Occurrence and distribution of plankton-associated and free-living toxigenic Vibrio cholerae *in a tropical estuary of a cholera endemic zone*

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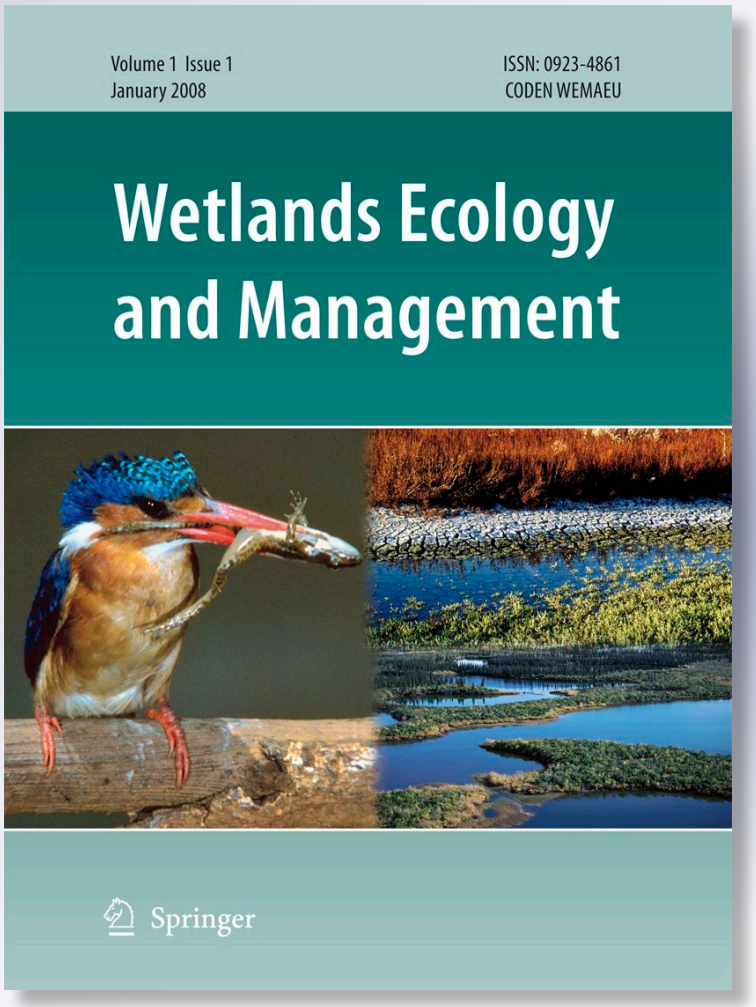

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ORIGINAL PAPER

Occurrence and distribution of plankton-associated and free-living toxigenic *Vibrio cholerae* in a tropical estuary of a cholera endemic zone

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Abstract Cholera epidemics are thought to be influenced by changes in populations of estuarine *Vibrio cholerae*. We investigated the abundance and distribution of this bacterium, as "free-living" (<20 μm fraction) and associated with microphytoplankton (>20 μm) or zooplankton (>60 μm), in the Karnaphuli estuary of Bangladesh during pre- and post-monsoon seasons. Cultivable *Vibrio* populations were $\sim 10^2$–10^4 colony forming units (CFU) ml^{-1} in the high saline zone (19–23 practical salinity unit, PSU) and declined in freshwater (<10^1 CFU ml^{-1}). Culture independent detection of toxigenic *V. cholerae* O1 and O139 serogroups revealed a higher abundance of "free-living" (10^4–10^5 cells l^{-1}) than those attached to plankton (10^1–10^3 cells l^{-1}). However, "free-living" O1 and O139 cells were sometimes absent in the medium saline and freshwater areas (0.0–11 practical salinity unit [PSU]). In contrast, plankton samples always harbored these serogroups despite changes in salinity and other physico-chemical properties. Microphytoplankton and zooplankton were dominated by diatoms and blue-green algae, and copepods and rotifers, respectively. Toxigenic *V. cholerae* abundance did not correlate with plankton abundance or species but had a positive correlation with chitin in the <20 μm fraction, where suspended particulate matter (SPM), *V. cholerae* and chitin concentrations were highest. C:N ratios indicated that organic matter in SPM originated predominantly from plankton. The differential occurrence of "free-living" and attached *V. cholerae* suggests a pivotal function of plankton in *V. cholerae* spreading into freshwater areas. The probable association of this pathogen with organisms and particles in the nanoplankton (<20 μm) fraction requires validation of the concept of the "free living" state of *V. cholerae* in aquatic habitats.

Keywords *Vibrio cholerae* · Salinity · Chitin · Phytoplankton · Zooplankton

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Introduction

Global warming and changes in hydrology will likely reshape coastal wetlands and estuarine dynamics, with increasing risk for diarrheal diseases such as cholera and other *Vibrio*-related illnesses (Lipp et al. 2002; IPCC 2007). Cholera is caused by the ingestion of toxigenic *Vibrio cholerae* which is part of the autochthonous flora of brackish and estuarine environments (Colwell et al. 1977). In the coastal areas of the Bay of Bengal, seawater intrusion is a major concern. Because of the low altitude of this region, even a small increase in sea-level may have a significant impact on the dynamics of the large Sunderban mangrove in the southern part of Bangladesh. However, in this cholera endemic zone, little has been done to monitor the ecology of toxigenic *V. cholerae* in different but related coastal environments such as mangroves, marshes and estuaries, despite the notion that this could provide important information for early warning and understanding human vulnerability to the disease (Constantin de Magny et al. 2008). Changes in the biogeochemical patterns of brackish ecosystems may modulate coastal *V. cholerae* dynamics and the seasonality of cholera. The 1991 El Niño event in Peru coincided with the resurgence of cholera in the region and the year-to-year variability of cholera outbreaks in Bangladesh is correlated with the El Niño Southern Oscillation (Colwell 1996; Pascual et al. 2000). Cholera still causes millions of human cases, especially in the developing countries having tropical climate, and thousands fatalities (Gaffga et al. 2007).

Vibrio cholerae is a member of marine gamma-Proteobacteria which can also persist in freshwater environments. Among more than 200 known serogroups of *V. cholerae*, strains belonging to the O1 and O139 serogroups are the predominant causes of cholera epidemics. Most of the cholera pandemics originated in the Ganges–Brahmaputra delta (Sack et al. 2004). In Bangladesh, cultivable *V. cholerae* O1 and O139 strains can be isolated from aquatic sources only during cholera epidemics (pre-monsoon, April–May, and post-monsoon, October–December) when plankton blooms often occur in many freshwater ponds or lakes (Islam et al. 1993). *Vibrio cholerae* can be found as a non-symbiotic 'free-living' organism in the water column and also attached to phytoplankton, zooplankton and other aquatic organisms (Tamplin et al. 1990; Islam et al. 1994a). *Vibrio cholerae* can secrete extracellular enzymes like chitinase and mucinase which can aid in the adherence and growth of the bacterium on chitinous or mucilaginous organisms like copepods, shrimps, blue green algae, etc. (Nalin et al. 1979; Huq et al. 1983; Islam et al. 1990, 2002). Chitin (polymer of *N*-acetylglucosamine) is a major component of the exoskeleton of zooplankton and cell wall of many species of phytoplankton. Members of the *Vibrio* genus can form biofilms on plankton or suspended particulate matter (SPM) as a survival strategy in the aquatic environment (Watnick and Kolter 1999; Islam et al. 2007). The persistence of *V. cholerae* within the environment is also facilitated by entering a dormant state in which it remains viable but becomes non-cultivable (VBNC) in conventional laboratory media (Colwell et al. 1985). Monoclonal antibody-based counting of the metabolically active viable cells is a useful method to understand the ecology of predominantly non-cultivable *V. cholerae* in nature (Kogure et al. 1979; Hasan et al. 1994).

High temperature, salinity and pH can favor survival of vibrios in aquatic environments (Miller et al. 1984; Louis et al. 2003; Mahmud et al. 2008). However, field-based investigations on the interaction between *V. cholerae* abundance and natural substrates i.e., providing carbon, nitrogen and energy needed for bacterial metabolism are limited. The driving force influencing a *V. cholerae* population is likely an integrated outcome of changes in physico-chemical factors, availability of suspended substrates for inorganic or organic nutrients, and abundance of biological hosts or reservoirs. Some studies have investigated the dynamics of estuarine and coastal *V. cholerae* but most were conducted in regions where cholera is not endemic and did not systematically observe the change of "free-living" and plankton-associated populations of the toxigenic O1 and O139 strains from high saline to freshwater zones (e.g. Colwell et al. 1981; Barbieri et al. 1999; Gil et al. 2004; Louis et al. 2003; Castañeda Chávez et al. 2005; Lizárraga-Partida et al. 2009). The concept of "free living" is often based on an operational definition using size fractionation, and does not necessarily exclude association of bacteria to particles smaller than the cut-off limit (usually 20 μm net), such as nanoplankton. In a study of the role of plankton and SPM in a Sunderban mangrove estuary, we observed the occurrence of *V. cholerae* in different size fractions (Lara et al. 2011). However, during that post-monsoon study, water salinity was exceptionally low (0–4 PSU)

throughout the whole wetland, and thus potential salinity gradient effects on the size distribution patterns of *V. cholerae* and particulate nutrients were not evident.

The El Tor cholera pandemic reached the coastal Chittagong region of Bangladesh near the Karnaphuli estuary 2 years after its first emergence in Indonesia in 1961 (Rizvi et al. 1965). The present study explored the Karnaphuli estuary, a coastal habitat nearby the Sunderban mangrove, from high saline (~20 PSU) to freshwater zone during both pre- and post-monsoon seasons. The abundance and distribution of free-living and plankton-associated populations of the toxigenic *V. cholerae* along this tropical estuary were correlated with physico-chemical factors and various nutrients serving as sources of carbon and nitrogen, including particulate chitin for better understanding the influences of changing biogeochemical patterns and plankton dynamics in coastal wetlands on *V. cholerae*.

Materials and methods

Sites and sampling

The Karnaphuli estuary in the southern part of Bangladesh traverses the port city Chittagong and ends in the Bay of Bengal (Fig. 1). The estuary has a semidiurnal tidal oscillation range of 2–4 m and an average channel depth of 8–10 m. The sampling sites were selected following the salinity gradient with increasing distance from the estuary's mouth. Samples were collected during four expeditions in December 2004, January and February 2005 (post-monsoon) and May 2007 (pre-monsoon). In all these expeditions, water and plankton samples were collected from a total of nine sites, three in each of the sectors representing high, medium and low saline or freshwater (Fig. 1). In May 2007, five water samples from each salinity zone were collected to observe the relationship among toxigenic *V. cholerae* and dissolved and particulate nutrients serving as sources of carbon and nitrogen. An attempt to carry out a pre-monsoon expedition during May 2006 was hampered due to a cyclone and early onset of monsoon rains. To compare the change in estuarine ecology between the pre- and post-monsoon, the two seasons when cholera epidemics occur every year in this region, a pre-monsoon expedition was carried out in May 2007.

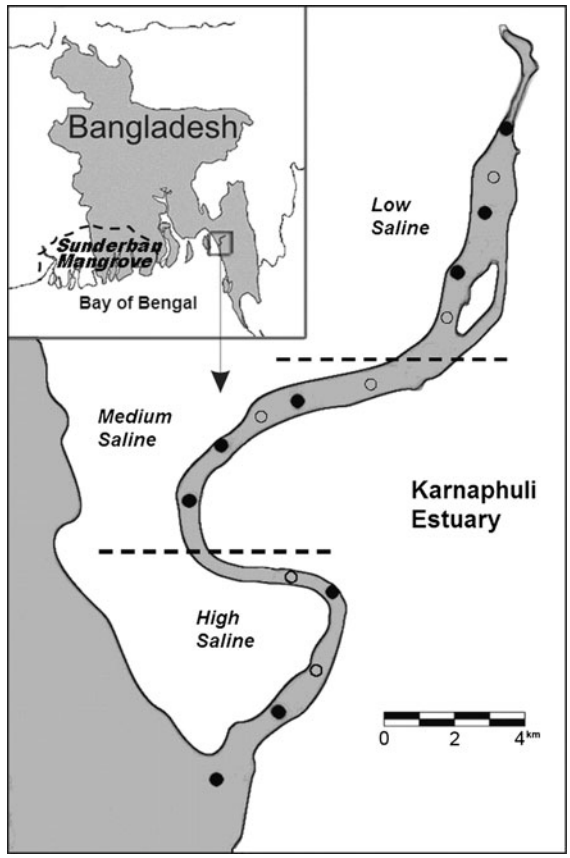

Fig. 1 Study area in the Karnaphuli estuary, Chittagong district, southeast Bangladesh. *Closed circles* indicate the sites where water and plankton samples were collected during all four expeditions. *Open circles* indicate the sites from where additional water samples were collected during the pre-monsoon. The *horizontal dotted lines* demarcate the estuarine zones based on salinity ranges

Sampling was carried out beginning at high saline water in the estuary's mouth sailing against ebb to about 30 km inland reaching the freshwater sector. At each station, three sub-samples of surface water (0.5 m depth, in the middle of the channel) were collected with sterilized buckets and pooled together. Plankton samples were collected using nets of 20 μm mesh for phytoplankton and 60 μm mesh for zooplankton (Millipore, Massachusetts, USA), concentrating 150 l water to 15 ml. The filtrate <20 μm was collected in sterile plastic bottles. All samples were transported in an insulated box with ice packs and processed within 24 h. Water samples were filtered through GF/F filters (Whatman, precombusted at 450°C, 3 h) and the filtrates were poisoned with $HgCl_2$ and stored at 4°C in 50 ml PE bottles for later

nutrient analyses (Kattner 1999). Filters with trapped suspended particulates were stored frozen at −20°C until analysis for SPM, particulate organic carbon (POC) and nitrogen (PON), and chitin.

A portion of each plankton sample (5 ml) was homogenized with a tissue grinder (Teflon-tipped, Wheaton Scientific, New Jersey, USA) for microbiological analyses. For the detection of viable *V. cholerae* by fluorescence microscopy, yeast extract (0.002%) and nalidixic acid (0.025%) were added to the water and plankton homogenates (Kogure et al. 1979), incubated overnight and then preserved with formaldehyde (4%).

Physico-chemical properties and dissolved and particulate nutrients in estuarine water

Water temperature, pH and dissolved oxygen (DO) were recorded in situ with portable field meters (sensION HACH, Colorado, USA). Salinity was determined by electric conductivity (WTW 340i with TetraCon325, Weilheim, Germany) and expressed in practical salinity units (PSU). Nitrate, nitrite, ammonium, silicate and phosphate were determined in a Skalar-SAN-plus autoanalyser (Skalar Analytical BV, Breda, the Netherlands) according to standard spectrophotometric methods for seawater (Kattner and Becker 1991).

SPM was determined gravimetrically after drying filters at 50°C until constant weight. POC was determined after removing inorganic C by acidification with 1 N HCl. POC and PON were quantified with an elemental analyzer (Fisons, NA 2100). Standard Reference Material 1515 was used for calibration. Chitin concentration was only measured in the samples collected in May 2007. A portion of the filter containing suspended particulates was re-suspended in 3 ml borate buffer (0.1 M, pH 7.4) and labeled with wheat germ agglutinin (WGA) and fluorescein isocyathionate (FITC) (Vector Laboratories, CA, USA) after Montgomery et al. (1990). The method was calibrated with purified crab chitin (Sigma-Aldrich, St Louis, MO, USA) as standard and quantified fluorimetrically (Turner 450; excitation 426 nm, emission 520 nm).

Plankton counting and identification

Part of the plankton samples (20–60 μm and >60 μm fractions) were fixed with formaldehyde (4%). The samples were then transferred into a Sedgewick-Rafter cell for taxonomical identification and counting following standard procedures (APHA 2005).

Enumeration of total bacteria, fecal coliforms and *Vibrio* spp.

For quantification of culture-independent total bacteria (TBC), water samples (5 ml) were fixed with formaldehyde (4%) and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 30 min according to the manufacturers' manual (Sigma-Aldrich, MO, USA). Counting was carried out after Porter and Feig (1980) using epifluorescence microscope (DM2500, Leica Microsystems, Tokyo, Japan).

Heterotrophic cultivable bacterial counts (CBC) were determined on nutrient agar (Difco, Detroit, Mich.). Water samples (10 and 100 ml) were passed through 0.2 μm filters (Millipore), and fecal coliforms (FC) were quantified using mFC agar (Difco) following methods described elsewhere (Islam et al. 1994b; APHA 2005). Sample aliquots were inoculated onto selective thiosulfate citrate bile salts sucrose (TCBS) agar media (Difco), and cultivable *Vibrio* counts (CVC) were determined after overnight incubation at 37°C. These counts were confirmed by specific biochemical tests for identification of *Vibrio* species (West and Colwell 1984). The samples were also subjected to enrichment overnight in alkaline peptone water followed by plating on TCBS for the presence of cultivable *V. cholerae*. The NaCl concentration in each medium was adjusted to sample salinity. All microbiological analyses were done in triplicate.

Direct fluorescence antibody (DFA) detection of toxigenic *V. cholerae*

The VBNC cells of *V. cholerae* O1 and O139 were enumerated by the Direct Fluorescence Antibody (DFA) technique using the Cholera and Bengal DFA kits, respectively (New Horizon Diagnostics Corp., Maryland, USA). Briefly, a 10 μl portion of concentrated sample was placed into a well of a PTFE (polytetrafluoroethylene) coated glass slide, air-dried and then labeled with monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-O1 and anti-O139 antibodies following manufacturer's instructions. Finally, slides were examined under an epifluorescence microscope (DM2500, Leica Microsystems, Tokyo, Japan).

Data interpretation and statistical analyses

Data from the three sites of each salinity zone were used to compare the abundance and distribution of bacteria and plankton in each sampling expedition. Differences among bacterial populations, physico-chemical variables and nutrients were compared using student's t test or F test. Non-parametric Spearman rank correlations among toxigenic *V. cholerae* abundance, chitin and nutrients were carried out with data obtained from May 2007. Probability values of <0.05 were considered significant. Statistical analyses were performed using Xact 7.21d (SciLab GmbH, Germany) and Statistica 10.0 (StatSoft Inc., USA). Linear regression was used to explore relationships between variables.

Results

Physicochemical parameters and dissolved nutrients

Average values of various physico-chemical parameters and nutrients are shown in Table 1. In the post-monsoon samplings (December'04–February'05), water temperature ranged from 23 to 25°C while in the pre-monsoon sampling (May'07) it increased to 31°C. In the zones named high, medium and low saline, salinity ranged from 19 to 23, 2.5–11 and 0.0–1.3, respectively. Dissolved oxygen (DO) and pH ranged from 3.6–6.6 mg/l to 6.9–8.0 respectively. In the high saline zone, pH was comparatively higher ($df = 13$; $P < 0.05$) than other zones. During the pre-monsoon sampling the waters of the medium and low saline zone exhibited comparatively lower DO ($df = 4$; $P < 0.001$) than post-monsoon samplings.

Ammonium and nitrite ranged between ~2.5–22 and ~0.1–8.5 μM N, respectively, and increased towards inland, while nitrate ranged from 15 to 50 μM N. The contribution of nitrate to total dissolved inorganic N (DIN) was >80% in most samples. Phosphate ranged from 1 to 4 μM. The low saline zone had higher ($df = 13$; $P < 0.01$) silicate concentration (65–228 μM, average 182 μM), which gradually decreased in the high saline zone (11–115 μM, average 52 μM).

SPM, POC, PON and chitin

POC varied between 12 and 36 μM C, on average ~20 μM C in both high and medium saline zones (Table 1). It was higher ($df = 13$; $P < 0.05$) in the low saline zone with an average of ~50 μM C and a range of 25–110 μM C. POC was 2–3 times higher ($df = 14$; $P < 0.0005$) during the pre-monsoon than post monsoon expeditions in all salinity zones. PON showed a similar trend, averaging ~3.1 μM C in both high and medium saline zones and ~7.2 in the low saline zone. The average C:N ratio of the particulate organic matter (POM) from all the sampling events was 6.4, however, it was lower ($df = 14$; $P < 0.001$) during the post-monsoon (Dec'04 to Feb'05, range 4.9–6.9, average 6.0) than in the pre-monsoon samplings (May'07, 7.3–8.8, average 7.8).

In the size fraction <20 μm, SPM and chitin concentrations were at least 100 and 10 times higher ($df = 8$; $P < 0.001$), respectively, than in the plankton (>20 μm) samples (Fig. 2). SPM concentrations (mg l^{-1}) in the <20 μm fraction ranged from 115–700 (average 270), 35–170 (average 67) and 60–165 (average 96) in the high, medium and low saline zones, respectively. Chitin concentrations (μg l^{-1}) in this fraction were 310–1,560, with average values of 1,030, 580 and 510 in these three sampling zones, respectively. In the phytoplankton and zooplankton samples, SPM values (mg l^{-1}) were 0.2–1.9 (average 0.74) and 0.1–0.7 (average ~0.33), respectively, while chitin concentrations (μg l^{-1}) were 11–31 (average 23) and 12–38 (average 25), respectively. The SPM and chitin concentrations of phytoplankton samples were higher ($df = 4$; $P < 0.005$ and $P < 0.05$, respectively) in the low saline zone (average ~1.4 mg l^{-1} and ~28 μg l^{-1}, respectively) than in the other zones. However, in zooplankton samples, these components showed higher concentrations ($df = 4$; $P < 0.01$ and $P < 0.005$, respectively) in the high saline zone (average 0.54 mg l^{-1} and 35 μg l^{-1}, respectively). Most of the chitin (92–95%) occurred in the <20 μm fraction, while the larger size fractions contained only 1.5–5% of the total chitin. Nevertheless, chitin represented only 0.7% by weight of total SPM in the <20 μm fraction. This percentage increased significantly ($df = 8$; $P < 0.005$) to ~5 and ~10% in microphytoplankton and zooplankton fractions, respectively.

Table 1 Average values of physico-chemical parameters, nutrients and bacterial abundance at different salinity zones of the Karnaphuli estuary

Parameters	High saline zone				Medium saline zone				Low saline zone			
	Dec'04	Jan'05	Feb'05	May'07	Dec'04	Jan'05	Feb'05	May'07	Dec'04	Jan'05	Feb'05	May'07
Temperature (°C)	24.2	23.4	23.8	30.9	24.3	23.6	24.0	31.2	24.6	23.5	24.1	31.3
Salinity (PSU)	19.3	21.5	22.9	19.5	8.6	9.3	10.6	7.7	0.1	0.1	1.3	0.0
pH	7.67	8.02	7.85	7.91	7.65	7.54	7.51	7.11	7.68	7.75	7.57	6.93
DO (mg/l)	6.15	5.73	6.46	6.61	6.05	6.37	6.30	3.61	6.37	5.81	5.73	4.03
Nitrate (μM)	29.4	16.5	30.1	46.7	24.1	21.7	19.2	30.1	18.9	20.9	21.1	16.6
Nitrite (μM)	0.79	0.12	0.78	0.36	0.97	8.53	2.11	4.05	0.37	4.69	5.03	1.73
Ammonium (μM)	5.0	2.5	3.5	2.6	4.1	12.9	20.9	4.1	3.2	11.4	21.9	9.1
Phosphate (μM)	1.58	1.24	1.62	3.80	1.52	2.12	1.85	3.84	1.16	2.07	1.98	2.49
Silicate (μM)	48.8	10.9	32.4	115	66.2	124	80.9	132	228	223	211	64.9
POC (μM)	15.7	16.7	16.6	26.1	12.6	19.6	16.3	35.6	33.6	30.3	26.5	107
PON (μM)	2.83	3.41	2.84	2.96	2.32	3.05	2.64	4.83	4.91	4.52	4.57	14.6
C:N of POM	5.55	4.90	5.85	8.82	5.43	6.42	6.17	7.37	6.85	6.71	5.79	7.30
TBC (cells/ml)	2.9×10^6	4.3×10^6	6.3×10^6	5.1×10^6	4.3×10^6	1.5×10^6	2.4×10^6	1.9×10^6	2.5×10^6	3.1×10^6	3.2×10^6	2.7×10^6
CBC (CFU/ml)	2.4×10^5	5.5×10^4	7.1×10^5	6.9×10^5	1.0×10^5	9.2×10^4	4.9×10^3	3.9×10^4	1.6×10^4	4.9×10^4	2.2×10^4	3.4×10^5
FC (CFU/100 ml)	5.7×10^4	2.0×10^3	4.0×10^3	9.8×10^0	3.0×10^3	1.0×10^3	1.3×10^4	1.2×10^4	8.0×10^3	1.5×10^4	1.3×10^4	6.0×10^3
CVC (CFU/ml)	2.4×10^4	2.2×10^3	2.9×10^4	4.7×10^2	5.5×10^3	2.0×10^1	6.0×10^1	2.4×10^2	4.0×10^0	<3	2.0×10^0	3.0×10^o

DO dissolved O_2; *POC* particulate organic C; *PON* particulate organic N; *TBC* total bacterial count by DAPI staining; *CBC* cultivable bacterial count; *FC* fecal coliform count; *CVC* cultivable *Vibrio* count; values given are arithmetic means of the observed values in three study sites in each salinity zone

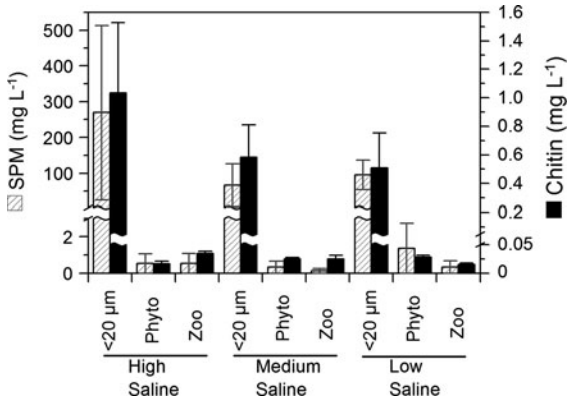

Fig. 2 Distribution of chitin and suspended particulate matter (SPM) in different size fractions in the high, medium and low saline zones. *Phyto*: microphytoplankton, 20–60 µm fraction; *Zoo*: Zooplankton, >60 µm fraction. Results are the average (±SD, shown by *error bars*) of the sampling sites in each salinity zone

Abundance and diversity of phytoplankton and zooplankton

The abundances and species composition of phyto- and zooplankton of the Karnaphuli estuary are shown in Table 2. Microscopic observations showed that the >60 µm and 20–60 µm fractions were comprised mostly (>95%) of phytoplankton and zooplankton, respectively. The low saline sector had higher ($df = 11$; $P < 0.05$) phytoplankton counts ($>10^4$ individuals l^{-1}) than the high and medium saline sites ($\sim 10^2$–10^3 indiv. l^{-1}). The phytoplankton populations were dominated by *Bacillariophyceae* (diatoms, e.g., *Coscinodiscus, Melosira, Synedra, Biddulphia, Nitzschia, Stephanodiscus, Pleurosigma, Ditylum* spp.), followed by *Cyanophyceae* (blue green algae, e.g., *Oscillatoria, Anabaenopsis, Microcystis, Anabaena, Nostoc, Rhaphidiopsis* spp.) and *Chlorophyceae* (green algae, e.g., *Ankistodesmus*, *Pediastrusm, Scenedesmus* spp.). The abundance of *Dinophyceae* (e.g., *Ceratium, Peridinium* spp.) and *Euglenophyceae* (e.g., *Trachelomonas, Ceratium, Euglena* spp.) was low and infrequent. Zooplankton abundance was 10–100 times lower ($df = 11$; $P < 0.05$) than phytoplankton. A relatively higher ($df = 11$; $P < 0.05$) occurrence of zooplankton ($\sim 10^2$ indiv. l^{-1}) was observed in the high saline zone than in the other zones. The zooplankton populations were dominated by copepods (e.g., *Acartia, Diaptomus, Calanus, Cyclops, Mesocyclops, Oithona* spp.) followed by rotifers (e.g., *Brachionus, Filinia, Asplanchna, Polyarthra, Trichocerca, Keratella, Rotaria* spp.) and protozoans (*Difflugia, Arcella, Paramoeceum* spp.). Cladocerans (e.g., *Bosmina, Daphnia* spp.) and ostracods (e.g., *Cypris* spp.) were occasionally observed, in low abundances.

Spatio-temporal variations of total bacteria, fecal coliforms and *Vibrio* spp. in unfractionated water

The cultivable bacterial counts (CBC) in water samples ranged from $\sim 10^4$ to 10^5 CFU ml^{-1}, while the culture independent DAPI counts revealed a nearly stable ($\sim 10^6$ cells ml^{-1}) total bacterial populations (Table 1). Comparatively higher ($df = 13$; $P < 0.01$) CBC in the high saline sites indicated predominance of halophilic bacteria. CBC comprised 0.5–14% of DAPI counts. The faecal coliform (FC) counts ranged from $\sim 10^1$ to 10^4 CFU 100 ml^{-1}. During the post-monsoon samplings, the variation of FC due to the influence of salinity was not noteworthy, while during the pre-monsoon sampling their counts clearly diminished ($df = 4$; $P < 0.005$) in the high saline zone.

Cultivable *Vibrio* counts (CVC) showed a decreasing trend from the high saline zone towards the freshwater area, ranging from $\sim 10^4$ CFU ml^{-1} (up to 0.85% of TBC) to $<10^1$ CFU ml^{-1} (<0.0005% of TBC) (Table 1). In the high saline zone, CVC showed a comparatively higher ($df = 4$; $P < 0.05$) abundance in the post-monsoon than pre-monsoon samplings. Among the identified vibrios (n = 150), the dominant species were *V. parahaemolyticus* (15%), *V. splendidus* (13%), *V. cholerae* (8%) and *V. mimicus* (4%); whilst *V. vulnificus*, *V. campbelli, V. harveyi, V. alginolyticus* and *V. fluvialis,* were also present. However, the occurrence of cultivable *V. cholerae* isolates was very low and enrichment-based most probable number (MPN) analysis indicated their populations between 50 and <1 ml^{-1} (data not shown).

Distribution and abundance of toxigenic *V. cholerae* in water and plankton samples

Vibrio cholerae O1 and O139 populations in the <20 µm fraction mostly ranged from 10^4 to 10^6 cells l^{-1}, while in the microphyto- and zooplankton fractions ranged from 10^1 to 10^3 cells l^{-1} (Fig. 3). Most of these populations remained in VBNC form as quantification of cultivable O1 and O139 was beyond

Table 2 Average abundance of phytoplankton and zooplankton communities at different salinity zones of the Karnaphuli river estuary

Plankton	High saline sites				Medium saline sites				Low saline sites			
	Dec'04	Jan'05	Feb'05	May'07	Dec'04	Jan'05	Feb'05	May'07	Dec'04	Jan'05	Feb'05	May'07
Phytoplankton (>20 μm)												
Total	4.1×10^3	5.4×10^3	1.4×10^2	1.2×10^3	4.4×10^2	1.5×10^3	1.4×10^4	4.7×10^3	5.7×10^4	1.4×10^5	1.2×10^3	2.3×10^4
Bacillariophyceae	2.0×10^3	1.9×10^3	9.0×10^1	7.2×10^2	1.6×10^2	1.4×10^3	1.2×10^4	1.1×10^3	4.2×10^4	8.1×10^4	7.4×10^2	9.6×10^3
Cyanophyceae	8.9×10^2	3.3×10^3	2.0×10^1	9.1×10^1	4.0×10^1	2.5×10^1	1.0×10^3	2.6×10^3	4.7×10^3	6.0×10^4	2.0×10^2	1.1×10^4
Chlorophyceae	8.4×10^2	1.5×10^2	2.0×10^1	6.0×10^0	2.4×10^2	2.1×10^1	8.3×10^2	8.3×10^2	9.8×10^3	1.5×10^3	2.1×10^2	1.9×10^3
Dinophyceae	3.6×10^2	5.0×10^1	<1	1.9×10^2	<1	<1	<1	4.8×10^1	<1	<1	<1	5.1×10^1
Euglenophyceae	<1	<1	<1	1.8×10^2	<1	5.0×10^1	1.0×10^2	7.1×10^1	<1	9.0×10^2	4.0×10^0	1.2×10^3
Zooplankton (>60 μm)												
Total	1.5×10^2	1.9×10^2	1.2×10^1	1.9×10^2	9.4×10^1	6.8×10^1	3.1×10^1	5.8×10^1	1.2×10^2	8.3×10^1	6.0×10^0	2.9×10^1
Copepoda	6.0×10^1	1.3×10^2	3.0×10^0	8.1×10^1	4.6×10^1	5.5×10^1	2.0×10^0	4.4×10^1	8.5×10^1	2.8×10^1	<1	1.2×10^1
Rotifera	4.2×10^1	1.8×10^1	1.0×10^0	2.7×10^1	3.0×10^1	5.0×10^0	4.0×10^0	3.5×10^1	2.1×10^1	2.3×10^1	1.0×10^0	8.0×10^0
Protozoa	4.0×10^1	4.3×10^1	8.0×10^0	4.0×10^1	1.1×10^1	9.0×10^0	4.0×10^0	7.5×10^1	5.0×10^0	2.5×10^1	5.0×10^0	6.5×10^0
Cladocera	1.0×10^0	<1	<1	4.1×10^1	<1	<1	<1	2.5×10^1	1.0×10^1	6.0×10^1	<1	1.5×10^0
Ostracoda	3.0×10^0	<1	<1	1.5×10^0	<1	<1	2.1×10^1	<1	<1	2.0×10^0	<1	0.5×10^0

Values given are arithmetic means of the observed values in three study sites in each salinity zone

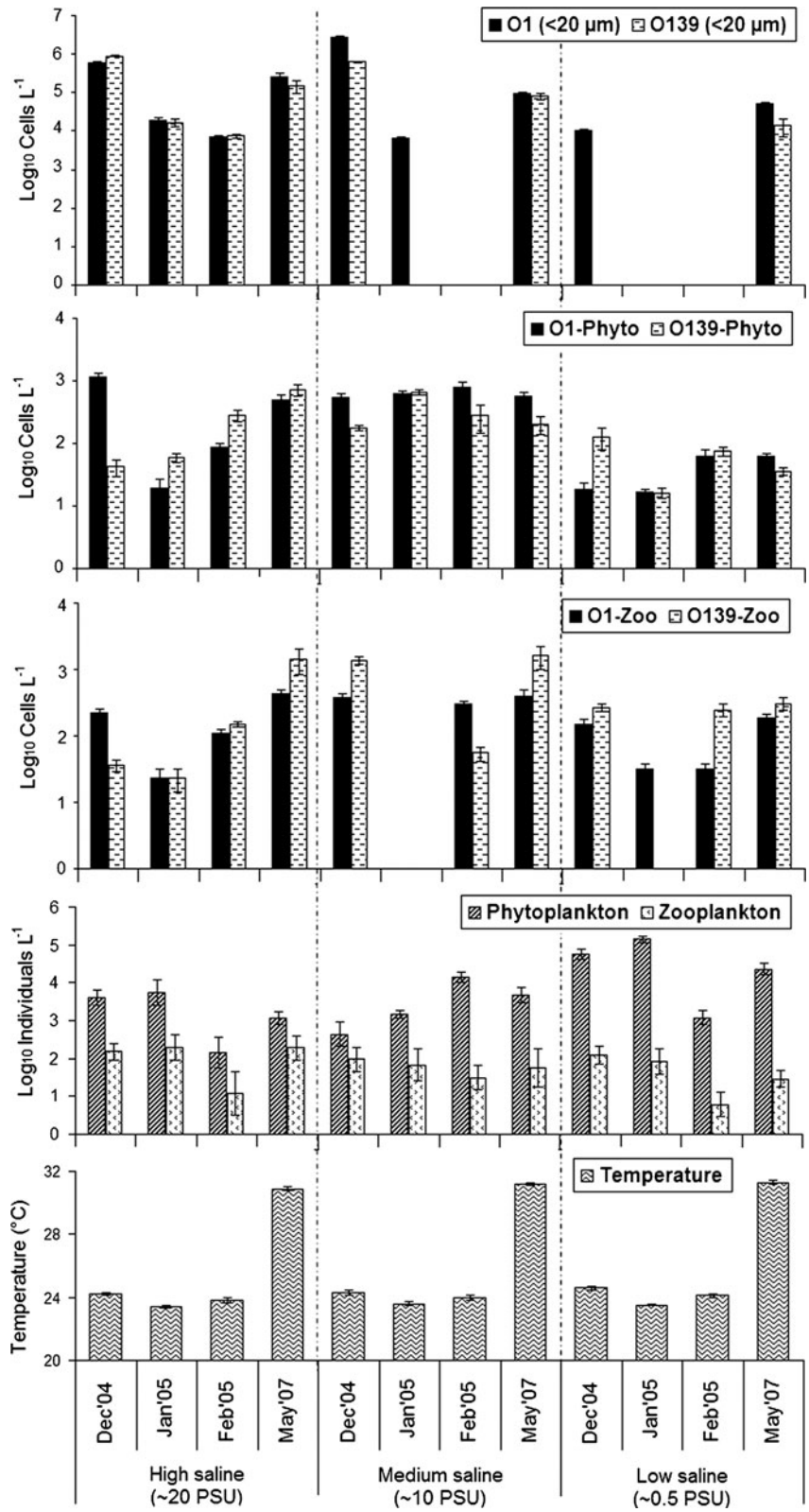

Fig. 3 Comparison of toxigenic *V. cholerae* O1 and O139 abundance in different size fractions of water along with phytoplankton and zooplankton counts and water temperature during sampling times. Culture independent direct viable counts of O1 and O139 cells as "free-living" (<20 µm fraction), in association with microphytoplankton (Phyto, 20–60 µm fraction) and zooplankton (Zoo, >60 µm fraction). Results are the average of three sites (±SD, shown by *error bars*) in each salinity zone

detection by conventional culture techniques. In comparison to O139, the occurrence of O1 strain was more frequent in the <20 μm fraction. Both O1 and O139 strains were observed in association with microphyto- and zooplankton in all sites.

In the high-saline zone, the populations of both "free living" O1 and O139 in the <20 μm fraction decreased gradually after the start of post-monsoon season (December'04–February'05) and were not frequently detected in the mid- and low saline zones. The populations of both serotypes increased again in the <20 μm fraction in the pre-monsoon sampling (Fig. 3). However, a fraction of the populations of these toxigenic bacteria were consistently present in microphyto- and zooplankton samples throughout the estuary. The abundance of *V. cholerae* O1 and O139 did not have any obvious influence of total microphytoplankton or zooplankton populations (Fig. 3). These bacterial populations also did not correlate ($df = 11$; $P > 0.05$) with the counts of any individual plankton species or groups in any salinity zone.

Relationship of "free-living" *V. cholerae* O1 with biogeochemical and bacteriological parameters

During the pre-monsoon, pH and DO did not have any discernible effect on *V. cholerae* O1 or O139 abundance in water, but a highly significant correlation existed between water salinity and these toxigenic strains ($r = 0.70, df = 14, P < 0.005$, Fig. 4). Among other biogeochemical parameters, phosphate showed a positive correlation ($r = 0.57$, $df = 14$, $P < 0.05$) with O1 abundance, although this might be produced by the positive correlation between salinity and phosphate ($r = 0.61$, $df = 14$, $P < 0.05$) (Table 3). Other inorganic nutrients such as DIN and Si did not have any direct influence on O1 and O139 abundance. The abundance of *V. cholerae* O1 in the fraction <20 μm showed a significant correlation with chitin ($r = 0.62$, $df = 14$, $P < 0.05$, Fig. 4). Chitin was positively correlated with SPM ($r = 0.72$, $df = 14$, $P < 0.05$, Fig. 4), which was correlated with POC and PON ($r = 0.58$ and 0.55, respectively, $df = 14$,

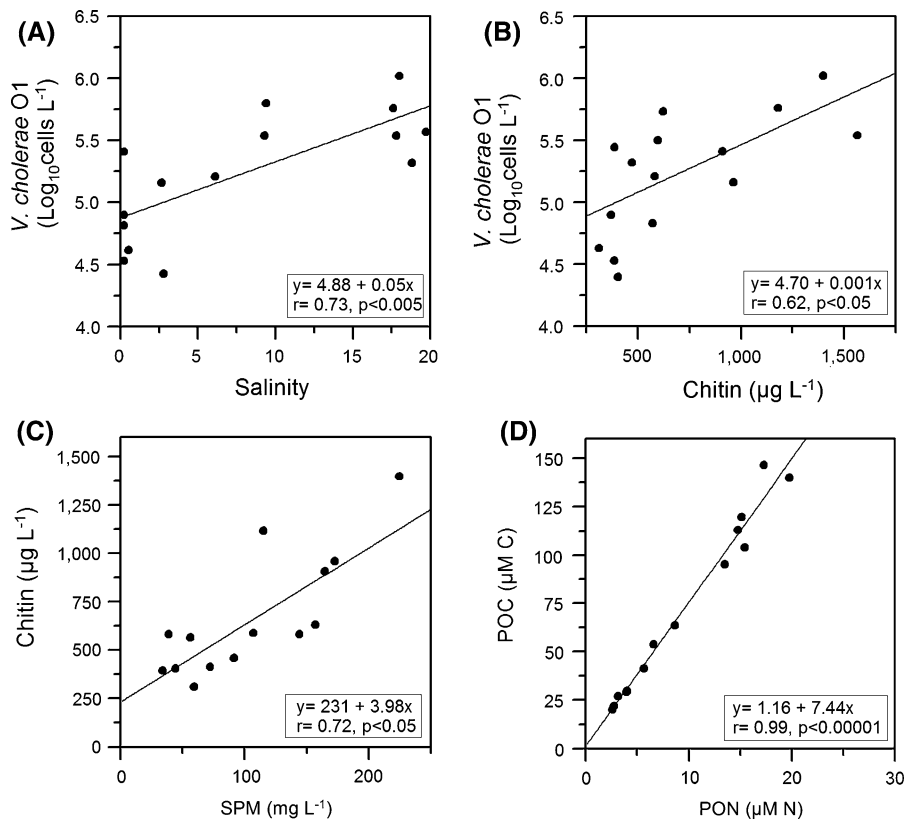

Fig. 4 Correlation among *V. cholerae* O1 abundance and biogeochemical parameters in <20 μm fraction. Observations (n = 15) were from pre-monsoon sampling. *SPM*, suspended particulate matter; *POC*, particulate organic C; *PON*, particulate organic N

Table 3 Spearman rank correlations among *V. cholerae* O1, salinity, chitin and nutrients

Variables	O1		Salinity		Chitin		SPM		POC	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Salinity	***0.73***	***0.003***								
Chitin	***0.62***	***0.017***	−0.44	0.100						
SPM	0.37	0.177	0.29	0.293	***0.67***	***0.009***				
POC	−0.13	0.648	−0.50	0.060	0.43	0.111	***0.58***	***0.025***		
PON	−0.14	0.612	0.41	0.132	0.38	0.164	***0.55***	***0.034***	***0.99***	***<0.001***
DIN	0.32	0.243	0.44	0.097	0.38	0.168	−0.19	0.508	−0.21	0.459
PO_4	***0.57***	***0.027***	***0.61***	***0.016***	0.19	0.491	−0.36	0.182	***−0.66***	***0.008***
Si	0.11	0.694	***0.54***	***0.039***	0.36	0.182	0.12	0.676	−0.06	0.820

SPM suspended particulate matter; *POC* particulate organic carbon; *PON* particulate organic nitrogen; *DIN* dissolved inorganic nitrogen (comprising nitrate, nitrite and ammonia, with overwhelming dominance of nitrate)

Statistically significant correlations are indicated in bold italics

$P < 0.05$, Table 3). Also POC had a strong correlation with PON ($r = 0.99$, $df = 14$, $P < 0.001$, Fig. 4).

During the post monsoon, salinity showed a correlation ($r = 0.61$, $df = 8$, $P < 0.05$) with toxigenic *V. cholerae* but no other biogeochemical parameters showed any direct influence (data not shown). Unfortunately, chitin concentrations were not measured during the post monsoon expeditions.

The abundance of the both *V. cholerae* O1 and O139 populations were not correlated ($df = 14$, $P > 0.05$) with DAPI counts or CBC. In contrast to CVC, there was no reduction of total bacterial population with decreasing salinity (Table 1). In this study and in a previous one (Lara et al. 2009), high fecal coliform counts were observed in the medium and high saline sectors (Table 1), pointing to a sewage pollution. This may also have contributed to increasing the *V. cholerae*, in addition to those belonging to the natural estuarine populations.

Discussion

The present study demonstrates that estuarine habitats near a cholera endemic zone can harbour high amounts of toxigenic *V. cholerae*. In the brackish coastal environment, these toxigenic strains can mostly persist as "free-living" organisms in the water (<20 μm fraction). However, both microphyto- and zooplankton may provide vital support to these potentially harmful bacterial populations, particularly during unfavourable conditions (e.g. low salinity), which may hamper their existence in the "free-living" state.

Influence of physico-chemical parameters on *V. cholerae* dynamics

The abundance of CVC in the high saline zone is comparable to that in seawater having temperatures >20°C in other coastal regions (Barbieri et al. 1999; Mahmud et al. 2008). Variations in physico-chemical parameters had no obvious effect on TBC ($\sim 10^6$ cells/ml), which were typical water-column values in the estuarine zone (e.g., Heidelberg et al. 2002a). However, a gradual decrease of CVC, O1 and O139 counts towards the freshwater end of Karnaphuli estuary is consistent with the general preference of vibrios for higher salinity (Miller et al. 1984). A salinity range of 2–10 is considered favorable for the growth and persistence of *V. cholerae* in coastal habitats (Louis et al. 2003). Yet, the data observed here show that in a cholera endemic region higher numbers of toxigenic *V. cholerae* can be present in estuarine waters with higher salinities around 20. Besides salinity, higher temperature (>20°C) may also contribute to *Vibrio* proliferation (Mahmud et al. 2008). Nevertheless, we observed less influence of water temperatures on *Vibrio* populations in this tropical estuary, probably due to the persistence of >20°C temperature throughout the year. Although there was no direct influence of pH on toxigenic *V. cholerae*, the high saline zone had higher pH (average 7.9) than the other zones. Laboratory

experiments have shown that *V. cholerae* has better survival capacity at pH between 7.5 and 8.5 (Miller et al. 1984; Huq et al. 1984).

Pivotal role of plankton in toxigenic *V. cholerae*'s survival and persistence

In contrast to the occasional disappearance of "free-living" *V. cholerae*, the plankton-associated population of this pathogen remained almost stable in all sites of the Karnaphuli estuary despite changes in the physico-chemical properties, notably in salinity (Fig. 3). Both microphyto- and zooplankton seem to provide a survival advantage for *V. cholerae* during non-favorable conditions (e.g. very low salinities), increasing the chance of dispersal in freshwater habitats and affecting human populations, particularly those who use freshwater for household purposes. The bacterium primarily remains in the VBNC stage and only during favorable conditions (e.g. increase in water pH, plankton bloom, low abundance of *Vibrio* phages, etc.) or upon ingestion by a host organism, may it rapidly flourish and become cultivable (Colwell et al. 1985; Islam et al. 1993; Colwell 1996; Faruque et al. 2005).

Several marine and freshwater plankton have been reported to provide shelter and survival advantage to *V. cholerae,* e.g. in association with duckweed (*Lemna minor*), a green alga (*Rhizoclonium fontenum*) and the water hyacinth (*Eichhornia crussipes*) the bacterium can survive for up to 1 month as a cultivable form (Islam et al. 1994a). Association of VBNC *V. cholerae* has also been observed with diatoms species often found in coastal wetlands, e.g. *Stigeoclonium* and *Nitzschia* spp. (Seeligmann et al. 2008). Blue green algae, particularly *Anabaena* spp, may act as a long-term reservoir of *Vibrio cholerae* O1 in the aquatic environment. Microcosm studies have observed that the toxigenic bacterium can multiply and maintain progeny inside the algal mucilaginous sheath even 2 years after inoculation (Islam et al. 1990, 1999). Among various zooplankton types, copepods (e.g. *Acartia* spp.) have been implicated as a potential vector of *V. cholerae* (Huq et al. 1983; Lizárraga-Partida et al. 2009). Microcosm studies have also shown association of toxigenic *V. cholerae* strains with cladocerans (*Bosmina*, *Ceriodaphnia*, *Diaphanosoma*, etc.), and rotifera (e.g., *Brachionus* spp.), as well as green algae (*Pediastruam* spp., *Volvox* spp., etc.) (Tamplin et al. 1990). Degradation of chitinous and mucilaginous parts of the plankton by the chitinase and mucinase enzymes can provide nutrients to aid in the better survival of the bacterium (Nalin et al. 1979; Islam et al. 1993).

In the eutrophic coastal wetlands, changes in the composition of the phytoplankton or zooplankton community may influence the seasonal occurrence of toxigenic vibrios. A few investigations have studied the relationship between *Vibrio* abundance and composition of the plankton community (Heidelberg et al. 2002b; Lizárraga-Partida et al. 2009). In the present study, there was no correlation between *V. cholerae* abundance and phytoplankton or zooplankton numbers or species. However, there was no plankton bloom that could have had a significant effect on *Vibrio* numbers. In addition, not all plankton types may support the growth or survival of toxigenic *V. cholerae* and the commensal association may also depend on plankton life stages. For example, during juvenile stages, many zooplankton species can frequently molt and shed chitinous exoskeletons along with attached vibrios (Tamplin et al. 1990). *Vibrio cholerae* strains have a higher capability of colonization of dead plankton compared with that of live plankton (Mueller et al. 2007). Only a small portion of total population of a plankton species may interact with toxigenic *V. cholerae* strains. Coastal wetlands also harbour other *Vibrio* species having chitinase or mucinase activity which may outcompete the toxigenic *V. cholerae* strains for association with plankton. Conversely, a rise in *Vibrio* population can occur during the decay of plankton and subsequent release of nutrient-rich organic matter (Middelboe et al. 1995). In particular, cyanobacterial-derived organic matter can act as an important growth-stimulating factor for *V. cholerae* (Eiler et al. 2007). Therefore, a complex relationship may exist between toxigenic *V. cholerae* and the temporal shifts in plankton composition and abundance.

Chitin in suspended particulates: an important determinant of "free-living" *V. cholerae* populations

The positive correlation between chitin and toxigenic *V. cholerae*, as observed in the present study, indicates that the bacterial populations might have utilized chitin as an important source of C and N while surviving as "free-living" in estuarine water. Interaction with

chitinous plankton may support the bacteria with some survival advantages including food availability, adaptation to nutrient gradients, and protection from predators (Pruzzo et al. 2008). A chitin-binding protein (GbpA) secreted from *V. cholerae* can play an important role in colonizing both aquatic plankton or particulate chitin and the human intestine (Kirn et al. 2005). However, no other studies have observed the relationship between chitin and *V. cholerae* abundance in aquatic habitats except in a recent study in the Sunderban mangrove (Lara et al. 2011). Further, the present study unveils for the first time that chitin concentration can influence the distribution of toxigenic *V. cholerae* in an estuarine environment (Fig. 4).

It is plausible that chitin in the water column is mostly associated with other suspended particles, and likewise we observed a significant correlation among these parameters (Table 3). Thus, although O1 counts did not correlate with SPM, POC and PON, correlations of these parameters with one another and chitin suggest their possible influence on the relationship between chitin and O1. The prevalence of particle-associated bacteria in an estuary is probably an adaptation strategy in the highly unstable coastal environment. We have recently observed that an increase in suspended sediments or other particulate matter can augment total cultivable *Vibrio* abundance (Lara et al. 2009). Further, settling experiments in microcosms (Neogi et al., unpublished) clearly indicated an association of cultivable *Vibrio* population (>90%) with particulate matter in estuarine water samples having a similar salinity range as in the present study. Benthic sediments may also act as a potential ecological niche for *V. cholerae* (Vezzulli et al. 2009). Nevertheless, little is known about chitin concentration and toxigenic *V. cholerae*'s occurrence or survival in the benthic environment. Re-suspension events of bottom sediment by turbulences can augment the concentration of SPM and associated chitin in the estuary, which may increase the chances of survival and persistence of "free-living" *V. cholerae*.

The overall average C:N ratio of 6.4 in SPM of Karnaphuli estuary points to phytoplankton and zooplankton as the source of organic C and N (Redfield et al. 1963). However, this ratio can deviate substantially from the Redfield value (6.6) depending on plankton composition (Daly et al. 1999). We also observed comparatively lower C:N ratios (4.9–6.9) during the post-monsoon but relatively higher values (7.3–8.8) during the pre-monsoon expeditions. The elevated C:N can be attributable to the irradiance-nutrient interaction and higher abundance of large sized diatoms and copepods (Daly et al. 1999). Among chitinous copepods, *Acartia* spp. have lower C:N ratio, averaging ~4.5 compared to *Pseudocalanus* spp., averaging ~7.5 (Walve and Larsson 1999). In coastal water, the predominant occurrence of "free-living" *V. cholerae* can be linked to occurrence of most of the SPM and chitin (>98 and >92%, respectively) in the <20 μm fraction (Figs. 2, 3), similar to the Sunderban mangrove waters (Lara et al. 2011). This and the C:N ratio of SPM supports the relevance of plankton-derived chitin as a nutritional substrate for *V. cholerae* populations. Further, chitin can induce *V. cholerae* to acquire genetic material from the environment, thus facilitating its diversification and greater ecological fitness (Meibom et al. 2005). However, it is not clear whether attachment to the abundant chitinaceous nanodetritus can allow *Vibrio* survival at unfavourable environmental conditions such as very low salinities, in the way microplankton does. This is supported by the frequent absence of O1 and O139 counts in the fraction <20 μm at low salinities (Fig. 3). The "free-living" cells of both serogroups were detectable in the low saline zone only when water temperature was highest. This seems to stress the "shelter" function of microphytoplankton under all the environmental conditions observed in this study.

Conclusion and outlook

The present study shows that both microphyto- and zooplankton in estuaries can provide survival advantage for *V. cholerae* populations and thereby assist in spreading it from saline into freshwater regions where it can more directly affect human health. Although *V. cholerae* O1 and O139 did not correlate with plankton abundance or species composition, a significant positive correlation existed with chitin concentration in the <20 μm fraction, where SPM, toxigenic *V. cholera* and chitin were highest. This observation and the distribution of *V. cholerae* in the <20 μm fraction along the estuarine gradient point out the necessity of long term studies on assumed "free-living" and plankton-attached *V. cholerae* dynamics for a deeper understanding of the seasonal occurrence of cholera in an endemic area. Similarly, the driving forces, types and persistence

of the association of this pathogen with organisms and particles in the nanoplankton (<20 μm) fraction requires a closer look in order to clarify the validity of the concept of "free living" state of *V. cholerae*.

There remains much to be understood about the mechanisms promoting the association of plankton and chitin with toxigenic *V. cholerae*. Mangroves are environments naturally rich in chitin, in the form of plankton, detritus, shrimps, crabs, etc. Further, shrimp aquaculture in or close to wetlands is increasing worldwide. As chitin abundance can favor genetic diversification, the high shrimp density of aquaculture ponds may trigger the emergence of new and highly pathogenic *Vibrio* types. Increasing aquaculture, eutrophication and salinization of tropical estuaries may pose a threat to human health of yet unknown dimension.

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