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Different approaches and limitations for testing phytoplankton viability in natural assemblies and treated ballast water

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ABSTRACT

Shipping is recognised as an unintentional efficient pathway for spreading non-native species, harmful organisms and pathogens. In 2004, a unique IMO Convention was adopted to control and minimize this transfer in ship's ballast water. This Convention entered into force on 8th September 2017. However, unlikely the majority of IMO Conventions, the Ballast Water Management Convention requires ships to comply with biological standards (e.g. concentration of organisms per unit of volume in ballast water discharges). This study aimed to apply different techniques developed to measure concentrations of viable phytoplankton in natural and treated ballast water samples and compare them with the established flow cytometry method and vital staining microscopy. Samples were collected in the English Channel over one year and on-board during ballast water shipboard efficacy tests. Natural abundance of live phytoplankton varied from 23% to 89% of the total, while for cells larger than 10 µm (a size defined by the BWM Convention) the percentage varied from 3% to 60%. An overall good correlation was seen between the measurements taken with the two fluorometers and in comparison with the flow cytometry analysis, as found in previous studies. Analysis of treated ballast water samples showed a large variation in the number of viable cells, however indicating a low level of risk on all occasions for regulatory purposes. One of the key aspects to bear in mind when sampling and analysing for compliance is to be aware of the limitations of each technique.

1. Introduction

The International Maritime Organization's Ballast Water Management Convention (-BWMC) entered into force on 8th September 2017, after a delay of > 13 years from its adoption on 13th February 2004. The Treaty was preceded by two sets of guidelines developed during the 1990s while progressing its work towards the development of an international convention; The International Guidelines for Preventing the Introduction of Unwanted Aquatic Organisms and Pathogens from Ballast Water and Sediment Discharges (resolution MEPC.50(31)) in 1991 (subsequently adopted as the IMO Assembly resolution A.774(18) in 1993) and the IMO Assembly resolution A.868(20) - Guidelines for the Control and Management of Ships Ballast Water to Minimize the Transfer of Harmful Aquatic Organisms and Pathogens (1997).

Also during the 1990s a landmark step was taken, with recognition by the United Nations (UN) Conference on Environment and Development, on the ballast water issue as a major international concern. With the adoption of the Convention on Biological Diversity by the UN (Rio 92) the threat represented by the transfer of non-native species was explicitly identified as one of the four greatest threats to the world's oceans.

The shipping industry is an extremely efficient pathway/vector for the spread of species worldwide (Ruiz et al., 2000; Bax et al., 2003; Coutts and Taylor, 2004; Drake and Lodge, 2004; Castro et al., 2017). There are many emblematic examples of invasive species recorded during the 1980s and early 1990s around the globe e.g. the golden mussel (*Limnoperna fortunei*) in South America (Darrigran and

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Table 1
IMO's Ballast Water Management Convention regulation D-2 (IMO, 2004).

Organisms/indicators and size class	Maximum allowable number in discharged water according to the Regulation (CFU = Colony Forming Unit)
Viable organisms ≥50 μm in minimum dimension	< $10/m^3$
Viable organisms ≥10 < 50 μm in minimum dimension	< 10/ml
Toxicogenic *Vibrio cholerae* (O1 and O139)	< 1 CFU/100 ml
Escherichia coli	< 250 CFU/100 ml
Intestinal Enterococci	< 100 CFU/100 ml

Pastorino, 1995), the zebra mussel (*Dreissena polymorpha*) in North America (Hebert et al., 1989) and the comb jelly (*Mnemiopsis leidyi*) in Europe (Kideys, 1994). Within the BWMC, a ballast water performance standard known as the D-2 standard defines maximum allowable concentrations of viable organisms in the discharged ballast water according to their size or group (Table 1). Unilateral regulations have also been adopted in some countries (e.g. Standards for Living Organisms in Ships Ballast Water Discharged in U.S. Waters, 2012, United States Coast Guard (USCG)) with similar requirements.

To meet the requirement for minimising the numbers of viable organisms within ballast water tanks, a variety of ballast water management systems (BWMS) have been developed which are mainly based on an initial filtration step plus a chemical or physical treatment. Electrochlorination and treatment using ultra-violet irradiation are the two main secondary treatments. Both treatments have pros and cons and their use needs to be evaluated together with the ship type, trading route and environmental aspects.

UV-C systems are often recommended as environmentally friendly systems as no potentially toxic by-products are release to the environment during the discharge (Batista et al., 2017).The main disadvantage however is related to the regrowth of many species of phytoplankton after a period varying from six to twelve days regardless the UV-C radiation dose (Martínez et al., 2012; Martínez et al., 2013; Stehouwer et al., 2015). In addition, UV-C systems have lower biological efficacy in high turbidity waters because UV light transmission is considerable reduced. Finally, there is a 'delayed kill effect' on organisms (Werschkun et al., 2014; First and Drake, 2014; Stehouwer et al., 2015).

Electrochlorination based ballast water treatment relies on the process of producing hypochlorite (a powerful oxidant) when an electric current is run through water containing a minimum concentration of salt. Yet electrochlorination is usually more efficient when used in waters of high turbidity (Batista et al., 2017). In contrast to UV-C irradiation systems, the hypochlorite generated in these systems may need to be neutralized before discharge and the dose is applied just once during the treatment (while UV-C treatment usually takes place during water uptake and discharge). These systems also generate disinfection byproducts, such as trihalomethanes, bromate, among others, and in particular bromoform and dibromoacetic acid, which are a cause of concern (Werschkun et al., 2012). Other concerns are related to the influence of lower temperatures on a system's efficacy and on the acceleration of tank corrosion (Morris, 1966; Lysogorski et al., 2011).

Marine ecosystems comprise only about 1% of Earth's photosynthetic biomass, yet are responsible for about 50% of our planet's annual net primary production (Geider et al., 2001; Falkowski et al., 2004). Photosynthetic activity in the oceans is carried out by a very diverse range of organisms including phytoplankton and macroalgae (Falkowski et al., 2004).

The fluorescence properties of the chlorophyll *a* of plants is a useful tool for studying photosynthesis as it occurs in all photosynthesizing plants and algae (Guilbault et al., 1973; Genty et al., 1989; Govindjee, 2004). Fluorescence occurs when a light photon is absorbed and an electron is excited. The electron subsequently returns to the non-excited state resulting in the emission of longer wavelength (than that used to cause excitation). In photoautotrophic organisms this process occurs in chloroplasts which have two photosystems (known as PSI and PSII), PSII is where oxygen is released as a by-product and PSI is where carbohydrates are formed. When light is absorbed by chloroplasts it can be used to drive photosynthesis, dissipated as heat or it can be re-emitted as chlorophyll fluorescence (Bradbury and Evennett, 1996; Maxwell and Johnson, 2000). From the perspective of photosynthetic organisms, fluorescence represents a waste of energy; however the amount is low with a maximum of circa of 3% of the absorbed light (Guilbault et al., 1973).

Due to the fact that it is non-destructive, expeditious and precise, chlorophyll *a* fluorescence has become a routine technique for measuring biomass as well as the photosynthetic activity of photoautotrophic organisms (Govindjee, 1995; Govindjee, 2004). Many techniques have been developed based on this principle of using chlorophyll fluorescence as a measure of photosynthetic primary production and photochemical efficiency e.g. 1 Hz Fluorometers, Pulse-Amplitude Modulated Fluorometers (PAM), Dual-Modulation LED Kinetic Fluorometers and the Fast Repetition Rate Fluorometers (FRRF) (Kolber and Falkowski, 1995; Schreiber, 1998; Wilhelm, 2003). Principles employed in the different techniques basically differ in how the photochemistry is saturated to generate the maximum fluorescence yield (F_m) (Röttgers, 2007). In addition to the dark-state (defined as the dark-adapted state of a molecule that cannot absorb or emit photons) ground fluorescence (known as F_0), maximum fluorescence (known as F_m) and consequently variable fluorescence (F_v) can be measured ($F_v = F_m - F_0$). The ratio of F_v to Fm (Fv/Fm) is often used as an indicator of the vitality of the phytoplankton.

An alternative method to assess the vitality of organisms is based on the bio-physical properties of the cells. Techniques using stains that can penetrate and once intracellular bind to cell DNA have been developed that allow the investigation of viability in the marine environment (Agustí and Sánchez, 2002). These stains have also been applied to the measurement of cell viability in ships ballast water. Stains that fluoresce yellow/green under excitation by certain wavelengths of light, mostly blue, have been generally adopted or proposed because they do not interfere with the red fluorescence of the chlorophyll (Veldhuis et al., 1997; Tang and Dobbs, 2007). The ability to measure the viability of phytoplankton cells helps, for instance, in distinguishing viable cells in the water column from non-viable cells that are still capable of fluorescing but contribute to over estimation of viable cells based only on chlorophyll *a* biomass (Veldhuis et al., 2001; Agustí and Sánchez, 2002; Steele, 2014). Previous studies have detected an occasionally large number of dead cells in the water column (ca. 95%) at certain periods of the year (Veldhuis et al., 2001; Agustí and Sánchez, 2002), highlighting the importance of discriminating viable from non-viable cells particularly when determining regulatory compliance.

Phytoplankton biomass and size distribution is of paramount importance to understanding the ecology of marine ecosystems and the fate of chemicals elements and particles within the oceans (Llewellyn et al., 2005). This study examines the use of different fluorescence techniques to measure viability and abundance of phytoplankton, being the dominant group in the IMO D-2 size range ≥ 10 < 50 μm. The pattern of distribution of viable and non-viable cells was investigated over one year in a natural assembly using a flowcytometer as well as two fluorometers (measuring the number of cells and the chlorophyll *a* biomass. Likewise, ballast water samples from commercial efficacy testing were also measured with both fluorometers and the results compared with those from flowcytometry (FCM) and epifluorescence