Changes in Marine Bacterial Populations in Ballast Water and Sediment of a LNG Carrier Bound for Qatar from Japan

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Abstract

Changes in colony-forming eutrophic marine bacterial populations in a ship's ballast water and sediment were examined for 7 voyages in 2005 to 2006. In the ballast water taken in Japan, total numbers of colony-forming cells were highly variable in the range of $10^{3.1}$ to $10^{5.6}$ colony-forming units (CFU) ml^{-1} regardless of the season. The ballast water loaded in Japan was exchanged in the high seas in the Indian Ocean. During the exchange of ballast water, the values in the samples taken from discharging and reloading ballast water varied from $10^{3.2}$ to $10^{4.7}$ CFU ml^{-1} and $10^{2.7}$ to $10^{5.2}$ CFU ml^{-1} , respectively. When the reloaded ballast water was discharged at the Ras Laffan port in Qatar, the values varied from $10^{2.6}$ to $10^{5.1}$ CFU ml^{-1} . These results indicate that the ballast water exchange in the high seas is not effective as for the reduction of marine bacterial populations in ballast water. In the samples of sediment, the values between $10^{5.2}$ and $10^{7.2}$ CFU ml^{-1} were estimated for all seasons in which voyages took place, indicating the importance of management of sediment in ballast tanks as well as ballast water of ocean going vessels.

As for the numbers of *Vibrio* sp. cells, which made colonies on the selective agar plate at 20°C, in the ballast water and sediment samples, the values varied from zero to $10^{5.4}$ CFU ml^{-1} , and so far examined, we did not detect pathogenic *Vibrio cholerae*, which can grow even at 37°C, throughout all the seven voyages.

Keywords : ship's ballast water, sediment, colony-forming marine bacteria, *Vibrio* sp.

1. Introduction

The ballast water management of ocean going vessels is very important for the conservation of marine biodiversities and the diffusion prevention of the infectious diseases caused by pathogenic *Vibrio cholerae* invaded in a ship's ballast water⁽¹⁾. The discharged ballast water at a foreign port seems to give some economical damage to the fish farming because a ship's ballast water is possibly contained some toxic dinoflagellates⁽²⁻⁴⁾.

The European zebra mussel, the European shore crab, the fishes of Round Goby and Ruffe, the tropical green algae, the Northern Pacific kelp, the Northern Pacific seastar, the American comb jelly, and the giant fan worm have been pointed out as another major invasive species by the International Maritime Organization (IMO)⁽⁵⁾. In those species, the European zebra mussel has spread most successfully⁽⁶⁾.

We have previously examined ballast water and sediment in ballast tanks of a liquefied natural gas (LNG) carrier (110,000 gross tons) bound for Qatar from Japan for six voyages throughout 2002 to 2003 in relation to the changes in marine bacterial populations and pathogenic *V. cholerae*⁽⁷⁾. Through the experiments, we found that the colony-forming marine bacterial populations were highly valuable in ballast water loaded on and discharged from the ship. Any of *V. cholerae* was not detected from all the samples.

In ballast tanks, some of marine bacteria could survive by making biofilm⁽⁸⁾ as well as in pooled ballast water and sediment on the bottom of ballast tanks⁽⁷⁾.

In this paper, changes in populations of colony-forming marine bacteria and pathogenic *V. cholerae* were examined in the LNG carrier's ballast water and sediment. In addition, we investigated the total number of temperature sensitive marine *Vibrio* species cells, which made colonies on the selective plate at

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20°C, but not at 37°C, in those samples.

2. Materials and Methods

2.1 Navigation route of the LNG carrier

The navigation route between Japan and Qatar is shown in Fig. 1. It takes approximately two weeks to go from a port in Japan to the Ras Laffan port in Qatar. The LNG carrier used in the experiments has one forward- and one after-peak ballast tanks and ten side ballast tanks located tandem on the starboard and port sides. Total capacity of the tanks is 55,000 m³.

Fig. 1. Navigation route between Qatar and Japan.

2.2 Sampling of ballast water and sediment on board

Ballast water was loaded on the ship at four different local ports such as the Osaka port (twice), the Yokkaichi port (twice), the Niigata port (twice), and the Himeji port (once) before leaving for Qatar (Fig. 2). When ballast water was loaded aboard, it was sampled at given time.

Fig. 2. The local ports where ballast water was loaded prior to leaving for Qatar.

After arriving in the high seas in the Indian Ocean, the ballast water, which had been taken at the original port, was discharged prior to re-loading ballast water (Fig. 3). The exchange of ballast water was continuously carried out for each ballast tank. The ballast

Fig. 3. Overview of ballast water exchange area in the high seas on the way to Qatar from Japan.

Table 1. Ballast water exchange sites in the high seas

Times of voyages and sampling date	Ballast water exchange site
1. January 26, 2005	05-56N, 089-06E (Discharging site) 05-50N, 087-16E (Reloading site)
2. May 7, 2005	05-59N, 089-59E (Discharging site) 05-59N, 089-34E (Reloading site)
3. September 27, 2005	05-34N, 091-59E (Discharging site) 05-35N, 091-28E (Reloading site)
4. October 31, 2005	05-34N, 089-36E (Discharging site)
4. November 1, 2005	05-33N, 084-48E (Reloading site)
5. February 5, 2005	05-48N, 092-58E (Discharging site) 05-35N, 091-52E (Reloading site)
6. April 10, 2006	05-34N, 089-46E (Discharging site) 05-34N, 087-34E (Reloading site)
7. August 17, 2006	05-34N, 083-34E (Discharging site) 05-35N, 082-45E (Reloading site)

water discharged from and re-loaded on the ship was sampled at given time.

It took approximately 10 days to reach the high seas in the Indian Ocean. The area was located in 5 degrees north and 82 - 93 degrees east. The exact site where the ballast water was exchanged for each voyage is shown in Table 1.

The IMO requires that ballast water be exchanged in the high seas, i.e., the open ocean at least 50 nautical miles from the nearest land and 200 meters in depth⁽⁹⁾.

The ballast water exchanged in the high seas was transported and discharged at the Ras Laffan port in Qatar. The ballast water discharged was sampled at given time.

Sampling of ballast water was carried out at the outlet of the ballast pump located in the engine room. After the sampled ballast water was poured into an

autoclaved bottle (250 ml, NALGENE®), it was stored in the refrigerator at 4°C until use.

On the return trip from Qatar to Japan, no ballast water was in the ballast tanks because LNG was fully loaded on board; therefore, sediment in ballast tanks was available to sample. The samples of sediment, which were taken from several sites in a ballast tank, were placed into each autoclaved bottle (250 ml, NALGENE®) and kept at 4°C until use.

Seven to 10 days after departing Japan, a surface water and a middle-depth water at 6 - 8 m from the surface of the ballast water in a tank were sampled into a Van Dorn Water Sampler (5026, RIGOSHA&Co., Ltd.) and then poured into the autoclaved bottle (250 ml, NALGENE®). The samples thus obtained were stored at 4°C until use.

2.3 Enumeration of colony-forming marine bacteria in ballast water and sediment

The samples, if necessary, were serially diluted with autoclaved natural seawater, and then the diluted sample (0.1 ml) was spread onto the agar plates containing 5 g Bacto peptone (Difco, Michigan, USA), 1 g yeast extract (Difco, Michigan, USA), and 15 g agar per liter of natural seawater. Incubation was carried out at 20°C for 6 days, and colonies made on the plates were counted. The number of colony-forming cells was shown as colony-forming units (CFU) ml⁻¹.

2.4 Enumeration of colony-forming marine *Vibrio* sp.

A thiosulphate-citrate-bile salts-sucrose (TCBS) selective medium (Pearlcore TCBS Agar "Eiken," Eiken Chemical Co., Ltd.) was used for counting colony-forming marine *Vibrio* sp. The TCBS selective medium contained 10 g peptone, 5 g yeast extract, 20 g sucrose, 5 g bile powder, 3 g sodium cholate, 10 g NaCl, 10 g sodium citrate, 7 g sodium thiosulfate, 1 g iron (III) citrate, 0.04 g bromothymol blue, 0.04 g thymol blue, and 15 g agar per liter of distilled water, pH 8.2. After serial dilution with autoclaved natural seawater, if necessary, the sample (0.1 ml) was spread onto TCBS selective agar plates, and incubation was carried out at 20°C for two days.

2.5 Identification of isolates that made yellow-colored colonies on TCBS selective agar plate

The isolates that made colonies on TCBS selective agar plates even at 37°C were identified based on their

morphological, biochemical, and physiological properties. Identification based on the partial 16S rDNA sequences was also carried out for the isolates. It was performed at the National Collections of Industrial, Food, and Marine Bacteria Japan Co., Ltd. (Shimizu, Shizuoka). Briefly, a partial 16S rDNA gene fragment (about 500 bp) at 5'-end was amplified by PCR with the MicroSeq® 500 16S rDNA kit (Applied Biosystems Japan), and the amplified gene fragment was sequenced with a sequencer. Homology analysis was carried out by the use of sequencing data with MicroSeq® Microbial Identification System Software and the database of MicroSeq® Bacterial 500 Library (Applied Biosystems, CA, USA) and GenBank/DDBJ/EMBL. When the partial rDNA sequence obtained from the isolate showed the homology percentage of less than 97% in comparison with those in database, it was deposited in GenBank/DDBJ/EMBL.

3. Results and Discussion

3.1 Changes in colony-forming marine bacterial populations in ballast water loading and discharging aboard

Ballast water was sampled when it was loaded aboard at an original port in Japan, discharged and re-loaded in the high seas in the Indian Ocean, and discharged at the Ras Laffan port in Qatar (Fig. 4). Regardless of the sampling time and season, variable numbers of colony-forming cells were observed.

As for the numbers of colony-forming cells in the samples taken at the original port in Japan, the values varied, and the minimum and the maximum values obtained were 10^{3.1} CFU ml⁻¹ in September 2005 (Fig. 4C, the third voyage) and 10^{5.6} CFU ml⁻¹ in October 2005 (Fig. 4D, the fourth voyage), respectively, throughout all the voyages. When the ballast water, which had already been loaded in Japan, was discharged in the high seas, the value varied from 10^{3.2} CFU ml⁻¹ in October 2005 (Fig. 4D, the fourth voyage) to 10^{4.7} CFU ml⁻¹ in August 2006 (Fig. 4G, the seventh voyage). After the ballast water was discharged, new ballast water was re-loaded in the high seas; the number of colony-forming cells varied from 10^{2.7} CFU ml⁻¹ in February 2006 (Fig. 4E, the fifth voyage) to 10^{5.2} CFU ml⁻¹ in November 2005 (Fig. 4D, the fourth voyage). At the Ras Laffan port, the re-loaded ballast water was sampled at given time when it was discharged. Again, the value varied from 10^{2.6} CFU ml⁻¹ in February 2006 (Fig. 4E, the fifth voyage) to 10^{5.1} CFU ml⁻¹ in May 2005 (Fig. 4B, the second voyage).

Fig. 4. Changes in the number of colony-forming cells in ballast water while it was loaded on and discharged from the ship. Ballast water was loaded at the Osaka port in January 2005 (A, the first voyage), the Yokkaichi port in April 2005 (B, the second voyage), the Niigata port in September 2005 (C, the third voyage), the Himeji port in October 2005 (D, the fourth voyage), the Osaka port in January 2006 (E, the fifth voyage), the Yokkaichi port in March 2006 (F, the sixth voyage), and the Niigata port in August 2006 (G, the seventh voyage). Ballast water was sampled when it was loaded at a port in Japan (open circle), discharged (closed circle) and re-loaded (open triangle) in the high seas, and discharged from the ship at the Ras Laffan port in Qatar (closed triangle). Enumeration of colony-forming cells in the samples was carried out twice for each sample, and the averaged value is shown here. The difference between the individual and the averaged values was less than 0.2 log cycle for all the samples.

We compared the variation of the number of colony-forming cells in ballast water in relation to the sampling time. For the ballast water taken in Japan, the maximum difference was $10^{1.3}$ CFU ml⁻¹, which was obtained from the fourth voyage (Fig. 4D). The maximum value of $10^{1.4}$ CFU ml⁻¹ (Fig. 4D) was obtained from the ballast water samples taken from discharged ballast water in the high seas. While ballast water was re-loaded, the value was $10^{1.3}$ CFU ml⁻¹ (Fig. 4E). For the samples taken at the Ras Laffan port, the value was $10^{1.2}$ CFU ml⁻¹ (Fig. 4A).

The number of colony-forming cells varied in ballast water samples when ballast water was taken aboard at the original port and the Indian Ocean as well, indicating that the distribution of marine bacteria in seawater is heterogeneous.

Regardless of the exchange of ballast water in the high seas (Fig. 3 and Table 1), the number of colony-forming cells in ballast water discharged at the Ras

Laffan port varied considerably and remained nearly identical to that in the samples taken in Japan.

In relation to the changes in the number of colony-forming marine bacterial populations, the poor effectiveness for the exchange of ballast water in the high seas was consistent with the results reported by Drake et al.⁽¹⁰⁾. However, the exchange of ballast water acts well as for the reduction of the number of planktonic cells in ballast water taken in Japan. In the previous data, about 10^2 planktonic cells in one ml of the ballast water, which had been taken in spring 2002 in Japan, was reduced to less than one-tenth by the exchange of ballast water in the high seas located in the Indian Ocean⁽¹¹⁾.

3.2 Changes in colony-forming marine bacterial populations in ballast water stored in a ballast tank

In the four ports of call such as Himeji, Osaka, Yok-

kaichi, and Niigata ports, ballast water was loaded. The pooled ballast water was originated from one of four ports. We believe that the quality of seawater around the coastal area of Japan is roughly the same. Actually, Himeji and Osaka ports are closely located in the Inland Sea. Comparing each data seems to be available primarily.

There were seasonal variations in the number of colony-forming cells in the samples obtained from the pools of ballast water in the ballast tanks (Fig. 5). The numbers of colony-forming cells in the summer samples taken from the surface of the body of water in September 2005 and August 2006, were $10^{4.9}$ and $10^{5.0}$ CFU ml⁻¹, respectively. On the other hand, the winter sample taken from the surface of the body of water in January 2005 contained $10^{3.3}$ CFU ml⁻¹. The difference of the values between the surface of the body of water and the middle depth of water taken at the same voyage was within a $10^{0.5}$ CFU ml⁻¹ for all the samples. Wave action during the voyage might contribute to diffuse marine bacteria within the ballast tanks.

Fig. 5. Seasonal variations in the number of colony-forming cells in the ballast water stored in a ballast tank. The surface (open column) and a 6-8 m depth water in ballast tank (closed column) were taken from the ballast water stored in a ballast tank. Experiments were carried out twice for each sample, and the averaged value is shown here. The difference between the individual and the averaged values was less than 0.2 log cycle for all the samples.

3.3 Total number of colony-forming marine bacterial populations in sediment in ballast tanks.

As for the number of colony-forming cells in sediment in the ballast tanks, a 5 - 7 log cycle of the value was estimated on the basis of the water content in the sample (Table 2). The values varied regardless of the season in which the voyage took place. All the values estimated were higher than those obtained from the pools of ballast water in ballast tanks.

Marine bacterial growth in sediment seems to be

easier than that in ballast water because dead marine small living things, in general, precipitate on the bottom of ballast tanks, and marine bacteria can utilize some biomaterials composed of the dead cells as nutrients for their growth. Therefore, the sediment management as well as that of ballast water is important⁽⁷⁾.

3.4 Changes in populations of colony-forming marine *Vibrio* sp. in the ballast water and sediment in ballast tanks

Total numbers of viable cells, which made colonies on TCBS selective plates at 20°C, are shown in Table 3. For the numbers of colony-forming marine *Vibrio* sp. cells in the samples of ballast water, the values varied from 0-2300 CFU ml⁻¹ in January to February 2005 (the first voyage), 0-340 CFU ml⁻¹ in April to May 2005 (the second voyage), 0-1980 CFU ml⁻¹ in September to October 2005 (the third voyage), 0-560 CFU ml⁻¹ in October to November 2005 (the fourth voyage), 0-140 CFU ml⁻¹ in January to February 2006 (the fifth voyage), 0-420 CFU ml⁻¹ in March to April 2006 (the sixth voyage), and 0-40 CFU ml⁻¹ in August 2006 (the seventh voyage) on the way to Qatar from Japan.

Colony-forming marine *Vibrio* sp. cells were not detected from the samples taken at re-loading ballast water in the high seas and discharging it at the Ras Laffan port throughout the five voyages out of seven voyages, except for the first and the second voyages. As for the reduction of the number of *Vibrio* sp. cells in ballast water, the exchange of ballast water seems to work effectively.

As for the samples of sediment taken on the return trip to Japan, the values varied: 0-843 CFU ml⁻¹ (the first voyage), 0-8048 CFU ml⁻¹ (the second voyage), 165-331 CFU ml⁻¹ (the third voyage), 0-1831 CFU ml⁻¹ (the fourth voyage), 13275-322708 CFU ml⁻¹ (the fifth voyage), 1750-268215 CFU ml⁻¹ (the sixth voyage), and 0-5866 CFU ml⁻¹ (the seventh voyage).

The populations of the yellow-colored colonies were relatively small in total number of colonies on TCBS selective plate for both samples taken from ballast water and sediment.

3.5 Effect of storage period of sampled ballast water on the population changes

The ballast water sample taken aboard at the Himeji port in Japan on October 21, 2005 (the fourth voyage) was used in this experiment. When the sample was arrived in the laboratory, 39 days had already passed

Table 2. Total number of colony-forming cells in the sediment of ballast tanks.

Times of voyages and sampling date	Colony-forming cells (log (CFU) ml ⁻¹) ^a	Sampling site
1. February 14, 2005	5.2 - 6.2 (5 samples)	No. 8 ballast tank (port side)
2. June 1, 2005	5.7 - 6.1 (4 samples)	No.2 ballast tank (starboard side)
3. October 15, 2005	5.9 - 6.5 (5 samples)	No. 9 ballast tank (port side)
4. November 16, 2005	6.7 - 7.2 (5 samples)	No.2 ballast tank (starboard side)
4. November 17, 2005	6.2 - 6.7 (2 samples)	No.8 ballast tank (starboard side)
5. February 21, 2006	6.3 - 6.6 (4 samples)	No. 5 ballast tank (starboard side)
5. February 21, 2006	5.9 - 6.6 (4 samples)	No. 6 ballast tank (starboard side)
6. April 21, 2006	5.7 - 6.4 (3 samples)	No. 8 ballast tank (starboard side)
6. April 21, 2006	5.3 - 5.9 (3 samples)	No. 9 ballast tank (starboard side)
7. September 2, 2006	6.0 - 7.0 (5 samples)	No. 4 ballast tank (starboard side)
7. September 2, 2006	5.6 - 6.4 (4 samples)	No. 8 ballast tank (starboard side)

^aSediment was taken from two to five different points in a ballast tank when the ship was carrying fully loaded LNG and sailing on the way back to Japan. The sediment (1 g) was diluted with distilled natural seawater (9 ml). Serial dilution, when necessary, was carried out, and the sample (0.1 ml) was spread onto non-selective agar plates. The number of viable cells were calculated on the basis of the water content of each sample. For example, the viable number of cells, 5.2 log(CFU) ml⁻¹, obtained from the sample in February 2005, was calculated by the following equation: $107 \text{ (number of the colonies on the plate)} \times (9.69/0.69) \times (10/1) \times (1/0.1) = 1.5 \times 10^5 \text{ CFU ml}^{-1}$, where 0.69 ml was the water content in 1 g of the sample. The minimum and the maximum values are shown here.

since the sample was taken aboard and stored. The number of colony-forming cells in the sample stored for 39 days was $10^{4.3}$ CFU ml⁻¹ (Fig. 4D and Fig. 6). The change of population in the sample was continuously examined until 117 days since the sample had been stored on board. Total number of colony-forming cells was linearly increased in response to the storage period. Consequently, the value just after the sample was taken aboard was estimated to be $10^{4.0}$ CFU ml⁻¹ by the extrapolation based on the changes in the numbers of colony-forming cells.

It was found that marine bacterial population gradually increased in the bottle in response to the storage period even when the sample was kept at 4°C, indicating that the data shown in this paper are maximally two times higher than those just after sampled ($10^{4.3}/10^{4.0} = 2$).

3.6 Identification of isolated *Vibrio* sp.

Pathogenic *V. cholerae* can make a yellow-colored colony on TCBS selective agar plate even at 37°C. Therefore, all the yellow-colored colonies on the TCBS selective agar plates at 20°C were picked up and streaked onto new plates, and the colony-forming

Fig. 6. Change in marine bacterial population in the ballast water sample in response to the days stored. The arrow shows the total days of 39 when the ballast water sample was arrived at the laboratory since the ballast water had been taken aboard at the Himeji port in Japan on October 21, 2005 (the fourth voyage) prior to leaving for Qatar.

Table 3. Number of colony-forming cells on TCBS selective plates.

Times of voyages and sampling date	Number of colony-forming cells (CFU ml ⁻¹) ^a	Sampling time and site
1. January 16, 2005	0 - 1400 (0)	While loading at the Osaka port in Japan
1. January 25, 2005	40 - 70 (0 - 10)	Surface of ballast water
1. January 25, 2005	50 - 70 (10 - 30)	7 m depth of ballast water
1. January 26, 2005	0 - 20 (0)	While discharging in the high seas
1. January 26, 2005	250 - 970 (10 - 50)	While reloading in the high seas
1. February 1, 2005	100 - 2300 (0 - 200)	While discharging at the Ras Laffan port in Qatar
1. February 14, 2005	0 - 843 (0)	Sediment in No. 8 side tank at the port side
2. April 24, 2005	0 - 40 (0 - 20)	While loading at the Yokkaichi port in Japan
2. May 5, 2005	270 - 340 (270 - 340)	Surface of ballast water
2. May 5, 2005	20 - 40 (0 - 10)	7 m depth of ballast water
2. May 7, 2005	0 - 30 (0)	While discharging in the high seas
2. May 7, 2005	0 - 20 (0)	While reloading in the high seas
2. May 21, 2005	0 - 180 (0)	While discharging at the Ras Laffan port in Qatar
2. June 1, 2005	0 - 8048 (0 - 7512)	Sediment in No. 2 side tank at the starboard side
3. September 17, 2005	60 - 1980 (0 - 60)	While loading at the Niigata port in Japan
3. September 26, 2005	0	Surface of ballast water
3. September 26, 2005	0	6 m depth of ballast water
3. September 27, 2005	0	While discharging in the high seas
3. September 27, 2005	0	While reloading in the high seas
3. October 5, 2005	0	While discharging at the Ras Laffan port in Qatar
3. October 15, 2005	165 - 331 (0 - 165) ^b	Sediment in No. 9 side tank at the port side
4. October 21, 2005	0 - 560 (0)	While loading at the Himeji port in Japan
4. October 30, 2005	0 - 10 (0)	Surface of ballast water
4. October 30, 2005	0	8 m depth of ballast water
4. October 31, 2005	0	While discharging in the high seas
4. November 1, 2005	0	While reloading in the high seas
4. November 10, 2005	0	While discharging at the Ras Laffan port in Qatar
4. November 16, 2005	0 - 432 (0 - 168) ^c	Sediment in No. 2 side tank at the starboard side
4. November 17, 2005	0 - 1831 (0 - 1549)	Sediment in No. 8 side tank at the starboard side
5. January 28, 2006	0	While loading at the Osaka port in Japan
5. February 5, 2006	40 - 140 (10 - 30)	Surface of ballast water
5. February 5, 2006	40 - 90 (0)	7 m depth of ballast water
5. February 5, 2006	0	While discharging in the high seas
5. February 5, 2006	0	While reloading in the high seas
5. February 11, 2006	0	While discharging at the Ras Laffan port in Qatar
5. February 21, 2006	13275 - 322708 (129 - 1952)	Sediment in No. 5 side tank at the starboard side
5. February 21, 2006	30057 - 49506 (0 - 1559)	Sediment in No. 6 side tank at the starboard side

6. March 31, 2006	0	While loading at the Yokkaichi port in Japan
6. April 9, 2006	410 - 420 (50 - 110)	Surface of ballast water
6. April 9, 2006	70 - 90 (0)	8 m depth of ballast water
6. April 10, 2006	0	While discharging in the high seas
6. April 10, 2006	0	While reloading in the high seas
6. April 16, 2006	0	While discharging at the Ras Laffan port in Qatar
6. April 21, 2006	1750 - 5531 (1750 - 5531)	Sediment in No. 8 side tank at the starboard side
6. April 21, 2006	160040 - 268215 (1482 - 9085) ^d	Sediment in No. 9 side tank at the starboard side
7. August 6, 2006	0	While loading at the Niigata port in Japan
7. August 15, 2006	0	Surface of ballast water
7. August 15, 2006	0	7 m depth of ballast water
7. August 17, 2006	0 - 40	While discharging in the high seas
7. August 17, 2006	0	While reloading in the high seas
7. August 23, 2006	0	While discharging at the Ras Laffan port in Qatar
7. September 2, 2006	1355 - 5866 (0 - 2406) ^e	Sediment in No. 4 side tank at the starboard side
7. September 2, 2006	0 - 765 (0 - 612)	Sediment in No. 8 side tank at the starboard side

^aTCBS selective plates were kept at 20°C for two days. Then total number of colonies on the plates were counted. For seawater samples, they were taken one to three times at given sampling time in a sampling site, and each sample was spread twice onto TCBS selective plates. The minimum and the maximum numbers of colonies are shown here. For sediment samples, which were taken from two to five different sampling points in a ballast tank, each sample of sediment (1 g) was diluted with autoclaved natural sea water (9 ml), and an aliquot (0.1 ml) was spread onto TCBS selective plates. For example, the viable number of cells, 843 ml⁻¹, obtained from the sample in February 2005, was calculated by the following equation: 6 (number of the colonies on the plate) × (9.69/0.69) × (1/0.1) = 843 CUF ml⁻¹, where 0.69 ml was the water content in 1 g of the sample. The minimum and the maximum numbers of colonies in sediment samples taken from a ballast tank are shown here. In parentheses, the minimum and the maximum values for yellow-colored colonies grown at 20°C were shown.

^bOne strain, which made a yellow-colored colony at 20°C on the TCBS selective plate, could grow at 37°C. The isolate was named as SB0307.

^cOne strain, which made a yellow-colored colony at 20°C on the TCBS selective plate, could grow at 37°C. The isolate was named as SB0407.

^dTotal five strains, which made yellow-colored colonies at 20°C, could grow at 37°C. One of those strains grew even at 45°C. The isolate was named as SB0610. For remaining 4 colonies, we named as SB0611, SB0612, SB0613, and SB0614.

^eTwo strains, SB0708 and SB0709, made yellow-colored colonies at 20°C on the TCBS selective plate, and could grow even at 37°C.

ability was checked at 37°C. As a result, we obtained seven isolates. They were identified based on the biochemical, physiological, and partial 16S rDNA sequences (Table 4). The partial 16S rDNA sequence of the isolate SB0307 showed a high homology percentage of more than 99% to that of the type culture of *V.*

alginolyticus (ATCC 17749) (99.6%), *V. parahaemolyticus* (ATCC17802) (99.6%), and *V. campbellii* (ATCC 25920) (99.6%), when homology analysis was carried out by using the database of GenBank/DBJ/EMBL. According to the molecular phylogenetic tree made by the neighbor-joining method, the isolate

SB0307 was gathered the same phylogenetic branch with *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802), where the bootstrap values for *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802) were the same as 59%. Since *V. parahaemolyticus* is not able to produce acid metabolites from sucrose, the color of colonies made on TCBS selective agar plate shows green. Consequently, the isolate SB0307 was identified to be *V. alginolyticus*.

As for the isolate SB0407, the partial sequence of 16S rDNA showed a highly homologous percentage of 95.6% to the type culture of *Photobacterium damsela* subsp. *damsela* (ATCC 33539). In general, the identification of species seems to be difficult when the homologous percentage in database is less than 97%, indicating that the isolate is a novel strain. The partial rDNA sequence of the isolate, SB0407, was deposited in the DDBJ/GenBank/EMBL under the accession number AB274303.

The isolate SB0610 showed 99.3% of sequence homology with *V. alginolyticus* (ATCC 17749) in database of GeneBank/DDBJ/EMBL. In the molecular phylogenetic tree, the isolate SB0307 was gathered the same phylogenetic branch with *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802), where the bootstrap values for *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802) were 48 and 39%, respectively. As described above, the color of colony for *V. parahaemolyticus* on

TCBS selective agar plate is green, so that the isolate SB0307 was identified to be *V. alginolyticus*.

All of the isolates, SB0611, SB0612, SB0613, SB0611, SB0708, and SB0709, were identified to be *V. harveyi*. The homologous percentage of the partial rDNA sequence for each isolate was 99.4% to *V. harveyi* (ATCC 35084) in the database of GenBank/DDBJ/EMBL, and the bootstrap value in the phylogenetic branch was to be 91%.

Pathogenic *V. cholerae* invaded into ballast water and sediment in ballast tanks is a major concern for public health of the people who are living around the coastal area⁽¹²⁾. Pathogenic *V. cholerae* O1 can survive in seawater more than 50 days⁽¹³⁾. Therefore, regulations adopted by IMO in 2004 placed restrictions on acceptable microbial populations in ballast water as human standard⁽¹⁴⁾. In the regulations, *V. cholerae* O1 and O139 were limited to less than one CFU 100 ml⁻¹ or less than one CFU (gram (wet weight) zooplankton sample)⁻¹.

We should use more than 100 ml of ballast water samples in order to try to detect pathogenic *V. cholerae* because the minimum unit proposed by IMO was 100 ml. In the experiments, we just spread 100 μ l of each sample onto TCBS agar plates (n=2). This experimental protocol might not be suitable for the complete detection of pathogenic *V. cholerae* in the ship's ballast water and sediment, although it is obviously impossible to examine total volume of ballast water and sediment in ballast tanks.

Table 4. Identification of yellow-colored colonies made on TCBS selective plate at 37°C.

Sampling date	Sampling site	Strain	Identification ^a
October 15, 2005	Sediment	SB0307	<i>Vibrio alginolyticus</i> (99.6%) ^b
November 16, 2005	Sediment	SB0407	<i>Photobacterium damsela</i> (95.7%) ^{b, c}
April 21, 2006	Sediment	SB0610	<i>Vibrio alginolyticus</i> (99.3%) ^b
	Sediment	SB0611, SB0612, SB0613, SB0614	<i>Vibrio harveyi</i> (99.4%) ^{b, d}
September 2, 2006	Sediment	SB0708, SB0709	<i>Vibrio harveyi</i> (99.3%) ^{b, c}

^aIdentification was carried out based on the partial rDNA sequence (about 500 bp) as well as the biochemical and physiological properties.

^bHomologous percentage obtained from the homology analysis using the data base of GenBank/DDBJ/EMBL is shown in parentheses.

^cIn general, identification based on 16S rDNA requires the homologous percentage of more than 97.0% in the database. Therefore, the sequence was deposited in GenBank/DDBJ/EMBL, and the isolate was listed as a newly isolated *Photobacterium* sp. SB0407. Accession number is AB274303.

^dThe data of the sequence and the homologous percentage obtained from each isolate were the same as those of other three isolates.

^eThe data of the sequence and the homologous percentage obtained from two isolates were quite the same.

Conclusions

Japan imports large amounts of crude oil and LNG from the Middle East, and very large crude oil carriers and LNG carriers sailing from Japan to the countries export ballast water, the amount of which is about a half to almost the equal of the fully loaded cargos of crude oil or LNG. Therefore, investigation for detecting pathogenic *V. cholerae* in the ports and along the coastal area of Japan is very important. Fifty-one *V. cholerae* O1 strains, which were identified to be El Tor biotype, Ogawa serotype, have been isolated in a port of Osaka, Japan in 1987 to 2001⁽¹⁵⁾. All of the isolates were cholera toxin (CT) negative and CT gene negative. Continuous investigations should be carried out to ensure that coastal seawater is not polluted by pathogenic *V. cholerae*.

The convenient detection and pasteurization technologies, which are rather than time consuming and available to use on board, are required for the management of ships' ballast water and sediment in ballast tanks.

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Questions and Answers

Shinya Yukihiro (Kyushu University)

You have emphasized the importance of the management of sediment in ballast tanks as well as ballast water prior to discharging ballast water from the vessel. Actually, how should ballast water and sediment be managed?

Shuhei Okuyama

Ballast water pooled in ballast tanks is necessary to be treated completely having no living things prior to discharging from vessels. The prevention of outflow of untreated sediment also requires on the voyage.

Masanori Tsugane (Tokai University)

Although you mentioned that the seasonal changes in colony-forming marine bacterial populations were observed in the pooled ballast water samples which had been taken in Japan throughout seven voyages, ports of call in Japan varied in your experiments. What do you think about the influence of the different

sampling sites in Japan on the seasonal changes of the number of colony-forming cells in the pooled ballast water?

Why was not the number of colony-forming cells in the ballast water samples taken in Japan reduced even when the ballast water was exchanged in the Indian Ocean?

Shuhei Okuyama

As you pointed out, ballastwater was loaded at four different ports such as Himeji, Osaka, Yokkaichi, and Niigata ports in the seven voyages. We believe that the quality of seawater around the coastal area of Japan is roughly the same. Actually, Himeji and Osaka ports are closely located in the Inland Sea. Comparing each data seems to be available primarily.

In general, marine bacteria can survive several weeks in dark and cool environments. That is why the obvious reduction of the number of colony-forming marine bacterial populations was not observed at the ballast water exchange site in the Indian Ocean.