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Research article

# Simulation experiments to elucidate variable fluorescence as a potential proxy for bulk microalgal viability from natural water, sediments and biofilms: Implication in ships ballast water management

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ABSTRACT

The variable fluorescence fluorometry measuring microalgal biomass (initial fluorescence - $F_0$, a chl-*a* proxy) and photosynthetic efficiency ($F_v/F_m$) has been suggested as a potential tool in ballast-water assessment. In ballast tank, microalgae can be found in contiguous compartments i.e., in water, sediment, and biofilms. Therefore the utility of $F_0$ and $F_v/F_m$ depends upon proper background corrections, which is straightforward for water samples but not for sediment and biofilms. This study proposes procedures for correcting $F_0$ values from sediment and biofilms. Irrespective of the saturation flash protocol used on any sample types the outcome of the results from viable and non-viable microalgae will remain same. Stress experiments (continuous darkness and biocide treatments) confirm that variable fluorescence ($F_v$) can be used as a potential proxy for viable cells as the values were negligible for non-viable cells and increased with an increase in abundance. Through this study, the utility of $F_v$ and $\sigma_{PSII}$ (functional-absorption-cross-section of photosystem II) along with $F_0$ and $F_v/F_m$ in providing additional information on cell-viability and algal-size group during assessment is discussed. The findings will have implications not only from the perspective of ballast water but also in testing/assays of specific interest (e.g. toxicity, water treatments, antifouling) and ecological studies involving microalgae.

## 1. Introduction

The discharge of water, sediments, and biofilm from ships' ballast-water tanks is widely considered as the most important vector for unintentional translocation of nonindigenous organisms from diverse taxonomic groups (such as viruses, bacteria, algae, plants, invertebrates, and vertebrates) across their bioregions. Such unintentional spreading has caused detrimental impacts to coastal communities and ecosystems (Ruiz et al., 1997, 2015; Gollasch et al., 2000, 2015; Carlton and Ruiz, 2003; Muirhead et al., 2014). Further due to trade shift, Holzer et al. (2017) findings also highlighted how 21$^{st}$-century global energy markets could dramatically alter opportunities for seaborne introductions and invasions by non-native species. Concern over the impacts of such nonindigenous species, International Maritime Organisation (IMO) has adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO, 2004) to curb the transfer of invaders by ballast water practices. As per IMO Convention estimation of viable organisms (i.e., living or ability to grow), in particular, autotrophic organisms, in addition to monitoring of composition is a prerequisite for efficient ballast water management practices. So far, several methods or techniques (eg. microscopy, FlowCAM and flow cytometry), in combination with viability stains or culturing method, and active fluorometry have been put forth as suitable methods for assessment (Veldhuis and Fuhr, 2008; Steinberg et al., 2011; Zetsche and Meysman, 2012; Cullen and McIntyre, 2016; Naik and Anil, 2017; Romero-Martinez et al., 2017; Holzer et al., 2017). Among them, the microscopy/flow cytometry-based assessment involves quantitative analysis of cells (and fits in to follow the compliance protocols with the D-2 regulation of the IMO convention, i.e., < 10 viable cells/ml for cell size > 10 μm) but are time- and resource-consuming procedure requiring highly qualified experts. Whereas the active fluorometers, with initial relatively higher capital investment, provides a quick microalgal viability assessment. Former methods provide both qualitative and quantitative evaluation whereas the latter method provides only the bulk microalgal viability assessment. The active fluorometers added advantages are its capability to offer real-time monitoring of microalgal viability check, which is the need of the hour for efficient and robust assessment.

At present, an array of fluorometers (for both *in situ* and bench top versions) are commercially available to measure variable chlorophyll

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fluorescence under a wide range of conditions and for various applications. Each of these fluorometers is based on one of a few basic operational principals. For example, fast repetition rate fluorometer (FRRF: based on single turnover i.e. ST protocol), pulse amplitude modulated fluorometer (PAM: based on multiple turnover i.e. MT protocol), pump and probe, fluorescence induction and relaxation (FIRe; both single and multiple turnovers) and Induction Fluorometer/ Continuous fluorometer. Over the years, the measurement of chlorophyll fluorescence by using these active fluorometers has become an important tool for photo-biologists. These fluorometers measures fluorescence transients induced by a rapid sequence of excitation flashlets and the interpretation of the same allows calculation of photosynthetic parameters initial–$F_0$, maximum–$F_m$ and variable–$F_v$ ($=F_m–F_0$) components of photosystem II (PSII) fluorescence, quantum yield ($F_v/F_m$), functional absorption cross-section ($\sigma_{PSII}$–only with single turnover protocol) and the kinetics of electron transfer on the acceptor side of PSII. Interpreting the measured parameters along with the environment (temperature, salinity, light and nutrients) or stressors (darkness, anoxia, fluctuating salinity, temperature and nutrient availability) or treatments (biocide and radiation treatments) with respect to time will lead to decipher the underlying mechanisms required to understand the vital physiological process (Gorbunov et al., 2001; Lesser and Gorbunov 2001; Levy et al., 2004; Patil and Jagadeeshan, 2011; Patil et al., 2017).

Since variable fluorescence fluorometry provides rapid estimation of both chl *a* concentration ($F_0$, proxy for chl *a*) and the physiological status of the organisms (in particular $F_v/F_m$) in a sample non-destructively in real time, it has been suggested as a potential tool to evaluate ballast water (Stehouwer et al., 2009; IMO, 2015; Drake et al., 2014; First et al., 2018; Gollasch and David, 2018). It is to be noted that all such claims are based on the assessment of natural seawater or microalgal cultures in the laboratory using a PAM fluorometer, which uses MT flash protocol. Most of these studies used only two metrics $F_0$ and $F_v/F_m$ for total concentrations and physiological status of the algae. Unfortunately, the variable component of the fluorescence ($F_v$), contributed only by live cells (chlorophyll attached to reaction center), was not given attention and live cell enumeration is a prerequisite as per IMO guidelines. Further, the utility of fluorometer with ST flash protocol is not much explored in this direction. The measurement of $\sigma_{PSII}$ (describes the functional 'target area' of the light-harvesting antenna) in addition to variable fluorescence measurements is an added advantage with ST fluorometer (Kolber et al., 1998; Suggett et al., 2004; Osmond et al., 2017). For instance, $F_v/F_m$ and $\sigma_{PSII}$ in natural populations represent unique taxonomic signature within the phytoplankton community that is further modified according to the physiological status *viz* adaptation, acclimation, and inhibition (Suggett et al., 2009). Hence, considering $F_v$ and $\sigma_{PSII}$ along with $F_0$ and $F_v/F_m$ will lead to better interpretation and assessment. In view of this, the utility of fluorescence induction and relaxation (FIRe) fluorometer, which uses both ST and MT flash protocol was explored in this investigation.

Generally, within a ballast tank, microalgae can be found in contiguous compartments, that is, in water, sediment and biofilm (Drake et al., 2007). Therefore the utility of $F_0$ and $F_v/F_m$ without proper background corrections (especially for the dissolved organic matter) will be misleading (Fuchs et al., 2002). First et al. (2018) also recommended validation exercises for the interpretation of variable fluorescence measurements of samples that contain mixed assemblages of live and dead cells and high concentrations of dissolved and particulate organic matter. The correction procedure for the water sample measurements is available but not for biofilms and sediments, which also contains a good amount of organic matter. In view of this, the study proposes $F_v$ could be a better viability indicator over the $F_0$ as this is contributed by both dead and live chlorophyll. Here, the elucidation of fluorescence parameters for the assessment of bulk microalgal viability from natural water, sediments, and biofilms was undertaken through simulation approach using a FIRe fluorometer. Further, the appropriate procedure for viability assessment of microalgae from different contiguous compartments of the ballast tank is also proposed.

## 2. Materials and methods

In this study, fluorescence induction and relaxation (FIRe) fluorometer (a variant of active fluorometer) was used for the viability assessment of microalgae by testing water, sediments and biofilm samples obtained during simulation experiments. The rationale for using FIRe are as follows:

i) FIRe uses both single (ST) and multiple (MT) turnover protocol there by offering information on the key parameters such as variable fluorescence (both ST and MT) and $\sigma_{PSII}$ (only by ST).
ii) In addition to cuvette-based operation for liquid samples, FIRe comes with a fiber optic probe as an accessory, which was put in use for assessment of microalgae in biofilms after careful standardization. While taking measurements extreme care of the expensive probe (which is not waterproof) should be taken to avoid damages.

Altogether three tests were performed separately with water (mixed assemblage and microalgal cultures), sediments and biofilms. However, the experimental design for each test was different, and details of the same are provided with a schematic illustration (Fig. 1 and supplementary material) in the following sections.

### 2.1. Testing with water samples

The testing with water was performed using the natural seawater collected from the Dona Paula Bay (Goa, west coast of India) and the laboratory-grown cultures of *Tetraselmis* sp (Fig. 1a and supplementary material). The experimental setup for seawater and cultures were same, and the incubation was carried out by suspending in an outdoor pond experiencing natural photo-cycle of 12:12 h light: dark and a mean water temperature of 31 ± 0.13 °C with a mean daily variance of 3.8 ± 0.13 °C. On the day of the experiment the collected natural seawater (salinity of ~35 PSU) was pre-filtered with 100 μm to remove larger organisms. The results on the phytoplankton abundance, chlorophyll *a* and inorganic nutrient levels from this experiment are published elsewhere (Carney et al., 2011). This study aims only to explore the utility of the fluorescence parameters ($F_0$, $F_m$, $F_v$, $F_v/F_m$, and $\sigma_{PSII}$) for the bulk assessment of microalgal physiological status using FIRe fluorometer (Satlantic LP, Halifax, Canada). Before measurements, the samples were dark adapted for 30 min. The gain was adjusted depending upon the chl *a* concentration of the sample taken for measurement. Curves were fitted using the FIRePro software.

### 2.2. Testing with sediments

Unlike with water samples, testing the sediment samples is not straightforward and requires preparation of the samples for appropriate assessment. To the best of our knowledge FIRe or any other available fluorometer does not have the capability to assess the microalgae in the ballast tank sediments directly. Further, the microalgae harboring sediments, comprise of both active as well as inactive cells however upon exposure to prolong darkness (e.g., in ships ballast tank) one would expect a decline in activity of the cell. Under such a scenario only possible way for assessment is first to prepare the inocula of the sediment samples followed by suspension in a known volume of sterilized filtered seawater or growth media and incubation in appropriate growth condition for several days till the signal of the growth is observed. FIRe measurement of the resuspended sample (day 0) will indicate the presence of viable cells and the measurements done during incubation at frequent intervals (day 1… day t) will determine the time (t) taken for growth. The same approach is adopted in this study, and