Research status of ship’s ballast water detection

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Abstract. Marine ballast water is the main way of marine biological invasion and poses a serious threat to marine ecological security. In order to effectively prevent biological invasion and protect marine ecological safety, the International Maritime Organization (IMO) has adopted the International Convention for The Control and Management of Ship’s Ballast Water and Sediments. In this paper, the progress of the implementation of the convention is briefly introduced, with emphasis on the detection methods of ballast water according to the requirements of regulation D-1 and regulation D-2, such as salinity analysis method, colored dissolved organic matter content analysis method, microscope detection method, chlorophyll A detection method, flow cytometry detection method and flow imaging detection method.

1. Introduction

“Ballast water” means water with its suspended matter taken on board a ship to control trim, list, draught, stability or stresses of the ship. [1] Ballast water is of great significance to ship handling and navigation safety, but a large number of plankton, microorganisms, bacteria, larvae or spores of various species contained in ballast water will be transferred from a certain primary sea area to other sea areas with ballast water. In the process of ballast water transfer, some organisms cross the geographical isolation, grow and reproduce in a new ecosystem with a suitable environment and no natural enemies, and establish a population, which poses a threat to the original species diversity and even the ecosystem. The Global Environment Fund (GEF) has defined hazardous aquatic organisms and pathogens in ballast water as one of the four major threats to the oceans after fully recognizing their catastrophic impact on the global Marine ecosystem.

In order to better manage and control ship ballast water, IMO adopted the International Convention on The Management and Control of Ship Ballast Water and Sediment in February 2004, which provides internationally binding regulations for the management and control of ship ballast water worldwide. The convention entered into force on September 8, 2017, and entered into force for China on January 22, 2019. It aims to prevent, reduce and ultimately eliminate harmful aquatic organisms and animals through the control and management of ship’s ballast water and sediments. The transfer of pathogens causes harm to the environment, human health, property and resources. It is the world's first international convention to deal with the invasion of alien species carried by ballast water.

The Ballast Water Convention requires that ballast water can meet the D-1 standard through replacement or meet the D-2 standard through treatment. The specific requirements of the ballast water D-2 standard are shown in the table. Ballast water replacement is only a transitional management measure before ballast water treatment technology matures. In order to meet the D-2 standard, ships need to install a ballast water management system, and perform physical, chemical or biological
treatment of the ballast water when the ballast water is loaded, in the ballast tank or before the ballast water is discharged, so as to discharge. The number of surviving organisms and indicator microorganisms contained in the ballast water meet the specified requirements.

Table 1. The regulation D-2 of the BWM Convention Annex.

| Indicator microbes                  | Standard                      |
|------------------------------------|-------------------------------|
| Greater than or equal to 50µm in minimum dimension | < 10 viable organisms/ m³   |
| Less than 50µm in minimum dimension and greater than or equal to 10µm in minimum dimension | < 10 viable organisms/mL |
| Toxicogenic Vibrio cholera (O1 and O139) | < 1 cfu/100 mL              |
| Escherichia coli                   | < 250 cfu/100 mL             |
| Intestinal Enterococci            | < 100 cfu/100 mL             |

2. Progress in the implementation of the Ballast Water Convention

Although the Ballast Water Convention has entered into force, it is still in the experience accumulation period. The implementation process of the experience accumulation period is divided into three stages: data collection, data analysis, and convention review and revision. The Maritime Environmental Protection Committee of the International Maritime Organization held its 72nd meeting (MEPC72) in London on April 19, 2018. The meeting adjusted the timetable for the data collection and analysis plan of the Convention’s experience accumulation period and required it in 2019. The summary data will be submitted to the MEPC74 meeting, and a proposal for the amendment of the convention will be submitted to the MEPC79 meeting in 2022.

The experience accumulation period requires the collection of numerous data, including sampling analysis methods and results used during the trial of BWMS. Therefore, there are two forms of sampling analysis during PSC inspection: one is sampling for a large number of detailed analyses to collect data, which can be called “voluntary sampling”. Such a non-mandatory sampling analysis conclusion cannot be used as a basis for determining whether the results meet regulation D-2. The other is based on Article 9 of the Ballast Water Convention, which allows PSCO to conduct sampling and analysis when necessary in accordance with the guidelines of the Convention during port state inspections. This can be called “mandatory sampling”. Proving that the ship does not meet the requirements of the Convention can be used as the basis for PSCO to punish the ship.

3. Detection method for regulation D-1

3.1. Contents of regulation D-1 of ballast Water Convention

In order to meet the D-1 standard, the ship should adopt the replacement method, the direct flow method or the dilution method during the voyage, so that the replacement rate of the ballast water in the tank can reach at least 95% of the ballast water volume. This is an alternative measure for some ships until regulation D-2 is fully applied. Regulation D-1 requires ships to replace ballast water at least 200nmile from the land and at least 200m deep. If it is really not feasible, it shall be as far away from the land as possible and under all circumstances at least 50nmile from the land and a depth of at least 200m, or the ballast water shall be replaced in the sea area designated by the port state. Under the Ballast Water Convention, a port state shall not require a ship to deviate from its intended course or delay its voyage in order to replace ballast water.[2]

3.2. Detection method for regulation D-1

The D-1 replacement standard of the Ballast Water Convention does not involve the quantitative requirements of individual organisms in the ballast water, and is mainly to verify whether the ballast water has been effectively replaced in the deep sea. Therefore, the detection method is mainly based on
the different characteristics of coastal waters and deep sea waters. Generally, detection and analysis are performed from different aspects such as salinity, turbidity and organic matter.

3.2.1 Salinity analysis method
As fresh water and rivers flow into the sea, the salinity of coastal waters is generally lower than that of deep waters. The salinity of deep-sea waters is generally higher than 30 PSU. If the salinity of detected ballast water is much lower than 30 PSU, it indicates that the ship may not have effectively replaced ballast water. In practice, conductivity meters or salinometers are commonly used to measure the salinity of ballast water. However, the salinity of some ports is similar to that of deep-sea waters. Ships carrying ballast water in these ports should not take salinity analysis as the only evidence for whether ballast water is replaced, but should combine or use other detection methods.[3]

3.2.2 Colored dissolved organic matter content analysis method
Another feature that clearly distinguishes coastal waters from deep water is the content of colored dissolved organic matter (CDOM). CDOM is an optical measurement of dissolved organic matter in water. Ballast water with high CDOM content has a strong absorption spectrum in the short-wave band, such as blue to ultraviolet. Ballast water with low CDOM content or without CDOM has absorption spectrum in long wave band, such as red. Therefore, the ballast water with a high CDOM value is green to yellow-green to brown in color, while the ballast water with a low CDOM value is blue.[4]

However, coastal waters are greatly affected by human activities, such as industrial wastewater, municipal wastewater, and wastewater discharged from agricultural activities, which will lead to a high CDOM value in coastal waters. With the increase of offshore distance and water depth, the CDOM value decreases gradually. The threshold value of CDOM can be accurately measured by Excitation-emission Matrix Spectrometry. The threshold value of CDOM can be used as a strong evidence to judge whether ship ballast water is replaced or not. In practice, portable nutrient sensor is generally used to detect the CDOM value of ship ballast water.

4. Detection method for regulation D-2

4.1. Traditional microscope detection method
The basis of plankton taxonomy is the microscopic analysis of samples. Microscope detection is also the basis to test whether other methods are effective. The microscope can distinguish the species and dominant species of samples while counting, and it is also convenient to observe the plankton morphology and measure accurately.[5] Therefore, the microscopic detection method is often used in the basic research of ballast water, such as the study on the plankton diversity of ship ballast water and the study on the treatment effect of ballast water. Usually in the inlet or outlet of the ship, through 10μm true screen or 50μm nylon mesh filtration of a certain amount of water, with filtering seawater washing filter screen 3 times, the bottom of the net tube of the sample into the sample bottle, can also use dye to dye the plankton.

4.1.1 Direct microscopy
Direct microscopic inspection means that no dye is added, and the inspection is performed directly under the microscope, eliminating many pre-processing steps, but there will be some problems in detecting the living aquatic organisms in the ballast water. Since no dye is added, plankton activity can only be judged by the phytoplankton cytochrome content and pigment degradation, and the movement state of zooplankton. This method requires the inspectors to have a wealth of experience, and requires a high degree of sample cleanliness. Therefore, this method is not suitable for the detection of plankton in ballast water.
4.1.2 Neutral red (NR) staining
The neutral red dye will stain the living plankton and present different depths of red. However, the dyeing state is related to the concentration of the dye, the dyeing time and the type of plankton. After copepod zooplankton is dyed with neutral red dye, some organisms will be completely dyed red, while some organisms can only be partially dyed; in addition, some plankton will be dyed red by neutral red even if they lose their vitality. For crustacean larvae, it is difficult for the neutral red stain to enter the organism and be stained due to the existence of the crustacean. The cell walls of phytoplankton such as dinoflagellate also prevent the entry of neutral red, and it is impossible to judge the survival status by whether it is stained.

4.1.3 The vital stain method
The vital stain method uses fluorescein stain and a fluorescence microscope to evaluate the number and status of organisms in ballast water with a size of 10-50 $\mu$m.

Commonly used fluorescent known as Fluorescein Diacetate (FDA) and 5-methyl chloride Fluorescein stain (CMFDA-5). CMFDA is FDA chlorine methyl derivatives, this kind of Fluorescein stain, also known as "tracers" of living cells. FDA is mainly used to test enzyme activity and vitality. For bacteria, diatoms, green algae and some herbaceous plants, when the fluorescein stain passively enters the cell, the lipophilic groups in the stain will be hydrolyzed by the non-specific esterase in the living cell, and the non-permeable fluorescent products will be retained in the cell membrane.[6] But not all organisms can be stained by the FDA, and the fluorescent signals vary. While CMFDA and FDA have the same fluorescence spectrum, combined use can make up for these defects. Cells that labelled by cmfda produced 5-chloromethylfluorescein that emitted green fluorescence, which was stable and could be distributed to two progeny cells for multigenerational tracking of cell movement.[7] In vivo staining is more effective for detecting living aquatic organisms in ballast water and can distinguish between cells that are "live" or "dead".

The microscopic method can detect the concentration of plankton, but it takes a long time and requires a high level of expertise, requiring that the inspectors can distinguish the species of plankton under the microscope and count them. The plankton in the field of view takes on different morphology due to the action of the slide and cover glass, and the cell angles observed under the microscope are varied. It is also a difficult problem how to accurately distinguish plankton species from debris and impurities in samples. In addition, 10-50$\mu$m plankton requires higher magnification and quality of the microscope, and the limitation of magnification brings difficulties to the identification of plankton. The high density of phytoplankton in certain algae blooms makes microscopic detection difficult. Therefore, timely detection is required after sampling to prevent the gradual death of plankton in the samples from causing inaccurate data.

4.2. Chlorophyll A detection method
As the main photosynthetic pigment in phytoplankton, chlorophyll A is an important indicator that reflects the biomass of phytoplankton. In the study of phytoplankton in ship ballast water, the situation of phytoplankton in the sample can be reflected by measuring the content of chlorophyll A in the sample. The content of chlorophyll A can be measured by spectrophotometry, chlorophyll A fluorescence, high performance liquid chromatography (HPLC) and other methods to quickly determine whether the emission requirements are met.

In the detection of chlorophyll A content, the water sample was first filtered through the filtration membrane, and then acetone was added to the sample to grind and extract chlorophyll A. The absorbance of the extract was measured by spectrophotometer at different wavelengths, and the value of chlorophyll A was calculated by formula.[8] When the chlorophyll concentration is low, high performance liquid chromatography can be used to analyze the chlorophyll content of surface seawater.

However, some studies showed that the content of chlorophyll A could not be accurately measured by spectrophotometry and chlorophyll fluorescence method, and the measured value was higher than the actual value. [9-10] The detection limit of spectrophotometry is only 1.0$\mu$g/mL, and the sensitivity
is low. In comparison, the HPLC method can avoid the interference of other pigments in the spectral detection, and the detection limit is 10ng/L. However, it can only detect the content of chlorophyll A, and cannot distinguish the species of algae.

4.3. **Flow cytometry**

Flow cytometry (FCM) provides high-speed analysis of tens of thousands of cells, rapid classification count and measurement of cell morphology and fluorescence characteristics. The size and shape of algae were obtained by scattering signal of algal cells to horizontal and vertical light. The chlorophyll fluorescence intensity and characteristic excitation spectra of different algae vary greatly. In 1981, Trask et al. [11] established a series of optical parameters based on this principle to ensure the determination of algae by flow cytometry, and proved the feasibility of using flow cytometry to analyze and measure algae.

However, the flow cytometry method is more suitable for studying fresh water samples with not too low sample concentration due to the need to mix the samples evenly during sample injection. Flow cytometry measurements are affected to some extent by the type of counting and sorting equipment used by the instrument, such as the laser intensity of the instrument and the difference in the filter. The operator also has some influence on the test results. However, the concentrations of living aquatic organisms required by the Convention are very low, which makes direct detection by flow cytometry difficult and subject to the limitations of instrument size and price. Therefore, flow cytometry is rarely used in the detection of plankton in ship ballast water, especially in the detection of discharge ballast water.

4.4. **Flow imaging method**

Flow imaging technology, together with image analysis software, are prepared for Flow imaging. In recent years, FlowCAM began to be applied in plankton count. According to the optical and morphological features of the plankton in the samples, compared with the image library, automatic species identification counting was realized and images were taken. To some extent, this automatic detection method can improve the accuracy of results and avoid the influence caused by sample preservation. Flow imaging replaces human eyes with imaging system, which is further improved on the basis of flow cytometry detection and can replace the traditional microscopic imaging detection method to some extent.

After studying the measurable particle size range of FlowCAM, Alvarez et al. [12] found that the measurement results of FlowCAM were more accurate than the traditional microscope method and could effectively avoid the influence of sample preservation on the results. See et al. [13] used FlowCAM to detect the plankton in the water sample that greater than 15μm, while Baptiste et al. [14] even used FlowCAM to detect the metazooplankton community of 80-1000μm. Based on the rapid detection of the structure and morphology of plankton, FlowCAM can also perform species diversity detection of plankton.

Because the FlowCAM method can be used directly after sampling to avoid sample storage, more accurate results can be obtained during plankton classification identification and community structure study. It is suitable for the detection of living aquatic organisms in ballast water and can photograph each cell for easy observation of morphological characteristics. Therefore, in the research work, it is necessary to constantly improve the plankton atlas, prevent missing detection, and get more accurate analysis results. Sample should be evenly mixed to reduce the error caused by uneven mixing and ensure the sample concentration is appropriate.

Due to the different morphologic angles of the plankton captured by FlowCAM and the limited resolution of the photos obtained, there is a small morphological difference in the cells of some similar species, and the plankton species are extremely abundant, so it is difficult to accurately analyze the samples.
5. Conclusions
At present, detection methods of ballast water still have great complexity and technical limitations. Before the IMO has established a standard detection method, researchers have studied and tried a variety of detection methods, but there are still some shortcomings and defects. First of all, for ballast water testing on ships, on-site testing requires fast and accurate testing methods that can adapt to the ship's environment. The use of chlorophyll rapid detectors, flow cytometers and other equipment can adapt to the ship environment and provide rapid detection of plankton, but the detection capabilities of pathogenic microorganisms such as Escherichia coli and toxic Vibrio cholerae are insufficient. Secondly, for ballast water testing in the laboratory, laboratory testing is a relatively accurate testing method, but at present, it is still necessary to focus on solving the problems of economy, speed and simplicity of operation.

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