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Characterisation of Microbial Community in Ballast Water

Lakshmi Eleyadath*, Priya M and Sivanandan Achari V

Department of Environmental Studies, Cochin University of Science and Technology, Kerala, India

***Corresponding author:** Lakshmi Eleyadath, Department of Environmental Studies, Cochin University of Science and Technology, Kerala, India, Tel: 9495675644; E-mail: dr.e.lakshmi@gmail.com

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Abstract

Majority of the trade around the world is transferred through ships. Buoyancy in ships is maintained by filling in ballast water. Ships during its voyage discharge invertebrates, plants, microorganisms, non-indigenous vertebrates along with the ballast water, creating an ecological imbalance, threatening the naturally evolved biodiversity in the ports. Here, in this study ballast water samples were collected from a ship running in the offshore waters of Indian ocean near Mosel Bay port in South Africa.

Both chemical and microbiological parameters were analyzed for the ballast water samples. On characterization, 5 bacterial strains were isolated. Microbial analysis showed that there is presence of coliform bacteria. From this study alone we cannot conclude that conducting ballast water exchange is not sufficient to meet the D2 standard. More studies must be done to come to conclusion that, with Ballast Water Exchange (BWE), ballast water treatment or with combination of both, D2 standard can be met.

Keywords: Ballast water; Ballast Water Exchange (BWE); Bio-invasion; Characterization; Marine environment

Introduction

Almost two thirds of traded goods worldwide are transported by ship [1]. To ensure ship buoyancy, stability and maneuverability, oceangoing ships need ballast water. Based on estimation, the world seaborne trade in 2013 amounted to 9.35 billion tons of cargo and the global ballast water discharges in 2013 are estimated to about 3.1 billion tonnes [2].

Ballast water in ships contains different invertebrates, non-indigenous vertebrates, phytoplanktons, zooplanktons and microorganisms. One of the major concerns regarding the ballast water is the introduction of these organisms which can be invasive and pathogenic to the new environment [3]. Species transfer by ballast water concerns the spreading of organisms with potentially harmful effects on human health, such as toxin releasing algae or pathogenic bacteria. Marine bio invasion by ballast water threatens the endemic biodiversity creating an ecosystem imbalance. Most of the organisms will not survive longer transit through ballast water, but some smaller organisms like microorganisms survive. Salinity, dissolved oxygen and temperature are the major limiting factors affecting the survival of organisms [4]. If the introduced environment is conducive, organisms will survive and become dominant destroying the endemic organisms. Majority of the ships carry out ballast water exchange for small voyage and it is observed that there is higher probability of organisms being transmitted through small transit. So, characterizing and identifying organisms that are transmitted and discharged to the port of call by the ship is very much important. Introduced species can be lethal to the ecology and socio-economic aspects of the new environment. Here, in this study ballast water samples were collected from a ship running in the offshore waters of Indian ocean near Mosel Bay port in South Africa [5].

Significance of the work

Shipping traffic is one of many human activities in the ocean that has heavy impact on the marine environment. The effects include air pollution, noise pollution, oil spills and spread of invasive species [6]. The ships operating in the offshore sectors undergo ballast water exchange for prevention of bio-invasion through ballast water. During the exchange many organisms, especially invasive species enter the port environment creating an ecological imbalance, competition with the endemic and other already stressed organisms. According to the 2004 convention on ballast water, vessels' operating in the sea has to comply with the D2 standard. IMO in its latest declaration has notified that by 2024 all the ships operating in the sea has to comply with the D2 standard [7]. So, characterization of ballast water is very much important to understand the effect of ballast water exchange used in the ship for an overall ballast water management. In this paper, we analyze the physicochemical parameters of ballast water and characterization of bacteria from ballast water using biochemical tests and 16S rRNA gene sequencing.

Materials and Methods

Sampling of ballast water

Ballast water samples were collected from a ship running in the offshore sector of Indian ocean waters near Mosel Bay port,

South African (Ship is a supply vessel to a nearby rig from the Mosel bay port, cannot reveal the name of the ship). (lat -34.1747° S, long-22.0834° E). The Indian Ocean occupies an area of 74.92 x 106 km^2 including the marginal seas [8]. Its average depth is 3873 m. It is a huge sea area ranging from Eastern Africa to Western Australia and bordered on the North bythe Asian subcontinent. The area between 25° N and 30° S latitude and between 40° E and 98° E longitude. Samples were collected from the ships pump outlet for discharge. Three samples were collected during 18/12/2018 (Sample A), 5/5/2019 (Sample B), 26/12/2019 (Sample C) from the same ship. Grab sampling was done to collect ballast water samples. Samples were transported to the laboratory in a sealed plastic container in an ice box within two days of sampling [9]. Physico-chemical analysis and microbiological analysis were done to the samples.

Physico-chemical analysis

Ballast water samples were analyzed for physico-chemical parameters [10]. pH, dissolved oxygen, conductivity, salinity and chloride parameters were analysed. Analysis was done based on procedures from American Public Health Association (APHA-Manual on water and waste water quality analysis), 2012.

Morphological characterization of isolated bacteria

To differentiate the bacteria between gram-positive or gram-negative, grams staining was done. Made a thin smear of culture on glass slides, dried the smear and heat fix, covered the smear one by one with crystal violet (60 sec), gram's iodine (60 sec), 95% ethanol (20 sec) and safranin (40 sec). Air dried the slides after washing with distilled water and observed under microscope [11].

Biochemical analysis

The biochemical tests was used to determine the ability of the organism to produce catalase test, coagulase, gram staining, urease, indole, methyl red test, Voges Proskauer test, citrate test for identification of isolates [12].

Microbiological analysis

Ballast water samples collected from the ship was used for the isolation and culture of bacteria. Pour plate was done for the characterization of ballast water using sea water agar kept at 37°C incubation for 48 hours. The colonies formed were isolated by streak plate using sea water agar as medium [13]. DNA was extracted from the isolated bacteria colony. The ballast water temperature during sampling was less than 26°C.

DNA extraction, PCR amplification of 16S rRNA sequencing

Cells were procured by centrifugation (12,000 x g for 1 min at 4°C) and chromosomal DNA was isolated from cell pellets. DNA was extracted from the isolated colonies using the genomic DNA kit (origin diagnostics and research) following the spin column protocol for isolation and purification of genomic DNA from cells. Quality and quantity of DNA extracts were estimated using UV spectrophotometer at a wavelength range of 260/280 nm. Optical Density (OD) of DNA extracts were measured and samples showing OD value between 1.7 and 2.0 were selected for PCR thermal amplification [14,15].

After quantification, good quality DNA extracts were stored at 20°C for performing molecular analysis. In order to amplify 16S rRNA gene sequence, PCR amplification kit, master mix (origin diagnostics and research) and 16S rRNA primer pair (27F 5'-AGAGTTTGATCCTGGCTCAG-3' (Forward primer) and 1514R 5'-AAGGAGGTGATCCAGCC-3' (reverse primer)) were used. PCR amplification was carried out with a 50 µl reaction mixture using a gradient thermal cycler (Bio-Rad, T-100). The reaction mixture consisted of 5 µl of template DNA (DNA extracts from samples), 2 µl of each primer (forward and reverse), 25 µl of PCR master mix with DNA polymerase (origin) and 16 µl dH_2O [16]. The thermal profile comprised of an initial denaturation of the DNA carried out at 95°C for 3 mins, followed by 34 repeats of denaturation at 95°C for 30 secs, annealing at 55°C for 30 secs and extension at 72°C for 30 secs, along with a final elongation carried out at 72°C for 7 mins [17]. PCR products exhibiting intense bands after agarose gel electrophoresis (1.5%) were analyzed to ensure their size with the help of a DNA ladder. These amplicons after gel documentation were selected and outsourced for purification and sequencing [18].

16S rRNA sequence analysis

Manual editing was done on chromatogram of 16S rRNA gene sequences using bio edit 7.0.9 to select evenly spaced peak regions without baselines. Basic Local Alignment Search Tool (BLASTn) was utilized for an initial screening of nucleotide sequences for identifying homologous sequences in public database like NCBI [19]. 16S rRNA sequences were aligned using Clustal X and phylogenetic tree based on Neighbour Joining (NJ) analyses with 1000 bootstraps were generated using MEGA X. In order to confirm the findings of NJ tree, pair wise sequence distance data based on Kimura 2 Parameter model was generated using the same software [20].

Results

General water quality

The physico-chemical characteristics pH, temperature, Eh, dissolved oxygen, total dissolved solids, conductivity and salinity of ballast water were analyzed and recorded (Table 1).

Table 1: Physico-chemical parameters analyzed for ballast water.

No.	Parameters	Results		
		Sample A (18/12/2018)	**Sample B (5/5/2019)**	**Sample C (26/12/2019)**
1	pH	7.85	8.04	7.62
2	Temperature (°C)	20	22	20
3	Eh (mV)	-44.1	-23.8	-40.1
4	Dissolved oxygen (mg/l)	8.59	8	8.41
5	Total dissolved solids (ppt)	52.62	24.79	50.21
6	Conductivity (mS)	56.45	26.53	54.32
7	Salinity (ppt)	27.54	26.92	27.32
8	*E.Coli*	Present	Present	Present

Coliform test

Ballast water samples showed the presence of coliform bacteria in all the samples analyzed (MPN method) and the result was confirmed with the streak plate confirmatory test (Figure 1 and Table 2).

Figure 1: Streak plate confirmatory test and MPN test for coliform bacteria.

Table 2: Result of MPN test for coliform bacteria.

Sample No.	24 hrs			48 hrs			MPN cfu/ml
	10 ml	**1 ml**	**0.1**	**10 ml**	**1.0 cqml**	**0.1 ml**	
Sample A (18/12/2018)	3	1	0	3	3	2	1100 cfu/ml
Sample B (5/5/2019)	3	3	2	3	3	2	1100 cfu/ml
Sample C (26/12/2019)	3	3	3	3	3	3	1100 cfu/ml

The biochemical tests was used to determine the ability of the organism to produce catalase test, coagulase, gram staining, motility test, nitrate reduction, shape, spore formation, urease, indole, Methyl Red test, voges-proskauer test, citrate test for identification of isolates [21]. From the tests the species identified are *Staphylococcus* sp, *Bacillus* sp, *Cyto bacillus* sp (Table 3 and Figure 2).

Table 3: Results of Biochemical test.

	BW 1	BW 2	BW 3	BW 4	BW 5
Gram staining	+ve	+ve	+ve	+ve	+ve
Voges-proskauer test	+ve	+ve	+ve	+ve	-ve
Urease test	-ve	-ve	-ve	-ve	-ve
Citrate test	+ve	+ve	-ve	+ve	+ve
Methyl red	+ve	+ve	+ve	+ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve
Species identified	*Bacillus cereus*	*Staphylococcus caprae*	*Cytobacillus kochii*	*Bacillus licheniformis*	*Bacillus amyloliquefaciens*

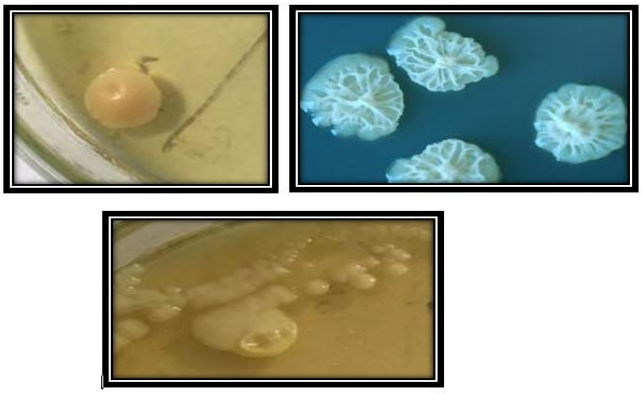

Figure 2: Bacteria isolated from the sample.

DNA barcoding, phylogenetic tree and genetic distance data

16S rRNA sequences were successfully generated for bacteria samples cultured from ballast water samples using the mentioned primer pair and thermal profile [22-25]. Homology of these nucleotide sequences with respect to the corresponding species in public database like National Centre for Biotechnology Information (NCBI) were evaluated using BLASTN annotations and they showed more than 99% identity score towards homologous sequences of *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus kochii* (*Cytobacillus kochii*) and *Staphylococcus caprae* [26].

Generated 16S rRNA sequences were submitted in National Center for Biotechnology Information (NCBI) database (Table 4). In order to perform molecular analysis, additional homologous 16S rRNA sequence of the above mentioned species were acquired from NCBI and their details are also given in Table 4 [27].

Table 4: Details regarding 16S rRNA sequences used for molecular analysis.

Sl No.	Species	Status	Accession number
1	*Bacillus cereus*	Generated for this study	MW287217
			MW287219
		Acquired from NCBI	D16266
2	*Bacillus amyloliquefaciens*	Generated for this study	MW287224
			MW287225
			MW287228
		Acquired from NCBI	HE610886
3	*Bacillus licheniformis*	Generated for this study	MW287223

		Acquired from NCBI	NR_074923
4	*Cytobacillus kochii*	Generated for this study	MW287222
		Acquired from NCBI	FN995265
5	*Staphylococcus caprae*	Generated for this study	MW287221
		Acquired from NCBI	NR_024665

Phylogenetic tree based on Maximum Likelihood (ML), 1000 bootstraps and Kimura 2 parameter and genetic distance data were generated using these sequences (Figure 3).

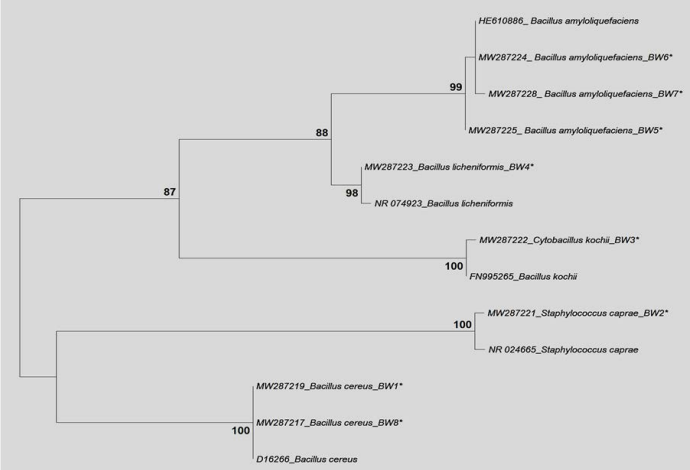

Figure 3: Maximum likelihood tree generated based on 1000 bootstraps (K-2-P Method). Bootstrap values less than 70 are not considered.

ML tree exhibited genetic congruency of the incorporated bacteria species as all of the homologous sequences got arrayed with respect to their speciation. From the top, first clade was formed by four sequences of Bacillus amyloliquefaciens within which three sequences (with accession numbers MW287224, MW287225 and MW287228) generated for this study got arrayed with its homologous sequence of *B. amyloliquefaciens* with accession number HE610886 (acquired from NCBI) with higher bootstrap value (99%). Second clade was formed by two sequences of *Bacillus licheniformis* within which, one sequence (MW287223) represented the generated one for this study while the second one (with accession number NR_074923), acquired from NCBI [28,29]. Their genetic congruency was evident from its higher bootstrap support (98%). Third clade was also formed by two sequences of *Cytobacillus kochii* (*Bacillus kochii*) (with accession numbers MW287222 (generated for this study) and FN995265 (acquired from NCBI) with 100% bootstrap support. Genetic congruency between generated (MW287221) and acquired (NR_024665) sequences of *Staphylococcus caprae* was witnessed in the fourth clade as they possessed a bootstrap value of 100 [30]. All the members of *Bacillus cereus* (MW287217, MW287219 and D16266) also exhibited genetic congruency which was evident from their bootstrap value (100%) [31].

Genetic distance data also showed lower intraspecific divergence within the selected species, confirming the findings of ML tree. For *Bacillus cereus*, there were no genetic distances within the generated sequences as well as the acquired one as the accounted intraspecific divergence was 0. In case of *B. amyloliquefaciens* intraspecific divergence ranged from 0 to 0.30% [32]. For *Bacillus licheniformis* and *Cytobacillus kochii* (*Bacillus kochii*), the maximum accounted intraspecific divergence was 0.10%. *Staphylococcus caprae* also exhibited intraspecific divergence up to 0.30%. However, all these intraspecific divergences were lower and contained under the threshold value of 98.6% for delineation of bacterial species [33]. As expected, interspecific divergence was higher for all the selected species. Intraspecific divergences are highlighted in yellow color (Table 5).

Table 5: Intraspecific divergence of the species.

MW287219-*Bacillus cereus*-BW1*												
MW287217-*Bacillus cereus*-BW8*	0											

D16266-*Bacillus cereus*	0	0										
MW287225-*Bacillus amyloliquefaciens* BW5*	0.073	0.073	0.073									
HE610886-*Bacillus amyloliquefaciens*	0.074	0.074	0.074	0.001								
MW287 224-*Bacillus amyloliquefaciens*-BW6*	0.074	0.074	0.074	0.001	0							
MW287228-*Bacillus amyloliquefaciens*-BW7*	0.075	0.075	0.075	0.003	0.001	0.001						
MW287223-*Bacillus icheniformis*-BW4*	0.077	0.077	0.077	0.023	0.025	0.025	0.026					
NR-074923-*Bacillus icheniformis*	0.078	0.078	0.078	0.025	0.026	0.026	0.028	0.001				
MW287222-*Cytobacillus kochii*-BW3*	0.093	0.093	0.093	0.081	0.081	0.081	0.083	0.063	0.065			
FN995265-*Bacillus kochii*	0.091	0.091	0.091	0.079	0.079	0.079	0.081	0.062	0.063	0.001		
MW287 221-*Staphyl ococcus caprae*-BW2*	0.087	0.087	0.087	0.11	0.11	0.11	0.112	0.108	0.11	0.109	0.111	

NR-024665- *Staphyl ococcus caprae*	0.087	0.087	0.087	0.11	0.11	0.11	0.112	0.108	0.11	0.109	0.11	0.003

Discussion

The general water quality analysis of ballast water shows that the water is alkaline or neutral with dissolved oxygen value ranging from 8.0 mg/l to 8.59 mg/l. Five bacterial species have been identified from both biochemical analysis and 16S rRNA sequencing [34]. Identified species are *Bacillus cereus*, *Staphylococcus caprae*, *Cytobacillus kochii*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. From the analysis, it is observed that Bacillus is the dominant species identified from the sample.

The identified bacteria *Bacillus cereus*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* are mostly found in soil and marine waters. *Bacillus cereus* is found to have close identity with *Bacillus thurigiensis* whereas *Bacillus amyloliquefaciens* and *Bacillus licheniformis* is found to have identity, similar to *Bacillus sub ilis* [35]. The distinction of bacteria to the one identified from the ballast water sample was confirmed from the biochemical tests and 16S rRNA gene sequencing. Using 16S rRNA sequencing the notable differences in the number and location of prophages, transposable elements were identified to distinguish species from each other.

Initially from sequencing the isolated bacteria, 7 species were identified. Those two species other than 5 identified species were *Bacillus sub ilis* and *Bacillus velenzensis*. The species was found to have similarity with *Bacillus amyloliquefaciens*. On thorough analysis it was found that those isolates were mimicking the identity of B*acillus amyloliquefaciens [*36]. The ambiquity in identifying the *Bacillus amyloliquefaciens* from *Bacillus velenzensis* and *Bacillus sub ilis* has been reported by Sacchi et al, 2002. In his study found that one small difference in one base between sequences or partial matches at a single nucleotide position in the 16SrRNA gene is important for species differentiation [37]. They found that the three species *Bacillus amyloliquefaciens*, *Bacillus Velenzensis* and *Bacillus sub ilis* are different species and 16S rRNA has the potential to differentiate strains at the sub species level.

Within the selected species, the genetic distance data showed lower intraspecific divergence, which is confirmed by the ML tree findings. The intraspecific divergences were lower and contained under the threshold value of 98.6% for delineation whereas interspecific divergence was higher for the species identified [38].

The ballast water sample collected during sampling was having less than 26°C temperatures. The culturing and isolation of the colonies were done using sea water agar and was incubated at 37°C [39]. The low percentage of bacteria count from the sample can be corroborated to the difference inincubation temperature during culturing to that of the ballast water sample temperature [40].

The Ship vessel from which sample was collected, perform ballast water exchange to prevent bio invasion. All the species identified from the sample are within the 50 µm size (0.25 µm to 15 µm) [41]. According to the 2004 convention on ballast water, the vessels should meet the D2 standard that is, presence of less than 10 viable organisms with less than 50 µm size.

Quantitative estimation of *E. coli* was done using MPN method (Table 2). From the analysis, it is observed that all 3 samples had the presence of coliform in the range of 1100 cfu per 100 ml. According to the IMO regulations the group of specified indicator bacteria should be: <1 colony forming unit $100.ml^{-1}$ of Vibrio cholera; <250 cfu.100 ml^{-1} of *Escherichia coli* and <100 cfu·100 ml^{-1} of intestinal Enterococci during discharge of ballast water. IMO regulation D-2, 'ballast water performance standard' states that indicator microbes in water discharged by ships shall not exceed specified concentrations. These include, but are not limited to: *Vibrio cholera*, *Escherichia coli* and intestinal enterococci.

Conclusion

From the ballast water sample analysis 5 bacterial species were identified. *Bacillus cereus* strain, *Staphylococcus caprae* strain, *Bacillus Kochii* strain CN20-4, *Bacillus licheniformis* strain TB 212, *Bacillus amyloliquefaciens* strain. The analysis shows the presence of coliform bacteria. Depending on the bacterial profile we can understand the history of ballast water. The study shows that the Ballast Water Exchange (BWE) has not restricted the invasion or presence of bacteria in the ballast water while discharging. This study alone does not validate that the BWE is not an appropriate method for ballast water management. More analysis of samples from ships running in different sea waters and ports needs to be done to come to conclusion that whether BWE, some other ballast water treatment method or a combination of both is appropriate to meet the D2 standard for ships with short voyages.

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There is no funding source available for the study.

Conflicts of Interest

There is no conflict of interest

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