

Influence of salinity stress on the bacterial diversity from a marine bioinvasion perspective: Evaluation through microcosm experiments

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Abstract

Mid-ocean ballast water exchange (BWE) has been used for the past few years to control bioinvasion through ballast water (BW) which requires the ship to exchange part of its BW in the open ocean. The change in salinity is expected to be detrimental to coastal species. This study investigated the changes in bacterial diversity with respect to salinity stress when translocated into an environment with non-ambient salinity. Surface water samples from 3 coastal ports, namely hypersaline Kandla port (Gujarat), saline Paradip port (Odisha) and freshwater Kolkata port (West Bengal) were collected and aged in dark for 30 days to mimic BW tank conditions. The aged water samples were translocated into water collected from the estuarine mouth (Dona Paula), mid-estuary (Cortalim) and upstream location (Sanvordem) of Zuari Estuary, Goa and incubated in the laboratory. Quantitative polymerase chain reaction (qPCR) technique was used to assess major bacterial phyla. Culturable marine bacteria, and *Vibrio* spp. including γ - proteobacteria preferred higher salinity, whereas culturable freshwater bacteria including coliforms in conjunction with α -, β - proteobacteria, Bacteroidetes and Firmicutes preferred lower salinity. The freshwater port bacteria could withstand the salinity stress better than that from the hypersaline or saline ports. The species comprising these phyla could be region and niche-specific. The invasive potential of these bacterial clades would depend on the environmental suitability of the recipient port. Investigating the changes in the ecosystem of the port environment after ballast water discharge in real-time would provide meaningful insights on the invasive potential of these bacterial phyla and is a way forward.

Keywords: Marine bioinvasion, Ballast water exchange, Salinity stress, Bacterial community, qPCR.

Introduction

Oceanic transport accounts for more than 90% of the world's cargo and in addition to the intended cargo, the translocation of over 10 billion tonnes of ballast water also occurs annually¹. As a result, a plethora of organisms present in the ballast water, are introduced into non-native environments², which is estimated to be about 7000 living species³. Such introduction of alien organisms into a non-indigenous environment is termed as "Bioinvasion" and ballast water (BW) is considered to be one of the key vectors for marine bioinvasion. When introduced into a non-native environment, the impact of bioinvasion may result in changing the biodiversity thereby altering the food web and can have a direct impact on the socio-economic and human health^{2,4}. Marine bioinvasion through ships' ballast tanks has been recognized as one of the greatest threats to the oceans.

Many studies have reported a profusion of invasive organisms in the past few decades⁵⁻⁷. Microorganisms are spread in higher numbers than other macro-organisms given their abundance in any given ecosystem⁸⁻¹⁰. Additionally, ballast water tanks act as incubators for heterotrophic bacteria, which can withstand prolonged dark conditions, unlike other autotrophs¹¹. Thus, by virtue of their abundance, life-history characteristics, and potential pathogenicity or toxicity, microorganisms possess a great capacity to invade and cause detrimental effects in new environments¹². Hence the threat of bacterial invaders is a major concern when compared to other higher organisms. Specifically, the spreading of bacteria, including pathogenic strains on a global level through ships' ballast water (BW) is a phenomenon that is gaining increased importance¹³. There are documented evidences that the organism *V. cholerae*, which is the causative agent for cholera can be transported via ships' BW and has caused epidemics in areas where it was not reported earlier¹³⁻¹⁶.

The International Maritime Organization (IMO) has put forth guidelines to reduce the risk of bioinvasion through ships' ballast water. Mid-ocean exchange of ballast water (BWE) requires ships to exchange a percentage of coastal water with open ocean water (200 nautical miles from the coast). The idea is to release the organisms into an environment with non-ambient salinity to reduce the undesirable bacterial load in exchange for oceanic species with presumably lesser concern¹⁷. A study has reported that salinity is the most significant environmental factor that contributes to the bacterial diversity in ballast water tanks¹⁸. Hence, oceanic species are less likely to survive the environmental stress when released into coastal (marine or freshwater) environments¹⁹. There are many studies that have tried to assess the efficiency of BWE with respect to phytoplankton abundance²⁰, marine invertebrates²¹, zooplankton abundance²², diatoms and dinoflagellates²³ and virus like particles²⁴. The importance of bacterial invaders has taken precedence much more recently. Studies have

reported that the bacterial abundances did not change significantly in most of the cases after BWE and at the end of the voyage^{25,26}. Contrastingly, studies on the bacterial diversity during a voyage reported a marked change in the community due to BWE²⁷ and stressed that ballast water tanks act as incubators for the prevailing bacteria in the tanks which changed overtime during the transit duration²⁸. Moreover, it has been reported that the response of bacterial populations to changing salinity is unlike higher organisms²⁹. An investigation into both the culturable and unculturable segments of bacteria showed that although the abundance of culturable bacteria in ballast tanks remained unchanged during the voyage, there was a significant decrease in the unculturable bacteria and their DGGE profiles were different after ballast water exchange (BWE)³⁰. The bacterial diversity is ever-shifting as a response to environmental stress. Rather than relying on the variation in individual abundances which may not equate to significant shifts in the bacterial community, a community level approach would provide a more sensitive and meaningful understanding of their response.

The premise of BWE is to expose the coastal bacterial communities in the ballast water tanks to a non-ambient salinity (Open Ocean). But the assumption that salinity change plays an important role in reducing bacterial load during BWE is not yet fully explored and explained. Although the bacterial diversity is heavily influenced by the prevailing environmental conditions³¹, studies concentrating on the response of bacterial populations to changing environmental conditions are sparse. Studies have reported the importance of salinity in the virility and viability of pathogens like toxicogenic *Vibrio cholerae*³². But there is a paucity of knowledge on the influence of salinity as a major environmental factor in the context of BWE. In view of this, the objective of the present study was to understand the response of bacterial communities to changes in salinity using microcosm experiments. Surface water samples from coastal ports with varied salinity conditions (hypersaline, saline and freshwater) were collected, aged and translocated into water collected from different areas (salinity gradient) of an estuary. This incubation experiment allowed us to assess the response of bacteria from different environments to a sudden salinity change which is analogous to the release of ballast water by ships into non-indigenous environments.

Real time quantitative polymerase chain reaction (qPCR) approach was used to assess the contribution of major bacterial phyla (Proteobacteria subclasses [α , β , and γ], Bacteroidetes, Firmicutes, and Actinobacteria) to the bacterial diversity. The variation in culturable pathogenic abundance was also assessed using standard plating techniques. Since dark conditions would have immensely reduced the photosynthetic bacteria, we hypothesized that the bacterial diversity would be mostly heterotrophic and is expected to change significantly as a response to the salinity shock

after translocation. Extreme salinity shock is expected when the bacteria from the hypersaline port are translocated into freshwater from the estuary and vice-versa with the freshwater port bacteria. The ability of various bacterial clades to tackle the salinity stress would depend on the mechanisms they adopt to cope with this stress.

Materials and Methods

Sampling strategy

Surface water samples were collected from Kandla port, Gujarat, India (Local Fishing Craft Anchorage-2; 23° 01' 03" N, 70° 13' 19" E) for hypersaline water experiment (Experiment I) (salinity – 39.68) using a 5L Niskin sampler and transferred into a 50L Nalgene carboy and stored in the dark, static condition for 30 days to simulate the ballast tank condition. For translocation into water with varying salinities, surface water samples were collected from the Zuari estuary, Goa, India from the estuarine mouth, Dona Paula (DP; 15° 25' 16.9" N, 73° 47' 36.9" E), Mid estuary, Cortalim (CR; 15° 24' 32.0" N, 73° 54' 50.2" E) and Upstream station, Sanvordem (SV; 15° 16' 01.1" N, 74° 06' 36.0" E). Similarly, surface water samples were collected from Paradip port (Area adjacent to fertilizer berths; 20° 16' 27.8" N, 86° 40' 2.9" E) for saline water (Experiment II) (salinity – 29.4) and Kolkata port (Tidal Basin-1; 22° 32' 43" N, 88° 19' 07" E) for freshwater (Experiment III) (salinity – 0.22) experiments (Fig. 1).

Translocation and experimental setup

The port water (~20 L) collected from the three port environments was immediately incubated in dark for 30 days. Temperature during all three experiments was maintained between 25.0-26.0 °C. All the three experiments were conducted separately; a schematic representation of the experimental setup is provided in Fig. 2.

Experiment I – Aged hypersaline water from Kandla port was translocated into the estuarine water of three different salinities in a ratio of 1:5 in 20L polycarbonate tanks (dimensions – 43 x 27 x 18cm) (Tarsons, Cat no. 41080). The tanks were placed in static condition in the laboratory with natural light so as to provide natural day-night environment. The translocation of the aged port water into the water from estuarine mouth (DP) is hence forth referred to as high saline condition (HSC); the translocation of aged port water into the water from the mid estuarine station (CR) is termed as mid saline condition (MSC); and the translocation of the aged port water into the water from the upstream station (SV) is henceforth termed as low saline condition (LSC). Incubation was conducted for each condition in duplicate tanks (n=2) for 12 days.

The salinity of the aged Kandla port water was 39.38, while it was 31.1, 13.51 and 0.03 at the mouth (DP), mid-estuary (CR) and upstream (SV) respectively. During the course of the experiment (after mixing), average salinity was 32.36 ± 0.72 in HSC, 15.28 ± 0.35 in MSC and 6.35 ± 0.26 in LSC.

Experiment II – Aged saline water from Paradip port was used for this experiment. The salinity of the aged Paradip port water was 26.15, while at DP, CR and SV it was 32.3, 18.57 and 0.05 respectively. After translocation, during the incubation period (12 days), the average salinity was 27.13 ± 0.19 , 20.48 ± 0.29 and 4.54 ± 0.06 in HSC, MSC, and LSC respectively.

Experiment III – Aged freshwater from Kolkata port was used for this experiment. Salinity at Kolkata port was 0.35, whereas it was 34.49 at DP, 17.02 at CR and 0.14 at SV. The average salinity after the mixing was 29.70 ± 0.87 in HSC, 13.11 ± 0.59 in MSC and 0.13 ± 0.01 in LSC.

Physicochemical analyses and dissolved nutrients

Salinity measurements were done using autosal (Guildline Autosol 8400B). For suspended particulate matter (SPM) measurements, 50ml of water samples were filtered through pre-weighed, pre-combusted ($400 \text{ }^{\circ}\text{C}$ for 4 h), Whatman GF/F filter papers and dried in the oven for 48h at 60°C . Final weights were recorded as milligrams per liter (mg L^{-1})³³. The dissolved oxygen (DO) was analysed using standard Winkler titration method³⁴. Dissolved nutrients (silicate- SiO_4 , phosphate- PO_4 , nitrate- NO_3 , and nitrite- NO_2) from the water samples were analysed using (Skalar SAN^{PLUS} 8505 Interface v3.331, Netherland)³⁵. Sodium hexafluoro silicate (Na_2SiF_6), Potassium dihydrogen phosphate (KH_2PO_4), Potassium nitrate (KNO_3) and Sodium nitrite (NaNO_2) were used for preparation of standards according to manufacturer's guidelines for estimation of SiO_4 , PO_4 , NO_3 and NO_2 respectively.

Microbiological analyses

Samples for total bacterial counts (TBC) were collected in duplicate ($n=2$), preserved in 1% paraformaldehyde (final conc.) and kept at -20°C until analysis. Samples were thawed before passing through BD cell strainer cap (BD Biosciences, USA), stained with SYBR Green I (1:10,000 final conc.), and incubated in the dark condition for 15 minutes. The stained samples were analysed on BD FACS AriaTM II flow cytometer equipped with a blue laser of 488nm, using fluorescent beads ($1\mu\text{m}$, Polyscience) as an internal standard³⁶. The data were processed using BD FACS Diva software (BD Biosciences, USA) and expressed as cells per milliliter (cells ml^{-1}).

For total viable marine bacteria (TVC-ZMA) and total viable freshwater bacteria (TVC-NA), samples were appropriately diluted and spread on Zobell Marine Agar (ZMA, HiMedia) and Nutrient Agar (NA, HiMedia) respectively. Pathogenic bacteria were quantified on selective media according

to manufacturer's guidelines (HiMedia). Thiosulfate citrate bile salts (TCBS) agar was used for differentiating various *Vibrio* spp. (*V. parahaemolyticus* [VP], *V. alginolyticus* [VA] and *V. cholerae* [VC]). MacConkey agar was used for enumeration of total coliforms (TC) and HiCrome EC O157: H7 Agar was used for enumeration of *E.coli* O157: H7. Identification of the specific bacteria has been confirmed earlier using both biochemical tests and protein profiling through MALDI-TOF MS Biotyping³⁵. The ZMA and NA agar plates were incubated at room temperature, while the selective media for pathogenic bacteria were incubated at 37°C. Abundance is expressed as colony forming units per millilitre (CFU ml⁻¹).

Metagenomic bacterial diversity

For metagenomic analysis, 1 L of water sample collected from the tanks was filtered through 0.22 µm pore size filter paper (Millipore, USA) by suction under low vacuum using a vacuum PR pump (220/50Hz) connected to a PALL 47mm filtration unit. The filter papers were kept at -20°C until further processing. DNA extractions were performed using Power Water DNA Isolation Kit (MoBio lab. Geneworks, Australia). The DNA elutions were then diluted to 1:100 in autoclaved nuclease-free water (AmbionTM) and quantified for an initial estimate of the concentration which was 10-20 ng ml⁻¹ (Qubit 2.0, Thermofischer Scientific, USA). One µl of the diluted samples was then subjected to quantitative polymerase chain reaction (qPCR) using specific sets of primers for major bacterial phyla, namely, Proteobacteria subclasses (α , β and γ), Bacteroidetes, Firmicutes and Actinobacteria to obtain the bacterial diversity as described by De Gregoris et al.³⁷ and Ashelford et al.³⁸ (Table 1).

The qPCR assays were carried out on Rotor-Gene Q (Qiagen). All the qPCR reactions were carried out in triplicates (n=3). The PCR cocktail consisted of 10 µl 2x Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µl each of the forward and reverse primers and 1 µl of template and made up to 20 µl using nuclease free water. The cycling conditions were initial enzyme activation at 95°C for 5min, 45 cycles of denaturation at 95°C for 1min, annealing at 53°C for 1min and extension at 72°C for 1min. Tenfold dilutions of previously quantified target DNA were used as internal standards. The amplification curves were obtained by plotting the threshold cycle (C_T) value against the logarithm of fluorescence. Furthermore, to check the efficiency of the reaction (which was always between 0.98 and 1.0), a melt curve analysis was performed after each designed run which confirmed that the fluorescence signals were obtained from the target amplicon. The unknown samples were quantified from their C_T values on the amplification curves using Rotor-Gene Q software (v 2.3.4).

Graphical representation and statistical analyses

All the graphical representations and illustrations were done using Grapher™ (Golden Software, LLC, v12.3.734). The relationship between abiotic (DO, pH, SPM, TOC, SiO₄, PO₄, NO₃ and NO₂) and biotic (TBC, TVC-ZMA, TVC-NA, bacterial diversity) variables was evaluated by using Canonical correspondence analysis (CANOCO v4.5 for Windows software package) after log transformation, using Monte-Carlo test to assess the significance of the method ($p < 0.05$; 999 permutations). Prior to the analyses, unimodal characteristics of the data were checked by using detrended correspondence analysis (the length of the first axes were > 2 SD units)³⁹. CCA triplots were created to visualize the relationship between the abiotic and biotic parameters. The length and the orientation of the arrows denote their relative influence on the biotic variables. Significant ($p \leq 0.05$) environmental factors are denoted by dashed lines. The correlations between biotic and abiotic parameters were further confirmed using StatSoft, Inc. (2007) STATISTICA (data analysis software system), version 8.0. www.statsoft.com. Regression with p -value ≤ 0.05 , was considered significant and has been tabulated.

Results

Hypersaline port water experiment (Experiment I)

The suspended particulate matter (SPM), total organic carbon (TOC) and dissolved nutrients values in the aged port water and water from the different stations of the estuary representing the mouth (DP), mid estuary (CR) and upstream (SV) locations are provided in Table 2. After translocation, during the incubation period, SPM was lower than the aged port water due to addition of estuarine water with low SPM (Table 2a), but increased during the incubation period and was 549 ± 119 mg l⁻¹, 475 ± 139 mg l⁻¹ and 537 ± 277 in HSC, MSC and LSC respectively (Table 2a). A similar trend was observed in the TOC concentration. However, there was no significant change in phosphate (PO₄) concentration. Nitrate (NO₃) concentration decreased in all three conditions whereas vice versa was observed in the case of nitrite (NO₂) concentration (Table 2).

The TBC, TVC-ZMA, TVC-NA and pathogenic bacteria in the aged port water, DP, CR, and SV are indicated in Table 3. During the incubation period, average TBC which was $6.9 \pm 0.7 \times 10^7$ cells ml⁻¹ in the aged port water decreased over time. Within the three conditions, TBC was high in HSC ($1.2 \pm 0.1 \times 10^7$ cells ml⁻¹) and showed a negative correlation with NO₃ ($r = -0.973$). It was low in LSC ($4.4 \pm 2.4 \times 10^6$ cells ml⁻¹). TBC was also positively influenced by PO₄ (Fig. 6a). However, TVC-ZMA increased after the mixing and was high in HSC ($5.5 \pm 2.1 \times 10^3$ CFU ml⁻¹) whereas TVC-NA was high in LSC ($28.9 \pm 18.3 \times 10^3$ CFU ml⁻¹) (Table 3a). *E.coli* O157: H7 was only detected in LSC, whereas

Vibrio spp., abundance preferred and increased in higher salinity. *V. alginolyticus* (VA) was high in HSC (393 ± 459 CFU ml⁻¹) and *V. parahaemolyticus*(VP) was high in MSC (95 ± 123 CFU ml⁻¹) (Table 3a). VA showed a negative correlation with NO₃ in HSC ($r=-0.983$) and was positively influenced by NO₂ in MSC ($r=0.961$) (Table 4). TOC and SPM were the key factors that positively influenced the TVC-ZMA, TVC-NA and pathogenic bacteria (Fig. 6a).

Before aging, the port water was dominated by Actinobacteria (53.64%) (Fig. 3a). After aging, however, the bacterial diversity shifted in the aged port water with α -proteobacteria (55.19%) and Bacteroidetes (36.36%) being the dominant phyla (Fig. 3b). In the estuarine stations, the bacterial diversity was dominated by β -proteobacteria (80.04%) at DP; α -proteobacteria (55.45%) at CR; and by γ -proteobacteria (36.06%) and Bacteroidetes (33.78%) at SV (Fig. 3c, d, and e). After translocation, in HSC, the bacterial diversity was initially dominated by Bacteroidetes (63.47%) followed by β -proteobacteria (19.67%). However, there was a steady increase in Actinobacteria and γ -proteobacteria over time (Fig. 3f). In MSC, however, the bacterial diversity was dominated by α -proteobacteria (40.58%) but subsequently shifted to β -proteobacteria and Bacteroidetes (Fig. 3g). The bacterial diversity at LSC was initially dominated by Actinobacteria but a significant increase in Bacteroidetes during the later stages of the incubation was evident (Fig. 3h). Nitrate had a positive influence on all the major taxonomic phyla (Fig. 6a).

Saline port water experiment (Experiment II)

The SPM, TOC and dissolved nutrient values in the aged port water, DP, CR, and SV are depicted in Table 2b. During the incubation period after translocation, the SPM did not vary significantly in HSC and MSC but was low in LSC (79 ± 13 mg l⁻¹) where it showed a decreasing trend (Table 2b). The TOC, however, showed an increasing trend in all the conditions. There was a significant increase in PO₄ concentration after 5 days of incubation. NO₃ concentration was high in LSC (7.4 ± 9.4 μ M) but decreased over time in all three conditions (Table 2b).

The bacterial abundance before translocation in the aged port water and the estuarine locations are given in Table 3b. After translocation, the TBC reduced by one order of magnitude. Within the three conditions, it was high in HSC ($3.3\pm 0.7 \times 10^6$ cells ml⁻¹) and low in LSC ($8.4\pm 3.6 \times 10^5$ cells ml⁻¹) during the incubation period. It was positively influenced by PO₄ during HSC, and by nitrate during MSC (Table 4), whereas, SPM and TOC showed a negative correlation with TBC in all three conditions (Fig. 6b). TVC-ZMA showed an increasing trend in all three conditions and was negatively correlated with nitrate in HSC, while TVC-NA were high in LSC (Table 3b) and were negatively correlated with TOC, silicate, and phosphate in HSC (Table 4). There

was a significant increase in *E.coli* O157: H7 abundance in LSC during the incubation period. However, both VA and VP were high in HSC ($2.5 \pm 3.3 \times 10^3$ CFU ml⁻¹ and 623 ± 917 CFU ml⁻¹ respectively) (Table 3b). VP showed a negative correlation with silicate and was positively influenced by nitrate in MSC (Table 4). A strong positive correlation was observed between nitrate and TVC-ZMA, TVC-NA and pathogenic bacteria (Fig. 6b).

The Paradip port water bacterial diversity was initially dominated by α -proteobacteria (75.74%) (Fig. 4a). However, after aging, the bacterial diversity in the aged port water was dominated by γ - and β -proteobacteria (52.63% and 29.72% respectively) (Fig. 4b). In the estuarine stations, γ - and α -proteobacteria (36.34% and 31.86% respectively) were dominant in DP; Actinobacteria and γ -proteobacteria (49.78% and 36.28% respectively) in CR; and β -proteobacteria in SV (51.26%) (Fig. 4c, d, and e). After the translocation, the bacterial diversity was initially dominated by α -proteobacteria (76.73%) in HSC. However, Bacteroidetes (78.11%) were abundant by day 4 of incubation. There was a steady increase in β - and γ -proteobacteria throughout the incubation period which eventually dominated the bacterial diversity along with Actinobacteria (Fig. 4f). In MSC, γ -proteobacteria (78.65%) were dominant, while β -proteobacteria showed a steady increase, eventually dominating the bacterial diversity (Fig. 4g). In LSC, the bacterial diversity was initially dominated by Bacteroidetes (52.9%) and γ -proteobacteria (38.59%). Similar to MSC, β -proteobacteria increased significantly and dominated the bacterial diversity (73.46%) by the end of the incubation period (Fig. 4h).

Freshwater port water experiment (Experiment III)

The initial physicochemical and dissolved nutrient values are tabulated in Table 2c. After translocation during the incubation period, the SPM which was 199 mg l^{-1} increased significantly in all the conditions and was $403 \pm 55 \text{ mg l}^{-1}$, $299 \pm 108 \text{ mg l}^{-1}$ and $213 \pm 33 \text{ mg l}^{-1}$ in HSC, MSC and LSC respectively. TOC increased significantly and was high in LSC ($4.6 \pm 1.8 \text{ mg l}^{-1}$) (Table 2c). NO₃ concentration was particularly high in the aged port water ($82.59 \text{ }\mu\text{M}$) before the translocation and was high in MSC ($23.9 \pm 5 \text{ }\mu\text{M}$) and LSC ($23.8 \pm 3 \text{ }\mu\text{M}$) during the incubation period after translocation (Table 2).

The bacterial abundances in the aged port water and that of the estuarine stations before the translocation are tabulated in Table 3c. After translocation, the TBC reduced by one order of magnitude and was high in HSC ($2.5 \pm 0.7 \times 10^6$ cells ml⁻¹) and low in LSC ($2.3 \pm 0.6 \times 10^6$ cells ml⁻¹). However, it increased over time in all the conditions during the incubation period. SPM and TOC showed a negative correlation with TBC in all three conditions (Fig. 6c). There was a significant

increase in both TVC-ZMA and TVC-NA which were high in HSC ($1.1 \pm 1 \times 10^4$ CFU ml⁻¹ and $3.5 \pm 4 \times 10^3$ CFU ml⁻¹ respectively) during the incubation period. During the incubation period, *E. coli* O157:H7 and total coliforms (TC) thrived in lower salinities and showed a weak positive correlation with NO₃ (Fig. 6c). VA was also high in LSC, whereas VP and *Vibrio cholerae* (VC) were high in HSC (Table 3c). A negative correlation between SPM and VA during LSC was observed (Table 4), whereas VC showed a positive correlation with SPM (Fig. 6c).

The Kolkata port water bacterial diversity was initially dominated by Actinobacteria (59.96%) (Fig. 5a). After aging, the bacterial diversity in the aged port water was dominated by Bacteroidetes (72.06%) (Fig. 5b), while in the estuarine stations, the bacterial diversity in DP and CR were dominated by Bacteroidetes (62.24% and 75.44% respectively) and by γ -proteobacteria in SV (67.92%) (Fig. 5c, d, and e). During the incubation period, γ -proteobacteria (75.62%) were dominant in HSC, while a steady increase in α - and β -proteobacteria was observed over time. Firmicutes, in HSC, did not vary significantly during the whole course of the experiment (~13.5%) (Fig. 5f). In MSC, the bacterial diversity was also dominated by γ -proteobacteria (71.75%) initially but decreased subsequently with an increase in the abundance of α -proteobacteria (41.09%) and Bacteroidetes (34.58%) which dominated the bacterial diversity by the end of the incubation period (Fig. 5g). In LSC, α -proteobacteria (86.23%) was dominant initially after the translocation. However, it was later dominated by Firmicutes and Bacteroidetes (Fig. 5h). TOC had a positive influence on Bacteroidetes, Firmicutes, Actinobacteria, and β -proteobacteria, whereas α - and γ -proteobacteria were positively influenced by SPM (Fig. 6c).

Discussion

The present study attempted to mimic the process of ballast water discharge using microcosm experiments. The aging of port water in dark simulated the conditions in the ballast water tanks during a voyage. Previous studies have reported that BWE is effective during longer duration voyages (15 days to several months)⁴⁰. In this study, the aging of the port water in dark for 30 days resulted in a shift in the bacterial diversity with an increase in bacterial clades better adapted to the stress. The study was conducted using qPCR technique which has been widely used to assess the bacterial diversity by overcoming the disadvantages of standard culture techniques, thus including the viable but not culturable (VBNC) segment of bacteria.

Hypersaline port water experiment (Experiment I)

The aging of the port water in dark resulted in a decrease in the total bacterial count (TBC), and also a shift in the bacterial diversity from Actinobacteria to α -proteobacteria, which are known to

grow in aphotic deep ocean areas⁴¹. The bacteria (TBC) in this aged hypersaline port water when translocated to the estuarine mouth (DP), mid estuary (CR) and upstream (SV), preferred high saline condition (HSC) (Table 3), which could be due to higher stress on the bacteria when the aged port water was translocated to lower salinity estuarine water. However, after the initial shock following the translocation, the TBC continued to increase throughout the incubation period in all the three conditions. In addition to TBC, VA and γ -proteobacteria also preferred higher salinity where low NO_3 concentrations were detected and NO_2 concentrations showed an increasing trend. γ -proteobacteria (which include VA) have been known to play a major role in nitrate reduction in marine systems⁴², which could have led to the reduction of NO_3 to NO_2 . Most of the viable bacteria, including the pathogenic strains (VA, VP, and TC) were positively influenced by SPM (Fig. 6). The influence of SPM on both autochthonous and allochthonous bacteria has already been illustrated³⁶. SPM not only provides important nutrients but also harbors attached bacteria that contribute to the bacterial diversity. Additionally, it provides protection from UV radiation and predation, which is beneficial when such organisms are discharged into a new environment from ballast water tanks. Bacteria attached to the SPM are metabolically more active and are more resistant to environmental changes⁴³. Hence, SPM can influence the bacterial load (especially pathogens) and also aid in their survival during stressed conditions in the aquatic systems.

The bacterial diversity changed significantly over time in all three conditions. γ -proteobacteria preferred high salinity, whereas β -proteobacteria were dominant in the mid saline range. This is in congruence with a similar study on biofilm bacteria from coastal port environments⁴⁴ and a similar pattern was observed in a study along a salinity gradient in Zuari estuary, India⁴⁵. They were positively influenced by NO_3 but negatively by PO_4 concentrations (low in HSC) (Fig. 6). A study reported that many phosphate solubilizing bacteria (PSB) belong to Firmicutes and β -proteobacteria⁴⁶, which were dominant in the lower salinity conditions (MSC and LSC). It is possible that PSB were low in HSC due to which the PO_4 concentrations were low in that condition. Additionally, most pathogenic groups belong to γ -proteobacteria, whose contribution to the bacterial diversity in LSC was minimal. Although they were present in the upstream location (SV), the addition of hyper saline port water could have led to additional stress on the prevailing bacterial diversity, which could not cope with the stress after translocation. On the other hand, Firmicutes were high in LSC, which play an important role in organic matter turnover due to their enzyme secretion capacity⁴⁷. The bacterial diversity was not only different in different saline conditions but the response of certain phyla to salinity stress was also different. Hence, the response of the aged bacterial taxa from ballast tanks when exchanged in the open ocean during BWE will depend to a

great extent on the source of the ballast water and its ability to tackle the salinity stress. In the present study, when the aged water from the hypersaline port was translocated into low saline water from estuary (LSC), the change in bacterial diversity was significant. This was expected since the change in salinity is highest in LSC.

Saline port water experiment (Experiment II)

As a result of aging in the dark, the TBC increased and the bacterial diversity also showed a distinct change where the dominance shifted from α - proteobacteria to γ -proteobacteria. When the aged saline port water was translocated into the water from DP, CR, and SV, the resultant salinity changed, especially in LSC (aged saline port water translocated to water from upstream location), leading to a significant decrease in TBC (Table 3b). However, both viable marine (TVC-ZMA) and freshwater (TVC-NA) bacteria persisted in the change in salinity after translocation. Similar to the trend observed in experiment I, the TVC-ZMA were high in HSC. Whereas, TVC-NA were high in LSC and were negatively correlated with PO_4 and TOC. However, interestingly in LSC, although TVC-NA were high, there was no significant decline in TVC-ZMA. The marine bacteria in the aged saline port water could cope with the stress caused by the change in salinity. *Vibrio* spp. were higher than other pathogenic strains in all the three conditions. Additionally, γ -proteobacteria, which include *Vibrio* sp., were also prevalent in all the conditions. The versatility and adaptability of *Vibrio* sp. is well documented as they are euryhaline. Infact, toxicogenic *Vibrio cholerae* are known to survive a wide range of salinity³². They could not only be free-living but also plankton- and particulate matter-attached. Pathogenic bacteria are known to be associated with plankton³⁵, which protects the bacteria from predator feeding and harsh environmental changes.

The bacterial diversity in HSC was initially dominated by α -proteobacteria and Bacteroidetes, however, with an increase in the incubation time after translocation, β -, γ -proteobacteria, and Actinobacteria dominated the bacterial diversity. Similarly, the bacterial diversity in lower salinity ranges (MSC and LSC) were also dominated by β - and γ -proteobacteria. This increase in β -proteobacteria was accompanied by a significant increase in the PO_4 concentrations. The role of β -proteobacteria in phosphate solubilisation is well documented⁴⁶ and was evident in both these salinity conditions, where they could dominate the bacterial diversity. They were distinctly different from that of hypersaline water. Proteobacteria in this experiment showed a weak negative correlation with NO_3 and were positively influenced by NO_2 . Proteobacteria are known to play an important role in dissimilatory nitrate reduction⁴⁸. NO_3 concentrations were comparatively less which stresses the importance of nitrate reducers in determining the community structure in response to the

environmental stress. Under favorable conditions, such mechanisms provide the bacteria additional leverage to establish and colonize a non-indigenous environment disrupting the local ecosystem.

Freshwater port water experiment (Experiment III)

The aging of the port water resulted in the shift of bacterial diversity from Actinobacteria to Bacteroidetes (known to degrade complex organic matter) and Firmicutes (known for spore-forming ability), in addition to an increase in the total bacterial count (TBC). The translocation of this aged freshwater port water, showed significantly contrasting results when compared to the marine port water experiments. TVC-NA from the aged freshwater port water, unlike those from the previous experiments (aged hypersaline and aged saline port water), were high at higher salinities. Port ecosystems are different from other coastal ecosystems given the high intensity of anthropogenic perturbations. The shipping activity and in turn the ballast water uptake and discharge are high in port areas. Moreover, Kolkata port is a freshwater port unlike the hypersaline Kandla and saline Paradip ports. It receives continuous inputs from the Hooghly River⁴⁹ and the bacterial diversity from the freshwater port ecosystem (Kolkata port) was able to resist the salinity stress better than that of the hypersaline and saline port. Hence, the freshwater bacteria from the Kolkata port seemed to withstand the translocation into high saline condition. Additionally, *Vibrio* spp. were not observed in the aged port water and the estuarine stations, except for VA in the upstream estuarine station. However, after translocation, they were observed in all three conditions, especially HSC. Adaptation of *Vibriospp.* to high salt concentration, has increased their chances of survival under environmental stresses⁵⁰. *Vibrio* spp. are euryhaline and were able to cope with the salinity shock during the translocation experiment. They are known to be stable for long periods of time in ballast water tanks, although their viability might decrease once introduced to new environments³². The bacteria could have been in a dormant condition until translocation into a suitable environment and under favorable conditions, they carry the potential to have a direct impact on human health.

The bacterial diversity in the aged port water was dominated by Bacteroidetes (Fig. 5b). Bacteroidetes can be found in diverse marine habitats including coastal and offshore waters. They are one of the most abundant bacterial phyla in coastal ecosystems aided by their ability to degrade complex organic matter⁵¹. But the translocation into estuarine water has revealed that Proteobacteria were more resilient to the salinity change and hence were dominant after translocation. HSC was dominated by β - and γ -proteobacteria, whereas α - and γ -proteobacteria were dominant in mid saline range. Although a similar pattern in their distribution was observed in the hypersaline and saline water bacterial diversity, the influence of distinctly different environmental factors could be due to the presence of region-specific Proteobacterial clades. In lower salinity, however, Firmicutes and

Bacteroidetes were dominant. Firmicutes are generally not considered to be marine organisms⁴⁷, hence they flourished in LSC. Additionally, the contribution of Bacteroidetes to the bacterial diversity in upstream location (SV) was minimal, so the contribution of this phylum to the bacterial diversity in LSC could be majorly from the port water and they showed a strong positive correlation with TOC. Since they are known for their ability to degrade complex organic matter⁵¹, high TOC may have aided in the proliferation of this phylum.

Coliforms (TC and *E.coli* O157: H7) were observed in LSC and generally preferred lower salinity. Coliforms are allochthonous and are less tolerant to salinity change. They are known to proliferate in low saline conditions⁵². Rapid salinity change could cause cell inactivation⁵³. However, they were detected in higher salinity ranges in all three experiments. It seems that even with the reduced culturability, they could reach a resting stage and proliferate under suitable conditions. Studies have also reported an increase in antibiotic resistance of pathogenic bacteria during incubation in ballast water tanks¹⁰.

Bacterial community response to salinity stress

The β - and γ -proteobacteria from aged hypersaline port water (Experiment I) preferred high to mid saline condition after translocation (HSC and MSC), whereas α -proteobacteria preferred LSC (Fig. 3f, g, and h). However, in the aged saline port water experiment (Experiment II), β - and γ -proteobacteria preferred mid to low salinity conditions (MSC and LSC) after translocation. The β - and γ -proteobacteria in aged freshwater port water (Experiment III) preferred HSC, whereas α -proteobacteria preferred MSC. No confirmed relationship can be established for the abundance of β - and γ -proteobacteria in response to salinity stress. Each phylum proliferated in different salinity conditions in all three experiments. Additionally, the bacterial diversity in each experiment was influenced by different physicochemical factors. Despite their ubiquity, the species comprising these phyla could be different and occupy a highly specific niche in each geographical location and the species composition could be region-specific which could explain the different response to salinity stress in each experiment. Additionally, CCA triplots showed a grouping of all the major phyla comprising the bacterial diversity (Fig. 6). This could mean that instead of each individual bacterial clade reacting to the environmental stress after translocation, they tackle the stress as a community.

The environmental factors affecting the microbiota were also very different. Hence, the composition of the bacterial community was driven by environmental settings. The dispersal and bioinvasive potential of these microbes may be influenced by environmental factors which is in accordance with the Bass Becking hypothesis which states that “everything is everywhere, but the

environment selects". Since the introduction of bacteria into a new environment in comparison to higher organisms is tougher to observe, it is important to identify the key environmental factors that could aid in the establishment of non-indigenous bacteria. Although salinity is a critical parameter in determining the bacterial diversity at a given location, the cumulative effect of other factors, in particular, the nutrient dynamics could play a major role in determining the bacterial diversity which could be useful in devising ballast water treatment mechanisms that could prove better than BWE.

Conclusions

The aging of port waters (hypersaline, saline and freshwater) in the dark condition resulted in a shift in the bacterial diversity. Although there was a change in the bacterial diversity in all three experiments after translocation, due to the initial salinity shock, the resultant bacterial diversity after prolonged incubation withstood the salinity stress over time. But the effect of the salinity stress was distinctly different among the bacterial phyla which allowed the proliferation of a few bacterial clades well-equipped to withstand the stress. The bacterial diversity of the freshwater port could withstand the salinity stress better than that from the hypersaline or saline ports. In freshwater port water, Bacteroidetes which are known to degrade a wide range of organic matter proliferated after the aging. Further, when this aged port water bacterial diversity was translocated into estuarine water with different salinities, the initial osmotic stress resulted in a shift in the bacterial diversity with α - and γ - proteobacteria as the dominant species. However, after prolonged incubation, Bacteroidetes and Firmicutes (which includes members with specific endospore forming capability) proliferated, in the lower salinities, whereas γ - Proteobacteria generally preferred higher salinity.

Thus, the risk of bioinvasion and the extent of success or failure of the introduced bacteria does not solely depend on the source of the ballast water (hypersaline, saline and freshwater) but also on the environmental conditions, ecological health and resident biota of the recipient coastal port. This also holds true in terms of BWE, where the efficacy does not depend only on the source of the ballast water but also the environmental settings at the point of BWE. It would be interesting to study the changes in bacterial diversity and their response to environmental stress during a voyage which could provide an understanding of the invasive potential of the ballast water tank microbiota. Additionally, understanding the effects of the introduction of such resilient bacterial clades into the port environment through real-time observations would provide valuable insights on the risk of marine bioinvasion and is a way forward.

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Table 1. Primer sets for quantitative PCR (qPCR) used in the study.

Target group name	Annealing temp. (°C)	Primer name	Sequence (5'-3')	Reference
α-Proteobacteria	53	α -682F α -908R	CIAGTGTAGAGGTGAAATT CCCCGTCAATTCCTTTGAGTT	Bacchetti De Gregoris et al. (2011) ³⁷
β-Proteobacteria	53	β -359F β -682R	GGGGAATTTTGACAATGGG ACGCATTTCACTGCTACACG	Ashelford et al. (2002) ³⁸
γ-Proteobacteria	53	γ -1080F γ -1202R	TCGTCAGCTCGTGYGTGA CGTAAGGGCCATGATG	Bacchetti De Gregoris et al. (2011) ³⁷
Bacteroidetes	53	798cfbF cfb967R	CRAACAGGATTAGATACCCT GGTAAGGTTCCCTCGCGTAT	Bacchetti De Gregoris et al. (2011) ³⁷
Firmicutes	53	928FirmF 1040FirmR	TGAAACTYAAAGGAATTGACG ACCATGCACCACCTGTC	Bacchetti De Gregoris et al. (2011) ³⁷
Actinobacteria	53	Act920F3 Act1200R	TACGGCCGCAAGGCTA TCRTCCCCACCTTCCTCCG	Bacchetti De Gregoris et al. (2011) ³⁷

Table 2a Physico-chemical parameters and dissolved nutrients in hypersaline port water (source), aged hypersaline port water, estuarine stations (destination) and during incubation period after mixing in High saline condition (HSC), Mid saline condition (MSC) and low saline condition (LSC) in Experiment I.

a)		DO			pH			SPM (mg l ⁻¹)			TOC (mg l ⁻¹)			
Experiment I	Source	Kandla port (Day 0)	6.40			7.91			1141.7			6.22		
		Kandla port (Day 30)	6.30			7.72			1619			2.81		
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	
		3.66	7.83	3.98	7.80	7.83	7.48	144.2	111.6	47.6	2.30	2.13	1.68	
	Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC
	Incubation period	0	5.10	6.04	6.12	7.87	6.95	7.77	433	373.5	274	2.28	2.35	2.51
		1	5.12	5.70	6.08	7.78	7.84	7.95	NS	NS	NS	NS	NS	NS
		2	5.00	5.25	5.40	7.87	7.72	7.76	NS	NS	NS	NS	NS	NS
		3	5.38	5.37	5.62	7.83	7.82	7.82	NS	NS	NS	NS	NS	NS
		4	5.25	5.11	5.18	7.83	7.58	7.56	NS	NS	NS	NS	NS	NS
		5	5.30	5.40	5.88	7.89	7.75	7.77	482	402	424.5	2.97	3.18	3.50
		6	5.42	5.49	5.93	7.95	7.84	7.88	NS	NS	NS	NS	NS	NS
	7	5.59	5.26	6.00	7.66	7.59	7.63	NS	NS	NS	NS	NS	NS	
	8	5.44	5.57	5.78	7.67	7.60	7.63	NS	NS	NS	NS	NS	NS	
	9	5.24	5.84	5.95	7.77	7.72	7.77	NS	NS	NS	NS	NS	NS	
	10	4.84	5.42	6.02	7.79	7.72	7.64	577	446	527	2.96	3.07	5.18	
	11	4.73	5.06	0.00	7.75	7.65	7.58	NS	NS	NS	NS	NS	NS	
	12	5.57	5.82	5.58	7.88	7.81	7.71	704	678	921	3.42	4.10	5.73	
Average±SD		5.3±0.3	5.5±0.3	5.4±1.6	7.8±0.1	7.7±0.2	7.7±0.1	549±119	475±139	537±277	2.9±0.5	3.2±0.7	4.2±1.5	
Experiment I			Silicate (µM)			Phosphate (µM)			Nitrate (µM)			Nitrite (µM)		
	Source	Kandla port (Day 0)	34.26			3.48			17.64			7.6		
		Kandla port (Day 30)	41.15			11.91			133.29			3.74		
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	
		29.25	59.37	76.51	5.16	7.38	6.98	38.22	13.25	BDL	0.89	0.56	BDL	
	Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC
	Incubation period	0	18.43	27.00	59.88	4.35	4.17	5.74	31.25	16.56	13.89	1.06	0.69	0.39
		1	24.51	50.61	68.92	5.02	7.14	6.85	37.34	27.30	6.63	1.30	0.87	0.45
		2	27.23	45.07	71.00	4.82	5.79	7.00	45.02	23.91	8.91	1.33	0.66	0.35
		3	40.28	35.40	44.57	6.62	5.29	5.79	57.57	18.80	14.58	1.22	0.66	0.40
		4	22.80	22.54	41.44	5.13	3.68	5.16	64.38	67.76	27.09	1.17	0.59	0.61
		5	31.82	61.28	18.04	4.75	6.95	3.76	26.30	41.93	64.26	1.34	0.61	0.75
	6	40.35	39.82	30.11	6.30	5.90	6.00	46.60	26.32	64.16	1.15	1.01	0.65	
	7	20.61	31.65	28.87	4.25	5.17	3.48	31.82	51.77	13.09	1.86	1.24	0.75	
	8	33.62	46.94	21.67	4.74	5.35	4.78	51.28	28.63	81.51	1.55	1.06	0.58	
	9	43.99	35.02	50.77	6.49	5.75	6.16	54.90	4.70	6.41	1.49	1.76	0.93	
	10	21.69	34.16	43.17	4.67	5.34	5.20	11.50	4.07	4.84	1.68	1.82	0.91	
	11	18.27	37.32	60.00	3.63	5.47	7.02	9.88	5.40	6.07	1.53	2.12	1.61	
	12	23.18	35.07	52.03	4.55	5.74	6.40	11.31	6.48	5.29	1.11	1.99	1.12	
Average±SD		28.2±8.9	38.6±10.3	45.4±17.2	5±0.9	5.5±0.9	5.6±1.1	36.9±18.4	24.9±19.5	24.4±27	1.4±0.2	1.2±0.6	0.7±0.4	

DP – Dona Paula (estuarine mouth station); CR – Cortalim (mid estuarine station); SV – Sanvordem (upstream station); DO – Dissolved Oxygen; SPM – Suspended particulate matter; TOC – Total organic carbon; NS – Not sampled; BDL – Below detection limit; Average±SD – Average ± Standard Deviation.

Table 2b Physico-chemical parameters and dissolved nutrients in saline port water (source), aged saline port water, estuarine stations (destination) and during incubation period after mixing in High saline condition (HSC), Mid saline condition (MSC) and low saline condition (LSC) in Experiment II.

		DO			pH			SPM (mg l ⁻¹)			TOC (mg l ⁻¹)			
Experiment II	b) Source	Paradip port (Day 0)	4.90			7.74			105.3			NS		
		Paradip port (Day 30)	6.35			7.93			242			2.31		
	Destination		DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV
			4.44	4.09	4.44	7.89	7.78	7.67	218	216.5	223	2.56	2.83	4.03
		Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC
	Incubation period	0	3.04	2.98	3.62	8.10	7.92	7.51	192.5	224.5	72	2.66	2.89	2.36
		1	3.17	3.23	3.66	8.05	7.93	7.48	NS	NS	NS	NS	NS	NS
		2	3.31	3.26	3.74	7.98	7.88	7.45	NS	NS	NS	NS	NS	NS
		3	3.27	3.10	3.7	7.92	7.85	7.60	NS	NS	NS	NS	NS	NS
		4	NS	NS	NS	7.94	7.82	7.55	NS	NS	NS	NS	NS	NS
		5	NS	NS	NS	7.97	7.90	7.55	260.5	250	94.5	2.69	2.98	2.73
		6	3.50	3.51	3.93	7.95	7.90	7.57	NS	NS	NS	NS	NS	NS
7		NS	NS	NS	7.97	7.97	7.44	NS	NS	NS	NS	NS	NS	
8		NS	NS	NS	8.01	7.96	7.39	NS	NS	NS	NS	NS	NS	
9		3.56	3.55	3.665	7.99	7.97	7.36	NS	NS	NS	NS	NS	NS	
10		NS	NS	NS	7.98	7.97	7.51	234	253.5	82.5	3.71	3.72	2.84	
11		NS	NS	NS	8.05	8.01	7.52	NS	NS	NS	NS	NS	NS	
12	3.59	3.64	4.055	8.06	8.01	7.74	260.5	263.5	66.5	3.79	4.73	3.76		
Average±SD		3.4±0.2	3.3±0.3	3.8±0.2	8±0.1	7.9±0.1	7.5±0.1	237±32	248±17	79±13	3.2±0.6	3.6±0.9	2.9±0.6	
		Silicate (µM)			Phosphate (µM)			Nitrate (µM)			Nitrite (µM)			
Experiment II	Source	Paradip port (Day 0)	43.24			1.98			7.11			1.39		
		Paradip port (Day 30)	16.95			3.54			11.03			0.95		
	Destination		DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV
			6.31	6.94	49.39	2.75	11.93	9.06	3.82	1.08	6.50	1.25	0.75	0.44
		Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC
	Incubation period	0	7.53	16.70	61.81	2.58	4.05	5.41	BDL	0.15	6.12	0.92	0.87	0.25
		1	6.33	16.57	57.51	1.73	3.71	5.16	BDL	BDL	4.60	0.81	0.79	0.27
		2	9.17	12.44	44.98	2.72	2.37	3.98	4.14	1.48	4.41	1.04	1.01	0.17
		3	6.46	17.29	63.88	1.91	2.91	5.65	0.47	2.75	4.74	1.05	0.88	0.22
		4	7.80	14.56	36.80	1.57	3.06	4.72	3.10	0.50	5.13	1.09	0.81	0.17
		5	22.44	28.93	37.05	7.92	13.62	14.79	17.52	26.69	26.62	0.68	0.69	0.10
		6	16.99	26.84	111.52	13.94	13.96	17.11	12.41	18.46	3.25	0.66	0.74	0.10
7		58.21	78.66	73.31	15.98	13.31	15.20	BDL	BDL	BDL	0.67	0.68	0.15	
8		53.93	55.74	59.21	16.94	17.43	10.54	BDL	BDL	1.33	0.42	0.53	BDL	
9		44.72	61.28	70.77	13.42	14.11	14.11	0.37	0.39	27.64	0.44	0.47	0.05	
10		48.85	82.10	48.86	15.10	13.56	10.45	0.71	1.03	0.74	BDL	0.14	BDL	
11		31.46	26.99	34.42	11.41	14.89	13.37	BDL	BDL	0.80	0.26	0.35	0.11	
12	54.58	66.49	35.03	14.34	15.37	15.24	1.67	1.88	2.84	0.47	0.58	0.13		
Average±SD		28.3±21	38.8±26	56.6±21.4	9.2±6.2	10.2±5.8	10.4±4.9	5.1±6.4	5.9±9.7	7.4±9.4	0.7±0.3	0.7±0.2	0.2±0.1	

DP – Dona Paula (estuarine mouth station); CR – Cortalim (mid estuarine station); SV – Sanvordem (upstream station); DO – Dissolved Oxygen; SPM – Suspended particulate matter; TOC – Total organic carbon; NS – Not sampled; BDL – Below detection limit; Average±SD – Average ± Standard Deviation.

Table 2c Physico-chemical parameters and dissolved nutrients in freshwater port water (source), aged freshwater port water, estuarine stations (destination) and during incubation period after mixing in High saline condition (HSC), Mid saline condition (MSC) and low saline condition (LSC) in Experiment III.

c)		DO			pH			SPM (mg l ⁻¹)			TOC (mg l ⁻¹)			
Experiment III	Source	Kolkata port (Day 0)		8.10	7.68			32			NS			
		Kolkata port (Day 30)		5.99	7.77			199			2.66			
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	
		4.28	5.40	9.09	7.99	7.75	7.77	784	521	347	2.45	2.93	2.73	
	Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC
	Incubation period	0	2.27	2.65	3.88	8.02	7.88	7.67	419	225	219	2.75	3.13	2.90
		1	NS	NS	NS	8.13	8.13	7.89	NS	NS	NS	NS	NS	NS
		2	NS	NS	NS	8.08	7.98	7.51	NS	NS	NS	NS	NS	NS
		3	3.515	3.725	3.59	8.09	8.07	7.37	NS	NS	NS	NS	NS	NS
		4	NS	NS	NS	8.12	8.08	7.46	323	350.5	164.5	2.85	3.44	3.35
		5	NS	NS	NS	8.12	8.14	7.43	NS	NS	NS	NS	NS	NS
		6	3.395	3.61	3.75	8.12	8.13	7.41	NS	NS	NS	NS	NS	NS
	7	NS	NS	NS	8.06	8.05	7.43	NS	NS	NS	NS	NS	NS	
	8	NS	NS	NS	8.08	8.09	7.58	448	194.5	234.5	4.63	3.89	5.06	
	9	0.895	1.045	3.51	8.14	8.11	7.51	NS	NS	NS	NS	NS	NS	
	10	NS	NS	NS	7.79	7.79	8.09	NS	NS	NS	NS	NS	NS	
	11	NS	NS	NS	8.14	8.09	7.47	NS	NS	NS	NS	NS	NS	
	12	3.005	3.45	3.82	8.18	8.14	7.50	421.5	426.5	233	6.55	5.25	6.95	
Average±SD		2.6±1.1	2.9±1.1	3.7±0.2	8.1±0.1	8.1±0.1	7.6±0.2	403±55	299±108	213±33	4.2±1.8	3.9±0.9	4.6±1.8	
Experiment III		Silicate (µM)			Phosphate (µM)			Nitrate (µM)			Nitrite (µM)			
	Source	Kolkata port (Day 0)		145.01	6.85			175.08			6.58			
		Kolkata port (Day 30)		48.99	6.21			82.59			0.17			
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	
		15.87	23.36	41.06	3.63	5.32	4.03	3.9	13.18	12.43	1.38	4.44	0.33	
	Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC
	Incubation period	0	22.44	28.93	37.05	7.92	13.62	14.79	17.12	23.06	26.49	1.09	4.32	0.23
		1	18.67	26.84	64.12	13.94	13.96	14.81	12.17	15.93	24.96	0.89	3.27	0.48
		2	21.22	35.53	62.06	14.76	19.16	11.44	15.20	21.53	26.18	0.46	4.59	0.66
		3	22.78	36.69	63.01	11.63	9.38	5.54	14.86	23.09	25.75	1.18	5.12	0.82
		4	21.21	28.69	57.08	3.30	4.74	5.52	13.85	18.66	25.32	1.16	5.11	0.47
		5	22.57	26.51	54.95	4.03	4.66	5.45	12.80	19.94	19.85	0.87	4.53	0.35
	6	21.24	32.20	46.45	3.77	6.41	4.85	12.35	22.30	19.89	1.21	4.44	0.41	
	7	25.70	36.01	56.86	4.02	5.02	4.65	16.20	31.42	27.29	1.28	3.90	0.49	
	8	23.03	39.90	56.19	3.55	5.50	4.46	14.93	33.64	24.83	1.45	3.51	0.89	
	9	21.62	29.57	53.14	3.29	4.06	4.41	14.15	23.18	26.05	1.32	2.95	1.14	
	10	24.79	38.53	62.39	3.61	5.60	5.14	16.92	28.41	18.81	1.31	2.83	1.20	
	11	26.90	38.01	66.33	4.11	6.25	5.26	18.54	26.31	23.42	0.76	2.88	0.85	
	12	28.56	33.65	67.00	4.36	5.02	5.28	19.06	22.90	20.70	1.76	2.01	0.54	
Average±SD		23.1±2.7	33.2±4.7	57.4±8.4	6.3±4.3	8±4.7	7±3.9	15.2±2.2	23.9±5	23.8±3	1.1±0.3	3.8±1	0.7±0.3	

DP – Dona Paula (estuarine mouth station); CR – Cortalim (mid estuarine station); SV – Sanvordem (upstream station); DO – Dissolved Oxygen; SPM – Suspended particulate matter; TOC – Total organic carbon; NS – Not sampled; BDL – Below detection limit; Average±SD – Average ± Standard Deviation.

Table 3a Mean (\pm SD) values of total bacterial count (TBC), total viable marine bacterial count (TVC-ZMA), total viable freshwater bacterial count (TVC-NA) and pathogenic bacteria in hypersaline port water (source), aged hypersaline port water, estuarine stations (destination) and during the incubation period after mixing in High saline condition (HSC), Mid saline condition (MSC) and low saline condition (LSC) in Experiment I.

a)		Total bacterial counts (cells ml ⁻¹)			Total viable marine bacteria (CFU ml ⁻¹)			Total viable freshwater bacteria (CFU ml ⁻¹)									
Experiment I	Source	Kandla port (Day 0)	10.2 \pm 1.1x10 ⁷		12.4 \pm 1.3x10 ³			3.7 \pm 0.92x10 ³									
		Kandla port (Day 30)	6.9 \pm 0.7x10 ⁷		1.2 \pm 0.3x10 ³			1.5 \pm 0.01x10 ³									
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV							
		2 \pm 0.07x10 ⁷	2 \pm 0.06x10 ⁷	0.9 \pm 0.7x10 ⁷	13.6 \pm 4.6x10 ³	2.1 \pm 0.6x10 ³	60 \pm 28	4.2 \pm 0.01x10 ³	1.6 \pm 0.01x10 ³	468 \pm 138							
	Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC						
	Incubation period	0	1.0x10 ⁷	1.0x10 ⁷	3.1x10 ⁶	3.0 \pm 0.3x10 ³	1.5 \pm 0.2x10 ³	536 \pm 94	1.1 \pm 0.2x10 ³	5.2 \pm 2.0x10 ³	4.5 \pm 0.8x10 ³						
		1	1.2x10 ⁷	1.1x10 ⁷	2.0x10 ⁶	NS	NS	NS	NS	NS	NS						
		2	1.2x10 ⁷	1.2x10 ⁷	2.2x10 ⁶	NS	NS	NS	NS	NS	NS						
		3	1.1x10 ⁷	1.1x10 ⁷	3.1x10 ⁶	NS	NS	NS	NS	NS	NS						
		4	1.3x10 ⁷	1.1x10 ⁷	3.1x10 ⁶	NS	NS	NS	NS	NS	NS						
		5	1.0x10 ⁷	1.0x10 ⁷	2.3x10 ⁶	5.8 \pm 1.0x10 ³	4.1 \pm 0.3x10 ³	8.9 \pm 0.4x10 ³	5.2 \pm 1.0x10 ³	2.4 \pm 0.6x10 ³	33.6 \pm 0.9x10 ³						
		6	1.2x10 ⁷	NS	5.6x10 ⁶	NS	NS	NS	NS	NS	NS						
	7	NS	1.3x10 ⁷	NS	NS	NS	NS	NS	NS	NS							
	8	1.2x10 ⁷	1.3x10 ⁷	3.1x10 ⁶	NS	NS	NS	NS	NS	NS							
	9	1.2x10 ⁷	1.1x10 ⁷	5.4x10 ⁶	NS	NS	NS	NS	NS	NS							
	10	1.3x10 ⁷	1.3x10 ⁷	5.8x10 ⁶	5.1 \pm 0.2x10 ³	3.0 \pm 0.2x10 ³	8.0 \pm 0.4x10 ³	5.8 \pm 1.1x10 ³	3.2 \pm 0.2x10 ³	48.0 \pm 2.2x10 ³							
	11	1.1x10 ⁷	1.3x10 ⁷	7.3x10 ⁶	NS	NS	NS	NS	NS	NS							
	12	1.3x10 ⁷	1.1x10 ⁷	9.7x10 ⁶	8.1 \pm 0.04x10 ³	2.9 \pm 0.03x10 ³	3.1 \pm 1.3x10 ³	5.3 \pm 0.2x10 ³	1.5 \pm 0.03x10 ³	29.6 \pm 5.3x10 ³							
	Average \pm SD	1.2 \pm 0.1x10 ⁷	1.2 \pm 0.09x10 ⁷	4.4 \pm 2.4x10 ⁶	5.5 \pm 2.1x10 ³	2.9 \pm 1.1x10 ³	5.1 \pm 4.0x10 ³	4.4 \pm 2.2x10 ³	3.1 \pm 1.6x10 ³	28.9 \pm 18.1x10 ³							
Experiment I	Source	Kandla port (Day 0)	ND		635 \pm 99			309 \pm 105			15 \pm 21		ND				
		Kandla port (Day 30)	ND		20 \pm 14			30 \pm 28			ND		ND				
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	
		ND	ND	10	140 \pm 7	255 \pm 113	180 \pm 170	ND	15 \pm 7	15 \pm 7	15 \pm 7	ND	15 \pm 21	25 \pm 7	ND	ND	ND
	Mixing (1:5)	Days	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC
	Incubation period	0	ND	ND	ND	3 \pm 4	38 \pm 25	173 \pm 39	5 \pm 7	13 \pm 11	18 \pm 11	5 \pm 7	15 \pm 14	18 \pm 11	ND	ND	ND
		1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		5	ND	ND	15	13 \pm 11	10	5.3 \pm 0.5x10 ³	43 \pm 32	5 \pm 7	13 \pm 11	0	5	26 \pm 9	ND	ND	ND
		6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	7	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	8	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	9	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	10	ND	ND	ND	66 \pm 9	5 \pm 7	5.7 \pm 0.3x10 ³	968 \pm 318	160 \pm 57	23 \pm 18	5	271 \pm 178	33 \pm 46	ND	ND	ND	
	11	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	12	ND	ND	84 \pm 16	13 \pm 4	15 \pm 14	9.4 \pm 1.3x10 ³	555 \pm 31	96 \pm 37	25 \pm 7	0	90 \pm 25	0	ND	ND	ND	
	Average \pm SD	ND	ND	25 \pm 40	23 \pm 29	17 \pm 14	5.1 \pm 3.8x10 ³	393 \pm 459	68 \pm 73	19 \pm 6	3 \pm 3	95 \pm 123	19 \pm 14	ND	ND	ND	

CFU ml⁻¹ – Colony forming units per millilitre; DP – Dona Paula (estuarine mouth station); CR – Cortalim (mid estuarine station); SV – Sanvordem (upstream station); NS – Not sampled; ND – Not detected; Average \pm SD – Average \pm Standard Deviation.

Table 3b Mean (\pm SD) values of total bacterial count (TBC), total viable marine bacterial count (TVC-ZMA), total viable freshwater bacterial count (TVC-NA) and pathogenic bacteria in saline port water (source), aged saline port water, estuarine stations (destination) and during the incubation period after mixing in High saline condition (HSC), Mid saline condition (MSC) and low saline condition (LSC) in Experiment II.

b)		Total bacterial counts (cells ml ⁻¹)			Total viable marine bacteria (CFU ml ⁻¹)			Total viable freshwater bacteria (CFU ml ⁻¹)									
Experiment II	Source	Paradip port (Day 0) 9.35 \pm 1.4x10 ⁵			2.3 \pm 0.4x10 ⁴			2.6 \pm 0.2x10 ³									
		Paradip port (Day 30) 0.35 \pm 0.001x10 ⁷			370 \pm 42			10									
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV							
		3.0 \pm 0.2x10 ⁷	4.6 \pm 0.005x10 ⁷	3.4 \pm 0.15x10 ⁷	21.8 \pm 2.4x10 ⁴	3.2 \pm 1.0x10 ⁴	0.4 \pm 0.07x10 ⁴	10	2.9 \pm 0.002x10 ⁴	8.4 \pm 1.3x10 ⁴							
	Mixing (1:5)	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC							
	Incubation period	0	2.4x10 ⁶	0.95x10 ⁶	3.7x10 ⁵	1.3 \pm 0.2x10 ⁴	0.2 \pm 0.02x10 ⁴	111 \pm 25	3.3 \pm 0.4x10 ⁴	1.2 \pm .09x10 ⁴	61 \pm 12						
		1	3.8x10 ⁶	1.5x10 ⁶	3.3x10 ⁵	NS	NS	NS	NS	NS	NS						
		2	3.0x10 ⁶	1.4x10 ⁶	3.2x10 ⁵	NS	NS	NS	NS	NS	NS						
		3	2.5x10 ⁶	1.2x10 ⁶	5.4x10 ⁵	NS	NS	NS	NS	NS	NS						
		4	1.8x10 ⁶	1.6x10 ⁶	8.3x10 ⁵	NS	NS	NS	NS	NS	NS						
		5	2.7x10 ⁶	1.5x10 ⁶	12.7x10 ⁵	2.2 \pm 0.2x10 ⁴	0.9 \pm 0.04x10 ⁴	1.4 \pm 0.01x10 ⁴	2.6 \pm 0.2x10 ⁴	0.9 \pm 0.3x10 ⁴	2.26 \pm 0.1x10 ⁴						
		6	3.1x10 ⁶	1.5x10 ⁶	8.9x10 ⁵	NS	NS	NS	NS	NS	NS						
	7	3.7x10 ⁶	1.4x10 ⁶	8.1x10 ⁵	NS	NS	NS	NS	NS	NS							
	8	3.8x10 ⁶	1.2x10 ⁶	9.7x10 ⁵	NS	NS	NS	NS	NS	NS							
	9	3.8x10 ⁶	1.1x10 ⁶	8.9x10 ⁵	NS	NS	NS	NS	NS	NS							
	10	4.1x10 ⁶	0.98x10 ⁶	11.9x10 ⁵	2.3 \pm 0.6x10 ⁴	2.2 \pm 0.3x10 ⁴	4.1 \pm 1.7x10 ⁴	0.7 \pm 0.01x10 ⁴	0.9 \pm 0.2x10 ⁴	3.9 \pm 1.5x10 ⁴							
	11	3.9x10 ⁶	0.74x10 ⁶	11.6x10 ⁵	NS	NS	NS	NS	NS	NS							
	12	3.9x10 ⁶	0.84x10 ⁶	13.5x10 ⁵	2.1 \pm 0.3x10 ⁴	1.2 \pm 0.01x10 ⁴	1.6 \pm 0.01x10 ⁴	0.6 \pm 0.02x10 ⁴	0.5 \pm 0.01x10 ⁴	14098 \pm 163							
	Average \pm SD	3.3 \pm 0.7x10 ⁶	1.2 \pm 0.3x10 ⁶	8.4 \pm 3.6 x10 ⁵	2 \pm 0.4x10 ⁴	1.1 \pm 0.9x10 ⁴	1.8 \pm 1.7x10 ⁴	1.8 \pm 1.4x10 ⁴	0.9 \pm 0.3x10 ⁴	1.9 \pm 1.7x10 ⁴							
		<i>E. coli</i> O157: H7 (CFU ml ⁻¹)			Total coliforms (CFU ml ⁻¹)			<i>Vibrio alginolyticus</i> (CFU ml ⁻¹)			<i>V. parahaemolyticus</i> (CFU ml ⁻¹)			<i>V. cholerae</i> (CFU ml ⁻¹)			
Experiment II	Source	Paradip port (Day 0) ND			93 \pm 95			553 \pm 80			82 \pm 33			ND			
		Paradip port (Day 30) ND			ND			ND			ND			ND			
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	
		ND	ND	ND	5.5 \pm 1.5x10 ³	ND	40 \pm 28	10 \pm 14	75 \pm 7	15 \pm 21	440 \pm 9	48 \pm 11	ND	ND	ND	ND	
	Mixing (1:5)	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	
	Incubation period	0	ND	ND	ND	ND	ND	ND	492 \pm 94	28 \pm 25	ND	220 \pm 21	3 \pm 4	ND	8 \pm 11	ND	ND
		1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		5	18 \pm 4	5	928 \pm 16	335 \pm 99	11 \pm 64	ND	7.3 \pm 0.5x10 ³	1511 \pm 220	1.8 \pm 0.1x10 ³	2.0 \pm 0.004x10 ³	3 \pm 4	3 \pm 4	ND	5	23 \pm 4
		6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	7	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	8	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	9	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	10	ND	ND	ND	536 \pm 35	ND	ND	565 \pm 116	196 \pm 5	2.2 \pm 0.2x10 ³	183 \pm 11	ND	ND	ND	ND	ND	
	11	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	12	ND	ND	2.4 \pm 0.02x10 ³	213 \pm 81	ND	ND	1.6 \pm 0.05x10 ³	395 \pm 81	2.6 \pm 0.3x10 ³	93	ND	548 \pm 223	ND	ND	ND	
	Average \pm SD	4 \pm 9	1 \pm 3	834 \pm 1136	271 \pm 225	3 \pm 5	ND	2.5 \pm 3.3x10 ³	532 \pm 669	1.7 \pm 1.2x10 ³	623 \pm 917	1 \pm 2	138 \pm 273	2 \pm 4	1 \pm 3	6 \pm 11	

CFU ml⁻¹ – Colony forming units per millilitre; DP – Dona Paula (estuarine mouth station); CR – Cortalim (mid estuarine station); SV – Sanvordem (upstream station); NS – Not sampled; ND – Not detected; Average \pm SD – Average \pm Standard Deviation.

Table 3c Mean (\pm SD) values of total bacterial count (TBC), total viable marine bacterial count (TVC-ZMA), total viable freshwater bacterial count (TVC-NA) and pathogenic bacteria in freshwater port water (source), aged freshwater port water, estuarine stations (destination) and during the incubation period after mixing in High saline condition (HSC), Mid saline condition (MSC) and low saline condition (LSC) in Experiment III.

c)		Total bacterial counts (cells ml ⁻¹)			Total viable marine bacteria (CFU ml ⁻¹)			Total viable freshwater bacteria (CFU ml ⁻¹)								
Experiment III	Source	Kolkata port (Day 0)			1.2 \pm 0.04x10 ³			3.7 \pm 0.06x10 ³								
		Kolkata port (Day 30)			10			5 \pm 7								
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV						
		5.3 \pm 0.8x10 ⁷	9.2 \pm 1.2x10 ⁷	7.8 \pm 0.4x10 ⁷	158 \pm 32	60 \pm 14	20 \pm 14	40 \pm 28	10	105 \pm 49						
	Mixing (1:5)	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC						
	Incubation period	0	1.1x10 ⁶	1.4x10 ⁶	1.1x10 ⁶	825 \pm 247	0.3 \pm 0.04x10 ⁴	75 \pm 35	275 \pm 35	375 \pm 177	75 \pm 35					
		1	1.3x10 ⁶	2.3x10 ⁶	2.0x10 ⁶	NS	NS	NS	NS	NS	NS					
		2	1.8x10 ⁶	2.5x10 ⁶	1.6x10 ⁶	NS	NS	NS	NS	NS	NS					
		3	2.3x10 ⁶	2.7x10 ⁶	1.9x10 ⁶	NS	NS	NS	NS	NS	NS					
		4	2.4x10 ⁶	3.0x10 ⁶	1.9x10 ⁶	2.4 \pm 0.2x10 ⁴	1.1 \pm 0.2x10 ⁴	0.8 \pm 0.01x10 ⁴	9.3 \pm 0.3x10 ³	3.8 \pm 0.5x10 ³	9.3 \pm 0.3x10 ³					
		5	2.3x10 ⁶	3.1x10 ⁶	2.5x10 ⁶	NS	NS	NS	NS	NS	NS					
		6	2.6x10 ⁶	2.6x10 ⁶	2.3x10 ⁶	NS	NS	NS	NS	NS	NS					
	7	2.7x10 ⁶	1.9x10 ⁶	1.8x10 ⁶	NS	NS	NS	NS	NS	NS						
	8	3.4x10 ⁶	2.1x10 ⁶	2.7x10 ⁶	0.6 \pm 0.04x10 ⁴	1.2 \pm 0.2x10 ⁴	0.2 \pm 0.02x10 ⁴	1.6 \pm 0.1x10 ³	3.2 \pm 0.1x10 ³	4.6 \pm 0.3x10 ³						
	9	3.0x10 ⁶	2.5x10 ⁶	2.8x10 ⁶	NS	NS	NS	NS	NS	NS						
	10	3.4x10 ⁶	2.4x10 ⁶	2.9x10 ⁶	NS	NS	NS	NS	NS	NS						
	11	2.9x10 ⁶	2.3x10 ⁶	3.4x10 ⁶	NS	NS	NS	NS	NS	NS						
	12	3.1x10 ⁶	2.2x10 ⁶	2.9x10 ⁶	1.3 \pm 0.01x10 ⁴	0.7 \pm 0.03x10 ⁴	338 \pm 181	2.8 \pm 0.4x10 ³	476 \pm 86	229 \pm 58						
	Average \pm SD	2.5 \pm 0.7x10 ⁶	2.4 \pm 0.4x10 ⁶	2.3 \pm 0.6x10 ⁶	1.1 \pm 1x10 ⁴	0.8 \pm 0.4x10 ⁴	0.2 \pm 0.4x10 ⁴	3.5 \pm 4x10 ³	1.9 \pm 1.8x10 ³	3 \pm 4.3x10 ³						
Experiment III	Source	Kolkata port (Day 0)			698 \pm 73			ND			10			ND		
		Kolkata port (Day 30)			ND			ND			ND			ND		
	Destination	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV	DP	CR	SV
		ND	ND	ND	ND	ND	ND	ND	ND	5 \pm 7	ND	ND	ND	ND	ND	ND
	Mixing (1:5)	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC	HSC	MSC	LSC
	Incubation period	0	ND	ND	ND	ND	ND	ND	100	ND	ND	ND	ND	ND	ND	ND
		1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		4	ND	ND	5	241 \pm 65	134 \pm 23	1.7 \pm 0.2x10 ³	15 \pm 179	45 \pm 28	ND	80 \pm 7	ND	54 \pm 12	446 \pm 7	ND
		5	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	7	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	8	ND	ND	5	ND	ND	1.3 \pm 0.06x10 ³	ND	123 \pm 25	ND	ND	ND	5 \pm 7	64	ND	
	9	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	10	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	11	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	12	ND	5	ND	183 \pm 53	53 \pm 14	65 \pm 16	ND	13 \pm 11	ND	ND	ND	ND	295	ND	
	Average \pm SD	ND	1 \pm 3	4 \pm 8	106 \pm 125	47 \pm 63	752 \pm 839	4 \pm 8	ND	13 \pm 27	20 \pm 40	ND	ND	201 \pm 207	70 \pm 50	

CFU ml⁻¹ – Colony forming units per millilitre; DP – Dona Paula (estuarine mouth station); CR – Cortalim (mid estuarine station); SV – Sanvordem (upstream station); NS – Not sampled; ND – Not detected; Average \pm SD – Average \pm Standard Deviation.

Table 4. Correlation analysis between bacterial counts and physicochemical parameters in high, mid and low saline conditions of (a)Experiment I; (b)Experiment II and; (c)Experiment III.

	TBC	TVC-ZMA	TVC-NA	<i>E. coli</i> O157:H7	TC	VA	VP	VC
(a) Hypersaline port water experiment								
High saline condition								
pH	-	0.992**	-	-	-	-	-	-
Nitrate	-0.973*	-	-	-	-	-0.983*	-	-
Mid saline condition								
Total organic carbon	-	-	-0.988*	-	-	-	-	-
Phosphate	-	0.954*	-	-	-	-	-	-
Nitrite	-	-	-	-	-	0.961*	-	-
Low saline condition								
Total organic carbon	-	-	-	-	-	-	-	-
(b) Saline port water experiment								
High saline condition								
Total organic carbon	0.975*	-	-0.994**	-	-	-	-	-
Silicate	0.983*	-	-0.987*	-	-	-	-	-
Phosphate	0.986*	-	-0.954*	-	-	-	-	-
Nitrate	-	-0.966*	-	-	-0.980*	-	-	-
Mid saline condition								
Silicate	-	-	-	-	-	-	-0.965*	-
Phosphate	-	-	-	-	-	-	-	-
Nitrate	0.9532*	-	-	0.998**	-	-	0.998**	-
Low saline condition								
pH	-	-	-	-	-	-	0.999**	-
Nitrate	-	-	-	-	-	-	-	0.983*
(c) Freshwater port water experiment								
High saline condition								
Suspended particulate matter	-	-	-	-	-	-0.971*	-0.971*	-
Mid saline condition								
Suspended particulate matter	-	-	-	-	-	-	-	-0.973*
Low saline condition								
Suspended particulate matter	-	-	-	-0.977*	-	-0.977*	-	-
Phosphate	-0.955*	-	-	-	-	-	-	-

TBC – Total bacterial count; TVC-ZMA – Total viable marine bacteria; TVC-NA – Total viable freshwater bacteria; TC – Total coliforms; VA – *Vibrio alginolyticus*; VP – *V. parahaemolyticus*; VC – *V. cholerae*; *p≤ 0.05; **p≤ 0.01; No significant correlation has been marked as “-”

Legends to figures

Figure 1. Location map showing the collection sites of the water samples used in the experiment.

Figure 2. Schematic representation of experimental design (TBC – Total bacterial counts; TVC-ZMA – Total viable marine bacterial counts; TVC-NA – Total bacterial freshwater bacterial counts; TC – Total coliforms).

Figure 3. Relative abundance of gene copy numbers μl^{-1} in experiment I of α -proteobacteria (red), β -proteobacteria (light blue), γ -proteobacteria (dark blue), Actinobacteria (yellow), Bacteroidetes (grey) and Firmicutes (purple) in (a) Hypersaline port water; (b) Aged hypersaline port water; (c) water from estuarine mouth (DP); (d) mid-estuary (CR); (e) estuarine upstream (SV). Relative abundance of major bacterial phyla during the incubation period after mixing in (f) high saline condition (HSC); (g) mid saline condition (MSC) and (h) low saline condition (LSC).

Figure 4. Relative abundance of gene copy numbers μl^{-1} in experiment II of α -proteobacteria (red), β -proteobacteria (light blue), γ -proteobacteria (dark blue), Actinobacteria (yellow), Bacteroidetes (grey) and Firmicutes (purple) in (a) Hypersaline port water; (b) Aged hypersaline port water; (c) water from estuarine mouth (DP); (d) mid-estuary (CR); (e) estuarine upstream (SV). Relative abundance of major bacterial phyla during the incubation period after mixing in (f) high saline condition (HSC); (g) mid saline condition (MSC) and (h) low saline condition (LSC).

Figure 5. Relative abundance of gene copy numbers μl^{-1} in experiment III of α -proteobacteria (red), β -proteobacteria (light blue), γ -proteobacteria (dark blue), Actinobacteria (yellow), Bacteroidetes (grey) and Firmicutes (purple) in (a) Hypersaline port water; (b) Aged hypersaline port water; (c) water from estuarine mouth (DP); (d) mid-estuary (CR); (e) estuarine upstream (SV). Relative abundance of major bacterial phyla during the incubation period after mixing in (f) high saline condition (HSC); (g) mid saline condition (MSC) and (h) low saline condition (LSC).

Figure 6. Canonical correspondence analysis (CCA) showing the relationship between bacteria and environmental parameters in (a) experiment I [hypersaline port water experiment]; (b) experiment II [saline port water experiment] and; (c) experiment III [freshwater port water experiment]. H0-H12 (filled black circle) denotes incubation period of 12 days in high saline condition; M0-M12 (filled green triangle) denotes the incubation period of 12 days in mid saline condition; L0-L12 (filled pink square) denotes incubation period of 12 days in low saline condition. Black lines indicate the various environmental parameters. Dotted black lines are highly significant (Monte Carlo test, $p < 0.05$). The empty triangles indicate the species variables [DO – Dissolved oxygen; SPM – Suspended particulate matter; TOC – Total organic carbon; SiO₄ – Silicate; PO₄ – Phosphate; NO₃ – Nitrate; NO₂ – Nitrite; TBC – Total bacterial counts; ZMA – Total viable marine bacterial counts; NA – Total viable freshwater bacterial counts; O157 – E.coli O157:H7; TC – Total coliforms; VA – *Vibrio alginolyticus*; VP – *V. parahaemolyticus*; VC – *V. cholerae*; Alpha – α proteobacteria; Beta – β proteobacteria; Gamma – γ proteobacteria; Actino – Actinobacteria; Bacter – Bacteroidetes; Firmi – Firmicutes].

Figure 1

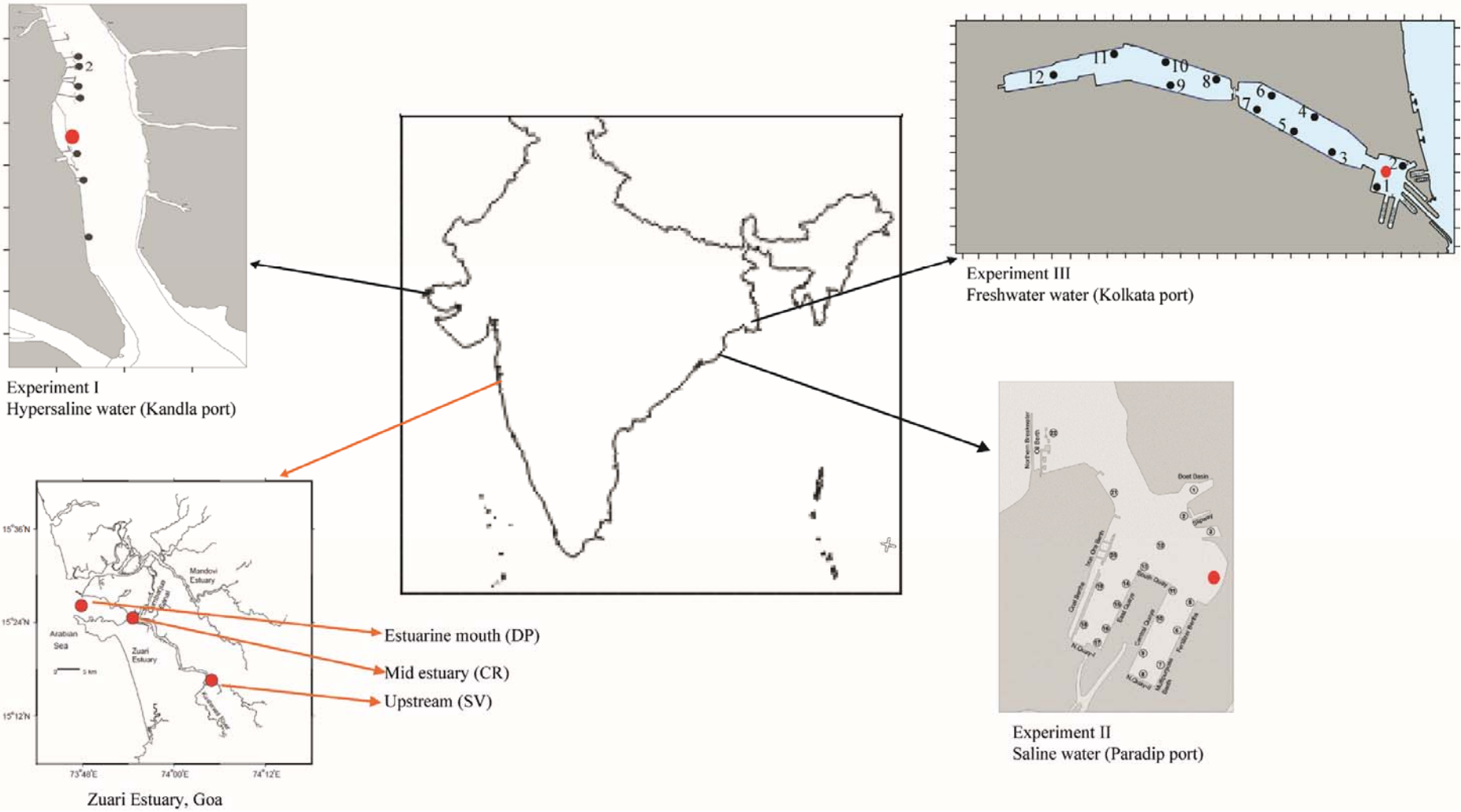


Figure 2

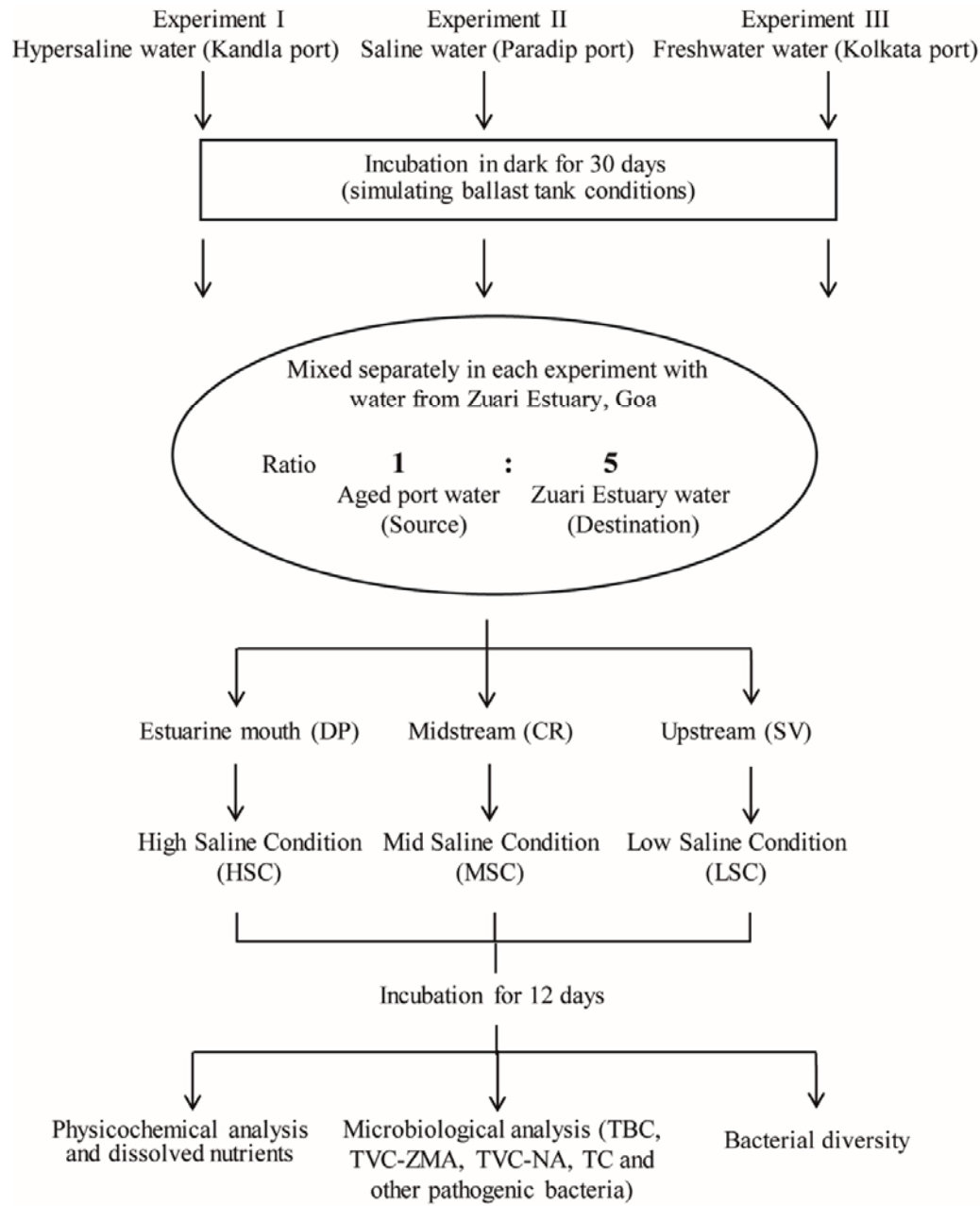


Figure 3

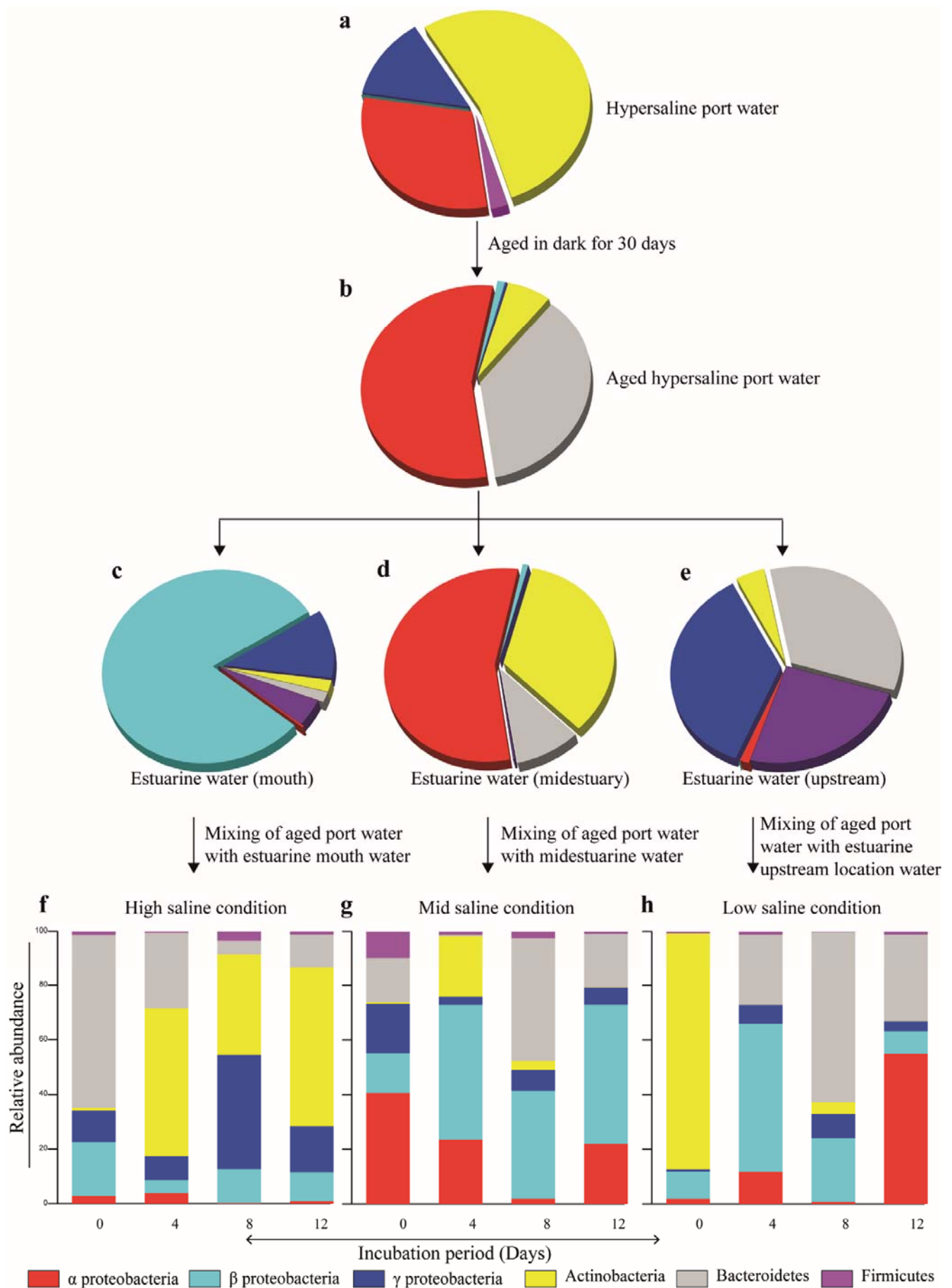


Figure 4

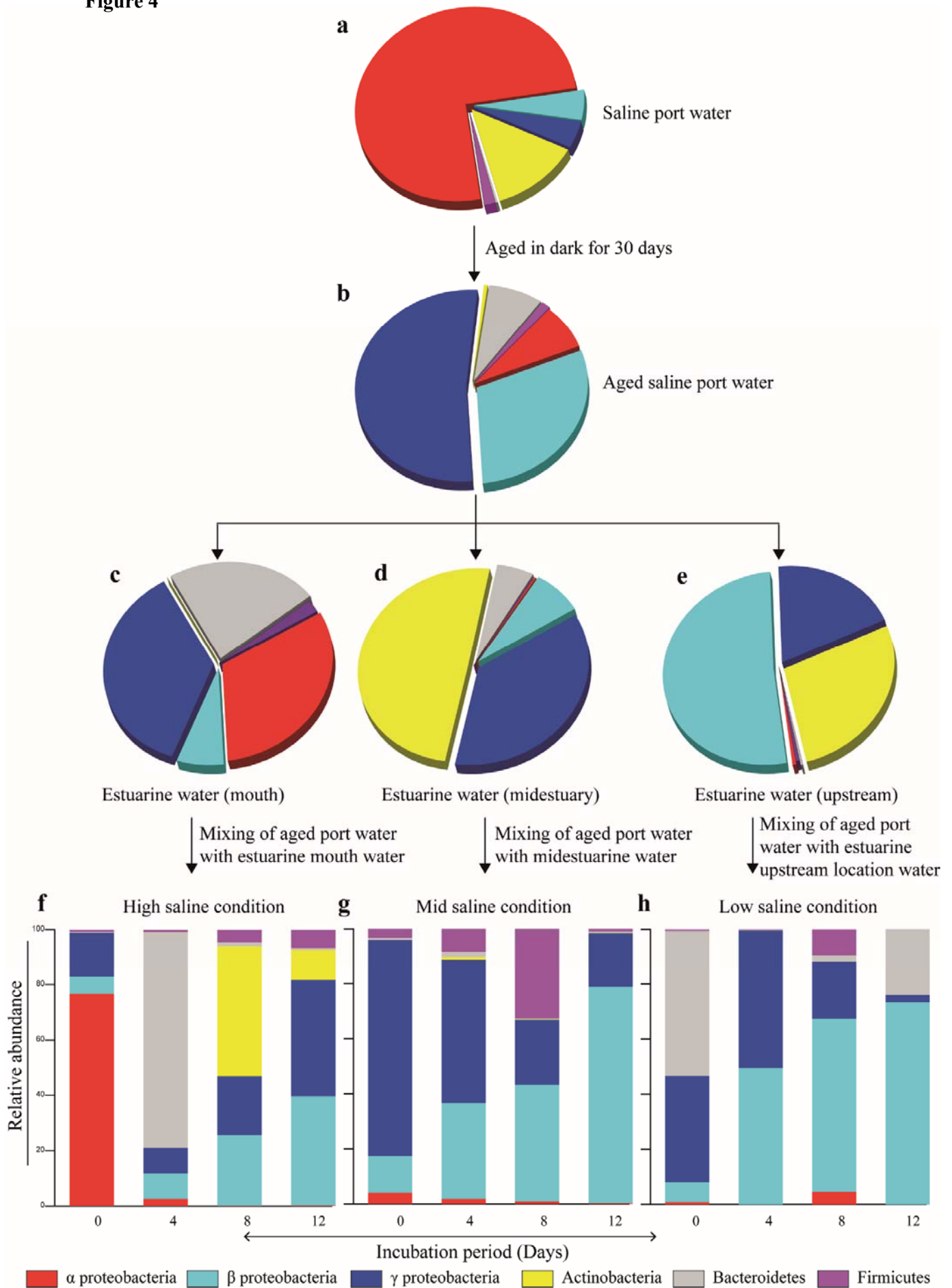


Figure 5

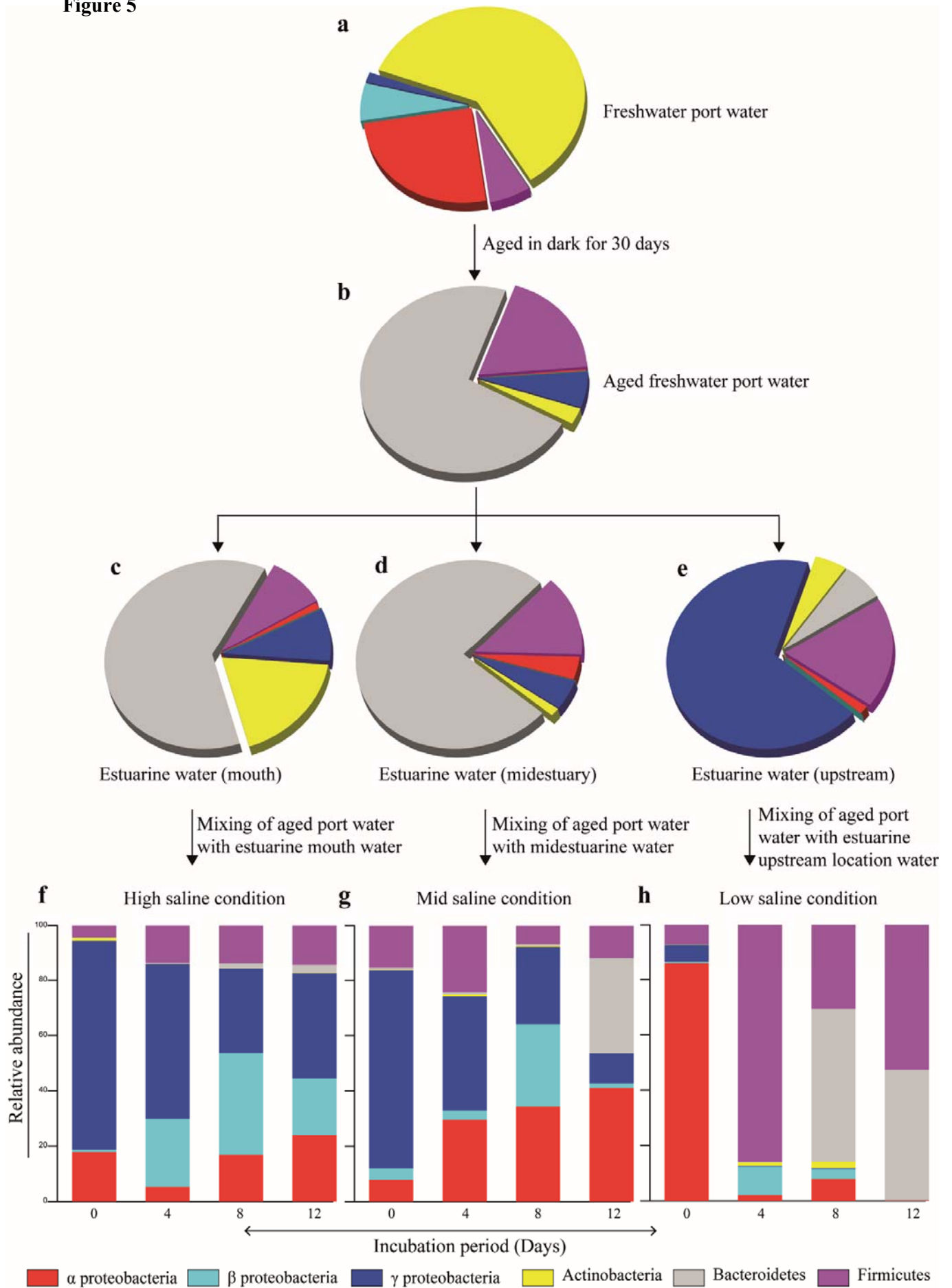
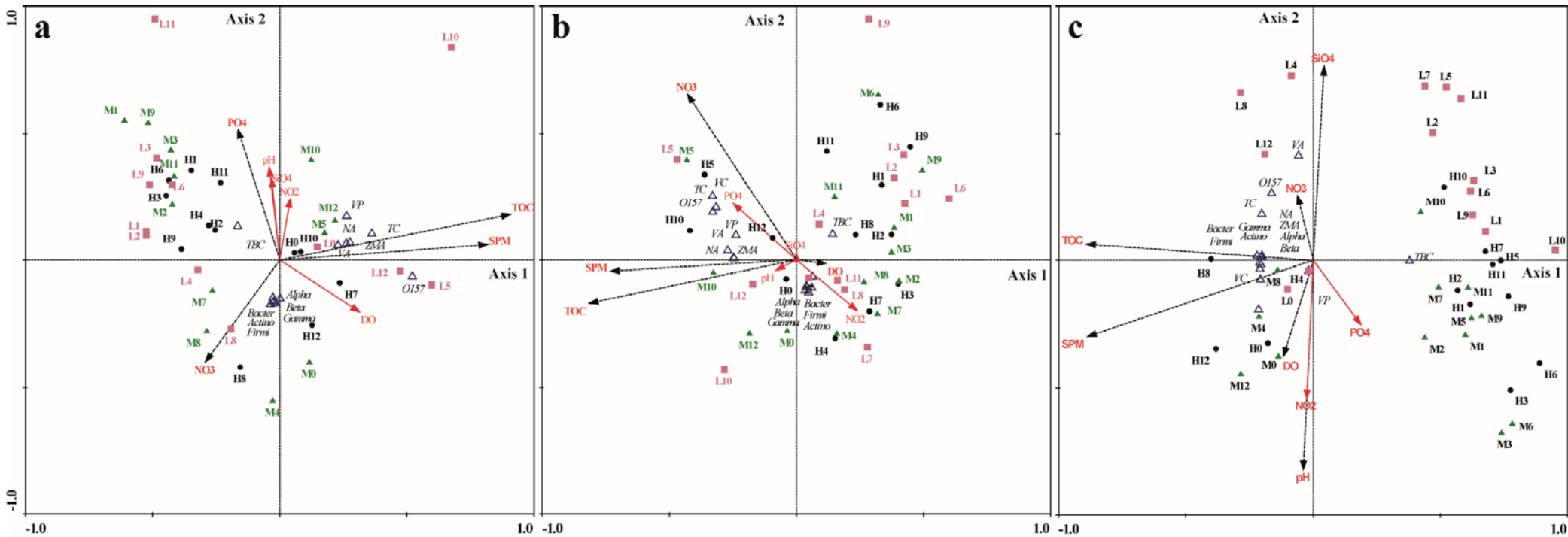


Figure 6



● H0- H12 High Saline Condition (HSC) Day 0- Day 12 ▲ M0-M12 Mid Saline Condition (LSC) Day 0- Day 12 ■ L0-L12 Low Saline Condition (LSC) Day 0- Day 12