culturable counts decreased from 10^3 $^{-}10^4$ CFU mL^{-1} in the high-salinity water to 10^2 $^{-}10^3$ CFU mL^{-1} in medium-salinity and $< 10^1$ CFU mL^{-1} in upstream freshwater. Compared with pre-tsunami data, fewer *V. cholerae* O1 and total *Vibrio* counts were obtained after 12 days of the tsunami. Concurrently, a decrease in SPM, POC, DOC, and DIN was observed after the tsunami in the high-salinity region. However, 42 days post-tsunami, SPM and POC increased, comparable with the pre-tsunami level and a similar recovering trend was observed for all major bacterial groups, including *Vibrio* spp., in this part of the estuary.

Effects of the cyclone on estuarine ecology and *Vibrio* populations

During pre- and post-cyclone sampling, the temperature (29.8–31.1 °C) and pH (~ 7.0–8.0) did not change significantly. A thrusting effect of seawater was observed after the cyclone, extending the high- and medium-salinity zones further inland, but salinity did not increase significantly in the high- and medium-salinity zones (Fig. 4b). DIN and dissolved phosphate did not change post-cyclone (Fig. 4c). Most of the DIN (> 85%) comprised nitrate (12–52 μM N) followed by ammonium (0.2–10.5 μM N) and nitrite (0.25–4.0 μM N). Higher ($p < 0.01$, $n = 10$) DOC concentrations were observed after the cyclone, with mean values of 70 ± 12 and 107 ± 18 in high-salinity environment pre- and post-cyclone, respectively.

Pre-cyclone turbidity was 30–70 NTU in high- and medium-salinity zones and 50–220 NTU in the low-salinity sites. After the cyclone, turbidity significantly increased (> 3 times, t test: $p < 0.05$, $n = 28$) and similarly, a significant (t test: $p < 0.05$, $n = 28$) rise in SPM (< 20 μm) was also observed for all salinity zones (Fig. 4d). A significant increase (> 2 times; t test: $p < 0.05$, $n = 28$) in chitin concentration was observed in all salinity zones after the cyclone, compared with pre-cyclone (Fig.4e). Chitin concentrations ranged from 300 to 1250 μg L^{-1} (mean 1470 μg L^{-1}) and 900–2100 μg L^{-1} (mean 683 μg L^{-1}) pre- and post-cyclone samplings, respectively, with highest concentration in the-high salinity zone after the cyclone. Both POC and PON increased significantly (t test: $p < 0.05$) in all salinity zones after the cyclone, notably > 10 times in the high-salinity zone (Fig. 4f). A positive and significant correlation of turbidity (SPM) with both POC and chitin concentrations ($r = 0.89$ and 0.53, respectively, $p < 0.005$, $n = 28$) was observed.

A strong influence of salinity on culturable *Vibrio* populations was observed in both pre- and post-cyclone sampling, reflected in higher counts (10^2–10^4 CFU mL^{-1}) at a salinity of > 5 PSU. In lower salinity water, only a few vibrios (< 10^1 CFU mL^{-1}) were culturable. Significantly higher (t test: $p < 0.05$, $n = 20$) *Vibrio* counts were observed post-cyclone, especially in the high- and medium-salinity zones, at each of the five sampling sites, in comparison with pre-cyclone (Fig. 4a). Culturable *Vibrio* and salinity showed a strong linear relationship ($r = 0.93$, $p < 0.0001$, $n = 20$; Online resource 2a (supplementary Figure)) for samples with salinity ranging 0–10 PSU but did not show correlation at higher salinities (10–20 PSU). Turbidity correlated ($r = 0.60$, $p < 0.01$, $n = 20$) with culturable *Vibrio* for samples collected from the high- and medium-salinity regions (2–20 PSU) but not at lower salinities (Online resource 2b, c (supplementary Figure)). However, field data and predicted values from a regression model, based on simple conversion of salinity and turbidity, showed a highly significant correlation ($r = 0.89$, $p < 0.0001$, $n = 28$) (Online resource 2d (supplementary Figure)). Direct viable counts (DVC) of *V. cholerae* O1 indicated presence of a large number (10^1–10^3 cells mL^{-1}) of viable but non-culturable (VBNC) *V. cholerae* O1 in the estuary, with higher abundance (> 10^2 cells mL^{-1}) in the high-salinity zone. Although only a few *V. cholerae* O1 could be cultured ($n = 6$ isolates), the *V. cholerae* O1 DFA-DVC count was higher in the high- and medium-salinity zones after the cyclone, compared with pre-cyclone (data not shown).

The drastic increase in *Vibrio* abundance after the cyclone coincided with significant ($p < 0.05$) increases in SPM (turbidity), chitin, and POC (Fig. 4). Multivariate analysis (MDS) of the pre- and post-cyclone data showed that culturable *Vibrio* counts in surface water clustered with particulate organic nutrients (SPM, POC, PON, and chitin) (Online resource 3 (supplementary Figure)). Spatio-temporal variation in culturable *Vibrio* counts pre- and post-cyclone were significantly correlated with variations in SPM ($r = 0.60$, $p < 0.05$, $n = 20$), POC ($r = 0.74$, $p < 0.001$, $n = 20$) and particulate chitin ($r = 0.64$, $p < 0.05$, $n = 20$). Dissolved inorganic nutrients (nitrate, nitrite, ammonia, and phosphate) had little, if any, effect on *Vibrio* abundance.

Comparative abundance of SPM, chitin, and vibrios in fractionated samples

Analysis of fractionated surface water samples collected pre-cyclone (May 2007) in the Karnaphuli estuary showed that, independent of salinity, the < 20-μm particulate fraction contained > 98% and > 90% of estuarine SPM and chitin, respectively, representing at least 50 and 10 times higher (t test: $p < 0.005$, $n = 14$) concentrations, respectively, compared with the plankton fraction > 20 μm. Similar results were also obtained post-monsoon in December 2007 for water samples collected in the Sundarban mangrove wetland (Fig. 5a). Neither chitin nor SPM concentrations in plankton fractions > 20 μm showed significant change after the cyclone.

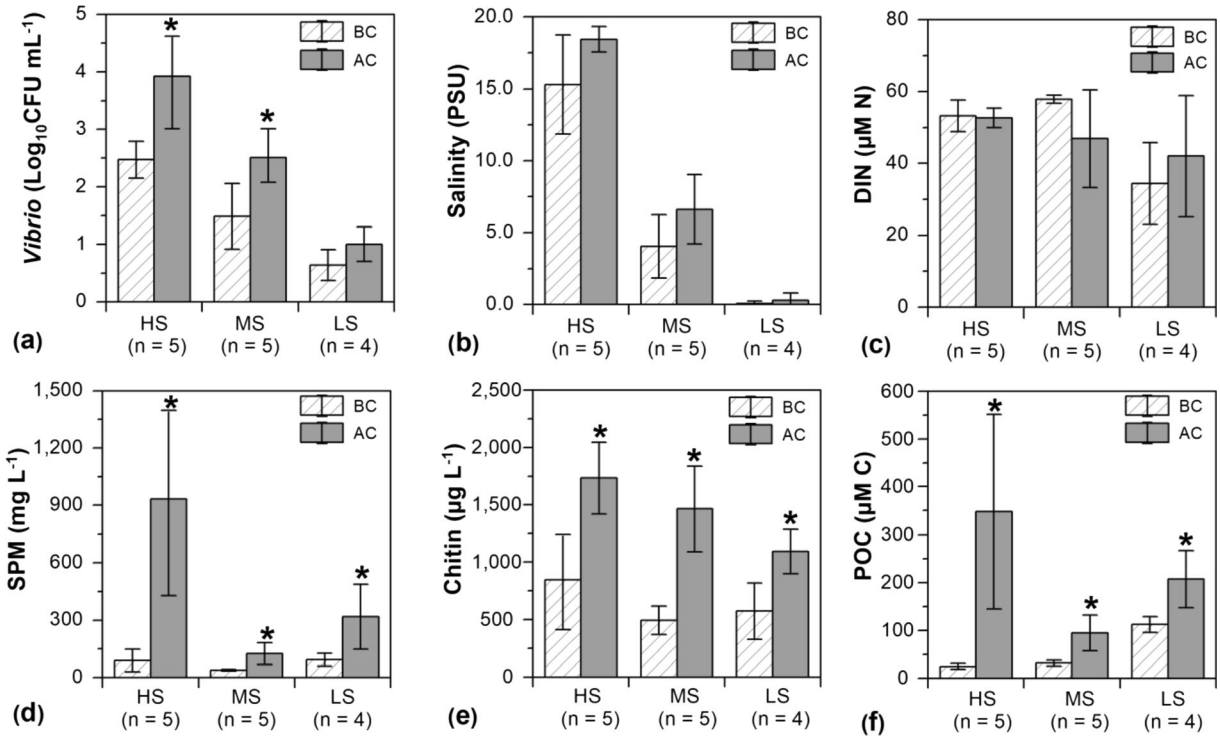

Fig. 4 Comparative abundance of *Vibrio* spp. and micro-ecosystem factors in high- (HS, 10–20 PSU), medium- (MS, 2–9 PSU), and low-salinity (LS, 0–1 PSU) environments of the Karnaphuli estuary before (BC) and after (AC) a cyclone in May 2007. Each column represents the sample mean; $n = 5$ for each of high and medium salinity and $n = 4$ for low salinity. The standard deviations of the mean values are shown in vertical bars. *$p < 0.05$, significant differences (*t* test, two-tailed) between mean values of the samples representing before and after cyclone scenarios. DIN, dissolved inorganic nitrogen; SPM, suspended particulate matter; POC particulate organic carbon

Culturable *Vibrio* counts in the < 20-µm particulate fraction (10^4–10^6 CFU L^{-1}) were 1–2 log higher (comprising > 90% of the CFU) than the larger microphyto- and zooplankton fractions in all salinity zones of the Karnaphuli estuary, as well as in the Sundarban mangrove creek. The trend of significantly (*t* test: $p < 0.005$, $n = 14$ and 5 in riverine estuary and mangrove wetland, respectively) higher counts for the < 20-µm particulate fraction than larger microplankton fraction was also observed for VBNC *V. cholerae* O1 (Fig. 5b). On average, > 98% of VBNC *V. cholerae* O1 were present in the < 20-µm fraction and in association with suspended sediment and organic nanodetritus.

Vibrio populations in benthic sediment and surface water

Suspended sediment or particulates (< 20 µm) in surface water eventually settle down by natural gravitation and accumulate as benthic sediment. At seven of the ten sites in Kuakata included in this study, independent of salinity, culturable *Vibrio* counts were significantly higher (*t*-test: $p < 0.05$, $n = 21$) in the benthic sediment (10^2–10^3 CFU g^{-1} wet sediment) than surface water (0–10^2 CFU mL^{-1}) (Fig. 5c). A seasonal impact of water salinity, with gradual increase from late monsoon (September) to post-monsoon (November) 2012 was observed. With increase in salinity, an increase in culturable *Vibrio* counts was noted for benthic sediment and surface water simultaneously (data not shown). The Karnaphuli estuary and Sundarban mangrove samples yielded similar results (Fig. 5c).

Biochemical screening and species-specific PCR used to assess species diversity among culturable *Vibrio* populations revealed Karnaphuli estuary isolates ($n = 272$) represented 14 species, with *V. parahaemolyticus* (19%) dominant, followed by *Vibrio splendidus* (16%), *Vibrio vulnificus* (15%), *Vibrio harveyi* (12%), and *V. cholerae* (9%). In the

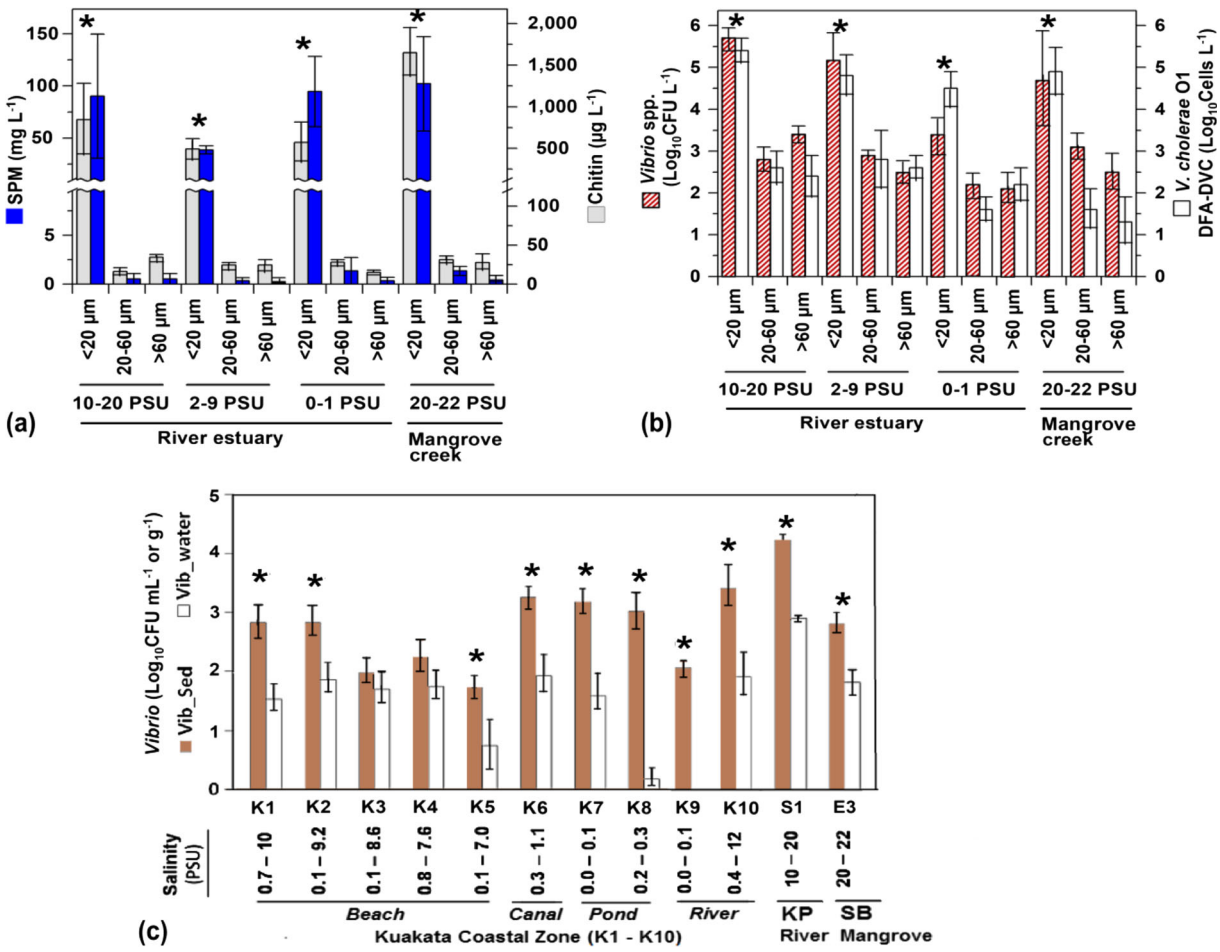

Fig. 5 Distribution of *Vibrio* populations in plankton, water, and sediment samples at different salinities in coastal regions of the Bengal delta. **a**, **b** Variation in suspended particulate matter (SPM), chitin concentration, culturable *Vibrio* counts, and *V. cholerae* O1 direct viable counts (DVC), determined by fluorescent antibody (DFA), in plankton samples (< 20, 20–60, and > 60 μm) of the Karnaphuli river estuary pre-cyclone (May 2007) and the Sundarban mangrove creek (December 2007) at different salinities. Each column represents the sample mean; $n = 5$ for each of high and medium salinity and $n = 4$ for low salinity. **c** Comparative abundance of culturable *Vibrio* in surface water and benthic sediment samples collected from the coastal sites at Kuakata (K1–K10, including beach, river, canal, and pond), Karnaphuli (KP) river, and Sundarban (SB) mangrove in Bangladesh. Culturable *Vibrio* counts in surface water (Vib_water), and benthic sediment (Vib_sed), represent mean values of three monthly samples (September–November 2012) for each location of Kuakata, and three tidal phases (low, inter-, and high) at a stationary point in the Karnaphuli river and Sundarban mangrove during June 2007 and December 2007, respectively. Standard deviations are shown as vertical bars; $*p < 0.05$, significant difference (*t* test, two-tailed) between mean values for < 20 μm plankton compared with the other plankton sizes (20–60 and > 60 μm) and between sediment and water samples

Sundarban mangrove, *Vibrio* isolates ($n = 102$) represented at least seven species, dominated by *V. cholerae* (48%), followed by *Vibrio mimicus* (20%) and *V. parahaemolyticus* (12%). *Vibrio* isolates ($n = 300$) from the Kuakata coastal region included *V. parahaemolyticus* (36%), *V. cholerae* (10%), *Vibrio alginolyticus* (7%), *V. vulnificus* (5%), and *Vibrio fluvialis* (3%).

Vibrio associations in microcosm sediment

The microcosm experiments, conducted to determine if and how particulate sediment acts as a major reservoir of vibrios and influences their population dynamics, showed that *V. parahaemolyticus*, the dominant *Vibrio* species isolated from the Karnaphuli estuary and Kuakata coast, remained culturable for a longer time in microcosms

amended with sediment and organic matter at all salinity concentrations, compared with sediment (S) alone and in control water (W) (Fig. 6a–c). *V. parahaemolyticus* populations (90%), incubated in artificial seawater microcosms for a week, were associated with sediment containing organic matter ($S + O$) and at all salinities (Fig. 6d). For natural water samples ($n = 20$), the majority of the *Vibrio* populations, e.g., > 97% in high-salinity water, also co-settled with suspended sediments (Online resource 4 (supplementary Table)).

Discussion

Salinity, temperature, plankton populations, sediment, rainfall, and dissolved nutrients all play a role in the dynamics of *V. cholerae* and V. parahaemolyticus in the Bengal delta (Lobitz et al. 2000; Huq et al. 2005; Constantin de Magny et al. 2008; Neogi et al. 2011, 2014; Akanda et al. 2013). Environmental parameters have been shown to regulate *Vibrio* populations at the micro-ecosystem level in the Bay of Bengal estuaries, notably, SPM and associated nutrients (Batabyal et al. 2014). In this study, seasonal observations made under a variety of hydroclimatic conditions in coastal regions of Bangladesh revealed commonalities, notably that SPM and related nutritional substrates, including chitin, POC, and DOC, are linked with *Vibrio* dynamics in the estuarine ecosystem of the Bengal delta.

Temperature and pH are known to promote growth of *Vibrio* spp. (Miller et al. 1984), with favorable ranges of 15–30 °C, and pH 7.5–8.5 (Louis et al. 2003; Mahmud et al. 2008; Julie et al. 2010). Both physicochemical factors were found to be within those optimum ranges in the tropical coastal region examined in this study. An elevated input of inorganic nutrient, e.g., phosphorous, is favorable for marine gammaproteobaceria, including vibrios, as shown for the offshore region of a tropical bay in Brazil (Gregoracci et al. 2012). A similar influence of phosphate

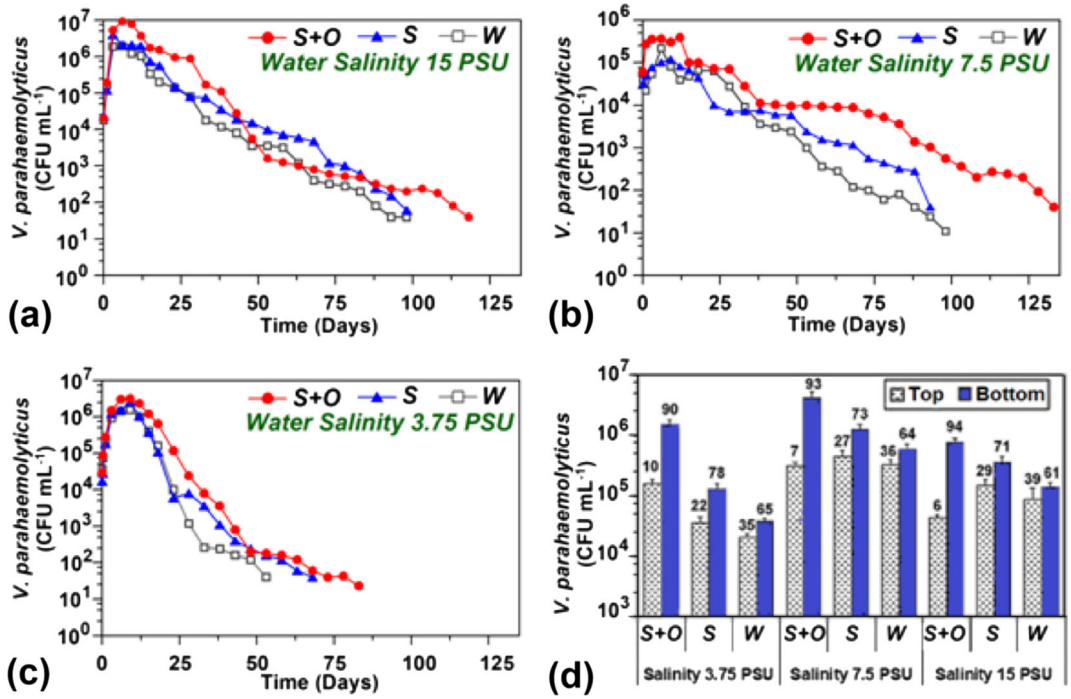

Fig. 6 Culturable counts of *Vibrio parahaemolyticus* in suspended sediment in microcosms with different salinities and incubated with shaking (50 rpm) at 25 °C. Microcosms were prepared using sterile artificial seawater (50 mL), inoculated with *V. parahaemolyticus* (~ 10^4 CFU mL^{-1}), and three treatments were employed: sediment (1 mg mL^{-1}) with organic matter ($S + O$), sediment (1 mg mL^{-1}) without organic matter (S), no sediment in control water (W), at each of the three salinities, **a** high (15 PSU), **b** medium (7.5 PSU), and **c** low (3.75 PSU). Mean *Vibrio* counts for 100 μL microcosm water in triplicate taken at different time intervals, are shown. **d** Culturable *Vibrio parahaemolyticus* counts in suspended sediment samples from a separate set of microcosms, with similar salinities and sediment treatments and incubated with shaking for a week. At day 7, the microcosms were maintained static for 12 h to settle out sediment. *Vibrio* counts were estimated using water pipetted from the top (no sediment) and bottom with settled sediment. Numbers above each column indicate average percentage of total *V. parahaemolyticus* count in top and bottom layers. Standard deviations of counts from triplicate experiments are shown as vertical bars

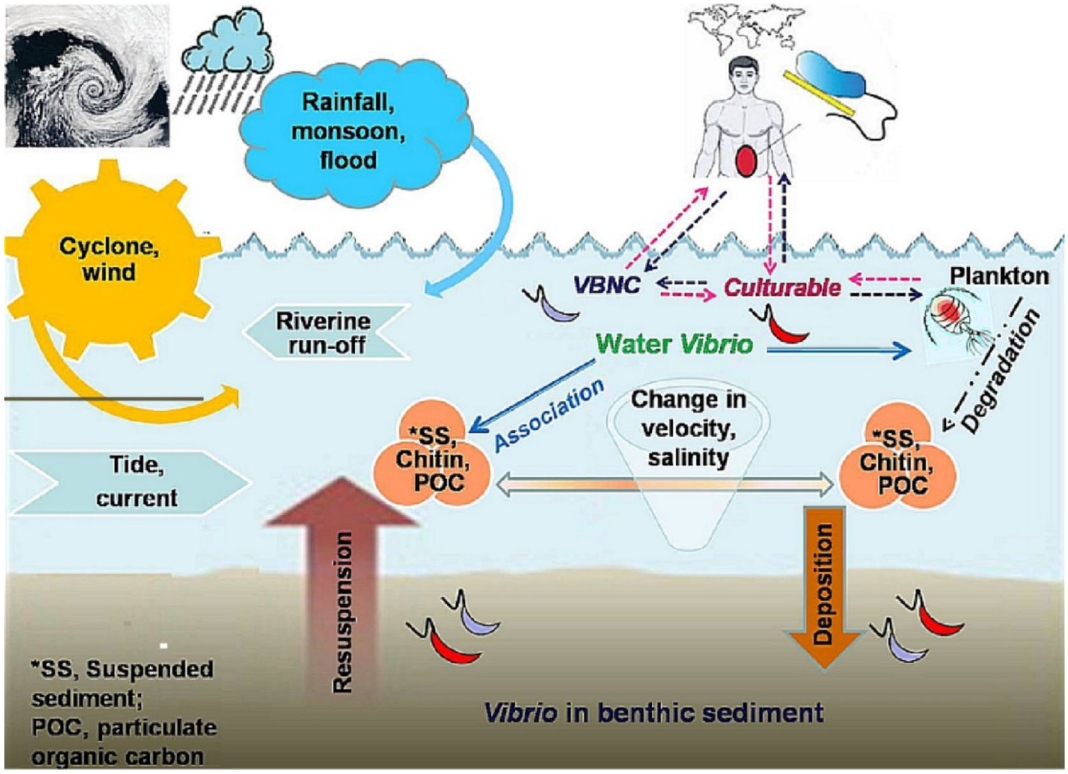

Fig. 7 Role of suspended and bottom sediment in *Vibrio* population dynamics. A majority of the *Vibrio* population remained associated with the top layer of sediment (0.25 m depth). Tide- and current-mediated seawater intrusion toward land and riverine runoff, rainfall (monsoon), and flood-mediated flushing of freshwater toward the sea, caused turbulence and re-suspension of sediment carrying vibrios. Cyclones and strong winds enhance re-suspension. Changes in water flow velocity and salinity play a role in deposition of suspended sediment (SS). In the water column, most of the *Vibrio* population remained VBNC and associated with SS. In favorable environmental conditions, e.g., when introduced to susceptible host (human, zooplankton), VBNC cells of vibrios may become culturable. Organic carbon from degraded plankton and chitin particulates accumulate in SS, facilitating growth of vibrios and their persistence in SS

and DIN on the *Vibrio* population was not evident at the inland estuarine sites of the Bengal delta. The positive role of salinity on *Vibrio* population size and distribution, within a range of 5 to 20 PSU, observed in this study, is in accordance with results of other investigators (Miller et al. 1984; Louis et al. 2003). The observed impact of daily tidal amplitude and seasonal rainfall, even though limited sampling was done, showed *Vibrio* populations can be correlated with salinity and tidal height in the low altitude estuarine regions of the Bengal coast (Fig. 2). This inference is supported by a similar role of tide and seasonal rainfall on the spatio-temporal variation in salinity and populations of *Vibrio* in the Hoogly estuary, West Bengal (Batabyal et al. 2014).

Large numbers of *ctx*$^{+ve}$ *V. cholerae* O1 in the VBNC state (10^1–10^3 cells mL^{-1}) were detected in estuarine water samples collected in the Karnaphuli, a cholera endemic region. The VBNC state appears to be a common feature of estuarine *V. cholerae* O1, ostensibly a mechanism to cope with changing salinity and nutrient concentrations in the natural environment (Roszak and Colwell 1987). It is known that culture-based methods detect only a minor fraction (0.01–10%) of the total population (Eilers et al. 2000), and such results were observed in this study, with salinity the major driver of the various physicochemical factors.

Chitinous microplankton, including zooplankton and diatoms, and their discarded exoskeletons have been shown to be favorable microhabitats for estuarine vibrios, including VBNC *V. cholerae* (Tamplin et al. 1990; Constantin de Magny et al. 2011). Results of this study showed *Vibrio* populations in estuarine surface waters are associated predominantly with nanoparticles of the < 20-μm fraction, carrying a higher load of chitin than the phyto- and zooplankton fractions (Fig. 5). *Vibrio* spp. colonize chitinous

sediment and plankton (Mueller et al. 2007; Neogi et al. 2014) and contribute to uptake of DOC released from POM, with organic matter acting as growth stimulant (Eiler et al. 2007). In this study, a coincident increase in DOC and *Vibrio* abundance in the Karnaphuli estuary was observed after the May 2007 cyclone. In the temperate oceanic environment, climate-mediated increase in temperature and plankton (> 20 μm) is considered a promoting factor for *Vibrio* populations (Neogi et al. 2011; Vezzulli et al. 2016). Increased occurrence in zooplankton, particularly copepods, cladocerans, and rotifers, has been shown to be associated with a larger number of pathogenic *V. cholerae* in coastal ponds, and thereby related to seasonal cholera outbreaks in the Bengal delta (Huq et al. 2005; Constantin de Magny et al. 2011).

It is concluded from our observations that suspended and benthic sediment, POM, and sedimentary chitin comprise major *Vibrio* reservoirs. The significant decrease or increase of *Vibrio* populations (both culturable and VBNC) after natural catastrophes, such as a tsunami or cyclone, was correlated with SPM in estuarine surface water of the Bengal delta (Figs. 3 and 4). Results of microcosm experiments confirmed an association of *Vibrio* populations with SPM, with removal of organic matter from sediment reducing bacterial colonization and persistence (Fig. 6; Online resource 4). Thus, shallow coastal benthic sediment in the Bengal delta harbors significantly larger populations of *Vibrio* spp. compared with surface water (Fig. 5). Increased sediment resuspended by wind-driven tidal surge supports growth of vibrios (Online resource 3 (supplementary Fig. 2)) and also introduces *Vibrio*-rich benthic sediment into the water column (Batabyal et al. 2014). Therefore, risk of infection with pathogenic *Vibrio* spp. may be related to their deposition and persistence in benthic sediment, as reported for the tropical Florida coast in the USA and the temperate beach and continental shelf of Europe (Williams and Larock 1985; Vezzulli et al. 2009; Böer et al. 2013). Tide-mediated variation in the concentration of POM in sediment particles was found to influence *Vibrio* populations (Fig. 2). Since the tidal impact on *Vibrio* populations is correlated with greater abundance, both salinity and/or turbidity can be concluded to play a significant role. Based on published data of other investigators and the results of this study, a hypothetical model of a tropical coastal zone, with vibrios persisting in association with benthic sediment, is provided in Fig. 7.

Conclusion

Suspended particulate matter, in combination with salinity, is concluded to be a key-driving factor influencing the population dynamics of vibrios in the estuaries of the Bay of Bengal. Benthic and suspended sediment serve as a reservoir of coastal *Vibrio* spp. and the quantity and quality of organic matter, notably chitin, in the suspended sediment fraction, affect growth and persistence of estuarine *Vibrio* populations in the Bay of Bengal estuaries. Changes in the coastal microecosystem as predicted by climate change models, namely increase in seawater intrusion and occurrence of more frequent storms, can be expected to increase the number and distribution of vibrios in the coastal zone of the Bengal delta, with a potential deleterious consequence for public health.

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