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# Changes of microbial populations in a ship's ballast water and sediments on a voyage from Japan to Qatar

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**Abstract**

Colony-forming eutrophic marine microorganisms in ballast water were counted in samples taken on board in 2002 and 2003. In the ballast water in Japan, viable cell numbers were highly variable but not by more than $10^{5.1}$ colony-forming units (CFU) $ml^{-1}$ regardless of season. Even when ballast water was discharged offshore, values varied but not by more than $10^{5.0}$ CFU $ml^{-1}$. The effectiveness of the ballast water exchange was unconfirmed, except for the February 2003 voyage. No microbial colonies were counted in the reloaded ballast water in the high seas on that voyage, which contributed to the reduction of the total number of viable cells sampled in the discharged ballast water at the Ras Laffan port in Qatar. In sediment samples, the values of $10^{5.2}$ - $10^{6.0}$ CFU $ml^{-1}$ were estimated for all seasons in which voyages took place. The maximum of the marine *Vibrio* species, 110 CFU $ml^{-1}$, was observed in the ballast water sample taken in July 2003. The estimated total viable cell numbers in sediments were higher than those counted in the ballast water throughout the experiments, indicating the importance of sediment management as well as ballast water management on vessels traveling from Japan.

*Key words***:** Ship's ballast water, Marine microorganisms, *Vibrio* species, Colony-forming units, Ballast water exchange, International Maritime Organization

## 1. Introduction

Ballast water is essential for ships to maintain stability and safety at sea when sailing without cargo. Generally, ballast water is taken aboard as cargo is unloaded and is discharged overseas before loading new cargo. Invasive marine species are transported by ship and spread in ballast water. Most, but not all, indigenous species taken in ballast tanks are killed during voyages as a result of temperature changes, reduction in concentrations of dissolved oxygen, and lack of food.

One species, *dinoflagellates*, has a strong survival technique. It forms cysts (Hallegraeff and Bolch, 1991; Hallegraeff and Bolch, 1992) and is activated when the ballast water is discharged at the destination. Some of the surviving organisms then invade local marine and estuarine ecosystems (Carlton and Geller, 1993; Ruiz et al., 2000a, b; Sakai et al., 2001). The potential for growth at above-normal temperatures has also been observed for some green flagellates loaded into ballast tanks in the Southern ocean when these organisms were released into Tasmanian waters (Lewis et al., 2003).

Drake et al. (2002) have started researching the microbial ecology in ballast water by the use of an overseas vessel. Moreover, experimental techniques to rapidly detect invading pathogens in ballast water are in progress (Lee et al., 2002; Aridgides et al., 2004). *Vibrio cholerae* has survived in ballast water (McCarthy and Khambaty, 1994; Ruiz et al., 2000a, b), and *Escherichia coli* is able to survive there as well (Rozen and Belkin, 2001). Therefore, the International Maritime Organization (IMO) has required overseas vessels to limit the viable cell numbers of *Vibrio cholerae* and

*Escherichia coli* in ballast water prior to discharging (IMO, 2004). The reduction of damage to fish farms by invading fish pathogens, e.g., *Vibrio anguillarum* (Martínez-Picado et al., 1996), is an important goal.

We investigated the changes in microbial populations in ballast water and sediments taken at ports in Japan by a vessel transporting liquefied natural gas. On route to Qatar from Japan, ballast water was exchanged in the high seas, as recommended by the IMO (IMO, 2004). The IMO also requires reducing or eliminating pathogens in ship ballast water and sediments in ballast tanks. Consequently, we investigated pathogenic *Vibrio cholerae* in samples taken from ballast water and sediments.

## 2. Materials and Methods

### 2.1. Navigation route and ship used for sampling ballast water

The navigation route from Japan to Qatar is shown in Fig. 1. It takes approximately two weeks to go from Japan to the Ras Laffan port in Qatar. The ship was a liquefied natural gas (LNG) carrier (110,000 tons gross) with one forward- and one after-peak ballast tank as well as ten side ballast tanks located tandem on the starboard and port sides. The total capacity of the tanks is 55,000 $m^3$.

### 2.2. Sites for exchanging ballast water

It took approximately 10 days to reach the high seas in the Indian Ocean, where ballast water

was exchanged (Figs. 1 and 2). The area is at position 5 – 6° north. The International Maritime Organization (IMO) requires that ballast water be exchanged in the high seas, i.e., open ocean at least 50 nautical miles from the nearest land and 200 m in depth (IMO, 2004).

**2.3. Sampling of ballast water and sediments on board**

Examination of marine microorganisms in ballast water and sediments in ballast tanks was carried out six times in the period between May 2002 and August 2003. Ballast water was taken aboard at the Yokkaichi port in Mie Prefecture or at the Niigata port in Niigata Prefecture.

After arriving in the high seas in the Indian Ocean, the original ballast water was discharged prior to reloading ballast water (Fig. 2). This procedure was repeated for each ballast tank. The ballast water thus exchanged was transported and discharged at the Ras Laffan port in Qatar.

The sampling of ballast water was carried out at the outlet of the air release valve on 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 arrival in Japan. On the return trip from Qatar to Japan, no ballast water was in the ballast tanks because LNG was fully loaded; therefore, the sediments in the ballast tanks were available to be sampled. Sediment samples were placed into an autoclaved bottle (250 ml, NALGENE®) and kept at 4°C until arrival in Japan.

Seven to 10 days after departing Japan, surface water and middle-depth water at about 10 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 were stored at 4°C until arrival in Japan.

**2.4. Total viable cell numbers and *Vibrio* species**

Counting the total number of viable cells of eutrophic microorganisms was carried out by the use of agar plates filled aseptically with a sterile medium containing 5 g Bacto peptone (Difco, Michigan, USA), 1 g yeast extract (Difco, Michigan, USA), and 15 g agar per liter of natural seawater. After serial dilution with autoclaved natural seawater, the samples, 0.1 ml, were spread onto the agar plates, and incubation was carried out for two days at 30°C.

A thiosulphate-citrate-bile salts-sucrose (TCBS) selective medium was used for the isolation of an estuarine *Vibrio* species (Pfeffer and Oliver, 2003). We used agar plates (Pearlcore TCBS Agar "Eiken," Eiken Chemical Co., Ltd.) filled in aseptically with a sterile medium containing 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, for detecting the *Vibrio* species in the sampled ballast water and sediments in ballast tanks. After serial dilution with autoclaved natural seawater, if necessary, the samples, 0.5 ml, were spread onto TCBS selective agar plates, and incubation was carried out for one day at 30°C.

**2.5. Identification of isolates on TCBS selective agar plate**

All the individual colonies on the TCBS selective agar plates were examined based on their

morphological, biochemical, and physiological properties (Krieg, 1984). Sensitivity to the antibiotic agent, 2,4-diamino-6,7-di-isopropyl pteridine phosphate (Barrow and Feltham, 1993), was examined by the paper disc method. After the antibiotic agent (150 μg) was adsorbed with filter paper (about 5 mm of diameter), it was placed onto an agar plate, on which a cell suspension of the isolate had been previously spread. After one day of incubation at 30°C, the zone of inhibition was checked.

As for two yellow colonies (see Table 2), identification based on the partial 16S rRNA gene sequences was carried out. It was performed at the National Collections of Industrial, Food, and Marine Bacteria Japan Co., Ltd. (Shimizu, Shizuoka). A partial 16S rRNA gene fragment (about 500 bp) for each isolate was amplified by PCR with the MicroSeq® 500 16S rDNA kit (Applied Biosystems Japan). Homology analysis was carried out by the use of sequencing data with MicroSeq® Microbial Identification System Software and MicroSeq® Bacterial 500 Library (Applied Biosystems, CA, USA).

**3. Results and Discussion**

Regardless of the sampling time and season, variable viable cell numbers were observed: $10^{2.3}$ - $10^{4.8}$ colony-forming units (CFU) $ml^{-1}$ in May 2002, $10^{4.2}$ - $10^{4.9}$ CFU $ml^{-1}$ in September 2002, $10^{3.9}$ - $10^{5.0}$ CFU $ml^{-1}$ in December, $10^{3.1}$ - $10^{4.8}$ CFU $ml^{-1}$ in February 2003, $10^{1.8}$ - $10^{3.4}$ CFU $ml^{-1}$ in April 2003, and $10^{2.7}$ - $10^{3.8}$ CFU $ml^{-1}$ in August 2003.

When ballast water taken aboard in Japan was discharged in the high seas, it was sampled, and the number of viable cells was counted (Fig. 3). Again, variable viable cell numbers were

observed: $10^{3.5}$ - $10^{3.8}$ CFU $ml^{-1}$ in May 2002, $10^{4.3}$ - $10^{4.9}$ CFU $ml^{-1}$ in September 2002, $10^{4.0}$ - $10^{4.8}$ CFU $ml^{-1}$ in December 2002, $10^{1.6}$ - $10^{4.3}$ CFU $ml^{-1}$ in February 2003, $10^{2.8}$ - $10^{3.2}$ CFU $ml^{-1}$ in April 2003, and $10^{4.4}$ - $10^{4.8}$ CFU $ml^{-1}$ in August 2003.

Until the ship arrived in the high seas in the Indian Ocean, the ballast water was continuously mixed in the tanks as a result of wave action. Nevertheless, the number of viable cells in the samples taken at any given time was variable. Since the port of the ballast water outlet pipes is located near the bottom of the ballast tanks, some sediment samples containing microbial populations that were approximately 10 times higher than those in the ballast water (Table 1) appeared to be mixed in with the discharged ballast water.

Samples were also taken each time when new ballast water was reloaded. With the exception of February 2003, there were no obvious reductions in the number of viable cells in samples taken in the high seas in comparison with samples taken from ballast water loaded in Japan. No colonies were detected from the samples taken in February 2003 (Fig. 3).

In the ballast water discharged at the Ras Laffan port, the number of viable cells in the samples varied from $10^{1.0}$ - $10^{4.5}$ CFU $ml^{-1}$ in May 2002, $10^{3.6}$ - $10^{4.6}$ CFU $ml^{-1}$ in September 2002, $10^{3.2}$ - $10^{4.3}$ CFU $ml^{-1}$ in December 2002, $10^{1.0}$ - $10^{2.2}$ CFU $ml^{-1}$ in February 2003, $10^{3.3}$ - $10^{3.9}$ CFU $ml^{-1}$ in April 2003, and $10^{4.3}$ - $10^{4.7}$ CFU $ml^{-1}$ in August 2003. An obvious reduction in the number of viable cells was observed in the samples from ballast water discharged at the Ras Laffan port in February 2003 in comparison with that in the samples taken before departing Japan. The effectiveness

of exchanging the ballast water in the high seas was only confirmed for the trip in February 2003.

Regardless of the exchange sites for ballast water in the high seas (see Fig. 2) and the season in which the samples were taken (Fig. 3), the total number of viable cells in ballast water reloaded in the high seas varied considerably and remained nearly identical to that in the samples from water taken aboard in Japan, except for the values obtained in February 2003.

There were seasonal variations in the number of viable cells in the samples obtained from the pools of ballast water in the tanks (Fig. 4). The maximum and minimum values on the surface of the body of water were $10^{4.9}$ in September 2002 and $10^{2.7}$ CFU $ml^{-1}$ in May 2002, respectively. At the middle depth of about 10 m from the surface, the maximum and minimum values were $10^{4.5}$ in September 2002 and $10^{2.6}$ CFU $ml^{-1}$ in April 2003, respectively. The difference in the values obtained from the samples taken at the same time from the surface and a 10 m depth was within a 0.5 log cycle, except for that in May 2002, indicating that the invading microorganisms might have been diffused within the ballast tanks during the voyage.

As for the number of viable cells in sediments in the ballast tanks, a 5 log cycle of the number was estimated on the basis of the water content in the sample regardless of the season in which the voyage took place (Table 1). All the values estimated from the sediment samples were higher than those obtained from the ballast water. These results indicate the importance of sediment management as well as that of ballast water.

Viable cell numbers on the TCBS selective plates are shown in Table 2. In five of the six

voyages, except for that in February 2003, colonies were detected from the ballast water and sediment samples. No colonies were detected in any of the samples taken from the ballast water loaded in Japan. Several colonies, however, were detected when the samples taken from the pooled ballast water in September and December 2002 and April and July 2003 were examined. Moreover, one colony was obtained from each sample taken as ballast water was discharged in the high seas in May 2002 and July 2003. No colonies were observed on the plates for the samples taken from ballast water reloaded in the high seas and discharged at the Ras Laffan port in Qatar.

Although the *Vibrio* species can be effectively detected by the use of the thiosulphate-citrate-bile salts-sucrose (TCBS) selective plate, selectivity is not quite complete (Pfeffer and Oliver, 2003). Therefore, we examined all the isolates that had been picked up from the individual colonies on the TCBS selective plates. As for the isolates obtained from ballast water samples, not from the sediment samples, every isolate was Gram-negative, rod-shaped, motile, and not spore-forming. The isolates had catalase, oxidase, and glucose fermentation activities. The zone of inhibition was confirmed on the plate for each isolate by exposure to a paper disc containing an antibacterial agent, 2,4-diamino-6,7-di-isopropyl pteridine phosphate. These results indicate that the isolates belong to the genus *Vibrio* (Krieg, 1984). On the TCBS selective plates, *Vibrio cholerae* was a yellow colony. Only two isolates, September 1 and December 1, taken in September and December 2002 (see Table 2), resulted in yellow colonies. Consequently, these two colonies were used for further identification. The remaining colonies were dark green. As for the isolates obtained from the

sediment samples, spore formation was observed in cells for all isolates with a phase-contrast microscope, indicating that the isolates are not a *Vibrio* species but a *Bacillus* species.

For isolates September 1 and December 1, further identification was carried out. Although we did not obtain 100% homologous sequences in the database (Applied Biosystems, CA, USA), the isolate September 1 was related to several species of the genus *Vibrio*, such as *V. campbellii* [99.26%], *V. alginolyticus* [97.03%], *V. mediterranei* [94.42%], and *V. tubiashii* [93.49%]. As for the isolate December 1, the related species of the genus *Vibrio* were *V. campbellii* [92.19%], *V. alginolyticus* [92.01%], *V. mediterranei* [91.45%], *and V. furnissii* [89.96%]. These results indicate that the isolates September 1 and December 1 differ from the type cultures of the *Vibrio* species listed in the database. The partial rRNA sequences of the isolates September 1 and December 1 were deposited in the DDBJ/GenBank/EMBL under the accession numbers AB195981 and AB195982, respectively.

The detection of hazardous *Vibrio* species by the use of the plate-counting method is time consuming; therefore, the development of an on-time detection system for eutrophic marine microorganisms that invade the ballast water is required for the effective management of ballast water.

Regulations adopted by the International Convention for the Control and Management of Ships' Ballast Water and Sediments in February 2004 (IMO, 2004) place restrictions on acceptable microbial populations in ballast water as a human health standard. *Vibrio cholerae* (serum types O1 and O139) is limited to less than one CFU $(100\ ml)^{-1}$ or less than one CFU (gram (wet weight)

zooplankton sample)$^{-1}$, *Escherichia coli*, to less than 250 CFU (100 ml)$^{-1}$, and intestinal *Enterococci*, to less than 100 CFU (100 ml)$^{-1}$. Throughout the investigation, toxicogenic *Vibrio cholerae* was not detected, indicating that the human health standard set at the Ballast Water Convention (IMO, 2004) had been adhered to at least in so far as *Vibrio cholerae* is concerned. Continuous investigations should be carried out to ensure this. Regardless of the political and economic considerations that arise from the implementation of the convention, a ship's ballast water must be pasteurized prior to discharge at foreign ports to maintain the indigenous marine environment.

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**Figure captions**

Fig. 1. Outline of the navigation route bound for Qatar from Japan.

The solid line shows the navigation route between the Niigata port or the Yokkaichi port in Japan and the Ras Laffan port in Qatar.

Fig. 2. Ballast water exchange sites on the way to Qatar.

Ballast water exchange sites for the voyage to Qatar in May 2002 (circles), September 2002 (triangles), December 2002 (squares), February 2003 (triangles, upside down), April 2003 (lozenges), and August 2003 (triangles, sideward) are shown. Open and closed symbols mean that sampling was carried out when ballast water was discharged from and reloaded onto a ballast tank, respectively.

Fig. 3. Changes in the number of viable cells in ballast water while loading and discharging from the ship.

Ballast water was loaded at the Niigata port in May 2002 (A). Except for that, it was loaded at the Yokkaichi port in September 2002 (B), December 2002 (C), February 2003 (D), April 2003 (E), and July 2003 (F). Ballast water was sampled when it was loaded at a port in Japan (open circle), discharged (closed circle) and reloaded (open triangle) in the high seas, and discharged from the ship at the Ras Laffan port in Qatar (closed triangle).

The experiment 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.

Fig. 4. Seasonal variations in the number of viable cells in the ballast water stored in a ballast tank.

The ballast water stored in a ballast tank was taken from the surface (open column) and at a 10m depth of the body of water (closed column).

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.

Fig. 1

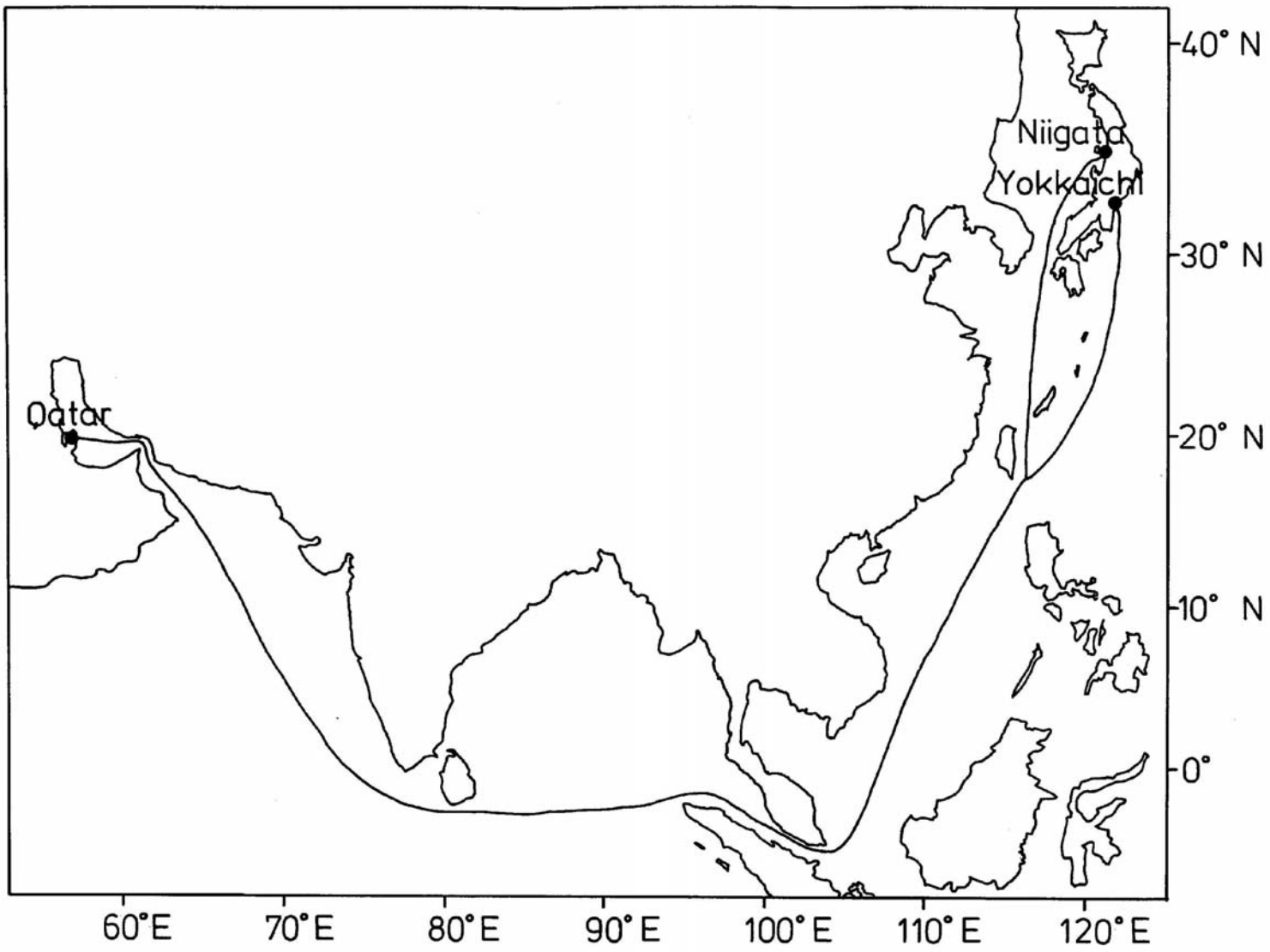
Niigata
Yokkaichi
Qatar
40° N
30° N
20° N
10° N
0°
60°E
70°E
80°E
90° E
100° E
110° E
120° E

Fig. 2

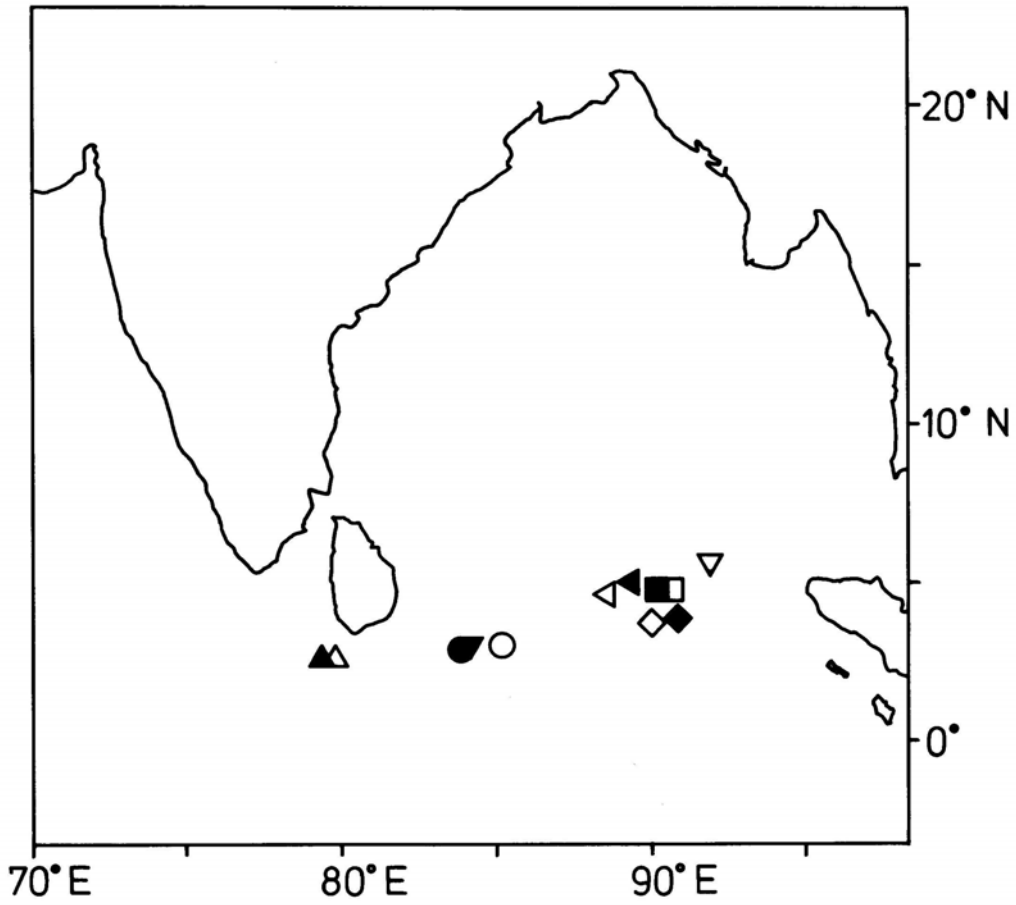

Fig. 3

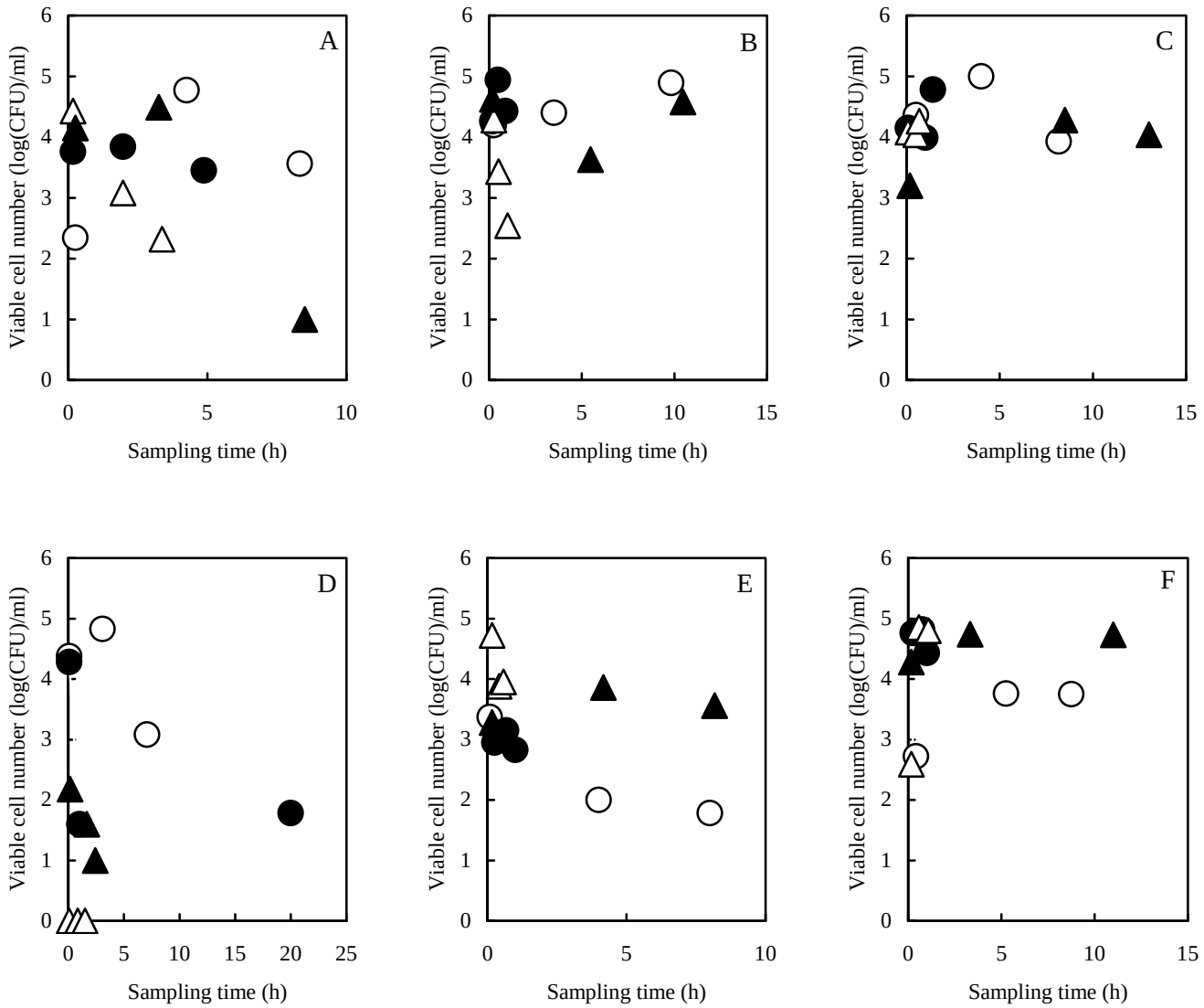

Fig. 4

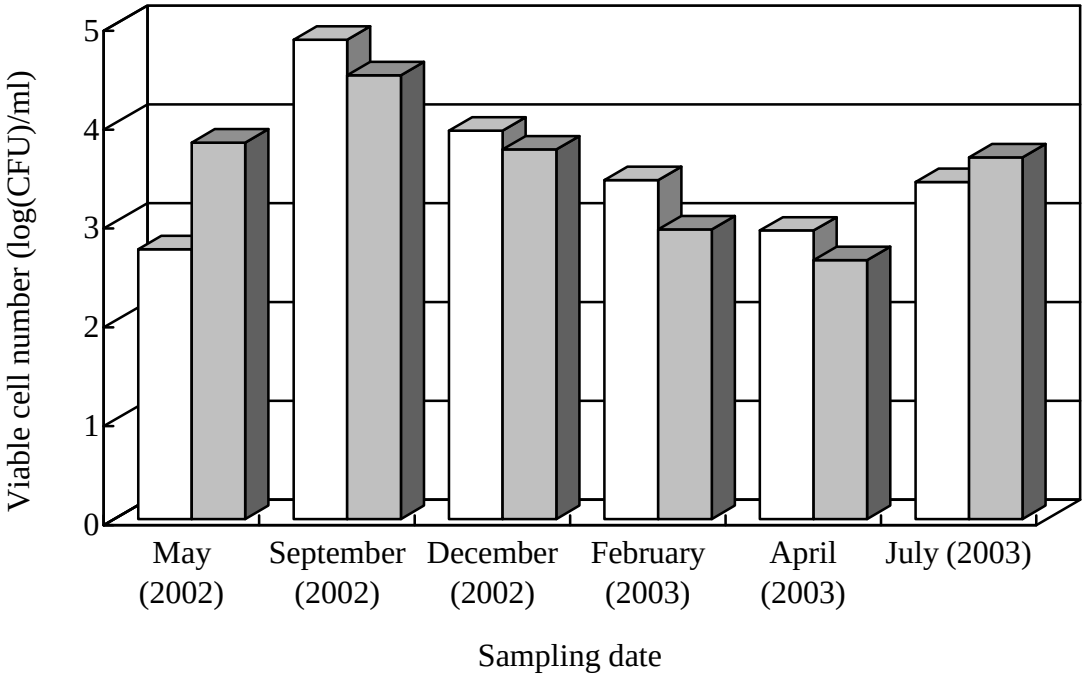
Viable cell number (log(CFU)/ml)
0
1
2
3
4
5
May (2002)
September (2002)
December (2002)
February (2003)
April (2003)
July (2003)
Sampling date

Table 1. Total number of viable cells in the sediments of ballast tanks.

| Sampling date | Viable number of cells (log (CFU) $ml^{-1}$)[a] |
|---|---|
| June 17 (2002) | 5.6 |
| September 16 (2002) | 5.8 – 5.9 |
| December 20 (2002) | 5.5 – 5.8 |
| February 24 (2003) | 5.2 – 5.3 |
| May 3 (2003) | 5.9 – 6.0 |
| August 10 (2003) | 5.7 – 5.9 |

[a]Sediments were taken from two different ballast tanks, except for June 2002. The sediment (1 g) was diluted with distilled seawater (9 ml). Serial dilution, when necessary, was carried out, and the sample (0.1 ml) was spread onto non-selective agar plates. The viable cells were calculated on the basis of the water content of each sample. For example, the viable number of cells, 5.6 log (CFU) $ml^{-1}$, obtained from the sample in June 2002 was calculated by the following equation: 180 (colony numbers on the plate) x (9.4/0.4) x (10/1) x (1/0.1) = 4.2 x $10^5$ CFU $ml^{-1}$, where 0.4 ml was the water content in 1 g of the sample.

Experiments were carried out twice for each sample by taking sediments from different sites, 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.

Table 2. Viable number of cells on the TCBS selective plates.

| Voyage date | Viable number of cells (CFU $ml^{-1}$) | Sampling time and site |
|---|---|---|
| May 19 (2002) | 0 | while loading at the Niigata port in Japan |
| May 27 | 0 | surface of ballast water |
| May 27 | 0 | 10 m depth of ballast water |
| May 30 | 2 | while discharging in the high seas |
| May 30 | 0 | while reloading in the high seas |
| June 5 | 0 | while discharging at the Ras Laffan port in Qatar |
| June 17 | 47[a, c] | sediment in a ballast tank |
| August 26 (2002) | 0 | while loading at the Yokkaichi port in Japan |
| September 5 | 2[b] | surface of ballast water |
| September 5 | 0 | 10 m depth of ballast water |
| September 6 | 0 | while discharging in the high seas |
| September 6 | 0 | while reloading in the high seas |
| September 10 | 0 | while discharging at the Ras Laffan port in Qatar |
| September 16 | 0[a] | sediments in ballast tanks |
| November 28 (2002) | 0 | while loading at the Yokkaichi port in Japan |
| December 5 | 4[b] | surface of ballast water |
| December 5 | 0 | 10 m depth of ballast water |
| December 7 | 0 | while discharging in the high seas |
| December 7 | 0 | while reloading in the high seas |
| December 13 | 0 | while discharging at the Ras Laffan port in Qatar |
| December 20 | 0 - 124[a, c] | sediments in ballast tanks |
| January 31 (2003) | 0 | while loading at the Yokkaichi port in Japan |
| February 8 | 0 | surface of ballast water |
| February 8 | 0 | 10 m depth of ballast water |
| February 8 | 0 | while discharging in the high seas |
| February 9 | 0 | while reloading in the high seas |
| February 15 | 0 | while discharging at the Ras Laffan port in Qatar |
| February 24 | 0[a] | sediments in ballast tanks |
| April 6 (2003) | 0 | while loading at the Yokkaichi port in Japan |
| April 14 | 0 | surface of ballast water |
| April 14 | 2 | 10 m depth of ballast water |
| April 16 | 0 | while discharging in the high seas |

| | | |
|---|---|---|
| April 16 | 0 | while reloading in the high seas |
| April 22 | 0 | while discharging at the Ras Laffan port in Qatar |
| May 3 | 0[a] | sediments in ballast tanks |
| July 14 (2003) | 0 | while loading at the Yokkaichi port in Japan |
| July 21 | 0 | surface of ballast water |
| July 21 | 110 | 10 m depth of ballast water |
| July 23 | 2 | while discharging in the high seas |
| July 23 | 0 | while reloading in the high seas |
| July 30 | 0 | while discharging at the Ras Laffan port in Qatar |
| August 10 | 48 - 144[a, c] | sediments in ballast tanks |

[a]Sediments were taken from two different ballast tanks except in June 2002. After dilution of the sediment (1 g) with distilled seawater (9 ml), the sample (0.5 ml) was spread onto TCBS selective plates. The number of viable cells was calculated on the basis of the water content of each sample. For example, the number of viable cells, 47 CFU $ml^{-1}$, obtained from the sample in June 2002 was calculated by the following equation: 1 (colony numbers on the plate) x (9.4/0.4) x (1/0.5) = 47 CFU $ml^{-1}$, where 0.4 ml was the water content in 1 g of the sample.

[b]Yellow colonies were named as September 1 and December 1.

[c]All the colonies were disc-shaped and dark green, and spore-forming in the cells was observed with a phase-contrast microscopic.

Experiments were carried out three times for each sample, and the maximum value is shown here.