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Evaluation of different sampling practices to achieve BWTS samples with realistic representativeness of plankton in ballast tanks

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Original Article

Abstract

Introduction of non-native organisms in to the oceans through ballast water is widely recognized as an ecological concern. The International Maritime Organization (IMO) has come up with stringent regulations for the release of ballast water into oceans and expect ships to install ballast water treatment system (BWTS) to satisfy the standards set. IMO D-2 regulations and United States Coast Guard- Environmental Technology Verification program are quantitative of the plankton in the ballast water. Critical care is needed in sampling as inappropriate sample with wrong representation will yield false negative results. In this study the effect of stirring the water and a central perforated tube in the sampling tank on the count of plankton are evaluated. On mixing the contents of the tanks before recovering the samples, the phytoplankton count in the samples enhanced by ~20% while the zooplankton count got doubled. Moreover, the electric pump used had 12-15% mortality rate on the zooplankton *(Artemia)*.

Keywords*: Ballast water, phytoplankton, Artemia, fluorescein diacetate, chloromethyl fluorescein diacetate.*

Introduction

Ships during their deballasting operations discharge humongous amount of ballast water into the sea. It is estimated that around 4 billion tonnes of ballast is discharged annually (Rick, 2011). This ballast water carries potentially invasive organisms which when discharged pose a threat to the survivability of the native biota, cause human health concerns and probably making financial losses too (David *et al*., 2007) especially via ballast water transport, may result in a change of biodiversity, alteration of ecosystems, negative impacts on human health and economic loss. Estimates show that annually more than 10,000 species of marine organisms are unintentionally introduced into new environment through ballast water transfer (NOAA, 2011). Since 1991, various measures, committees and guidelines had been framed to study, check and prevent the transfer of such organisms through ballast water. The most widely used strategy to reduce the risk of ballast-mediated foreign organism transfer is ballast water exchange (BWE). BWE can eliminate 99% of freshwater organisms when properly implemented (Gray *et al*., 2007). However, the efficacy of BWE seems to be variably low for marine organisms (Wonham *et al*., 2001); thus, BWE is not considered comprehensively protective. Consequentially, in

2004, IMO accepted a convention to prevent, minimize and possibly eliminate the risk to environmental and commercial risk from ballast transfer. Recently the Ballast Water Convention was ratified by 52 nations accounting to 35.14% of total tonnage and came into force on September 8, 2017. Hence, it becomes mandatory for all the countries to satisfy the ballast water performance standard Regulation D-2 of IMO which requires that during discharge, ballast water should not have more than a specific number of organism in a size class (IMO, 2004). The regulations of D-2 states that ships that satisfy the convention must discharge:

- Less than 10 organism of size ≥50 μm per cubic metre of the ballast water
- Less than 10 cells of size ≥ 10 and ≤ 50 μm ml-1 of the ballast water.

The earlier BWE regulation (D-1) was just qualitative with respect to the biological species (IMO, 2004). However, D-2 requires strict quantitative analysis of organisms in different size class. As wrong samples could indicate either higher or lower number of organism than the actual count in the tank, sampling the discharge water for compliance testing is of supreme significance. Ballast water sampling needs prior planning and must be executed with utmost care. It is important that the samples retrieved are a true representation of the whole tank (Gollasch and David, 2011) especially via ballast water transport, has raised considerable attention especially in the last decade due to the negative associated impacts. Ballast water sampling is important to assess the compliance with ballast water management requirements (i.e. compliance monitoring. This is needed because of the diversity of the plankton in the ballast water. Each and every species in the tank behave in a unique way and hence it is mandatory that the sampling practices followed are well suited for the plankton in the study.

The D-2 regulations has guidelines to plankton count but do not recommend sampling practices to be followed to achieve compliance. Sample procedure for ballast water treatment system, therefore, should be optimized for count repetition in order to provide complete representation of the tank.

The paper analyses the different methods followed in in-tank sampling of the indigenously assembled BWTS. The setup installed uses a filter and ultra violet radiation to remove plankton or render them inactive from the ballast water. During the treatment system, a sampling line from the discharge pipes drains into the 1 m^3 sampling tank. The sample tank is filled throughout the ballast water treatment duration. By this way, the water in the sampling tank becomes the complete representation of the water being treated. To check the biological efficacy of the system in eliminating the plankton, samples from this sampling tank has to be analysed. The objective was to end up with a sampling method that will yield an accurate sample that is most illustrative of the tank.

Material and methods

To study how the testing organisms perform at the treatment and sampling tanks, a phyto and a zooplankton was used. *Micractinium* sp. (minimum dimension of 26 μm) a freshwater microalgae was obtained from DHI Water and Environment (S) Pvt. Ltd., Singapore. The organism was maintained and grown in MLA growth medium (Bolch and Blackburn, 1996) under continuous aeration and lighting with white fluorescent light at an intensity of 40 μmol photon m^{-2} s^{-1} (3000 lux) at 25°C ± 2°C in a culture room. *Artemia* nauplii was hatched from the brine shrimp eggs a day before the experimentation. The brine shrimp eggs were added into artificial sea water prepared with a specific gravity of 1.0211-1.0264. The cysts were aerated rigorously for 24-36 hours with illumination. The aeration and illumination was stopped to harvest the developed organisms from the bottom of the tank. The developed nauplii measured around 400-700 μm in size.

Design

One metric cube volume sampling tanks were used in the study. The tanks have a detachable central pipe with holes at regular intervals throughout its height. The pipe is connected at the bottom to the drain of the tank. To study the effect of stirring the water in the tank while collecting samples, one tank was stirred and other tank was not stirred prior to sampling.

Known quantities of the plankton were added into the tank. The samples were collected using the sample drain at the bottom of the tank with and without the central pipe. The water in the tank was allowed to settle for 10 minutes. Then the samples were taken from the open surface and from water drain at the bottom. Similar samples were collected after removing the central perforated pipe.

Analyses

Epifluorescence microscopy using FDA and CMFDA stain: Fluorescein diacetate and chloromethylfluorescein diacetate (Thermo Scientific) were prepared in dimethyl sulphoxide (DMSO) with a final concentration of 5 and 2.5mM respectively. A total of 10 μL of working solution was added to 10 mL of sample water in a 15- mL glass vial for staining (Garvey *et al.,* 2007; Reavie *et al.,* 2010). Samples were incubated in the dark for 10 min; after which 1 mL was transferred to a 1-mL gridded Sedgwick- Rafter cell for observation under Primovert inverted fluorescent microscope (Zeiss, Germany). This set-up permitted visualization of only the living cells in green and were counted (Garvey *et al.*, 2007; Reavie *et al.*, 2010).

Zooplankton sample collection: A vertical zooplankton net of 25 inch in length and 6.5 inch in diameter, with 80μm pore size, was used to filter the zooplankton from one metric cube water sample. The filtered zooplankton are then counted in a Bogorov counting chamber using an inverted microscope. Only the live organisms (showing movement) were counted.

Statistical analysis: Each experiment was done thrice and the mean and standard deviation of the experimental results were calculated using Sigmaplot. The values plotted in the graphs are mean values of triplicate samples and the error bars denotes the standard deviation among the replicates.

Results and discussion

The sampling points in ballast water treatment system can be either (a) in-tank or (b) at-discharge. The in-tank method uses sounding or air pipes to drain water samples while at-discharge sampling done using the discharge points. This study analysed the efficiency of different sampling methods in producing samples that completely represents the treated tank.

Effect of the pump

To assess the effect of the pump on mortality of the larger sized zooplankton, developed *Artemia* nauplii of known concentration ($3.7 * 10^5$ *Artemia* per m^3) was passed through a pump and was filtered at the discharge with the help of a zooplankton net (Fig. 1a). The nauplii showing visual movement were counted as viable, while individuals with no movement even after poking were considered as dead and not counted.

The results revealed the negative effect of pump on the survival of the *Artemia* nauplii that 12% of the organisms were dead after passing through the pump. It is possible that this lethal effect could go up with pump capable of transferring huge volumes of ballast water as in actual ships. This is a serious issue in working with larger sized test organisms for compliance testing on BWTS as it will affect the initial count of the individuals in the challenge water. USCG and IMO states that the initial count should be 10^5 zooplankton in a metric cube of challenge water before the BWTS. Hillman *et al.* (2004) reported 70-80% mortality of *Artemia* nauplii after passing it through a pump (20 tonnes per hour), while Sassi *et al.* (2005) reported that most of the zooplankton added into the system were killed by the pump itself. Hence the addition of *Artemia* should take place in such a manner to bypass the pump and avoid loss of individuals to mechanical shear. Thus, the efficiency of the filter in BWTS can be accurately and effectively studied. If this could not be achieved, the test organisms must be added in a higher amount so that even after the lethal effect of the pump, required minimum amount of zooplankton remain in the system.

Sampling methods

The organisms, both phytoplankton and zooplankton, were taken in more than required concentration by the USCG and IMO guidelines (IMO, 2004). They were added into the two tanks; one tank was mixed (WS-with stirring) prior to collecting sample while other was not stirred (WOS without stirring). The samples from each tank was collected with and without the central perforated tube (WT and WOT). Fig. 1b depicts the different methods that were employed to draw samples from the tanks.

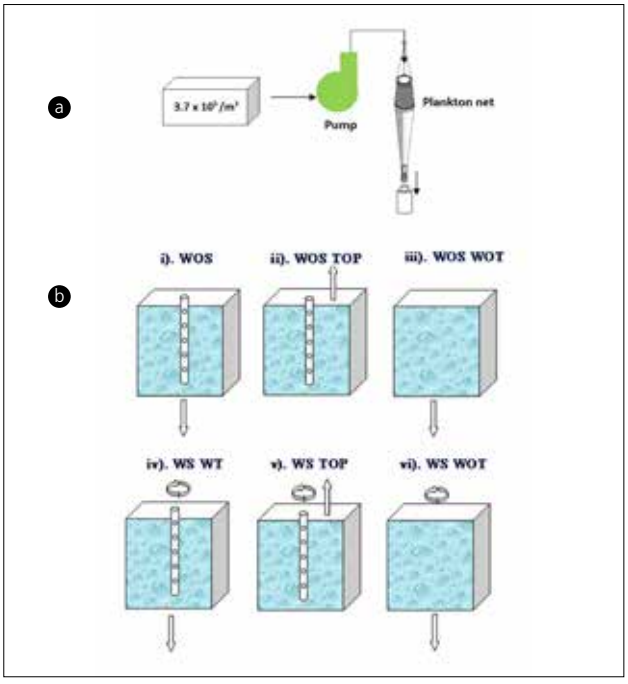

Fig. 1. Schematic representation of the experimental BWTS sampling methods performed to find an effective sampling practice. a. Setup and flow of the experiment carried out to detect the mortality rate of the pump on the zooplankton. b. Sampling points and practises followed to recover samples to optimize sampling for maximum representativeness of the tank. (i- iii)- without mixing the tank; (iv-vi) after mixing the tank.

Phytoplankton count

The samples were taken from both tanks (WOS and WS) and from the discharge drain at the bottom of the tank. The latter was done with and without the provision of central perforated tube. The samples were immediately stained with FDA and CMFDA and was counted in Sedgewick counting chamber. The number of cells that were added into the tank was 2316 cells ml^{-1} (Fig. 2). In the WOS tank, a total of only1208 cells were counted in the sample taken from top surface of the tank; while 1662 and 1412 number of cells were recorded with and without central tube respectively. Whereas, in the WS tank, 2108 cells were found in a millilitre of the sample taken from the surface, while 2116 and 1745 cells were present in the samples collected with and without the pipe (Fig. 2).

On comparing the values from both the tanks, it is evident

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that the samples collected from WS tank yielded values close to the initial count. Phytoplankton have the property of auto flocculating and it becomes faster in dark and non-aerated cultures. The ballast tanks will facilitate auto flocculation in dark and which will lead to uneven distribution of the plankton in the tank. Samples collected from the top, middle or bottom will not provide the actual concentration of algae in the tank. A combination of samples taken at different depth may be used; however one cannot be sure about the accuracy of the values. Hence, we propose the water in the tank be stirred or agitated before the sample is collected. The effect of the perforated tubes was also studied and it was found that the usage of this tube enhanced the count of the planktons even in WS tank. This could be due to the fact that the perforated tube takes sample from the complete height of the tank rather than only from the bottom. Summing it up, it is proposed to use the central tube for sampling after the tank is stirred to yield samples representative of the initial count.

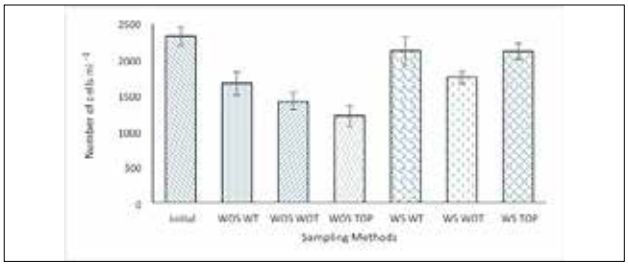

Fig. 2. Concentration of viable phytoplankton mL^{-1} in ≥10-μm to <50-μm size range estimated by FDA and CMFDA staining in the samples retrieved using different methods. The bars represent the standard deviation of the three replicates used per sample in the study.

Zooplankton

Artemia nauplii, because of its bigger size, settles at the bottom of the tank when there is no agitation or aeration. Hence, BWTS sampling with *Artemia* as test organism becomes tricky. After hatching the brine shrimp eggs in 30 ppt salt water, the developed nauplii tend to settle at the bottom on absence of aeration; while the broken shells of cysts float at the surface. Sampling a wrong section of the water would yield no organism in the analysis and leads to false negative counts.

Sounding pipes are used for sampling the ballast tanks. However, they are not very effective in producing a representative sample of the zooplankton in the tank. Samples taken from the manholes using nets were found to be more diverse than the sounding pipe sample (Sutton, 1998). Hence sampling the whole tank with a zooplankton net is recommended and the present study also yielded satisfactory results with the sampling accessory.

The WOS and WS tanks used were added with 1.5 x 10^5 individuals per m^3 of water. The samples from WOS tank yielded only 2.8 x 10^4 and 8.6 x 10^3 individuals per m^3 used with central tube and without the tube respectively (Fig. 3). While the WS tank yielded 6.0 x 10^4 and 1.1 x 10^5 nauplii per m^3 in the same conditions (Fig. 3). The presence of the central tube produced different cases in WOS and WS tanks. As the tank is filled, *Artemia* entering the central tube settles down and drained out during sampling. While when sampled without the tube, very few of the individuals settled would have come out of the drain.

In the WS tank, the central tube yielded only the individuals that filled in during the tank filling. This minimizes the number of individuals draining out. Whereas, without central pipe, the chance of *Artemia* coming out of the drain is more and hence the higher counts in WS-WOT. Mixing the tank and draining out the water without the central pipe was found to give good results with zooplankton.

In brief, for the present system and other similar systems that we have, the samples has to be mixed in the tank prior to draining for biological efficacy test. We also recommend the same for any BWTS with in-tank sampling that make use of the similar test organisms.

The study evaluates the difficulties in sampling ballast water tanks and choosing the right practice to get samples representative of the whole ballast tank. Plankton settling at the bottom is the major issue that was addressed in this study. The results showed that mixing the tank prior to sampling will enhance the representativeness by 20% and more than 100% in phyto and zooplankton respectively. The paper also concludes that, in biological efficacy testing for compliance, sampling method and practices need to be evaluated and optimized with specific reference to the BWTS installed and the test organisms. Consequently, improvement in design and suitable techniques will facilitate the rapid and accurate measurement of the residual plankton in the tank so as to get the compliance in relation to D-2 regulations of the IMO.

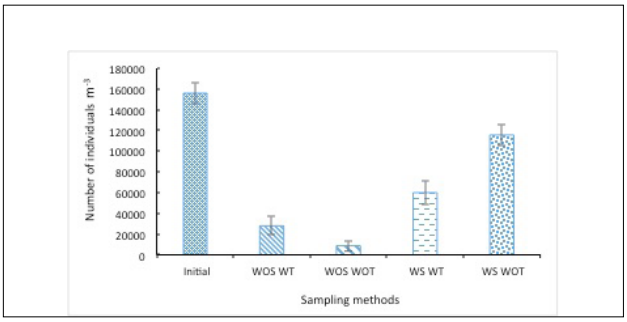

Fig. 3. Counts of live *Artemia nauplii* in the samples drawn from different sampling points. The bars represent the standard deviation (n=3).

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