D-4 Studies of Impact of Marine Organisms Introduced by the Ballast Water/ship Hull Community on Coastal Ecosystems and the Efficient Management of Ballast Waters (Abstract of the Final Report)

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(1) Assessment of the impact of organisms introduced by the ballast water/ship hull community on coastal ecosystems
(2) Study of succession in the ballast tank community and the management of ballast water
(3) Ecophysiological study on harmful phytoplankton cysts under simulated ballast tank environments

Key Words Ballast water, Introduced marine organisms, Harmful algae, Ship hull community

1. Introduction

Due to the globalization of ship transport systems, increasing trans-ocean introductions of marine organisms threaten coastal ecosystems. The organisms attached to the ships' hulls, and especially the discharge from large scale bulk carriers of ballast water containing numerous kinds of plant and animal plankton as well as microorganisms, are important elements in these trans-ocean introductions. Because Japan imports large amounts of primary resources such as oil, coal, and iron ore, Japan also exports large amounts of ballast waters. However, few studies have been done on the biodiversity and dynamics of ballast water communities. Similarly, our knowledge of the origins, as well as the primary and secondary dispersal mechanisms of the introduced (or exported) marine organisms comprising ship hull communities is rather scanty.

2. Research Objective

In order to elucidate the processes contributing to intercontinental introductions of marine organisms (e.g. seaweeds, benthic animals and harmful phytoplankton) and to assess their impacts on coastal ecosystems, we are monitoring the biodiversity and succession of the biota in the ballast tanks and on the hulls of bulk carriers, as well as investigating the genetic diversity of representative introduced taxa in the international port areas of Japan and overseas.

3. Research Methods

We investigated the genetic diversities of native and introduced populations of *Undaria pinnatifida* (Laminariales, Phaeophyceae) using mitochondrial molecular genetic markers (*cox3* and *tatC-tLeu* sites). We collected *Undaria* specimens from various localities in Japan, Korea, China, California (USA), Australia, New Zealand and France, and also obtained specimens from Baja California (Mexico) and Argentina from collaborators. We extracted total DNA from the specimens, amplified the targeted sequence regions, and sequenced by the direct sequence method. We also compared the genetic divergences between the Japanese, Korean and New Zealand populations using micro-satellite analyses of nuclear genes using the specific primers generated by French collaborator for the introduced populations of

Undaria in European.

We also investigated the genetic diversities of native and introduced populations of *Ulva/Enteromorpha* (Ulvales, Ulvophyceae) using molecular markers (nuclear rDNA ITS and spacer region between chloroplast *atp*H and *atp*I genes). We collected *Ulva/Enteromorpha* specimens from various localities in Japan, Korea, China, Australia, New Zealand and Europe, and also made seasonal samplings of dominant species in the Mikawa and Osaka Bays, typical enclosed water area with international ports in Japan. We also included the specimens collected from the ship hull and ballast water of intercontinental bulk cargo carriers.

We also investigated the genetic diversities of filamentous (ectocarpoid) brown algae belonging to Ectocarpales, typical fouling species of ship-hull communities, using *cox3* mitochondrial gene sequences, to establish basis for the taxonomy using molecular markers. We analyzed the culture strains of ectocarpoid species collected from worldwide localities and the ship-hulls, and those deposited in Kobe University Macroalgal Culture Collections.

We made morphological observations and molecular phylogenetic analyses of the *Sargassum* species newly recorded from California (USA) and Baja California (Mexico) coasts using mitochondrial cox3 gene sequences.

We investigated the genetic diversities of native and introduced populations of *Xenostrobus securis* using mitochondrial molecular genetic markers (CO1). We collected specimens from various localities in Japan, Australia and New Zealand, and extracted total DNA, amplified the targeted sequence regions, and sequenced by the direct sequence method. In order to compare the genetic divergences between the populations, we examined 708 specimens by PCR-RFLP method based on the sequence data. PCR-RFLP was detected on the amplified fragments with EcoT22I and AfaI restriction endonucleases. We also investigated the biodiversities of organisms attached on the hulls of bulk cargo carriers by samplings in dry-docks. We identified the collected specimens based on the morphology.

We examined a container vessel operating between Japan and USA via China. During a voyage from Japan to Hong Kong in August 2005, we monitored the biodiversity and environmental parameters in the ballast tank, and also made samplings of specimens from the same tank. Through a sounding pipe, we collected manually the ballast seawater samples; those were immediately divided for several treatments: fixation, incubations, cell counting, DNA analysis and EM observations. For continuous measurements of the temperature and chlorophylls, we set a sensor (Alec Compact-CLW) at the bottom of the ballast tank. We also investigated a bulk cargo carrier in June/July 2005, in the operation between a port in Tohoku Region, Japan and Australian ports, and obtained specimens form the Japanese port.

We collected surface marine sediments from Japanese coast to simulate the cysts response to ballast tank environment. We checked the cysts content from these sediments and their germination ability under favorable conditions. Aliquots of the same sediments were then used for ballast tank simulations with variable length of time and including temperature changes, complete darkness and turbulence effect. One simulation exactly reproduced the conditions for a voyage from Japan to Australia. Once the simulations of transit were over, the treated sediments were tested for cysts germination.

4. Results and Discussion

(1) Assessment of the impact of organisms introduced by the ballast water/ship hull community on coastal ecosystems

1) Biogeographical study of introduced macroalgae using molecular markers.

a. Genetic diversities of native and introduced populations of *Undaria pinnatifida*.

In its native distributional area (Japan, Korea and China), we found 27 haplotypes, which were classified into 4 genetic groups: 1) Continental-type found in Korea and China; 2)

Northern Japan-type distributed in Hokkaido and Pacific northern Honshu; 3) Pacific central Japan-type; and 4) Sea of Japan-type. Among the introduced populations, European and Mexican populations agreed with the northern Japan-type. In Australia, the Tasmanian population agreed with the Sea of Japan-type, whereas the Victorian population showed the continental-type. Very high diversities were found in New Zealand: nine haplotypes were found including both the northern Japan- and the continental-types. The haplotype found in California agreed with one of the central Japan-type found in Miura Peninsula. The Argentine population agreed with the continental-type. The fact that European populations showed the Northern Japan-type agreed with the notion that European *Undaria* was first introduced together with oyster spat for fishery purposes, and then spread by secondary dispersals. It is speculated that Californian and Mexican populations were recently introduced by ship transport. There have been many introduction events to New Zealand from the late 1980s, and the dominant populations changed from Japanese origin to Korean/Chinese origin in South Island. Introductions to Argentina could be secondary introductions from New Zealand/Australia populations, because transport within the same range of latitude is considered to be easier than ship transport crossing the tropics.

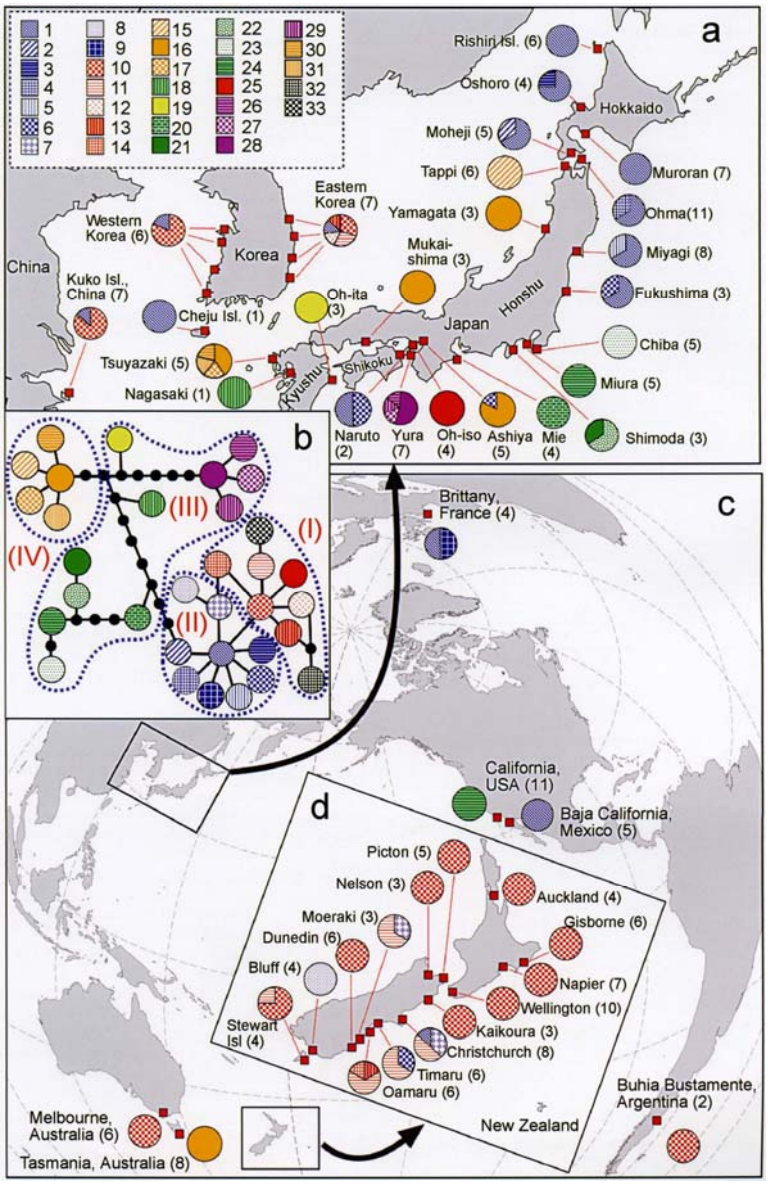

Fig. 1. Geographical distribution of mitochondrial DNA haplotypes (*cox3* and *tatC-tLeu* regions) of *Undaria pinnatifida* in native and introduced populations and the Spanning Network tree. Combinations of color and pattern represent different haplotypes. Numerals in parentheses represent the number of samples in each population. a. Native range; b. SPN tree. c. Worldwide introduced populations except New Zealand. D. New Zealand.

b. Genetic diversities of native and introduced populations of *Ulva/Enteromorpha* species.

i. Taxonomy of dominant *Ulva/Enteromorpha* species in the Mikawa Bay and the Osaka Bay, and the genetic diversity of introduced *Ulva pertusa* populations.

In the Mikawa Bay and the Osaka Bay, based on the taxonomy using rDNA ITS sequence data, the following species were dominant *Ulva/Enteromorpha* species: *Ulva pertusa*; *U. ohnoi*; *U. arasakii*; *U. fasciata*; *U. linza* (=*Enteromorpha linza*); *U. compressa* (=*E. compressa*);

U. flexuosa (=*E. flexuosa*); *U. tanneri*; *U. californica*; *U. scandinavica*; *U. armoricana.* Among the species, *Ulva pertusa* and *U. ohnoi* were most dominant from spring to early summer and from autumn to early winter respectively. *U. ohnoi* has been suggested to be an introduced species, but the origin is not clear. *Ulva flexuosa*, *U. armoricana, U. californica*, *U. scandinavica* are considered to be relatively recently introduced to Japanese coast, and the latter two were recorded for the first time in the present study.

ii. Origin of the introduced *Ulva pertusa* populations.

In *Ulva pertusa*, based on the chloroplast *atp*H-*atp*I sequence data, 15 haplotypes were recognized in the NE Asian populations (native range), but only one (Oceania) or two (Europe) haplotype(s) corresponding to those found in the Pacific coast and the Sea of Japan coast of Japan were found. Therefore, the *U. pertusa* populations in Oceania and Europe are considered to be based on introductions from NE Asia.

c. Genetic diversities of ectocarpoid brown algae deduced from mt *cox3* gene sequences.

Based on the *cox3* sequence data, *Ectocarpus* species were suggested to be classified into 9 species including *E. faciculata*. Ectocarpoid specimens isolated from the ship hull and ballast water clustered with one of the clades including South American and European strains. These data will become basis of species level taxonomy of world-wide *Ectocarpus* species.

d. Taxonomy and origin of *Sargassum filicinum* newly introduced to Pacific North America.

Morphological studies associated with the molecular analyses using mitochondrial cox3 gene showed that the *Sargassum* species newly introduced to California (USA) and Baja California was *S. filicinum* (Fucales, Phaeophyceae). Individuals from both of the populations had the same gene sequence as those from the Seto Inland Sea area in Japan, so that the introduced populations were considered to be originated from western Japan. Considering the reproductive features of the species (i.e. monoecism, annual nature and the ability to float by bladders), there is serious concern that *S. filicinum* would spread world-wide in near future as occurred in *S. muticum*.

2) Biogeographical study and origin of introduced *Xenostrobus securis* populations

The *Xenostrobus securis* populations of New Zealand were shown to be different from those of Japan. The present study concluded that *X. securis* was introduced to Japan from Australia. This is the first report to elucidate the origin of introduced marine invertebrates into Japan using DNA analyses. Based on the Afa I examination by PCR-RFLP, 6 and 3 haplotypes were recognized in the Australian populations and the Japanese populations, respectively. Only the Newcastle population didn't show significant difference from Japanese populations by the pair-wise fixation index Fst. The results, as well as the records of ship operations between Japan and Austraria suggested that *X. securis* was introduced from Newcastle to Japan attached to the ship hulls .

3) Biodiversity of hull communities on bulk cargo carriers, container ships and PCC ships

We investigated the biodiversities of organisms attached on the hulls of four intercontinental bulk cargo carriers, two intercontinental container ships and two PCC ships by sampling in dry-docks from 2004 to 2006. We identified the collected samples based on the morphology. We found 90 taxa of 7 phyla of benthic animals on the hull of eight ships. The bulk cargo carriers showed higher diversity and larger biomass than the container ships and the PCC ships. The highest diversity and largest biomass of benthic animal were those of the barnacle. The richness and community structure of attached organisms differed depending on the site on the hull of the bulk cargo carriers. The most dominant species was *Balanus*

trigonus followed by *Tetracilitella purpurascens* and *Balanus improvisus* on smooth hulls. On the other hand, the most dominant species was *Megabalanus tintinnablum* followed by *Austrobalanus imperator*, *B. rosa* and *M. volcano* on rudders, rope guards and sea-chest. These results contributed to controlling fouling animals on the ship hulls.

4) Biodiversity and succession of macroalgae and zooplanktons in the ballast tank

We also investigated a bulk cargo carrier operating between Japan (Port in the Tohoku region) and Australian ports, and we monitored the biodiversity and environmental factors in the ballast tank during the voyage. The investigation was conducted for 10-13 days during the voyage from Japan to Mackay or Newcastle, Australia in August 2004, December 2004 and June 2005. We found zooplankton in the samples such as Foraminiferida, Tintinnina, Radiolaria, Cladocera, Copepoda, Gastropoda larva, Bivalvia larva and Polychaeta larva. The zooplankton of samples dominated Copepoda species. We could detect 18 different Copepoda species throughout three voyages. The number of zooplankton showed a rapid numerical decrease during the voyage. Most of zooplankton should die during the voyage. However we found a few species until end of the investigation in June 2005.

(2) Study of the ballast tank community succession and the management of ballast water

a. Monitoring of microorganism biodiversity in the ballast water

The environmental factors (temperature, pH, salinity, dissolved oxygen) and chlorophylls were continuously monitored during voyages of the bulk carrier serving between Japan and Australia all the year round (Fig. 2a, b, d). Those data were used for designing laboratory experiments. Temperature was the most changeable factor and has been suggested to affect the biodiversity and the state of the living organisms. In addition to the continuous measurements, heavy metals in the ballast water were measured by ICP-MS. Samples were collected from the top of ballast tank at the beginning and the end of the voyage and before and after of re-ballast. Just after re-ballasting (exchange of ballast water content with open ocean seawater), the Cd concentration increased, while Pb concentration decreased. The Ni concentration remained steady through the voyage, and the Sn concentration was close to limit of detection. Zn concentrations increased in the tank before and after re-ballast procedure.

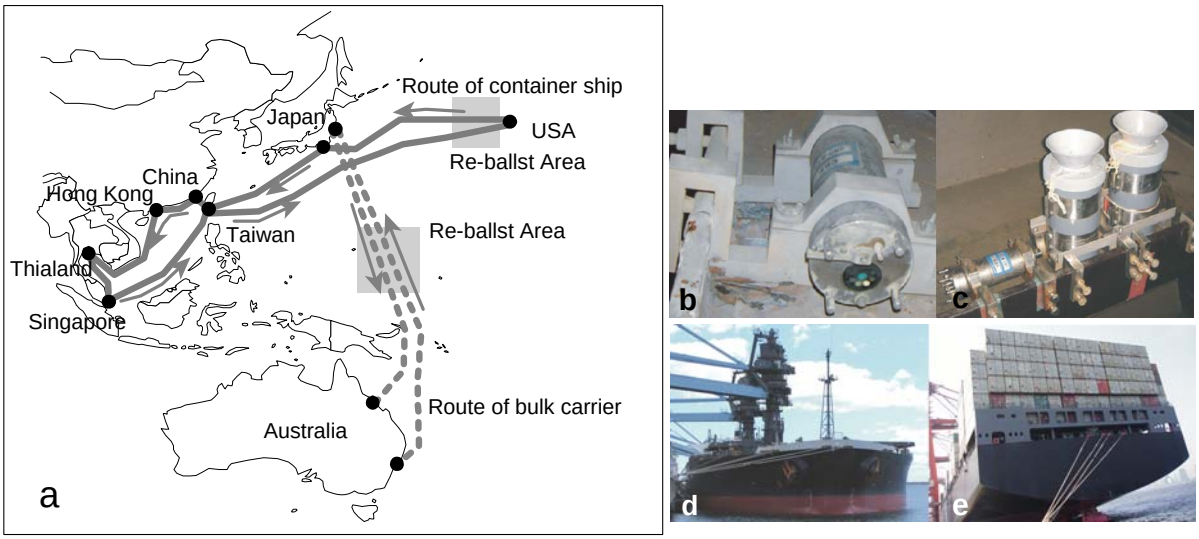

Fig. 2. The investigated route map (a) of bulk carrier (d) and container ship (e). The chlorophyll sensor (2b) and the sediment trap (c) placed in the ballast tank.

The continuous ~~This~~ monitoring was also done on the container ship serving between the

East Asia (Japan and China) and the west coast of USA. Chlorophyll and temperature were continuously recorded from August 2005 to January 2006, using a sensor placed at the bottom of a ballast tank (Fig. 2a, b, e). We could monitor in detail the phytoplankton biomass and found two succession patterns. The first case was the chlorophyll content decreasing daily and sharply right after the filling of the ballast tank, while the second case was the chlorophyll content remaining at a certain level till it was discharged in arrival port. This was observed during the route from China coast to the West coast of USA. Although we need to check the type of phytoplankton that was remaining in the tanks, this finding highlights the danger of introducing a huge number of living phytoplankton across the Pacific Ocean.

The dominant phytoplankton of samples from the second voyage of the bulk carrier (December 2nd to 21, 2004) were colonial diatoms such as *Chaetoceros* and *Asterionella*, and to a less extent solitary diatoms, dinoflagellates and *Dictyocha*. We could detect c. 30 different species by cell counts with sediment chamber and found a rapid numerical decrease during the voyage. With an initial cell number of 1,260 cells/mL on the first day, the cell concentration dropped to 217 cells/mL on the 6th day. At mid-voyage, the ship operated a reballasting in the open sea which showed a cell count of only 10 cells/mL, and the final number was 70,000 cells/Ton on the 15th day when the ship anchored at Newcastle offing. Though cell count attests for a general decrease, in detail, from the departure till the reballasting, the diatoms such as *Pseudo-nitzschia* and *Cylindrotheca* kept certain concentration; and an heliozoan and some ciliates showed active growth, suggesting their resistance to the unfavorable ballast water conditions. FlowCAM analysis showed that the total number of cells was kept around 4,000-6,000 cells/ml till the day 4. The detailed analysis for size fraction clarified that the size range of 5-30µm decreased the number in the period. While, the smaller sizes less than 5µm caused by fractionation of the colonial diatoms had increased, resulting the total number did not change a lot and kept 4,000-6,000 cells/ml. With FlowCAM, we could monitor changes of the cell states as well as the number of 1-5µm size particles, which were difficult to monitor with the sediment chamber counting. DGGE (Denaturing Gradient Gel Electrophoresis) method was also conducted to monitor the picoplankton diversity. The band pattern analysis indicated the proliferation of particular organisms in the ballast water. The major bands were sequenced, and the BLAST search was applied to clarify the identities. Among the proliferated organisms, some showed high homology to a pathogenic fungus or parasitic flagellates.

The dynamics of pico-phytoplankton in the ballast water was monitored during the voyage (16 days) by flow cytometer (Becton Dickinson, — Facscalibur). Before measurement, samples were melted in room temperature and filtrated by plankton net (41µm) . Initial numbers of both *Synechococcus* and pico-phytoplankton were almost on the same level, ca.110,000 cells/ml and ca. 140,000 cells/ml, respectively, while, *Synechococcus* was immediately decreased reaching ca. 60,000 cells/ml after loading sea water in the ballast tank. At mid-voyage, the ship operated a reballasting in the open sea. The open sea water where the ship operated reballasting contained ca.170,000 cells/ml of *Synechococcus*. When the ship anchored at coast of Australia offing after 16 days voyage, the number of *Synechococcus* was decreased to ca. 12,000 cells/ml. In the case of eukaryotic pico-phytoplankton, the number of cell was increased up to ca. 200,000 cells/ml after loading. The increase may be caused by fragmentation of colonial diatom. However, the number decreased rapidly after reballasting (ca. 50,000 cells/ml). The final number was ca. 13,000 cells/ml. The results suggested possibility that *Synechococcus* and eukaryotic picp-phytoplankton survived when the ship ballast water was discharged.

The container ship charges and discharges ballast water according to the load of the cargo, so that the ballast water pumping up and out varies from port to port. When we investigated

the phytoplankton diversity and biomass on the boat in August 2005, the total cell number of phytoplankton decreased from 15.0×10^6 cells/L (Tokyo bay) to 2.6×10^3 cells/L (China port) (0.02% of the initial number). The most dominant species was *Thalassiosira* spp. followed by *Skeletonema* sp. and *Pseudo-nitzschia* spp. In addition to the dominant species, 12 species of phytoplankton including some harmful species (e.g. *Heterosigma akashiwo* and *Prymnesium* ~~*sp*~~*parvum*~~.~~) were detected from the enrichment samples of the ballast water.

We also looked at the sediments accumulated in the ballast tank of this container ship. Vegetative cells of diatoms such as *Nitzschia* sp., green seaweeds and colorless dinoflagellates were detected in the enrichment samples and estimated at 45-96 cells/g-wet mud from the MPN method. From direct observations of the sediments, we could confirm the presence of a diversified group of cysts and cysts-like cells. The sediments examined should come from the sea water loaded at the west coast of USA, so that we can expect a risk of introducing marine organisms from USA to Japan. Reduction of the sediment load in the ballast tanks and elimination of the sediment dispersion into arrival ports must be an essential task to limit the introduction of marine species. We also tested the viability after a freshwater treatment for sediments containing vegetative cells and cysts. While 14 species of microorganisms (phytoplankton and protozoa) were found in the control, all phytoplankton and ciliates were undetectable after one hour treatment with freshwater (c. 0.03% salinity). But some flagellates such as *Cafeteria* spp., *Bodo* spp., *Gonyomonas* spp. were still detected. Though this result is a preliminary test, it gives an opening for a simple treatment to suppress some targeted microorganisms.

b. Effect of ballast water exchange on assemblage of virus like particles, bacteria and nanoflagellates in ballast water~~Monitoring of the virus like particles, bacteria and nanoflagellates in ballast water~~

Changes of microbial community in ballast tanks were observed at the voyages of a merchant ship in winter and summer seasons, in order to analyze the effect of ballast water exchange at high sea. Biomass of virus like particles (VLPs), bacteria and nanoheterotrophic flagellates (NHF) showed similar changing trends at both seasons. Total bacteria numbers measured by the direct count method always significantly decreased (Wilcoxon test. Winter: Surface; $W = 0$, $p < 0.01$: Bottom; $W = 0$, $p < 0.01$. Summer: Surface; $W = 24$, $p < 0.01$: Bottom; $W = 23$, $p = 0.02$) after the open sea exchange of ballast water in both seasons. In contrast, live heterotrophic bacteria numbers measured by the plate counting method were not clearly different after the open sea exchange in both seasons, because the number suddenly dropped after replacing coastal ballast water with open sea water and then bacteria gradually increased along the voyages. In the voyage in summer, pathogenic microbes indicated in the Ballast Water Management Convention (IMO, 2005) were observed. The results showed that these pathogenic bacteria predominantly lived in coast waters and were probably eliminated by replaced ocean water.

The observations of VLPs and NHF were carried out at summer season voyage, too. The number of VLPs significantly decreased (Wilcoxon test. Surface; $W = 24$, $p < 0.01$: Bottom; $W = 24$, $p = 0.01$) after the open sea exchange, and the pattern was similar to that of total count bacteria. Instead, the number of HNF significantly increased (Wilcoxon test. Surface; $W = 1$, $p = 0.02$: Bottom; $W = 1$, $p = 0.02$) after the exchange, and it had similar trend to that of living bacteria. It dropped after replacing ballast water with ocean water and gradually increased along the voyages, because they probably grazed bacteria and detritus and increased their number.

As the voyages in both seasons, denaturing gradient gel electrophoresis (DGGE) analysis

of bacteria from ballast tanks showed that; 1) the dendrogram resulting from cluster analysis showed mainly two clusters before and after open-ocean exchange, and 2) multidimentional scaling (MDS) ordination also separated into two groups by the exchange. These trends were similar in both seasons. These analyses showed that bacteria flora changed during the storage in ballast tanks along voyage. In contrast, the dendrogram based on cluster analysis of heterotrophic bacteria colonies, which were collected at live heterotrophic bacteria quantification using the plate counting method, showed unclearly separate clusters before and after ocean exchange. From this result, revival of bacteria originated from coastal waster in ballast tanks was suspected even after high sea ballast water exchange. It is necessary to confirm the revival by identification of bacteria species found both before and after the exchange.

c. Tolerance and succession of phytoplankton assemblage to darkness with different temperatures and zinc

Open-ocean exchange of ships' ballast water is designed to prevent coastal ecosystems from being invaded by exotic phytoplankton species. We studied the effect of prolonged darkness on phytoplankton and examined the possibility that released coastal phytoplankton affect the ecosystem through the open-ocean exchange. We used unialgal cultures of *Prorocentrum dentatum*, *Papiliocellulus elegans* and *Cylindrotheca* sp.. All cultures and coastal seawater were incubated in the dark and some of them were returned into the light. Furthermore, we studied the effect of zinc, which was measured in high concentrations in the ballast water, on phytoplankton survival. We studied the effect of zinc (1ppm) using unialgal cultures of *Phaeodactylum tricornatum* (CCMP630) and *Emiliania huxleyi* (CCMP373). The biomass of unialgal culture decreased immediately in the dark. Chlorophyll *a* concentrations of cultured coastal seawater were half of the initial concentration (0.2–0.3 μg/L) during 4 d darkness. Returning to the light led to an increase in biomass, except in *P. dentatum*. Cell numbers of the *Papiliocellulus elegans* and of *Cylindrotheca* sp. had increased to 3.0×10^5 and 3.0×10^4 cells/mL, respectively, by 7 d after the switch from dark storage. The biomass of both unialgal cultures with 1ppm zinc addition decreased immediately in the dark. However, there was no difference between control culture and zinc addition culture. These results suggest that some coastal phytoplankton may survive in ballast tanks under dark conditions during voyages, and that high concentrations of zinc in ballast water have not effect on phytoplankton decrease under the prolonged darkness.

d. Ballast water management method considering movement of aquatic organisms

Fresh water supplying experiment and the investigation of engine room arrangement

According to the data gained in the onboard experiments in last year it was confirmed that there was the possibility of effective management method of sediments to supply fresh water into the nearly empty tank. Two experiments were carried out on coal carriers. The first experiment result showed the effectiveness of fresh water but the second experiment did not show clear result. Those differences occurred by the different ways of supplying of fresh water into the tank. In first experiment the fresh water was supplied directly into the bottom of the tank though water hose but in second experiment the fresh water was supplied though the sounding pipe into the bottom of tank. In by-the -stern trim condition the supplied fresh water did not mix enough with the existing sea water because the water was supplied in the after part of the tank. This result showed that method of supplying fresh water should be considered depending on the trim condition. This fresh water supplying method can be conducted on the homeward voyage under full draft condition considering fuel consumption

during the voyage. By this work it is expected that living aquatic organisms will not be cist in the empty ballast tank and also expected that this treatment will be effective method for the management of sediment noted in the convention. The investigation was carried out on 2 coal carriers and one container ship to confirm the possibility of connection of fresh water line and ballast water line. The investigation result showed that one coal carrier and one container ship are available for the connection in the engine room. Therefore we understood that the connection of fresh water line with ballast water line is generally available on the existing ships.

Proposal of the optimum ballast water treatment

The ballast water treatment system should be installed when the convention will be in force. It is important to operate the system efficiently because the ballast water to be loaded is very large in quantity. The following proposal shall be taken into account to operate the system efficiently.

Timing of the operation of the system: Basically the system is operated when the ballast water is loaded in the discharging port. But the result of the study indicates that the timing of treatment shall be when the almost aquatic organisms are dead after a while the ship left the discharging port.

The monitoring of the inside environment of ballast tank: The monitoring of ballast water is stipulated in the convention. It is possible to carry out properly the ballast water management if the condition of ballast water is continuously checked and it is confirmed that the ballast water is clear of the criteria of regulation. The chlorophyll sensor which was used for our experiments is considered to be one of the equipments.

The fresh water treatment of sediments in ballast tanks: The convention requires the treatment of sediment in ballast tanks. It is recommended that ballast tank cleaning shall be conducted on every voyage. According to the experiment result the aquatic organisms in the remaining ballast water in the bottom of the ballast tank are killed by supplying of fresh water. It is confirmed that the fresh water pipe line can be connected with the ballast water line on board of some ships. It is considered that the fresh water treatment for the empty ballast tank is a very effective method to kill the aquatic organisms in the sediments and also is good for the crew members' health management.

The dry up treatment of sediments in ballast tanks: It is clear that dry up treatment of the ballast tank is the most effective method according to the study results which were obtained when the ship entered into the dry dock. Therefore the method to supply dry air into the ballast tanks and evaporate the remaining water is considered to be an effective method. The airing (hot air if possible) from the escape hatches into the ballast tanks in case of existing ships is recommended after the ballast water is discharged as completely as possible.

e. Development of microsatellite marker in the *Chattonella* species

We tried several methods to obtain the microsatellite marker necessary to analyze the genetic diversity of the *Chattonella* species at the local level population and finally succeeded to establish. This was applicable to *Chattonella antiqua*, *C. marina*, *C. obvata* and *C. minima*. Comparing the amplified fragments from this marker, the 4 species showed a highly genetic similarity, and 7 genetic polymorphisms were detected among 14 strains of 4 species. Interestingly, 8 strains (50%) have two DNA fragments different in length, indicating heterozygote. This would mean that *Chattonella* is a diploid organism and suggests the existence of a sexual reproduction.

(3) Ecophysiological study on harmful phytoplankton cysts under simulated ballast tank environments

Marine sediment contains a pool of phytoplankton resistance forms (i.e. dormant stages, called cysts), which have a high resistance to heat, desiccation, darkness and even anoxia. As a consequence of the use of ballast water, the dangerous bloom forming dinoflagellates *Alexandrium tamarense* and *A. catenella* causing paralytic shellfish poisoning that were unknown in the southern hemisphere before 1970, were well documented by 1990. And, unlike other forms of marine pollution, the impacts of invasive marine species are most often irreversible! When cysts are loaded into the ballast tanks, drastic changes in their environment happen, giving several stresses. The first obvious parameter is a sudden complete darkness. And since the ships are under the winds-waves influences, a high turbulence inside the tanks is a regular pattern. The temperature inside the tanks follows the temperature of outside seawater surrounding the ship; so that according to the route, the temperature changes constantly. The most stressful route is probably from one hemisphere to the other, with equatorial zone crossing that raises the temperature in the tanks to 31ºC or more. The experiments designed here were dedicated to elucidate the effects on dinoflagellates cysts of the combined stresses encountered in ballast tanks.

Using a statistical method after enrichment of surface sediment from Japanese ports, the number of potentially dangerous cysts species could be estimated at 200-500 cysts/g of wet sediment. For a single ship and for one voyage only, the amount of living cysts that could be transported to new places would be from 20 to 400 millions. From our experimental settings and germination results comparisons, it appeared that strong stresses or at least some combinations of stresses can enhance or delay the dinoflagellate cysts germination according the each genus/species. And tank simulations similarly enhanced the cysts germination for some of the species. A noticeable fact though is that the darkness seems an important factor in the combination of stresses. The cysts response to darkness and combined stresses are therefore dependent on the genus/species and most probably on its physiological state. But in any case, the transit in ballast tanks, even with severe conditions of stresses, does not kill the cysts. It may even have an opposite effect and increase their ability to germinate afterwards, regardless of the normal dormancy period of the cysts. This study highlights the great importance of drastic procedures to reduce the sediment load inside the ballast tanks and to avoid its dispersion in any cases.

Major Publications

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