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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, whilst 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 (IMO BWMC) entered into force on 8th September, 2017, after a delay of more than 13 years from its adoption on 13th February, 2004. The Treaty was preceded by two sets of guidelines developed during the 1990s whilst 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 &Taylor, 2004; Drake &Lodge, 2007; 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 & 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.

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	less than 10/m^3
Viable organisms ≥ 10 < 50 μm in minimum dimension	less than 10/ml
Toxicogenic *Vibrio cholerae* (O1 and O139)	less than 1 CFU/100ml
Escherichia coli	less than 250 CFU/100 ml
Intestinal Enterococci	less than 100 CFU/100 ml

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 reemitted as chlorophyll fluorescence (Bradbury & Evennett, 1996; Maxwell & 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. 1Hz Fluorometers, Pulse-Amplitude Modulated Fluorometers (PAM), Dual-Modulation LED Kinetic Fluorometers and the fast repetition rate Fluorometers (FRRF) (Kolber et al., 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 & Sanchez, 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 & 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; Augustí & Sanchez, 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; Augustí & Sanchez, 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 (each with different excitation techniques) 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 microscopy analysis using stains. The primary objective was to identify patterns on the phytoplankton size distribution with regards to the viability of cells in a natural assembly and possible benefits and limitations of the techniques in the context of the ballast water compliance issues.

2. Material and Methods

2.1. Area of study

Station L4 of the Western Channel Observatory (WCO), located in the English Channel, about 13 km from the coast (coordinates 50°15.0'N; 4°13.0'W), was used to investigate natural phytoplankton assemblages (see Castro & Veldhuis, 2018 for details). The WCO is well characterised through ongoing research projects conducted by the Plymouth Marine Laboratory (PML) and the Marine Biological Association (MBA). The L4 area is known to be influenced by inputs of nutrients from rivers together with oceanic influences (Pingree and Griffiths, 1978; Woodward et al., 2017).Weekly samples were collected from the surface with a bucket between June 2016 and July 2017.

2.2. Ballast Water Shipboard Biological Efficacy Tests

Eight on-board tests of a commercial ballast water treatment system were conducted between 2016 and 2017. All treatment systems were certified and based on UV-C disinfection (ultraviolet irradiation). On all occasions, sampling occurred during the discharge of ballast water while in port.

Samples were taken from the sampling point in the discharge line provided in each ship. A sterile sampling tube was fitted directly to the sampling valve on the BWTS. The ballast water discharge was run for 5 minutes prior to the first sample (to avoid debris in the ballast water lines). Ideally six samples are taken over typically 1 hour of discharge from a single tank or the simultaneous discharge of two tanks. The number of samples varied among tests from two to six (plus replicates) due to pumping rates and varying volumes of water in each tank.

2.3. Methods

2.3.1. Flow cytometry

Analysis usually started in 2-3 hours after samples were collected at L4 and as soon as they arrived at PML. They were analysed at the flow cytometry facility using a Bekton Dickinson FACSort ™ flow cytometer. Samples were analysed for five minutes in a high flow rate of approximately 225 µL min^{-1} (total volume 1.125 mL), as follows:

- 2 ml living samples;
- 2 ml living stained samples;
- 2 ml dead samples; and
- 2 ml dead stained samples.

Flow rates were calibrated with Beckman Coulter Flowset fluorospheres of a known size and concentration. SYTOX Green dye was used as a nucleic acid stain for live/dead determinations (See section 2.3.3). Samples were also killed by heating at 80ºC for five

minutes in a water bath before analysis followed by stained analysis as described. FCM data were analysed with the FCS Express Flow Cytometry Software, version 5 (Denovo Software).

FCM settings were set to display cells in the size range from 2 to 50 µm. The size was measured as the scattered light in the forward direction (FS), the measurement best related to size (Ormerod, 2012, Castro & Veldhuis, 2018). The red fluorescence from the phytoplankton chlorophyll *a* pigment (emission > 630 nm) was measured after excitation with blue laser light (488 nm) while stained samples fluoresced bright green (emission peak of 523 nm). Standard spherical beads with known diameters (9.7 and 50 µm, Polysciences) were used as an internal standard for instrument calibration. These beads are uniform in size with known coefficients of variation (C.V. <2%) and measurements should possess the same spread for size and fluorescence.

Data analysis was based on clustering (sub) populations with identical size and chlorophyll fluorescence properties. The total number of phytoplankton cells (total number of cells/ml) was derived from the analysis of stained living samples (living + dead cells) while viable phytoplankton cells (viable cells/ml) were identified by the red fluorescence of living samples.

2.3.2. Fluorometry

The two fluorometers used in this study, the FastBallast (FaB), from Chelsea Technologies and the Ballast Check 2 (BC2), from Turner Designs, use different approaches to measure fluorescence. The difference is mainly related to the way the light pulse saturates the Photosystem II Reaction Centre (PSII RC). Both instruments in principle provide an estimated number of cells in the sample, using a pre-set conversion factor of variable fluorescence (F_v) into cell numbers.

The BC 2 (Turner) procedure includes a filtration step (using a 10 µm mesh filter) to estimate the abundance of cells >10 µm based on the conversion of a fluorescence value divided by a fixed constant value of chlorophyll fluorescence per cell for the size range of ≥ 10 < 50 µm. In a separate run the total chlorophyll fluorescence of the sample was measured using a syringe filter of 0.2 µm mesh. Default results displayed on the screen of the equipment are the abundance of cells in the sample as well as the photochemical efficiency (F_v/F_m, a measure of the effects of stress/vitality on the cell). The BC2, using default settings, provides a risk indication with regards to the IMO D-2 BW performance standard: high or low, depending on the combination of the abundance (no of cells/ml) and photosynthetic activity. According to the equipment manual, high risk water samples give an abundance > 10 cells per mL and a $F_v/F_m > 0.25$. When the number of cells is < 10 cells per mL or $F_v/F_m < 0.25$,

then the equipment displays a low risk indication (Table 2). Another important aspect is that the photosynthetic activity (F_v/F_m) is reported as not-detected (ND) whenever its value is outside the range 0.01 to 0.75. The upper detection limit of the instrument is > 2,000 cells per ml and, when seen, a high risk is displayed in the screen.

Table 2: Ballast Check 2 risk assessment readings and advised action as recommended in the user manual (Ballast-Check 2 User Manual – Rev. 1, 5th Sept 2016).

Readings Risk	• Abundance (cells/ml) • Activity	• Interpretation • Advised action
LOW	< 10 < 0.25	Within D2 Guidelines Maintain BWTS performance
LOW	< 10 ≥ 0.25	Within D2 Guidelines Maintain BWTS performance
LOW	> 10 < 0.25	Within D2 Guidelines Maintain BWTS performance
HIGH	> 10 ≥ 0.25	Exceeds D2 Guidelines Retest from sample flow. Check BWTS performance. If results remain high, plan for a more detailed analysis at earliest opportunity.

In this study the equipment was connected to a laptop during analysis allowing the reading of all fluorescence parameters being measured (F_0, F_m and F_v/F_m) through the HyperTerminal software (Hilgraeve, Inc).

The FaB fluorometer (Chelsea) has two analysis steps. The initial level (Level 1) provides a numeric value that relates to cell density (usually equals to F_v*1000 or F_v*100 depending on the software version) where < 0.04 indicates a "pass" and a numeric value > 40 indicates a "fail". Whenever the sample produces results between these two values, the system will continue to a Level 2, where cell density is estimated from the distribution of Fv values within several hundred semi-discrete measurements, alternatively to the amplitude of Fv derived from a single measurement (Oxborough, 2017). After about six minutes (in addition to the two minutes for level 1 analysis) the actual cell density in the sample is displayed. The software FaBtest gives the user different possibilities for obtaining further information during the data acquisition and analysis. For this study, samples were measured with and without filtration giving total cells in the range of 2 – 50 µm and, using a 10 µm mesh filter and subtracting the results from the total, numbers of cells between 10 and 50 µm.

All samples were kept in dark (i.e. dark adapted) for at least fifteen minutes before analysis.

2.3.3. Vitality staining

To test the viability of phytoplankton cells, the nucleic acid specific stain SYTOX Green™ (S-7020; Molecular Probes, Inc.) was used to indicate cells with compromised membranes since this dye can only penetrate such cells which then fluoresce bright green when excited (Roth et al., 1997; Veldhuis et al., 2001). The SYTOX Green is available in a 5mM solution and that requires a 100x dilution before use. Working stocks were prepared by diluting 50 µL in 5 mL of ultrapure water (Mili-Q water). For flow cytometric analysis, 2 mL samples of seawater were mixed with 20 µL of the SYTOX Green working stock and kept in the dark for a minimum of 15 minutes prior analysis. As described by Veldhuis *et al*. (2001), cells exposed to the dye which stained bright green were classified as dead cells and together with the non-stained (viable) cells that exhibited red emission fluorescence were considered the total phytoplankton community in the sample.
Another fluorescent staining method used in this study for ballast water samples was the one recommended by the IMO and USCG for detecting viable cells in the < 50 ≥ 10 µm size group. This method uses a combination of two vital stains: Fluorescein Diacetate - FDA (Molecular Probes-Invitrogen) and 5chloromethylfluorescein diacetate – CMFDA (CellTracker™ Green; Molecular Probes-Invitrogen) (Steinberg, 2011). In contrast to SYTOX Green which is a dead-stain, FDA is a live-stain. All BWTS tested in this study were UV-C disinfection technologies, therefore, samples were stored for 24 hours at ambient seawater temperature in the dark before analysis, in order to provide enough time for the UV-C damage to take effect.

2.3.4. Statistical data analysis

All statistical analyses were carried out using IBM SPSS Statistics software (Version 23 and 24), Microsoft Excel (Analysis ToolPak) and Primer 7 (version 7.0.13) from Primer-e (Quest Research Limited).

3. Results

3.1. Annual field data

L4 samples collected at the surface from June 2016 until July 2017 showed an averaged abundance of 20,153 cells/ml from which 13,179 in average were viable cells (no/ml) in the size range of 2 to 50 µm . Within this cell size class, the majority of cells detected was between 2 and 10 µm of size (ca. 98%) (Table 3).

Table 3: Mean number of total and viable cells (no/ml) at surface considering cells between 2 and 50 µm and the fractions between 2 - 10 µm and >10 - 50 µm. Samples were collected from June 16 to July 17 at L4 sampling site, in the English Channel.

Size class	Mean (Total cells ± SD)	Mean (Viable cells ± SD)
Total cells 2 – 50 µm (no/ml)	20153 ±11718.9	13179 ±11401.2
>2 - < 10 µm (no/ml)	15974 ±9558.3	10392 ±9659.5
> 10 µm (no/ml)	1404 ±1575.4	203 ±171.9

During the winter at L4 (October to March) the lowest numbers of viable cells were found (23%; CV ± 10%) at surface. On the other hand, the highest values were found during the summer period at L4 (spring + summer) with a peak of 89% in September (CV ± 31%). For cells > 10 µm, abundance of viable cells dropped from 60% in September to 3% in February (M= 26.9; CV ± 96.2%) (Fig. 1).

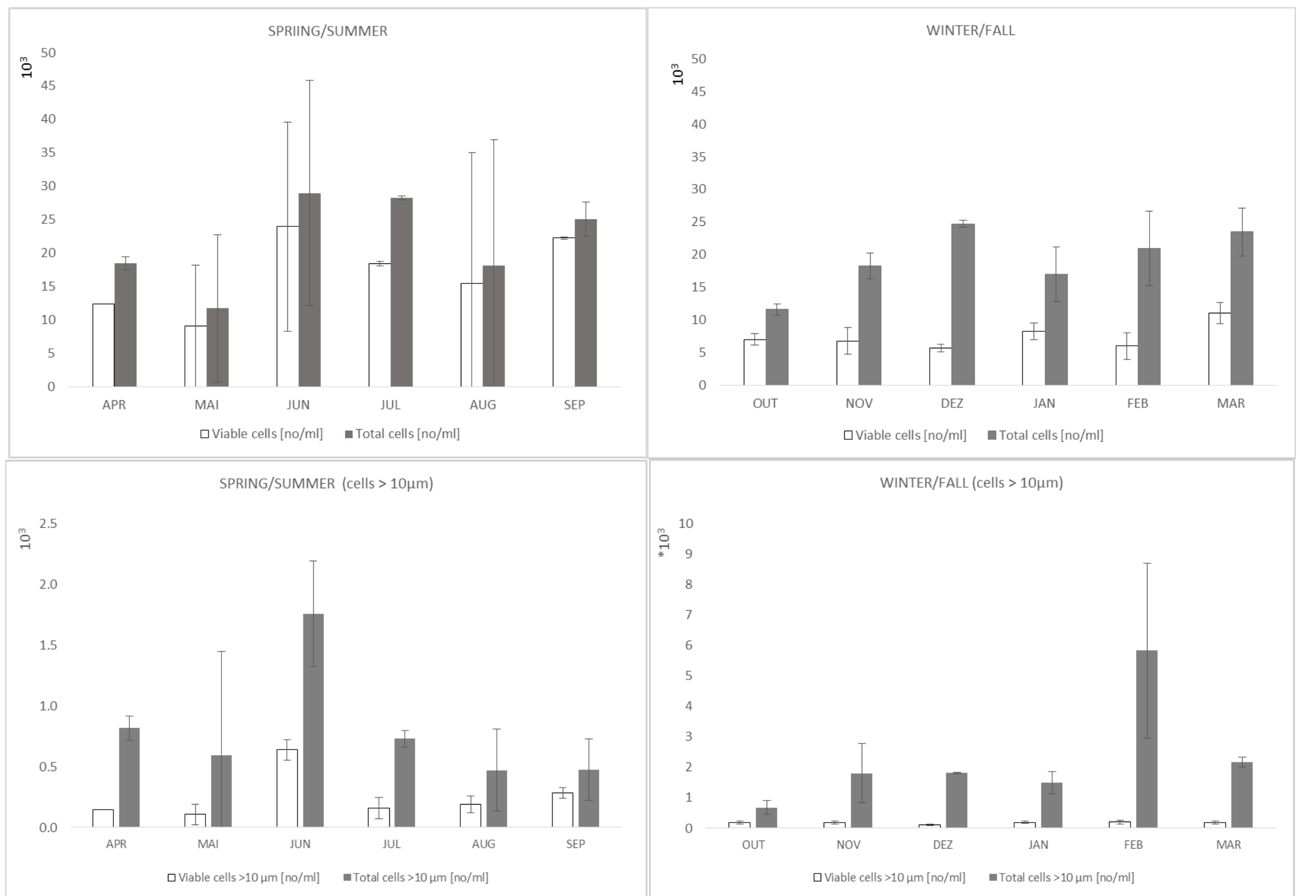


Fig. 1: Number of viable and total cells (no/ml) in the size range 2 to 50 µm and > 10 to 50 µm according to the season at L4. Samples collected at the surface at L4 sampling site from June 2016 to July2017.

Throughout the entire sampling period, fluorometers were available for analysis in conjunction with the FCM, from August/2016 to April/2017 uninterruptedly. A Draftsman plot (Fig. 2) and its correlations coefficients (Table 4) are presented to determine the covariation between the chlorophyll parameters F_0 and Fv measured with both fluorometers and the abundance of cells and the chlorophyll biomass (abundance of cells * red fluorescence) based on flow cytometrical measurements (cf Castro & Veldhuis, 2018). The latter was done to determine the variation in cellular chlorophyll concentration due to changes in cell size, since a co-variation is expected as demonstrated by Castro & Veldhuis, 2018.

Results from the correlation coefficient between F_0 and Fv measured using the two fluorometers showed a strong correlation (> 0.8); when compared to the number of cells/ml and the chlorophyll biomass detected with the FCM, results obtained with the fluorometers showed a moderate / relatively strong covariation (Table 4).

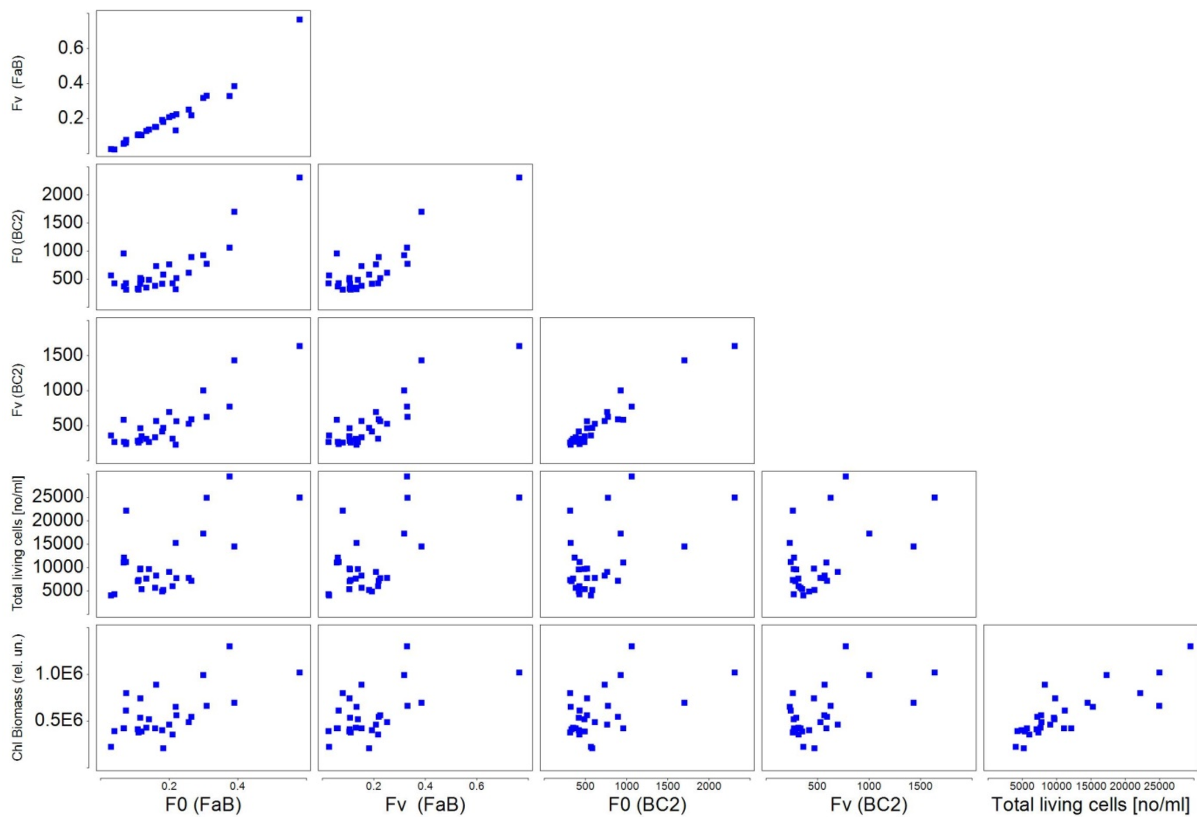


Fig. 2: Covariation between chlorophyll fluorescence (F_0 FaB and F_0 BC2) and variable fluorescence (Fv FaB and Fv BC2) measured with the two fluorometers and the number of viable cells between 2 and 50 μm [no/ml] measured with the FCM and chlorophyll biomass detected with the FCM (Chl biomass). L4 surface samples from August/2016 to April/2017.

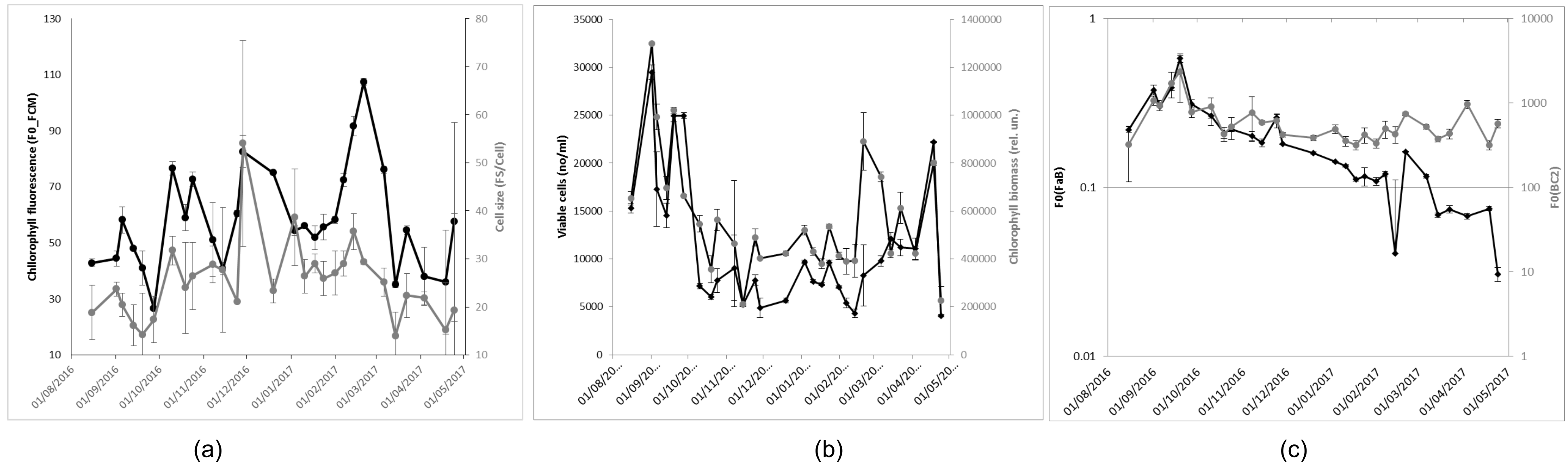
Table 4: Correlations coefficients among F_0 and Fv measured with the two fluorometers and the number of viable cells ([no/ml]) and chlorophyll biomass measured with the FCM (FCM Chl biomass). Surface samples August/2016 to April/2017 at L4 sampling site.

	F_0 (FaB)	Fv (FaB)	F_0 (BC2)	Fv (BC2)	Viable cells[no/ml]
F_0 (FaB)					
Fv (FaB)	0.97				
F_0 (BC2)	0.82	0.86			
Fv (BC2)	0.85	0.88	0.96		
Viable cells [no/ml]	0.61	0.58	0.52	0.51	
FCM Chl biomass	0.62	0.56	0.53	0.56	0.81

The estimated number of cells provided by the two fluorometers was based on the amount of chlorophyll detected in each viable cell (using a fixed value per cell set within the instrument firmware). Therefore, a good correlation between the chlorophyll fluorescence

measurements obtained with the two fluorometers and the chlorophyll biomass obtained with the flow cytometer is expected.

Chlorophyll content, cell size and the number of viable cells were investigated during the sampling period. The lower number of viable cells and therefore lower chlorophyll biomass measured with the FCM was found in the winter (Fig. 1, 3a). The lower number of cells was accompanied by bigger cells and therefore by higher chlorophyll *a* contents per cell measured with the FCM (Fig. 3b). F0 measured with the fluorometers in general were more sensitive to the trend observed for the number of cells in the period of sampling with higher chlorophyll fluorescence values obtained for the summer while lower values were seen in the winter (Fig. 3c).



(a) (b) (c)

Fig. 3. (a) chlorophyll fluorescence (F0 FCM) and cell size distribution (FS/cell) measured with the flow cytometer; (b) number of viable cells (no/ml) and the derived flowcytometric chlorophyll biomass (no of cells * red fluorescence); and (c) chlorophyll biomass measured with the fluorometers (F0(FaB) and F0(BC2)). Samples collected at the surface at L4 sampling site, from August 2016 to April 2017.

3.2. Ship-board data

Eight biological efficacy tests of BWM systems on board of ships were conducted between 2016 and 2017. Samples taken were analysed for the abundance of phytoplankton cells in the range between ≥ 10 - < 50 µm to ensure compliance with international regulations in place (IMO, 2004, USCG, 2012). Results obtained using the two fluorometers together with epifluorescence microscopy (FDA/CMFDA staining) were in all cases very different from results obtained with the flowcytomerically collected data, except on one occasion. Despite the small number of subsamples, co-variance analysis was conducted taking into account the results obtained with the two fluorometers and the epifluorescence microscopy; the latter considered the most accurate of the three. No linearity was observed between the abundance results found with the portable instruments and the number of cells counted using the epi-fluorescence microscopy vital staining assay. In addition, a very large variation between replicates was measured (Fig.4). Comparing F0 results obtained with the fluorometers and the FCM, significant results with a relatively moderate strength were found between FaB(F0) and FCM Chlorophyll biomass for the total population and for the fraction > 10 µm (r_s= 0.4).

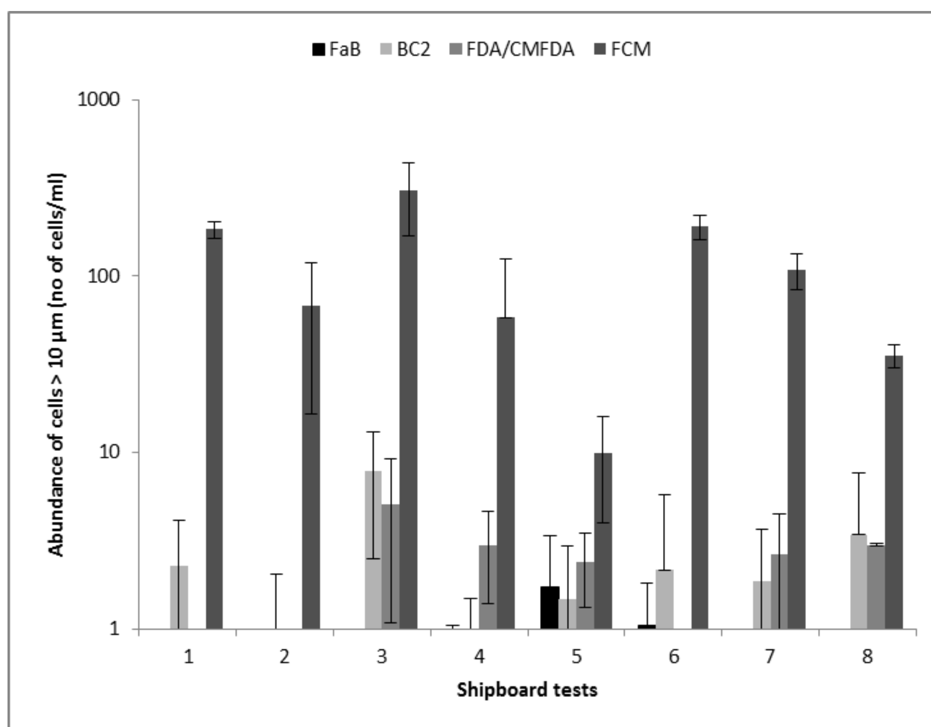


Fig. 4. Abundance results (no of cells/ml) obtained with the FastBallast ~~FaB~~, Ballast Check 2 ~~BC2~~, FDA/CMFDA assays and FCM (number of cells/ml) for eight shipboard tests conducted in 2016/2017 for cells equal or larger than 10 and smaller than 50 µm.

4. Discussion

Viability results from samples collected at L4 over a whole year showed the dominance of viable phytoplankton cells for most of the time except for during the winter period (October to March).The percentage of viable cells with a cell size > 10 μm of the total number of viable cells was always low but consistent (ca. 2% of total). The percentage of viable cells > 10 μm followed the trend observed for total viable cells with higher numbers during the summer however always lower than 40% of total, expect for a peak of 60% in September (CV ± 29%). Results from the correlation coefficient between F_0 and Fv measured with the two fluorometers showed a strong correlation (between 0.82 and 0.88), confirming earlier results using different types of fluorometers (Gollasch et al., 2015; Bradie et al., 2016). Compared to FCM results, moderate / relatively strong correlation coefficients were found (around r_s= 0.5 for $F0_{BC2}$ and r_s= 0.6 for $F0_{FaB}$).

Depending on the time of the year, a higher contribution of dead cells can be found among the marine phytoplankton populations as well as a fluctuating amount of chlorophyll *a* due to environmental changes (Veldhuis and Kraay, 2000; Veldhuis et al., 2001). In this study, abundances of viable cells in the water varied from 23% (December) to 89% (September) of the total, whilst for cells larger than 10 μm the percentage varied from 3% (February) to 60% in September (Fig. 1). However, a lower number of cells/ml covaried with a larger cell size and high a higher chlorophyll *a*. (Fig.3). Cell numbers determined with both fluorometers are based on the amount of variable fluorescence, i.e. active fluorescence. In contrast the flow cytometer measures a fixed fluorescence only varying with cell size. A lower number of viable cells would therefore result in a lower Fv and subsequently in a lower calculated cell number using a fixed conversion value. As a result, the flowcytometric numbers of total cells would over-estimate the actual number of viable cells. Therefore, fluctuations in the numbers of viable cells during the year (Fig. 1 and 3) might be expected to show up different responses according to technique used, and possibly strong deviations among replicates, certainly in the samples with a low number of cells.

Analysis of biological efficacy of ballast water samples applying different methods showed varying results, largely differing from the FCM results. This implies that for the present disinfection technology (UV-C) the remaining phytoplankton cells are dominated by intact but dead cells. In this regard, for low concentrations of viable cells an adequate calibration of the equipment is crucial considering all the other debris and contaminants that are to be expected in ballast water samples. This may be done by analysing, for instance, samples with known concentrations. Romero-Martínez *et al*. (2017) using a FlowCAM for blank samples of Milli-Q water detected ca. 100 contaminants / ml, enough to compromise the

analysis of samples where low concentrations of viable cells are expected (e.g. treated ballast water samples). Significant relationships (p-values <0.001) of relatively moderate strength (ca. r_s= 0.4) were observed between $F0_{FaB}$ and FCM number of cells/ml and chlorophyll biomass. Results for number of cells provided by the two instruments and counted on the microscope differed from one another and neither linear relationship nor significant correlation could be observed. The large variability may be seen as a sign that the detection methods are far from perfect. However, it should be noted that the fluorescence generated with the vital staining (from enzymatic activity) and the variable fluorescence of cells measured with the other techniques are not necessarily expected to co-vary or to be strictly correlated (The Danish Environmental Protection Agency, 2017). Another important aspect is the fact that the use of these stains do not necessarily work as assumed for some microorganisms including phytoplankton, depending on how stains interact with the target organisms (Hammes et al., 2010; MacIntyre et al.2016; Blatchley III et al., 2018).

Alliance for Coastal Technologies reports (Ref. No. [UMCES] CBL 2017-035 ACT VS17-11; Ref. No. [UMCES] CBL 2017-032 ACT VS17-08) using field trials, showed that the linear relationship between abundance and concentration of organisms can vary significantly due to the interplay of the environment. As a result the coefficient of determination is not necessarily highly correlated to the measured concentration of organisms ≥10 and <50 μm. The location of ballast water uptake and therefore the species composition present will be a challenge for regulatory compliance testing.

Density of cells at L4 varies due to the environmental conditions throughout the year; having that also the relationship between number of cells and fluorescence will vary depending on the chlorophyll content and cells size in the community (Veldhuis et al., 1997; Bradie, 2016; Bradie et al., 2017). For the fluorometers, considering that the calibration factor is defined by the manufacturer, different equipment, regardless measuring the same fluorescence, may provide different results (Bradie et al, 2017). The use of filters will also incur in error as observed by Castro & Veldhuis (2018), where smaller cells (<10 μm) overestimated the number of cells in the 10 to 50 μm size fraction by as much as a factor of 5.4.

5. Conclusion

Knowledge of phytoplankton viability allows the discrimination of functioning and non-functioning cells (non-viable) in the water column that is essential for regulatory ballast water issues. In the natural assemblage investigated (L4), the abundance of marine phytoplankton obeyed a seasonal pattern regardless of size (2 – 50 μm). A good correlation was observed between the fluorometers measurements and in comparison with the flow cytometer. Flow cytometry analysis showed a potential over-estimation of the number of cells in particular when a high number of dead cells was found. Its use with DNA-specific dyes should be

further investigated and precautions should be taken regarding the inclusion of contaminants and debris within the results, as suggested by Romero-Martínez *et al*. (2017).

For the shipboard trials, the techniques compared in this study (PAM fluorometer - Ballast Check 2, ST fluorometer - FastBallast and staining microscopy) showed a large variation in the number of viable cells and often between replicate samples. This variation implies that a sufficient high number of replicate samples need to be analysed. Nevertheless, the overall outcome indicated a low level of risk on all occasions for regulatory purposes.

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