

Changes in the ballast water tank bacterial community during a trans-sea voyage: elucidation through next generation DNA sequencing

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Abstract

Ballast water (BW) mediated bioinvasion is one of the greatest threats to the health of aquatic ecosystems. Bacteria, unlike higher organisms, are transferred in large numbers through BW. Owing to their abundance and potential pathogenicity, they pose a direct threat to the prevailing microbiome in the recipient waters and also to human health. This study investigated the changes in the BW tank bacterial community during a trans-sea voyage from Visakhapatnam port, located along the east coast of India (Bay of Bengal) to Mumbai port, located along the west coast of India (Arabian Sea). Next generation sequencing was used to explore the unculturable segment of bacteria. The BW tank conditions led to a decrease in photoautotrophs and non-spore forming bacteria. On the other hand, biofilm forming and antibiotic producing bacteria, nutrient limiting condition sustaining bacteria, and those capable of synthesizing enzymes prerequisite for active metabolism under stress, increased over time. The shifts in the bacterial community were dependent on mechanisms adopted by the clades to cope with the BW tank conditions. Functional prediction of the bacterial community revealed a significant increase in the core metabolic functions, which enabled the survival of such bacteria. As the voyage progressed, an increase in the total viable bacteria in BW tanks could be attributed to the decrease in the abundance of phytoplankton and zooplankton. At the end of the voyage, the bacterial community in the BW tanks was significantly different, and the species diversity and richness were higher than that of the natural seawater (source water). Pathogenic species were more abundant during mid-voyage than at the end of the voyage, suggesting that voyage duration influences the pathogenic bacterial community. Investigating the fate of the discharge bacterial population at the deballasting point is a way forward in the assessment of marine bioinvasion.

Keywords: Trans-sea voyage; Marine bioinvasion; Ballast water; 16S rRNA gene high-throughput sequencing; Bacterial community; Core metabolic functions

1. Introduction

When aquatic organisms are dispersed beyond their native ecosystems, it may lead to the invasion of non-native environments. This phenomenon, termed as 'Marine bioinvasion' may negatively impact aquaculture industries in addition to posing a risk to the environment and human health (Anil et al., 2002; Simberloff, 2011; Galil et al., 2014). Although BW is essential for ships to maintain their stability during a voyage, it has been identified as a significant vector for bioinvasion. Due to increase in maritime trade over the past few decades, cargo ships have been responsible for translocating more than 10 billion tonnes of BW annually (Elçiçek et al., 2013) along with the resident biota (Hulme, 2009; Seebens et al., 2013) into other aquatic environments. In addition to BW, the sediment (Mimura et al., 2005) and biofilms (Drake et al., 2007) in the BW tanks have also been identified as potential vectors for bioinvasion. Such translocations have resulted in the invasion of a plethora of species into ecosystems beyond their natural range, which is estimated to be over 7000 species (Carlton, 1985). In the past three decades, many organisms have invaded non-native marine environments around the world such as *Mnemiopsis leidyi* [Opaque comb jellyfish] (Vinogradov et al., 1989), *Dreissena polymorpha* [Zebra mussel] (Ludyanskiy et al., 1993), *Mytilopsis salsalei* [Black-striped mussel] (Field, 1999), *Asterias samurensis* [North Pacific Seastar] (Buttermore, 1994), and *Vibrio cholerae* O1 and O139 (Ruiz et al., 2000). As many as 450 invasive species are catalogued in the United States of America alone (Ruiz et al., 2015).

Studies have been carried out on the diversity of ballast tank water concerning marine invertebrates (Gray et al., 2007), zooplankton abundance (Gollasch et al., 2000; Olenin et al., 2000; Dibacco et al., 2012), harmful algae (Smayda, 2007), phytoplankton (McCarthy and Crowder, 2000; Choi, 2009), and invertebrate-associated bacteria (Khandeparker and Anil, 2013). Bacteria are much smaller organisms and are present in higher numbers in the BW tanks (Dobbs and Rogerson, 2005). Approximately 10^{19} bacteria are being translocated globally through ships' BW daily (Brinkmeyer, 2016). Given their potential pathogenicity, dispersal of marine bacteria is of great concern when compared to higher organisms (Drake et al., 2007). Thus, marine pathogenic bacteria and their transport through BW have been studied extensively (Joachimsthal et al., 2003; Drake et al., 2007; Seiden et al., 2011; Altug et al., 2012; Emami et al., 2012; Desai et al., 2018; Ng et al., 2018). However, most of these studies concentrated only on the culturable segment of bacteria. Modern molecular methods have

allowed the inclusion of unculturable bacteria, thereby providing a more holistic bacterial diversity in BW tanks (Khandeparker and Anil, 2017).

Metagenomic exploration of theseawater from the BW tanks has revealed new information on the changes in the bacterial community during a voyage. A study by Brinkmeyer (2016), revealed that the bacterial community in the BW tanks is unlike that of typical marine environments. The novel pathogenic forms of bacteria were also detected (Ng et al., 2015). Few studies have also assessed the efficacy of mid-ocean exchange, a technique used to reduce the risk of bioinvasion (Tomaru et al., 2014; Lympelopoulou and Dobbs, 2017) and identified biomarkers to assess the origin of BW (Gerhard and Gunsch, 2019). Studies have also evaluated the effectiveness of other BW treatment technologies (Lafontaine and Despatie, 2014; Moreno-Andrés et al., 2016; 2018; Romero-Martínez et al., 2017; Cullen, 2018; Petersen et al., 2019). Environmental DNA metabarcoding is being used to assess the efficacy of BW management systems (Rey et al., 2019). However, there are lacunae in the understanding of the modification in the structure and function of bacterial communities during a voyage, since marine bioinvasion continues to be a considerable threat to the health of aquatic ecosystems.

The objective of the current study was to acquire a holistic understanding of the changes in the bacterial community inside the BW tanks during a voyage in the seas surrounding the Indian peninsula. The voyage was undertaken from Visakhapatnam port, India (Bay of Bengal), to Mumbai port, India (Arabian Sea). The bacterial community inside the BW tanks was assessed using Ion Torrent PGMTM, a next generation sequencing technique. The sequences were acquired from a range of hypervariable regions on the 16S rRNA gene to improve the efficacy of the data. The functional diversity and the expression of core metabolic functions have been predicted using the same datasets. It was hypothesized that photoautotrophs and non-spore formers would decrease over time since the conditions in the BW tanks are not conducive for their growth. On the other hand, bacterial groups with strategies to overcome this stress and compete with the existing organisms are expected to survive or proliferate during the voyage. In such a case, an increase in the expression of core metabolic functions that could support their survival was expected.

2. Materials and methods

2.1. Voyage route

Sampling was carried out on a bulk carrier motor vessel, which had no BW treatment system onboard. The voyage was undertaken from Visakhapatnam port, Andhra Pradesh (East coast of India) (17.6885° N, 83.2974° E) to Mumbai port, Maharashtra (West coast of India) (18.9477° N, 72.8525° E) during June 2014. The BW was taken ~20 nautical miles from the Visakhapatnam port in two ballast tanks. The complete duration of the voyage was approximately five days, after which the BW was discharged ~45 nautical miles from the Mumbai port.

2.2. Sample collection

Two ballast tanks (top side tank port side -TSTP and top side tank starboard side-TSTS) were sampled. Each ballast tank had a capacity of 581.64 m³ and a depth of 5.05 m. The water samples from ballast tanks were collected through the manhole from both surface and bottom of the ballast tanks using a 5 L Niskin sampler daily at 09:00 AM until the end of the voyage. In TSTS, BW was partially discharged after 36 hours of the voyage, after which the water depth reduced to 2m. Subsequently, only surface water samples were collected from this tank. Additionally, natural seawater samples were also collected from the area of BW uptake (SW) (~20 nautical miles from the Visakhapatnam port) and the area of BW discharge (DW) (~45 nautical miles from the Mumbai port). Sample codes, age of the water, and sample description are provided in Table 1.

2.3. Physicochemical parameters and dissolved nutrients

Temperature, pH, and total dissolved solids (TDS) were measured using a multi-parameter probe (Oakton PCD 650, Cole-Palmer, USA). Samples for salinity were collected and stored in 50 mL airtight glass bottles and analysed on Guildline Autosol 8400B (within 10 days of collection). Dissolved oxygen was analysed on board (within 1 hour of collection) using Winkler's method, as described by Parsons et al. (1984). For chlorophyll *a* (Chl *a*) estimation, 500 mL of water sample was filtered through GF/F (0.7 μM) filter paper and stored at -20 °C until analysis. Subsequently, filter papers were extracted with 90% acetone and raw fluorescence units (RFU) were measured using a Fluorometer. For suspended particulate matter (SPM), water samples (500 mL) were filtered through pre-ashed, pre-weighed GF/F filter paper. The filter papers were weighed after drying for 48 hours in a hot air oven at 60 °C. Final readings were expressed as milligrams per litre (mg L⁻¹). Samples for dissolved

nutrients were collected in cryovials and preserved in liquid nitrogen. They were analysed using SKALAR SAN^{plus} ANALYZER within 10 days of collection.

2.4. Total bacterial count, total viable count, and culturable pathogenic bacteria

Water samples for total bacterial count were preserved in paraformaldehyde (1% final concentration) and stored at -20 °C until analysis. In the laboratory, they were thawed and stained with SYBR Green I (1:10,000 final concentration) prior to incubation in the dark for 15 minutes. The stained samples were analysed on the BD FACSVerse flow cytometer using a blue laser (488nm) (Khandeparker et al., 2017a). Cells with low nucleic acid content (LNA) and high nucleic acid content (HNA) were differentiated by gating against green fluorescence (FITC) versus the right angle light scatter (SSC). BD FACS Diva software (BD Biosciences, USA) was used to process the data, which were expressed as cells per millilitre (cells mL⁻¹).

For the enumeration of total viable bacteria, water samples were spread plated after appropriate dilution on Zobell marine agar (HiMedia), and incubated at room temperature for 24 hours before enumeration. *Vibrio* spp., *Escherichia coli* O157: H7 and total coliforms were quantified using spread plate technique on Thiosulfate citrate bile salts (TCBS) agar (Cat no. M189, HiMedia), HiCrome EC O157: H7 Agar (Cat no. M1574, HiMedia) and MacConkey agar (Cat no. M081B, HiMedia) respectively. The identification and enumeration of the specific bacteria were carried out after 24 hours of incubation at 37 °C, as provided in the technical data sheet from the manufacturer. This identification has been previously confirmed using protein profiling through MALDI-TOF MS and biochemical tests on randomly picked isolates from the respective selective media plates (Khandeparker et al., 2015). The estimation of total viable bacteria and pathogenic count was carried out onboard in a temporary laboratory set up inside the cabin.

2.4. DNA extraction, PCR amplification, and sequencing

Water samples for metagenomic amplicon analyses were collected on alternate days in triplicates. Two liters of the samples were filtered through sterile 47mm diameter, 0.22µm pore size filter papers (Millipore, USA) using low suction from a vacuum pressure pump (220/50Hz) and stored at -20 °C until DNA extraction was performed using the protocol given in Khandeparker et al. (2017b). The amplicons were prepared using the Ion 16STM metagenomics kit (Thermo Fischer Scientific; Cat. No.: A26216) following the manufacturer's guidelines. The proprietary primer pool uses 6 variable regions on the 16S rRNA gene (V2, V3, V4, V6-7, V8, and V9). Two amplification reactions were performed

(one pool amplifying V2, V4, and V8, and second pool amplifying V3, V6-7, and V9). Amplicons from both the primer sets were pooled and purified using magnetic beads (Agencourt AMPure XP beads, Beckman Coulter) before quantification (Qubit 2.0 Fluorometer). The pooling allowed the sequence-based identification of the microbial composition across all the variable regions (Sperling et al., 2017).

Libraries were prepared using Ion Plus Fragment Library Kit (Thermo Fischer Scientific, Cat. No.: 4471252) according to the manufacturer's instructions. The prepared libraries were pooled in equimolar concentration (25-30 picomoles) and loaded on Ion 316™ Chip V2 (Cat. No.: 4483188). Sequencing was performed on Ion Torrent PGM™ sequencer (Khandeparker et al., 2017b).

2.5. Filtering of reads and analysis on Ion Reporter™ software

Following the sequencing run, the acquired reads were subjected to signal processing, base calling, mapping, and quality control (Phred score) using Torrent Suite v5.0.5, which also categorized the reads based on barcodes. The tab-delimited text file containing sequence alignment data (.bam) files comprising the final reads were subjected to phylogenetic analysis by uploading on Ion Reporter™ software v5.2 (ionreporter.thermofisher.com/). A custom workflow that utilized both Curated MicroSEQ® 16S v2013.1 and Curated Greengenes v13.5 as reference libraries was created. For the taxonomic assignment, the parameters were set, as described by Sperling et al. (2017). The results were exported as tab-delimited text files (.txt) containing the abundance hits of operational taxonomic units (OTUs) at family, genus, and species levels for each sample.

2.6. Inferring the functions of the microbiome

The functional diversity of the metagenome was predicted from the existing 16S amplicon metagenomic data sets using iVikodak, a web-based platform (Nagpal et al., 2019). The platform is standardized to analyse data up to genus level; hence the genus OTU abundance data and metadata were uploaded as “.txt” files. The data were normalized and subjected to functional prediction based on existing algorithms using the Kyoto encyclopedia of genes and genomes (KEGG) database (Kanehisa and Goto, 2000). Simultaneous functional prediction was done to compare the functional diversity in the ballast tanks with that of the natural seawater (SW) and also to determine the functional diversity in the ballast tanks based on duration of incubation/voyage. Heat maps and box whisker plots depicting the abundance of core metabolic functions were obtained.

2.7. Zooplankton and phytoplankton abundance

For zooplankton sample collection, towcasts using Heron-Tranter net (mesh size 100 μm and mouth area of 0.5m) were made inside both the tanks. Using the initial and final flow meter readings the volume of water filtered was calculated as $V = \alpha N_R A$ (where, V – volume of water filtered in m^3 ; α – Flow meter calibration factor; N_R – Number of revolutions (read from the flowmeter); A – area of the mouth of the net). Aliquots of the sample were obtained by splitting the sample into two halves using a Folsom plankton splitter and taken on petri plates with grids. Live and dead zooplankton were counted using a stereo microscope (Olympus SZX16) under 4X and 10X magnification at the temporary laboratory setup inside the cabin on board and expressed as live and dead individuals m^{-3} . For the analyses of phytoplankton abundance, 1 litre of the water sample was preserved in Lugol's iodine and stored until analysis. In the laboratory, samples were allowed to settle for 48 hours, and the supernatants were siphoned out (Hasle and Sournia, 1978) until only 100 mL was remaining. Aliquots were taken in petri dishes and analysed using an inverted microscope (Olympus IX71) under 20X magnification and expressed as cells L^{-1} .

2.8. Graphical representation and statistical analyses

The relative abundance of OTUs was visualized in the form of cumulative bar charts (Golden Software, Inc. Grapher™ v8.4.696 Graphing software). Multidimensional scaling (MDS) was performed to summarize the substantial OTU abundance data. The reads were log transformed (normal distribution was confirmed using Shapiro-Wilk test of normality [SPSS Statistics, Version 22]) and clustered based on Bray-Curtis similarity (Primer 6, v6.1.10). MDS plots were obtained with clustering at 70% similarity at family and genus levels and 55% similarity at species level. The species diversity was assessed using Shannon's index [$H' = -\sum (P_i * \text{Log}_e (P_i))$], where “p” is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N). “i” is the limits to the summation ranging from 1 to “s”, where “s” stands for total number of species] and Simpson's index [$1 - \lambda' = 1 - \sum (N_i * (N_i - 1) / (N * (N - 1)))$], where N stands for the total number of individuals found and “i” is the limits to the summation from 1 to total number of species (s)]. Species richness has been assessed using the total number of OTUs. This analysis has been performed using Primer 6, v6.1.10. The correlation analyses between biotic and abiotic parameters were performed using StatSoft, Inc. (2007) STATISTICA (data analysis software system), version 8.0. www.statsoft.com. Regression with p-value ≤ 0.05 , were considered significant.

3. Results

3.1. Physico-chemical parameters and dissolved nutrients

Results of the physico-chemical parameters and dissolved nutrients are provided in Table 2. In brief, the temperature at SW was 32.1 °C, whereas it ranged from 30 – 31.5 °C inside the ballast tanks and did not vary significantly as the voyage progressed. Salinity was 33.85 in SW. After BW uptake, it ranged from 33.9 to 34.1 in both TSTP and TSTS and did not vary significantly during the voyage. The total dissolved solids (TDS) were 26.5 mg L⁻¹ in SW and were 26.5±0.09 mg L⁻¹ and 26.6±0.04 mg L⁻¹ in TSTP and TSTS respectively during the voyage. The concentration of Chl *a* which was 0.65 µg L⁻¹ at SW, showed a decreasing trend in TSTP and it was 0.24±0.1 µg L⁻¹ and 0.42±0.3 µg L⁻¹ in the surface and bottom. In TSTS it was 0.36±0.1 µg L⁻¹. The phosphate (PO₄) concentration in SW was 1.65 µM. After BW uptake, it was 1.39±0.18 µM and 1.63±0.32 µM in the surface and bottom waters of TSTP and 1.58±0.32 µM in TSTS. Nitrite (NO₂) concentration was 0.68 µM in SW. It was 0.71±0.05 µM in the surface water and 0.79±0.09 µM in the bottom water of TSTP, and 0.8±0.1 µM in TSTS. Nitrate (NO₃) concentration was 4.18 µM in SW. After the uptake it was 5.87±2.1 µM and 4.53±0.57 µM in surface and bottom of TSTP respectively and 4.63±0.4 µM in TSTS. All the dissolved nutrients except NO₃ showed an increasing trend over time.

3.2. Total bacterial count, total viable bacteria and pathogenic count

The total bacterial count was 44.1±18.7 x 10⁶ cells mL⁻¹ in SW. However, after the BW uptake, the average total bacterial count decreased in both TSTP (1.40±0.94 x 10⁶ cells mL⁻¹ and 1.66±0.87 x 10⁶ cells mL⁻¹ in surface and bottom respectively) and TSTS (0.67±0.39 x 10⁶ cells mL⁻¹) (Table 3). The cells with low nucleic acid content (LNA) which were 28.1±6.8 x 10⁶ cells mL⁻¹ in SW decreased to 0.92±0.63 x 10⁶ cells mL⁻¹ and 1.15±0.65 x 10⁶ cells mL⁻¹ in the surface and bottom of TSTP respectively (Table 3). In TSTS they were 0.49±0.34 x 10⁶ cells mL⁻¹. Similar trend was observed in cells with high nucleic acid content (HNA) which were 161±119 x 10⁵ cells mL⁻¹ in SW. They decreased in the surface (4.73±3.11 x 10⁵ cells mL⁻¹) and bottom (5.12±2.48 x 10⁵ cells mL⁻¹) of TSTP and TSTS (1.87±0.53 x 10⁵ cells mL⁻¹) (Table 3). Conversely, the total viable bacteria which were 290±99 CFU mL⁻¹ in SW, increased after the BW uptake and were 7.7±12.3 x 10³ CFU mL⁻¹ and 4.4±2.5 x 10³ CFU mL⁻¹ respectively in the surface and bottom waters of TSTP and 3.3±2.9 x 10³ CFU mL⁻¹ in TSTS (Table 3). No pathogens were detected in SW except for

Vibrio parahaemolyticus (10 CFU mL⁻¹). However, both *V. parahaemolyticus* and *Vibrio alginolyticus* were present in the ballast tanks during the voyage. The abundance of *V. alginolyticus* was 33±30 CFU mL⁻¹ and 79±94 CFU mL⁻¹ in surface and bottom water of TSTP respectively. It was 37±68 CFU mL⁻¹ in TSTS. Total coliforms were also detected in both the tanks (< 13 CFU mL⁻¹), whereas, *Vibrio cholerae* and *E. coli* O157: H7 were not detected (Table 3).

3.3. Metagenomic exploration of the bacterial community

Multiplex sequencing using barcodes yielded ~312 million bases (mb) of data corresponding to ~1.8 million reads. Figure 1 visualizes the major bacterial families at SW and in the ballast tanks during the voyage. SW was dominated by family Caulobacteraceae (16.11%), belonging to α -Proteobacteria (Fig. 1). The bacterial community in both the ballast tanks was different from the natural seawater and varied significantly during the period of voyage. Family Rhodobacteraceae increased in both surface (8.77% to 34.36%) and bottom (10.56% to 31.35%) of TSTP and in TSTS (5.57% to 23.61%) with respect to time (voyage duration) and dominated the bacterial community by the end of the voyage (Fig. 1). Additionally, Alteromonadaceae and Flavobacteriaceae also increased by the end of the voyage. Inversely, family Prochlorococcaceae decreased from 15.37% to 0.33% in the surface, 5.05% to 0.23% in the bottom of TSTP and from 12.36% to 0.26% in TSTS (Fig. 1). Similarly, a decreasing trend was observed in Sphingomonadaceae family (9.96% to 6.3% in the surface and 19.64 to 7.19% in the bottom) and Pseudoalteromonadaceae family (1.98% to 0.42% in the surface and 11.43% to 0.39% in the bottom). Family Mycobacteriaceae, which was very less in TSTP, showed a decreasing trend in TSTS (10.45% to 1.54%) (Fig. 1). Families Microbacteriaceae and Caulobacteraceae which were present in SW contributed minimally to the bacterial diversity inside the ballast tanks.

The major genera contributing to the bacterial community are presented in Figure 2. The results were concurrent with the observations at family level. At SW, the bacterial community was dominated by *Phenylobacterium* (22.12%), from family Caulobacteraceae followed by *Prochlorococcus* (12.12%), a marine cyanobacterium (Fig. 2). Other genera such as *Gordonia* (8.84%), *Alteromonas* (7.98%), *Nocardioides* (7.71%) and *Mycobacterium* (4.63%) contributed to the bacterial community (Fig. 2). In the ballast tanks during the voyage, Genus *Alteromonas*, a γ -Proteobacteria belonging to family Alteromonadaceae showed an increasing trend and dominated the community by the end of the voyage. It contributed 27.49% and 13.62% to the bacterial community in surface and bottom of TSTP,

respectively, and 10.64% to the bacterial community in TSTS (Fig. 2). Genus *Shimia* increased in the surface of TSTP (0.54% to 12.51%) and TSTS (0.19% to 4.59%). Furthermore, genus *Sulfitobacter* which contributed minimally to the bacterial community in SW increased in the bottom water of TSTP (16.71%) (Fig. 2). Genus *Tenacibaculum* was higher during the mid-voyage period in both the tanks. Inversely, genus *Prochlorococcus* decreased in both ballast tanks, and its contribution to the bacterial community was negligible by the end of the voyage (<1%). Additionally, genus *Erythrobacter* (α -Proteobacteria) decreased over time in TSTP (11.13% to 7.45% in the surface and 41.87% to 13.62% in the bottom). Genus *Mycobacterium* (Actinobacteria), which was less in TSTP, showed a decreasing trend in TSTS during the voyage (19.05% to 2.48%). Genus *Norcardioides* (Actinobacteria), which was present in both the tanks, also decreased during the voyage (Fig. 2).

Figure 3 depicts the major bacterial species at SW and in the ballast tanks during the voyage. At SW, *Phenylobacterium falsum* was the most abundant species (30.04%). Whereas, Actinobacterial species, *Rhodococcus corynebacteroides* (Nocardiaceae) and *Microbacterium schleiferi* (Microbacteriaceae), were equi-abundant (~8%) (Fig. 3). In the ballast tanks, *Shimiaisopora* and *Nautella indica*, both α -Proteobacteria belonging to Rhodobacteraceae, showed an increasing trend and dominated the bacterial community, especially in the surface water of TSTP (30.43% and 12.52% respectively) by the end of the voyage. *Sulfitobacter mediterraneus* increased in the bottom water of TSTP (3.15% to 18.69%). On the other hand, *Erythrobacter citreus* showed a significant decrease during the voyage in TSTP (18.03% to 9.73% in the surface and 42.52% to 11.29% in the bottom water). In contrast, a marginal decrease was observed in TSTS (5.11% to 4.98%). A similar trend was observed with *Phenylobacterium falsum* belonging to Caulobacteraceae (α -Proteobacteria). However, even after the decrease, *P. falsum* and *Tenacibaculum* sp. were still the dominant species at the end of the voyage (Fig. 3).

The bacterial community in DW was dominated by family Gordoniaceae (Actinobacteria) (20.83%) (Supplementary Fig. 1b). Families Caulobacteraceae (6.3%), Rhodobacteraceae (4.13%) were also observed, while Alteromonadaceae, Mycobacteriaceae, and Sphingomonadaceae were equi-abundant (~3%) (Supplementary Fig. 1b). Concurrently, *Gordonia* was the dominant genera (20.01%), followed by *Prochlorococcus* (19.07%) (Supplementary Fig. 1d). At the species level, however, the

bacterial community was dominated by *P. falsum* (18.71%), followed by *R. corynebacteroides* (14.42%) (Supplementary Fig. 1f).

3.3.1 Species richness, diversity and Multidimensional scaling (MDS)

The species diversity at SW (Shannon – 3.005; Simpson – 0.884) was much lower than the ballast tanks. In TSTP, the diversity increased mid-voyage in both the surface (Shannon – 3.875; Simpson – 0.959) and bottom (Shannon – 3.403; Simpson – 0.928) waters. In TSTS, the diversity increased over time and was high at the end of the voyage (Shannon – 3.558; Simpson – 0.945). The species richness was also considerably higher in the ballast tanks. 147-153 OTUs were detected in the surface water of TSTP, while 88-102 OTUs were observed in the bottom water. In TSTS, 91-113 OTUs were found, whereas, only 76 OTUs were found in SW (Table 4).

MDS analysis revealed two distinct clusters belonging to the bacterial community of the surface and bottom waters of TSTP, respectively, while the bacterial community in the natural seawater (SW) was an outlier (Fig. 4a, c, and e). The bacterial OTUs in TSTS, revealed one cluster belonging to TSTS, while that of SW was distinctly different at family and species hierarchical levels (Fig. 4b and f). At the genus level, TSTS2 and TSTS4 formed one cluster, while TSTS6 was significantly different (Fig. 4d).

3.3.2 Inference of functional potential of the bacterial community

Predictive inference of functions of the bacterial community revealed that with an increase in the voyage time, there was an increase in the expression of genes corresponding to core functions in both the tanks which included biosynthesis of various essential amino acids, biosynthesis and resistance to antibiotic compounds and active nucleic acid synthesis and repair (Fig. 5a and b). In TSTP, the core functions were expressed more in the surface than in the bottom (Fig. 5a). In both the tanks, key functions such as metabolism of nucleic acid, essential and non-essential amino acid, carbohydrate, lipid and energy were expressed to a greater extent by the end of the voyage (Fig. 5a and b). Their expression was also distinctly higher in the tanks when compared to natural seawater (Supplementary Fig. 2).

3.4 Phytoplankton and zooplankton abundance

The phytoplankton abundance was 5600 cells L⁻¹ in SW. After BW uptake, there was an initial increase in their abundance in TSTP (12700 cells L⁻¹). However, in both the tanks, the abundance showed a decreasing trend over time (Fig 6c).

The mortality of zooplankton after the uptake of BW was 7.14% and 13.59% in TSTP and TSTS respectively. During the voyage, the mortality continued to increase over time and was 74.27% in TSTP and 91.44% in TSTS at the end of the voyage (Fig. 6c).

4. Discussion

4.1. Variation in the total bacterial count, total viable count, and culturable pathogens

The total bacterial count, which was $44.1 \pm 18.7 \times 10^6$ cells mL^{-1} in SW reduced after the uptake in both TSTP and TSTS. The bacterial load is influenced by various factors such as the physico-chemical parameters within the ballast tanks and their mortality due to viral load and grazing pressure (Drake et al., 2007). It is also possible that the uncoupling of the microbial loop resulted in the failure to utilize the dissolved organic matter (DOM) released by zooplankton and phytoplankton mortality (Drake et al., 2002). Additionally, decrease in the bacterial count has been attributed to an increase in algal extracellular products such as toxins (Bell and Mitchell, 1972), which might have been released as a result of phytoplankton mortality. In the present study, a positive correlation was observed between total dissolved solids (TDS) and total bacterial count ($r=0.58$, $p \leq 0.05$); thus, a decrease in the total bacterial count was observed with decreasing TDS. On the other hand, total viable bacteria showed an increasing trend in both the tanks. This is in congruence with a study by Tomaru et al. (2014), wherein viable bacterial numbers increased during the voyage. Zooplankton and phytoplankton are known to harbour many bacteria (Khandeparker and Anil, 2013). The release of organic matter due to the death of zooplankton and phytoplankton, in addition to the associated bacteria, could result in an increase in the total viable bacteria. An increase in the viable bacteria was attributed to the higher suspended particulate matter as a result of sediment resuspension inside the BW tanks (Desai et al., 2018). Although, no such correlation was observed in this study, both TDS ($r=-0.68$, $p \leq 0.05$) and Chl *a* concentration ($r=-0.68$, $p \leq 0.05$) showed a strong negative correlation with total viable bacteria, indicating the utilization of plankton derived organic matter by the viable fraction of bacteria.

Vibrio cholerae and *E. coli* O157:H7 were not detected in the BW samples. However, *V. alginolyticus* (up to 175 ± 141 CFU mL^{-1}) and *V. parahaemolyticus* (<10 CFU mL^{-1}) were observed in the BW tanks, but decreased over time. *Vibrio* sp., are non-spore forming bacteria, and under stressed conditions, they can thrive on association with phytoplankton (Rehnstam-Holm et al., 2010) and zooplankton (Tang, 2005). Due to the decrease in these higher planktonic forms, such an association may not have been possible. Although toxigenic *V. cholerae* is euryhaline (McCarthy, 1996), other *Vibrio* spp. are also

known to prefer lower salinity, and are usually found in the coastal areas (Baker-Austin et al., 2012). The source of the BW was from the off-shore region, where the abundance of *Vibrio* spp. was low.

The unculturable bacterial community obtained through metagenomic amplicon sequencing in the ballast tanks changed over time and was significantly different from that of the natural seawater (Fig. 4). A study by Tomaru et al. (2014) also observed a continuous change in the bacterial community inside the ballast tanks. The richness and diversity in the tanks was also higher than the natural seawater. Similar results were observed by Gerhard and Gunsch (2019), where the diversity in the BW samples were higher than the diversity in the harbour. One of the reasons for the higher diversity could be the residual sediments and unpumpable water present in the tanks as a result of previous ballasting operations. The resuspension of this sediment and mixing of the unpumpable water during BW uptake could have contributed to the bacterial community in the water column inside the ballast tanks, leading to the higher diversity and richness. It is also possible that phytoplankton and zooplankton mortality could have released the bacteria associated with them, which could have influenced the bacterial diversity.

4.2. Variation in photoautotrophs and non-spore forming bacteria

In addition to the total phytoplankton abundance, which showed a decreasing trend inside the ballast tanks (Fig 6c), phylogenetic analysis of the bacterial community revealed that few bacterial clades also decreased over time due to the conditions inside the tanks. The bacteria that decreased during the voyage were phototrophs, a group of organisms unable to cope with the dark conditions inside the ballast tanks (Tomaru et al., 2014; Ng et al., 2015), and non-spore forming bacteria. In this study, *E. citreus*, a photoautotroph, was observed in both the tanks and dominated the bacterial community during uptake but decreased rapidly during the voyage. *Erythro bacter* sp., are aerobic phototrophs containing bacteriochlorophyll *a* and carotenoids which help them in harvesting light and also protect against radiation (Setiyono et al., 2019). Additionally, a decrease in *Prochlorococcus*, a photosynthetic cyanobacterium over time was also observed. Among the non-spore forming bacteria, Family Caulobacteraceae, incapable of forming resting stages during stress conditions (Lingens et al., 1985) decreased. Bacteria in the BW tanks usually transform to form resting stages (spores) during a voyage to battle the unfavourable (nutrient depleted, sub-oxic) conditions (Ruiz et al., 2000). Organisms incapable of undergoing such transformations showed a decreasing trend. *Phenylobacterium* sp., belonging to the family Caulobacteraceae, in addition to being a

non-spore former, has an extremely restricted nutritional range (Lingens et al., 1985). Due to high competition within the prevailing microbiome caused by the nutrient limiting conditions in the BW tanks (Seiden et al., 2011), there was a decrease in the abundance of *P. falsum* over time and its contribution to the bacterial community was lesser at the end of the voyage than at the time of uptake in both the BW tanks. *Phenylobacterium* sp., has been reported to cause skin infections in humans (Zhu et al., 2010). Although their abundance was low, the possibility of their proliferation under favourable circumstances could be inimical to human health. Additionally, under favourable conditions, it is possible that the genetic interactions between the invading pathogens and their non-native counterparts could lead to an increase in virulence gene expression resulting in disease outbreaks (Gennari et al., 2012).

4.3. Variation in bacteria with strategies to cope with stress

Bacteria that can adopt mechanisms to cope with the conditions inside the BW tanks survived and proliferated during the voyage. Bacterial clades that are antibiotic-producing, biofilm-forming, or which can grow under nutrient limiting conditions increased over time during the voyage. α - proteobacteria, especially family Rhodobacteraceae, one of the most dominant bacterial groups, especially in coastal ecosystems (González and Moran, 1997) play a significant role in biogeochemical processes (Pujalte et al., 2014). Members of the family Rhodobacteraceae are also known to grow under phosphate limiting conditions by employing multiple strategies to overcome the stress (Smith et al., 2019). They can also actively perform denitrification and nitrate respiration (Kraft et al., 2014). During a voyage, due to lack of fresh nutrients over time, the competition for available nutrients results in conditions that would only be favorable to certain bacterial species over others (Neyland, 2010). Family Rhodobacteraceae, due to its innate ability to grow under low nutrient conditions, flourished and dominated the bacterial community only at the end of the voyage. Members of this family are known to be the most common primary surface colonizers in coastal ecosystems and can establish themselves in non-native environments (Dang et al., 2008). They play a major role in biocorrosion and biofouling (Beech et al., 2005). Naturally, they could have a major economic impact if translocated into a new environment.

Among the species belonging to Rhodobacteraceae, *N. italica* showed an increase over time during the voyage. It was first isolated from a marine biofilm (Vandecastelaere et al., 2009) with an ability to produce antibiotics and other secondary metabolites to compete with other microorganisms (Martens et al., 2007). These strategies are not only useful in the BW tank environment where competition for nutrients is high, but they also aid in the

proliferation of such bacteria in a non-native environment after BW discharge. *Shimiaisopora*, from the same family, increased over time, eventually dominating the bacterial community by the end of the voyage. It was first isolated from reef-building corals on the coast of southern Taiwan (Chen et al., 2011). Another species belonging to the family Rhodobacteraceae, *Sulfitobacter mediterraneus*, was observed in the bottom water of TSTP, which dominated the bacterial community by the end of the voyage. It is a sulfate-oxidizing bacterium that was first isolated from eutrophic waters on the Mediterranean coast. It is a non-spore forming, strictly aerobic organism which can activate enzymes such as arginine hydrolase (amino acid hydrolysis), β -glucosidase (carbohydrate degradation), and gelatine hydrolase (proteolysis) after a prolonged incubation (>3 days) (Pukall et al., 1999). The delayed emergence of this bacterium in the bacterial community could be due to such a prolonged incubation, which is an essential condition for the activation of these enzymes. Thus, the capability to produce important enzymes required for active metabolism, may have influenced their growth in the ballast tanks. Emergence of such bacteria and their introduction through BW in non-native environments can influence and modulate the prevailing microbiome.

An increase in specific bacterial clades could be attributed to the core metabolic functions expressed by bacteria inside the BW tanks. Metabolic functional prediction of the bacterial community was carried out using iVikodak, a workflow designed to analyse and visualize the functional potential of bacterial assemblages (Nagpal et al., 2019). The core functions of the bacterial community in the ballast tanks were expressed to a higher extent when compared to that of the source of BW. These functions include antibiotic resistance (platinum drugs, cationic antimicrobial peptides, betalactam, and vancomycin), and biosynthesis of antimicrobials (Tetracycline, monobactam, acarbose and validamycin, phleomycin, kanamycin, and gentamycin). Functional pathways for central carbohydrate metabolism, including the TCA cycle, starch, and sucrose metabolism, propanoate metabolism, fatty acid metabolism, were also expressed. Additionally, metabolic functions such as essential and non-essential amino acid metabolism, energy metabolism (oxidative phosphorylation, methane, sulphur, and nitrogen metabolism), genetic functions (DNA excision, repair, and replication, mismatch repair, RNA degradation) and quorum sensing were also expressed. Moreover, the expression of most of the core functions in the BW tanks increased with voyage duration (Fig. 5). These mechanisms are rudimentary in the survival of the bacteria and subsequent propagation in new environments after discharge. Lv et al. (2018)

have reported a high prevalence of antibiotic resistance genes (ARGs) in the ballast tank sediments, and they attributed this to the high anthropogenic perturbations in the coastal waters. Activities such as sewage discharge and aquaculture that have intensified lately are reported to have significant copies of ARGs (Li et al., 2015) and some of the antibiotics are extensively used in mariculture (Muziasari et al., 2016). The ability of a bacterial community to invade a new environment should take into account not only the density of the inoculum but also the ability of the microorganisms to express mechanisms that might aid in their survival (Hess-Erga et al., 2019). From the marine bioinvasion perspective, introduction of bacterial communities which express mechanisms to cope with the competition, into non-native port environments, would be of concern not only to the prevailing microbiome, but can also cause economic loss to the aquaculture industry.

4.4. Variation in pathogenic bacteria

One of the critical reasons for understanding the invasive potential of bacteria is their pathogenicity. Translocation of pathogenic bacteria through BW could pose a threat to marine life and also to human health. In the present study, it was observed that *Pseudoalteromonas ruthenica*, belonging to family Alteromonadaceae increased briefly mid-voyage. They are pale orange pigment forming bacteria, first isolated from marine invertebrates (Ivanova et al., 2002). This family was first reported by Colwell et al. (1986) and includes a wide range of enteric organisms that are pathogenic to both humans and marine mammals (Esteve, 1995; Pereira et al., 2008). They carry substantial epidemiological importance, and the introduction of bacteria of this family into a non-native environment could have undesirable effects.

A species belonging to the family Flavobacteriaceae (Phylum Bacteroidetes), *Tenacibaculum* sp. showed a significant increase during the voyage. This family hosts several fish pathogens and is a known etiological agent for tenacibaculosis and flexibacteriosis in marine fish (Småge et al., 2016; 2018). It primarily holds an important economic significance in aquaculture (Avendaño-Herrera et al., 2006), which could pose a significant threat to marine life if introduced into new environments. *M. schleiferi* (Microbacteriaceae) and *R. corynebacteroides* (Nocardiodaceae), which belong to Actinobacteria, were observed in SW, however, their contribution to the bacterial community in the ballast tanks during the voyage was minimal. In general, coryneform bacteria, are pigment-producing and have been found in clinical specimens as opportunistic pathogens (Gneiding et al., 2008). Despite their low abundance in the BW tanks, there is a considerable risk to human health, in a scenario

where the environmental conditions at the point of discharge are conducive for their growth and proliferation.

An overall increase in most of the pathogens was evident, their abundance was high during the mid-voyage when compared to the end of the voyage. Hence, voyage duration seems to be an important factor that influences pathogenic bacterial species. Storage time of BW has been reported as a critical factor in determining the bacterial diversity inside the tanks (Wennberg et al., 2013) and a reduction in bacterial load was observed over a longer BW retention time (Seiden et al., 2010; Tomaru et al., 2014; Ng et al., 2015). Moreno-Andrés et al. (2018) reported that even after treatment of the ballast water, regrowth of bacteria is time-dependent and the modified bacterial community may pose a higher risk of invasion due to their capability to overcome the stress of the treatment technique. In addition to storage time, salinity is a critical factor in determining the bacterial diversity and richness (Lymeropoulou and Dobbs, 2017). However, this could be highly specific to the biogeography of the source of the BW. In the present study, there was a distinct shift in the bacterial community over time, although the salinity did not vary significantly in the ballast tanks during the voyage (33.9 – 34.1). Hence the key factors influencing the bacterial community could be region-specific. Recognising these factors would be valuable in developing strategies to reduce the risk of biological invasions.

Overall, the average total bacterial count in the BW tanks decreased at the end of the voyage (Fig. 6a). The composition of the bacterial community showed a differential response during the voyage. A sharp decline in photoautotrophic bacteria after BW uptake was observed, and their contribution to the bacterial diversity decreased over time. On the other hand, the bacteria which exhibit strategies to cope with the conditions inside the BW tanks increased during the voyage (Fig. 6a). The contribution of pathogenic bacterial species increased during mid-voyage. At the end of the voyage, however, their contribution to the bacterial community was low (Fig. 6a). In the culturable segment of bacteria, the total viable bacteria in the BW tanks did not increase significantly till day 3; however, after day 3, the total viable bacteria increased by two orders of magnitude at the end of the voyage (Fig. 6b). *V. cholerae* and *E. coli* O157:H7 were not observed during the voyage, however, *V. alginolyticus*, *V. parahaemolyticus* and total coliforms were observed. There was a decrease in the abundance of phytoplankton after the uptake of BW (Fig. 6c). The mortality of zooplankton also increased over time (Fig. 6b, c). However, *V. parahaemolyticus* and total

coliforms showed a minimal increase during day one and continued to decrease till the end of the voyage (Fig. 6b).

The bacterial community in the source water (SW) was different from that of the location of BW discharge (DW). Although, genus *Prochlorococcus* was abundant in both the environments, the species level bacterial community did not reflect the same. Instead, *P. falsum* was the most abundant species in both the environments (source and destination). *P. falsum*, which belongs to α -proteobacteria was first isolated in non-saline groundwater (Tiago et al., 2005) and has an extremely limited nutritional spectrum (Lingens et al., 1985). *M. schleiferi*, belonging to Actinobacteria was more at the source than at the destination and this bacterium was isolated from human clinical specimens and reported as an opportunistic pathogen (Gneiding et al., 2008). Conversely, *R. corynebacteroides*, belonging to Actinobacteria was higher at the discharge location than at the source. It is a red pigment forming bacteria reported to be an opportunistic pathogen in immunocompromised individuals (Yassin and Schaal, 2005; Kitamura et al., 2012). The Bay of Bengal (source) and the Arabian Sea (destination) are different in their hydrographic and biogeochemical characteristics (Anil et al., 2002). Since the bacterial community is distinctly different at the source and the destination of BW, its discharge into a non-native environment should be carefully considered. The fate of the bacterial community from the ballast tanks in the new environment would depend on the geographical features, environmental conditions, ecological health, and the resident biota of the recipient waters. Further studies should focus on identifying the key factors that influence the outcome of such introductions.

5. Conclusions

The diversity and richness of the bacterial community in the BW were higher than the natural seawater. Prolonged dark conditions in the BW tanks lead to a decrease in photoautotrophs (*E. citreus*, *Prochlorococcus*) and non-spore forming bacteria, *P. falsum*. Alternatively, biofilm forming and antibiotic producing *N. indica* and *S. isopora* (belonging to Rhodobacteraceae known for their ability to grow under nutrient limiting conditions) increased during the voyage. *S. mediterraneus* can synthesize and express essential enzymes required to maintain a metabolically active state, which aided in their proliferation over time. The shift in the bacterial community is governed by the ability of the bacteria to cope with the stress through mechanisms that could aid in their survival and allow them to compete with the other members of the microbiome. There was a substantial increase in the expression of core functions by the bacterial community in the ballast tanks with an increase in voyage

duration, which could provide insights on the ability of the bacterial community to compete with the indigenous biota at the point of discharge. Pathogenic species, *P. ruthenica* and *Tenacibaculum* sp. were higher during the mid-voyage period than at the end of the voyage, suggesting voyage duration to influence their community. Understanding the fate of the altered bacterial community after discharge into a new environment is crucial.

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Legends to figures

Figure 1. Relative abundance of major bacterial families of source ballast water (SW) and in ballast tanks on (a) port side (TSTP); (b) starboard side (TSTS).

Figure 2. Relative abundance of major genera of bacteria of source ballast water (SW) and in ballast tanks on (a) port side (TSTP); (b) starboard side (TSTS).

Figure 3. Relative abundance of major species of bacteria of source ballast water (SW) and in ballast tanks on (a) port side (TSTP); (b) starboard side (TSTS).

Figure 4. Cluster analysis with SIMPROF test based on Bray-Curtis similarity coefficient of source of ballast water (SW) and ballast tanks (TSTP and TSTS) classified based on (a, b) Family; (c, d) Genus and; (e, f) Species operational taxonomic units (OTUs).

Figure 5. Heat map representing the expression of core metabolic functions by the bacterial community over time in [a] TSTP and [b] TSTS. Numbers 1-38 denote the various metabolic functions (1. Betalactam resistance; 2. Beta alanine metabolism; 3. Vancomycin resistance; 4. Valine, leucine and isoleucine degradation; 5. Valine, leucine and isoleucine metabolism; 6. Tyrosine and tryptophan metabolism; 7. Tetracycline biosynthesis; 8. Sulfur metabolism; 9. Starch and sucrose metabolism; 10. Quorum sensing; 11. Propanoate metabolism; 12. Phleomycin, kanamycin and gentamicin biosynthesis; 13. Phenylalanine, tyrosine and tryptophan biosynthesis; 14. Peptidoglycan biosynthesis; 15. Oxidative phosphorylation; 16. DNA excision repair and replication; 17. Non homologous end joining; 18. Nitrogen metabolism; 19. Monobactam biosynthesis; 20. Mismatch repair; 21. Methane metabolism; 22. Lipopolysaccharide biosynthesis; 23. Glycine, serine and threonine metabolism; 24. Glutathione metabolism; 25. Fructose, galactose and mannose metabolism; 26. Folate biosynthesis; 27. Fatty acid degradation and biosynthesis; 28. Cysteine and methionine metabolism; 29. Cyanoamino acid metabolism; 30. Central carbon metabolism; 31. Cationic antimicrobial peptide resistance; 32. Carotenoid biosynthesis; 33. Carbon fixation pathway; 34. Butanoate metabolism; 35. Arginine biosynthesis; 36. Aminobenzoate degradation; 37. Aminoacyl tRNA biosynthesis; 38. Acarbose and validamycin biosynthesis).

Figure 6. Trend of (a) total bacterial count, photoautotrophs, pathogenic species and bacteria with strategies to cope with stress; (b) total viable bacteria and culturable pathogenic bacteria and; (c) phytoplankton abundance and zooplankton mortality with respect to voyage duration. (R^2 – Correlation coefficient; m – slope. The R^2 values are obtained by drawing a line of best fit using the linear model taking into consideration all the experimental data points. The slope was obtained from the equation of the line $y=mx + c$; where “ m ” is the slope)

Legends to supplementary Figures

Supplementary Figure 1. Relative abundance of major bacterial taxa in source water and destination water at (a, b) family; (c, d) genus; and (e, f) species levels.

Supplementary Figure 2. Predictive inference of functional diversity of the bacterial diversity. [a] Box whisker plots depicting the top (most abundant) functions at L1 level with respect to the location (SW and TSTP); [b] Box whisker plots depicting the top functions at L1 level with respect to the location (SW and TSTS); [c] Box whisker plots depicting the top functions at L2 level with respect to the location (SW and TSTP); [d] Box whisker plots depicting the top functions at L2 level with respect to the location (SW and TSTS). L1 and L2 represent the specific levels of functional hierarchy.

Legends to tables

Table 1. Details of the samples(sample ID), age of ballast water andvoyage duration (days).

Table 2. Physicochemical characteristics of source water (SW),(a) port side tank (TSTP) and (b) starboard side tank (TSTS) and destination water (DW). Temp – Temperature; TDS – Total dissolved solids; DO – Dissolved oxygen; SPM – Suspended particulate matter; NH₄ – Ammonia; PO₄ – Phosphate; SiO₄ – Silicate; NO₂ – Nitrite; NO₃ – Nitrate.

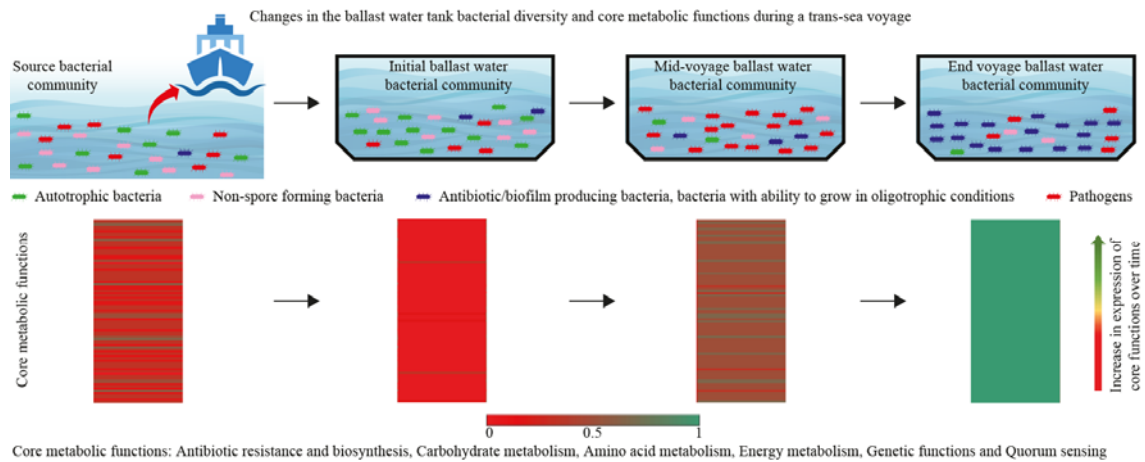
Table 3. Total bacterial count, total viable bacteriaand pathogenic abundance in source water (SW), (a) port side tank (TSTP) and (b) starboard side tank (TSTS) and destination water (DW). LNA – cells with low nucleic acid content; HNA – cells with high nucleic acid content; *Vibrio alginolyticus*; *V. parahaemolyticus*; VC – *V. cholerae*; TC – Total coliforms; EC – *Escherichia coli* O157: H7;ND – Not detected; CFU mL⁻¹ – Colony forming units per millilitre.

Table 4. Variation in the bacterial species (OTUs) richness and diversity in the source water (SW), ballast tank water (TSTP and TSTS) and destination water (DW).

Legends to supplementary table

Supplementary Table 1. Number of bases, \geq Q20 bases (Phred score), reads, mean read length of the samples.

Graphical Abstract



Highlights

- Bacterial metagenome in ballast tanks during tropical trans-sea voyage changed
- Autotrophs and non-spore formers decreased during the voyage
- Viable bacteria increased due to utilization of plankton derived organic matter
- Increase in culturable bacteria correlated with zooplankton mortality
- Voyage duration influenced pathogenic bacterial diversity
- Discharge water bacterial community characteristically different from source water

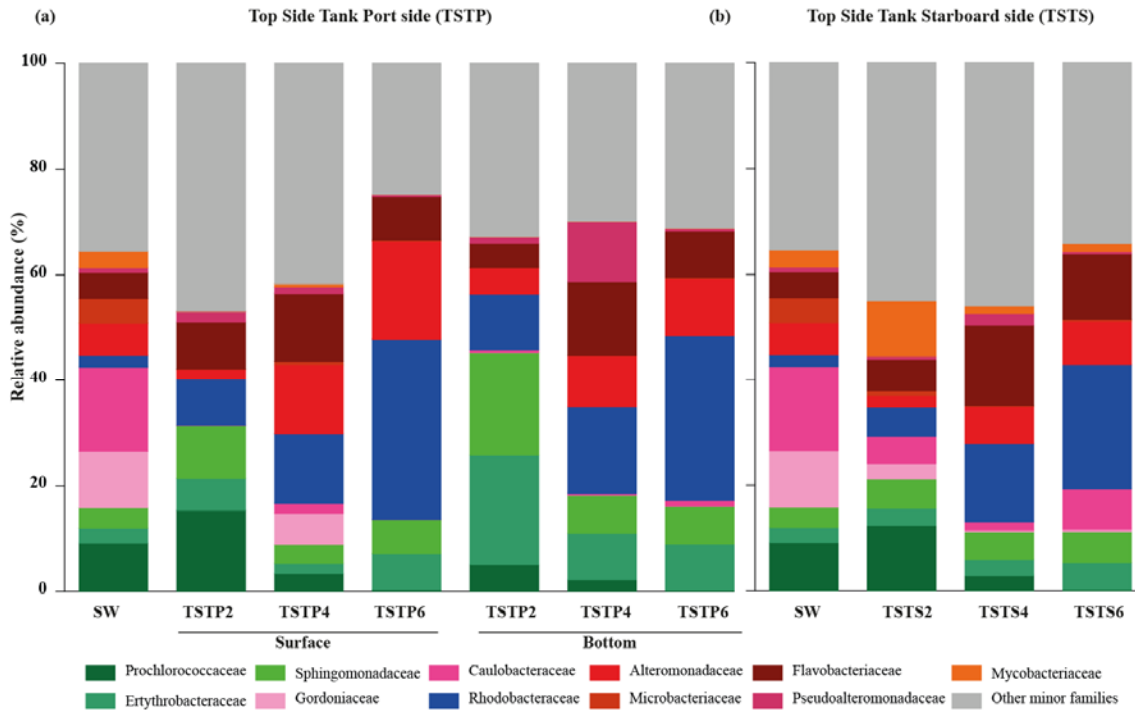


Figure 1

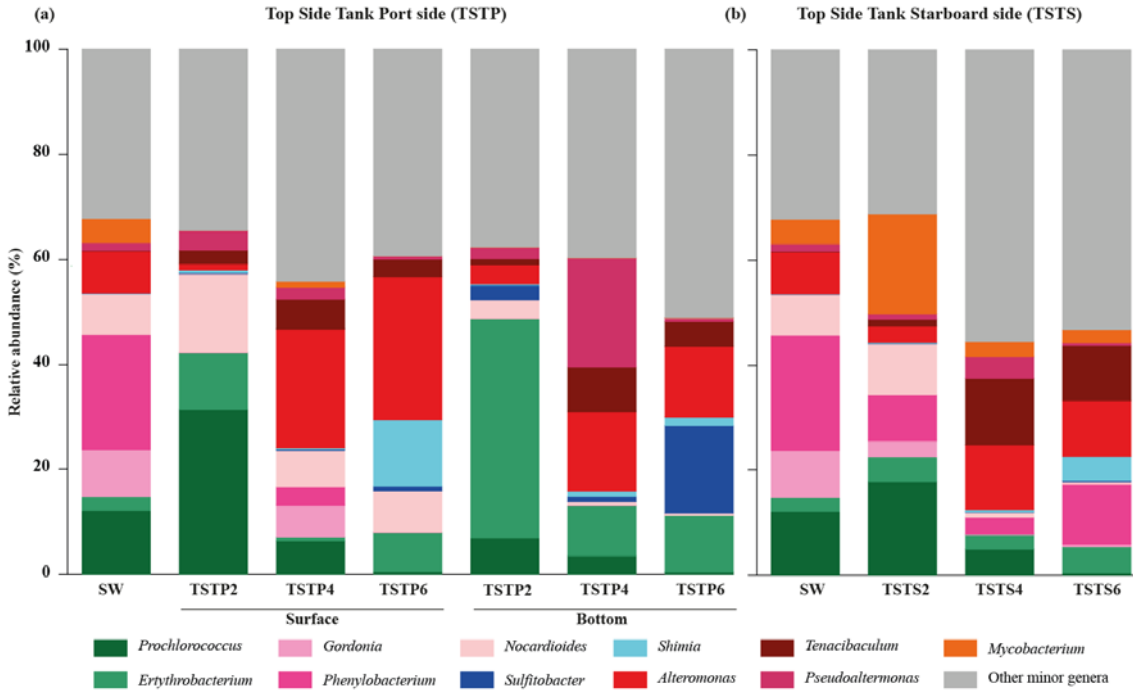


Figure 2

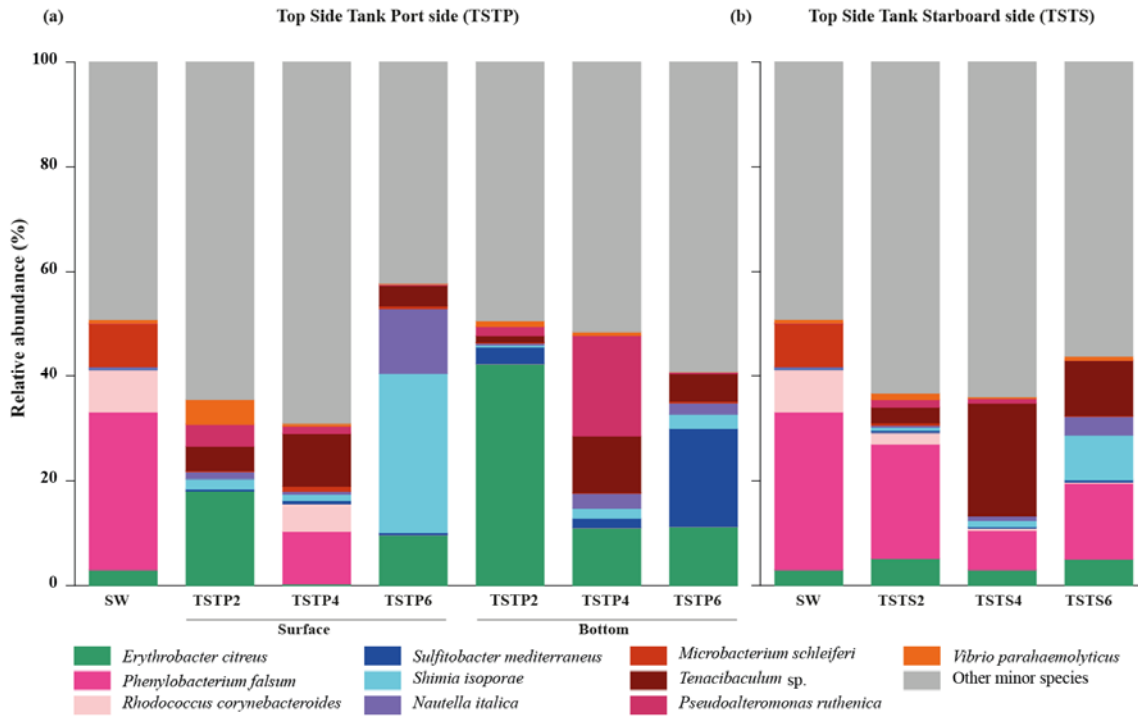


Figure 3

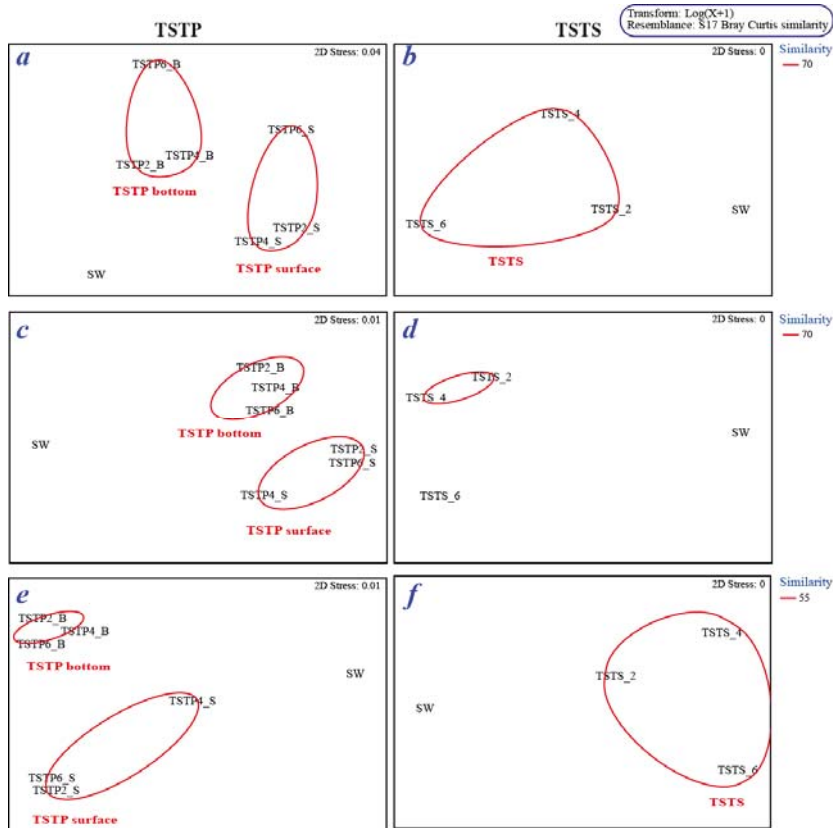


Figure 4



Figure 5

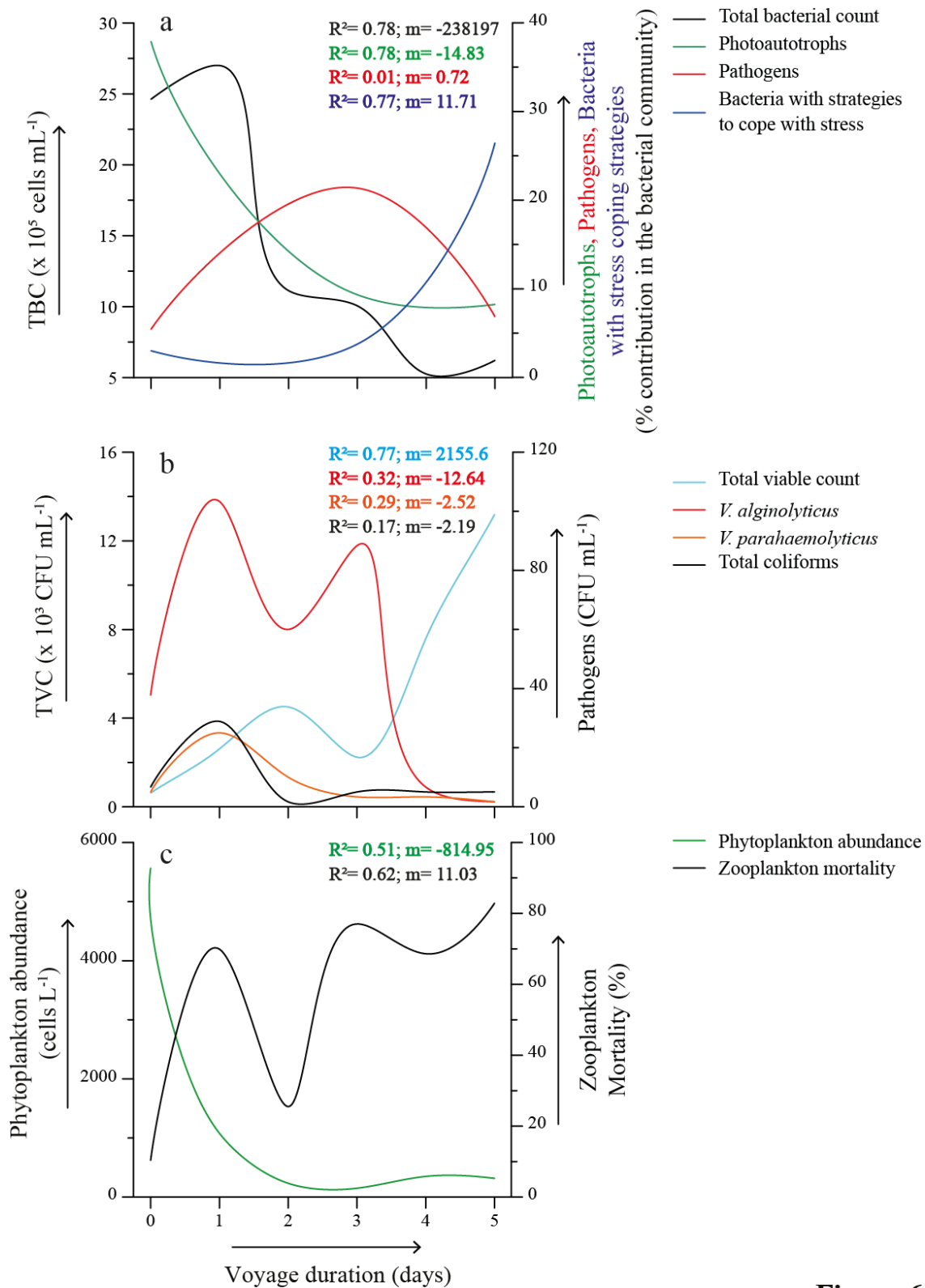
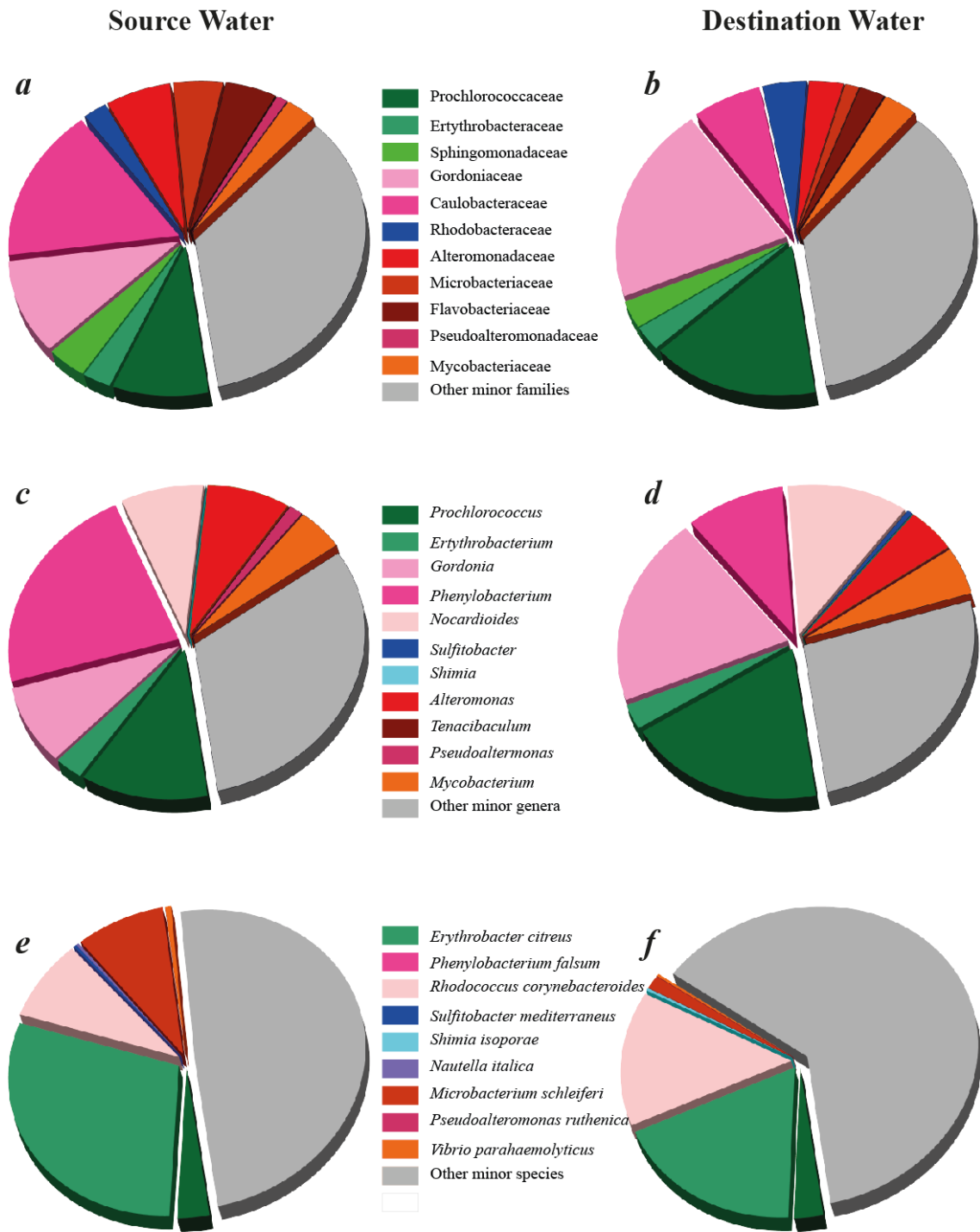
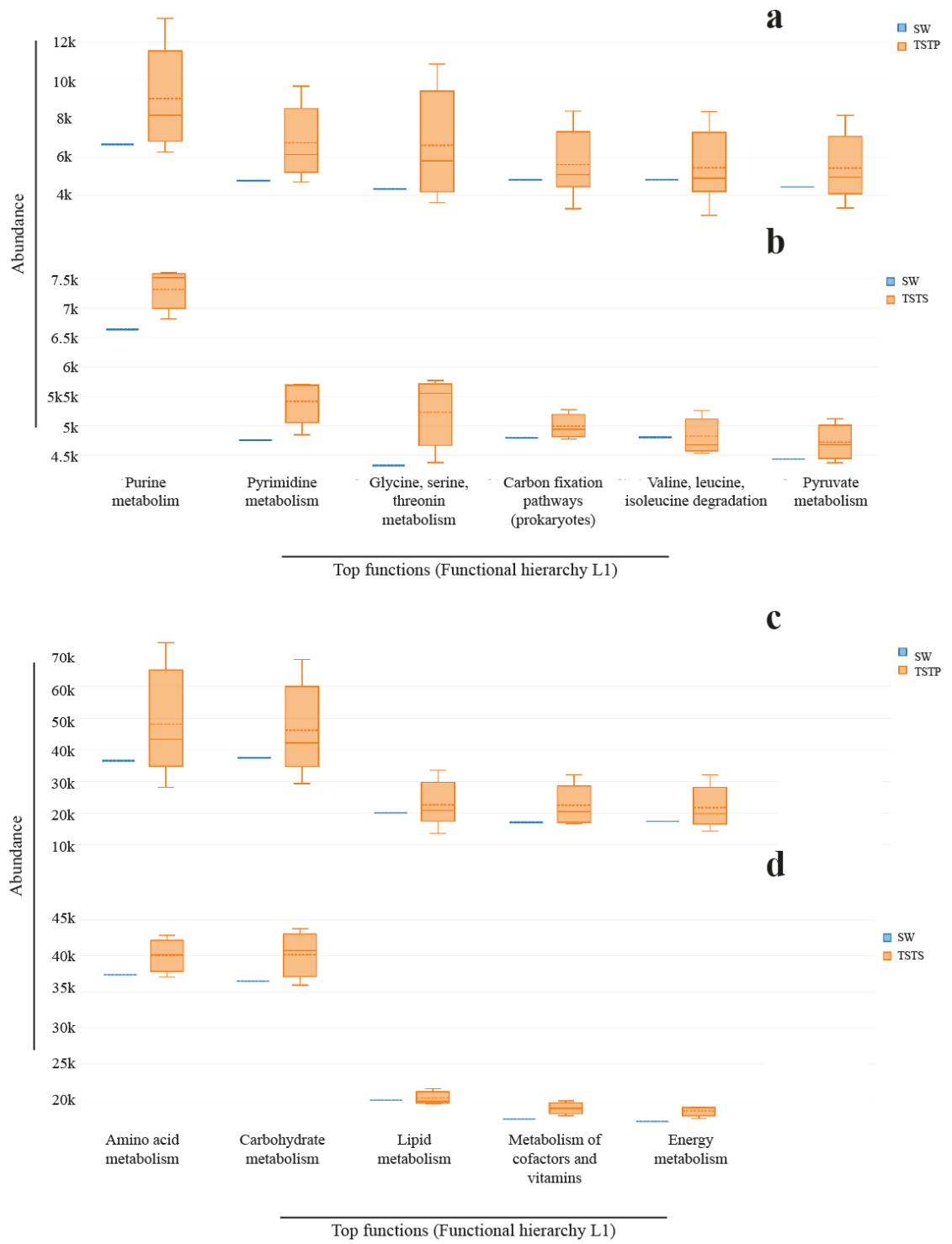


Figure 6



Supplementary Figure 1



Supplementary Figure 2

Table 1

| S. no. | Sample ID | Age of water sample (hours) | Voyage duration (Days) |
|--------|-----------|-----------------------------|------------------------|
| 1 | SW | - | - |
| 2 | TSTP1 | 0 | 0 |
| 3 | TSTP2 | 24 | 1 |
| 4 | TSTP3 | 48 | 2 |
| 5 | TSTP4 | 72 | 3 |
| 6 | TSTP5 | 96 | 4 |
| 7 | TSTP6 | 120 | 5 |
| 8 | TSTS1 | 0 | 0 |
| 9 | TSTS2 | 24 | 1 |
| 10 | TSTS3 | 48 | 2 |
| 11 | TSTS4 | 72 | 3 |
| 12 | TSTS5 | 96 | 4 |
| 13 | TSTS6 | 120 | 5 |
| 14 | DW | - | - |

SW – source water: natural sea water collected from the source (off-Visakhapatnam, India);
TSTP – Top side tank port side; TSTS – Top side tank starboard side; DW – destination
water: natural sea water collected from the destination point/ area of deballasting (off-
Mumbai, India)

Table 2.

| (a) | Sample ID | Temp (°C) | Salinity | pH | TDS (mg L ⁻¹) | DO (mg L ⁻¹) | Chla (µg L ⁻¹) | SPM (mg L ⁻¹) | NH ₄ (µM) | PO ₄ (µM) | SiO ₄ (µM) | NO ₂ (µM) | NO ₃ (µM) |
|---------|-----------|-----------|----------|-----|---------------------------|--------------------------|----------------------------|---------------------------|----------------------|----------------------|-----------------------|----------------------|----------------------|
| | SW | 32.1 | 33.85 | 8.3 | 26.5 | 2.5 | 0.65 | 25.5 | 10.47 | 1.65 | 6.37 | 0.68 | 4.18 |
| Surface | TSTP1 | 31 | 34.1 | 8.6 | 26.7 | 1.9 | 0.36 | 18.5 | 15.27 | 1.28 | 10.22 | 0.71 | 10.07 |
| | TSTP2 | 31.3 | 33.99 | 8.4 | 26.6 | 2 | 0.31 | 40.5 | 14.46 | 1.24 | 3.95 | 0.69 | 5.26 |
| | TSTP3 | 30.9 | 33.96 | 8.6 | 26.6 | 2.2 | 0.26 | 19 | 12.83 | 1.38 | 8.8 | 0.7 | 4.24 |
| | TSTP4 | 30 | 34 | 8.6 | 26.6 | 2.7 | 0.20 | 31 | 15.73 | 1.25 | 5.35 | 0.64 | 5.93 |
| | TSTP5 | 30.3 | 33.97 | 8.6 | 26.4 | 2.3 | 0.12 | 11 | 14.23 | 1.71 | 13.65 | 0.77 | 5.04 |
| | TSTP6 | 30.9 | 33.98 | 8.5 | 26.4 | 2.2 | 0.17 | 56 | 13.86 | 1.49 | 6.25 | 0.75 | 4.69 |
| Bottom | TSTP1 | 31.2 | 34.12 | 8.5 | 26.6 | 1.9 | 0.76 | 80 | 13.52 | 1.98 | 4.36 | 0.71 | 4.27 |
| | TSTP2 | 31.3 | 34.01 | 8.5 | 26.6 | 2.1 | 0.35 | 34.5 | 10.46 | 1.52 | 4.81 | 0.71 | 5.56 |
| | TSTP3 | 30.9 | 33.95 | 8.6 | 26.5 | 2.4 | 0.45 | 29 | 13.75 | 1.35 | 4.81 | 0.69 | 4.44 |
| | TSTP4 | 30.6 | 34 | 8.7 | 26.5 | 2.6 | 0.76 | 37 | 15 | 1.41 | 5.56 | 0.83 | 3.88 |
| | TSTP5 | 30.4 | 33.97 | 8.6 | 26.5 | 2.4 | 0.12 | 42.5 | 12.1 | 2.08 | 5.86 | 0.92 | 4.71 |
| | TSTP6 | 31.2 | 33.98 | 8.5 | 26.5 | 2.2 | 0.10 | 11.5 | 13.87 | 1.42 | 7.21 | 0.85 | 4.34 |
| (b) | Sample ID | Temp (°C) | Salinity | pH | TDS (mg L ⁻¹) | DO (mg L ⁻¹) | Chla (µg L ⁻¹) | SPM (mg L ⁻¹) | NH ₄ (µM) | PO ₄ (µM) | SiO ₄ (µM) | NO ₂ (µM) | NO ₃ (µM) |
| | SW | 32.1 | 33.85 | 8.3 | 26.5 | 2.5 | 0.65 | 25.5 | 10.47 | 1.65 | 6.37 | 0.68 | 4.18 |
| Surface | TSTS1 | 31.2 | 33.9 | 8.5 | 26.6 | 1.9 | 0.51 | 47.5 | 14.25 | 1.42 | 6.17 | 0.79 | 4.18 |
| | TSTS2 | 31.5 | 33.98 | 8.4 | 26.6 | 2.3 | 0.27 | 46.5 | 12.25 | 1.2 | 3.47 | 0.63 | 5.3 |
| | TSTS3 | 31 | 33.92 | 8.5 | 26.5 | 2.2 | 0.44 | 36.5 | 13.34 | 1.3 | 5.89 | 0.8 | 4.57 |
| | TSTS4 | 30.5 | 33.97 | 8.7 | 26.6 | 2.6 | 0.35 | 33 | 14.9 | 1.73 | 7.07 | 0.76 | 4.84 |
| | TSTS5 | 29.8 | 34.04 | 8.7 | 26.6 | 2.5 | 0.14 | 32 | 14 | 1.76 | 12.48 | 0.94 | 4.29 |
| | TSTS6 | 30.1 | 33.98 | 8.6 | 26.6 | 2.2 | 0.42 | 85 | 12.29 | 2.04 | 8.11 | 0.85 | 4.58 |
| | DW | 31.2 | 35.35 | 8.4 | 27.6 | 2.5 | 0.27 | 24.5 | 11.92 | 0.9 | 3.23 | 0.61 | 4.74 |

Table 3.

| (a) | Sample ID | TBC (x10 ⁶ cells mL ⁻¹) | LNA (x10 ⁶ cells mL ⁻¹) | HNA (x10 ⁵ cells mL ⁻¹) | TVC (CFU mL ⁻¹) | VA (CFU mL ⁻¹) | VP (CFU mL ⁻¹) | VC (CFU mL ⁻¹) | TC (CFU mL ⁻¹) | EC (CFU mL ⁻¹) |
|---------|-----------|---|---|---|--------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| | SW | 44.1±18.7 | 28.1±6.8 | 161±119 | 290±99 | ND | 10 | ND | ND | ND |
| Surface | TSTP1 | 2.46±0.08 | 1.57±0.04 | 8.92±0.35 | 683±32 | 55±7 | ND | ND | 15±7 | ND |
| | TSTP2 | 2.67±0.27 | 1.86±0.19 | 8.15±0.80 | 1.7±0.1 x10 ³ | 55±21 | 10±14 | ND | 15±21 | ND |
| | TSTP3 | 1.11±0.04 | 0.69±0.02 | 4.24±0.21 | 2.2±1.3 x10 ³ | 70±21 | 15±7 | ND | ND | ND |
| | TSTP4 | 1.0±0.14 | 0.65±0.11 | 3.45±0.26 | 1.4±0.04 x10 ³ | 10 | ND | ND | 5±7 | ND |
| | TSTP5 | 0.52±0.05 | 0.35±0.03 | 1.64±0.2 | 7.8±0.3 x10 ³ | 5±7 | 5±7 | ND | 5±7 | ND |
| | TSTP6 | 0.62±0.07 | 0.42±0.05 | 1.97±0.21 | 32.2±0.02 x10 ³ | 5±7 | ND | ND | 15±7 | ND |
| Bottom | TSTP1 | 2.53±0.03 | 1.78±0.02 | 7.48±0.01 | 1.1±0.02 x10 ³ | 38±2 | 10±14 | ND | ND | ND |
| | TSTP2 | 2.23±2.14 | 1.74±1.51 | 4.96±0.63 | 2.4±0.4 x10 ³ | 80±14 | 45±21 | ND | 12±12 | ND |
| | TSTP3 | 2.04±0.18 | 1.33±0.10 | 7.09±0.08 | 4.7±0.01 x10 ³ | 90±0 | 10 | ND | ND | ND |
| | TSTP4 | 2.02±0.33 | 1.34±0.16 | 6.80±1.72 | 4.4±0.01 x10 ³ | 255±14 | 10±14 | ND | 5±7 | ND |
| | TSTP5 | 0.38±0.04 | 0.24±0.02 | 1.45±0.19 | 8.3±0.3 x10 ³ | 10±14 | 5±7 | ND | 10±0 | ND |
| | TSTP6 | 0.78±0.07 | 0.48±0.04 | 2.91±0.29 | 5.5±0.1 x10 ³ | ND | ND | ND | ND | ND |
| (b) | Sample ID | TBC (x10 ⁶ cells mL ⁻¹) | LNA (x10 ⁶ cells mL ⁻¹) | HNA (x10 ⁵ cells mL ⁻¹) | TVC (CFU mL ⁻¹) | VA (CFU mL ⁻¹) | VP (CFU mL ⁻¹) | VC (CFU mL ⁻¹) | TC (CFU mL ⁻¹) | EC (CFU mL ⁻¹) |
| | SW | 44.1±18.7 | 28.1±6.8 | 161±119 | 290±99 | ND | 10 | ND | ND | ND |
| Surface | TSTS1 | 0.53±0.01 | 0.39±0.005 | 1.46±0.07 | 80±14 | 20±14 | 5±7 | ND | 5±7 | ND |
| | TSTS2 | 0.45±0.07 | 0.31±0.04 | 1.36±0.37 | 3.6±0.1 x10 ³ | 175±141 | 20±14 | ND | 60 | ND |
| | TSTS3 | 0.50±0.05 | 0.31±0.02 | 1.92±0.26 | 6.6±0.4 x10 ³ | 20 | 5±7 | ND | 5±7 | ND |
| | TSTS4 | 0.47±0.05 | 0.31±0.03 | 1.57±0.20 | 915±92 | ND | ND | ND | 5±7 | ND |
| | TSTS5 | 0.63±0.01 | 0.42±0.07 | 2.13±0.06 | 6.7±0.3 x10 ³ | 5±7 | ND | ND | ND | ND |
| | TSTS6 | 1.45±0.001 | 1.18±0.0001 | 2.78±0.009 | 1.8±0.01 x10 ³ | ND | 5±7 | ND | ND | ND |
| | DW | 26.2±1.12 | 16.1±0.04 | 102±10.9 | 2.5±0.2 x10 ³ | ND | ND | ND | ND | ND |

TBC – Total bacterial count; LNA – Low nucleic acid cells; HNA – High nucleic acid cells; TVC – Total viable count; VA – *Vibrio alginolyticus*; VP – *V. parahaemolyticus*; VC – *Vibrio cholerae*; TC – Total coliforms; EC – *Escherichia coli* O157: H7; ND – Not detected; CFU mL⁻¹ – Colony forming units per millilitre.

Table 4

| Sample ID | Number of species OTUs | Number of sequences | Shannon Diversity (H') | Simpson Index (1- λ) |
|---------------|------------------------|---------------------|------------------------|-------------------------------|
| SW | 76 | 8419 | 3.005 | 0.884 |
| TSTP2_Surface | 153 | 11343 | 3.829 | 0.947 |
| TSTP4_Surface | 151 | 13305 | 3.875 | 0.959 |
| TSTP6_Surface | 147 | 29516 | 3.062 | 0.875 |
| TSTP2_Bottom | 88 | 7232 | 2.774 | 0.804 |
| TSTP4_Bottom | 102 | 7224 | 3.403 | 0.928 |
| TSTP6_Bottom | 100 | 9785 | 3.384 | 0.932 |
| TSTS2_Surface | 91 | 4656 | 3.397 | 0.926 |
| TSTS4_Surface | 91 | 4607 | 3.454 | 0.929 |
| TSTS6_Surface | 113 | 10857 | 3.558 | 0.945 |
| DW | 65 | 6414 | 3.14 | 0.923 |

Supplementary Table 1

| Barcode name | Sample ID | Bases | ≥Q20 | Reads | Mean Read length (base pairs/bp) | Total bases obtained | Total reads |
|---------------------|------------------|--------------|-------------|--------------|---|---------------------------------|--------------------|
| IonXpress 002 | SW | 19,749,208 | 17,279,305 | 103,396 | 191 | 311,984,460 | 1,795,874 |
| IonXpress 004 | TSTP2_Surface | 58,067,723 | 51,742,550 | 297,161 | 195 | | |
| IonXpress 008 | TSTP4_Surface | 58,778,824 | 52,335,706 | 299,701 | 196 | | |
| IonXpress 011 | TSTP6_Surface | 63,804,743 | 57,226,502 | 321,145 | 199 | | |
| IonXpress 005 | TSTP2_Bottom | 20,581,162 | 17,931,573 | 106,182 | 194 | | |
| IonXpress 009 | TSTP4_Bottom | 23,253,054 | 20,411,880 | 116,784 | 199 | | |
| IonXpress 012 | TSTP6_Surface | 23,439,341 | 20,623,605 | 118,722 | 197 | | |
| IonXpress 006 | TSTS2 | 21,934,122 | 19,201,426 | 111,711 | 196 | | |
| IonXpress 010 | TSTS4 | 21,289,440 | 18,531,114 | 109,846 | 194 | | |
| IonXpress 013 | TSTS6 | 22,885,469 | 20,142,780 | 116,302 | 197 | | |
| IonXpress 003 | DW | 18,852,267 | 16,558,019 | 94,924 | 199 | | |