

**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.

**Keywords:** Microalgae, variable fluorescence, stress, viability, assessment, sediments, biofilms

## 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<sup>st</sup>-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 (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 >10µm) 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 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 measure 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 Jagadeesan 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, Osmund 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  $31 \pm 0.13$  °C with a mean daily variance of  $3.8 \pm$

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 microalgaeharboring 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 the details are provided in Fig. 1b and supplementary material. Duration of the experiment was 13 days, and the phytoplankton growth in each of the treatment was monitored using FIRE fluorometer as above at frequent intervals.

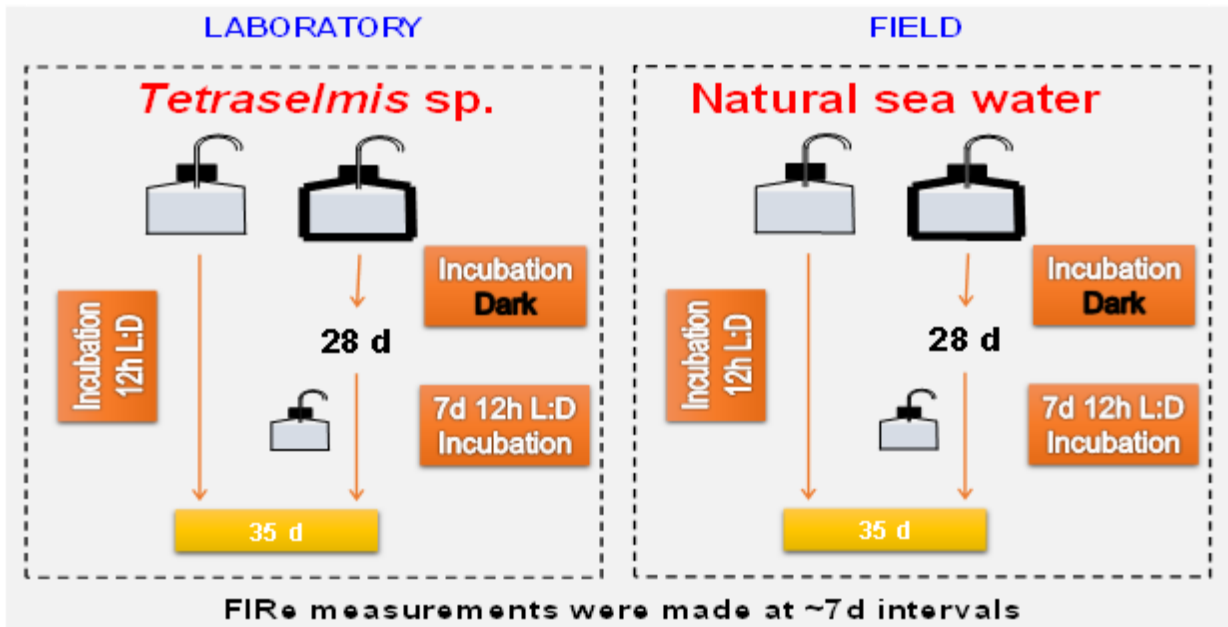
## **2.3. Testing with marine microalgal biofilms**

Biofilms testing using the active fluorometer is not straightforward and needs a different approach unlike with the water and sediment samples. Here the biofilms were developed in the laboratory and the same was utilized for conducting the experiments. Altogether two experiments were performed wherein the first and second experiments corresponds to the dark and biocide (i.e., chlorination) treatments on biofilms (Fig. 1c and supplementary material). However, both the experiments were run separately. In both experiments, the untreated biofilms were considered as the experimental controls. In both the experiments, fluorescence transients were measured using a FIRE fluorometer

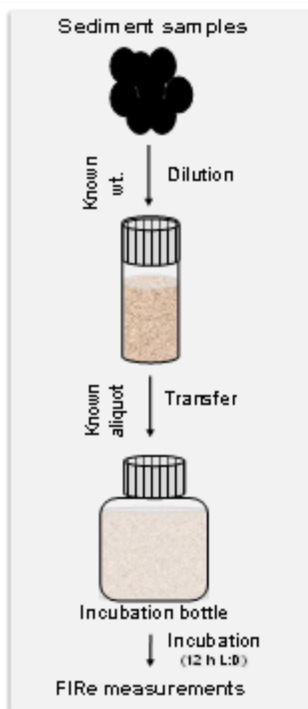
equipped with a fiber optic probe. Before measurements, the biofilms were adapted to darkness for 30 min. Curves were fitted using the FIREPro software.

## Experimental design

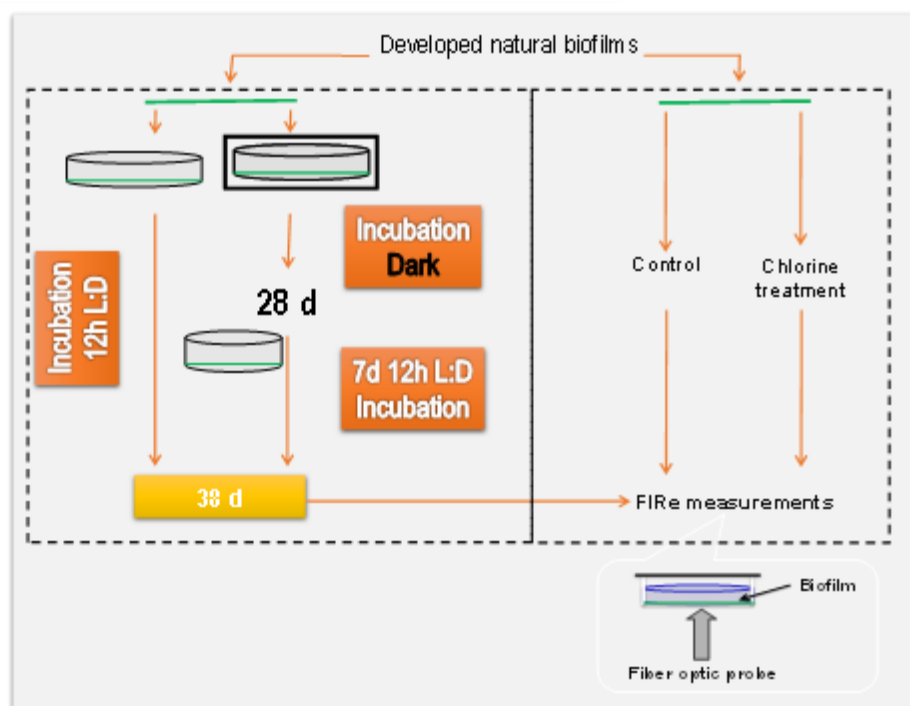
### a. Water samples



### b. Sediment samples



### c. Biofilms



**Figure 1.** Experimental design for the microalgal assessment from (a) water samples, (b) sediments and (c) biofilms. The detailed procedure is provided as supplementary.

### 3. Results

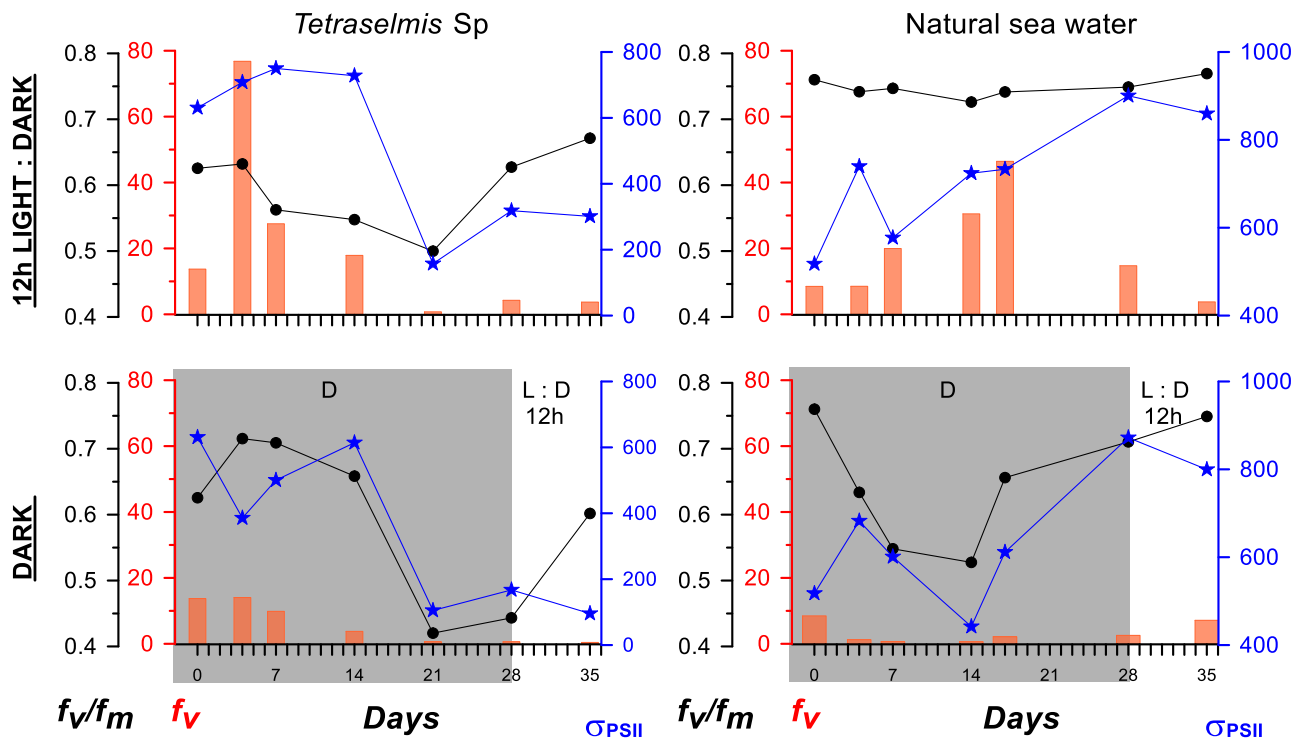
3.1. Fluorescence measurements of water samples exposed to continuous darkness, natural photocycle (NPC) and re-exposure from continuous darkness to light-dark conditions (LDC)

*3.1.1. Tetraselmis cultures as samples:* The curve patterns and trends in fluorescence parameters during 28 days incubation were different in the dark and LDC (Figs. 2 and 3). The  $F_0$  and  $F_v$  both showed a similar trend under the respective treatments. In LDC, both  $F_0$  and  $F_v$  increased initially up to 7 days and after that showed a decreasing trend. Whereas in dark condition both  $F_0$  and  $F_v$  showed a downward trend up to 28 days. However, upon re-exposure of these 28 days, dark-adapted cells to LDC a significant increase in the  $F_0$  and  $F_v$  values were not evident indicating no increase in biomass. However, similar trends were not evident with  $F_v/F_m$  and  $\sigma_{PSII}$ . The trends of  $F_v/F_m$  and  $\sigma_{PSII}$  concerning incubation days varied according to the treatments. In LDC,  $F_v/F_m$  showed a decreasing trend up to 21 days and after that increased up to 35 days. However,  $\sigma_{PSII}$  remained high up to 14 days (~650), decreased significantly on the 21<sup>st</sup> day (<200) and after that showed a marginal increase (300). Under dark conditions, both  $F_v/F_m$  and  $\sigma_{PSII}$  remained high up to 14 days indicating no alteration in the physiological state. On the 21<sup>st</sup> day, both the parameters showed a significant decline and remained at the same level for a week, i.e., until 28 days. Upon re-exposure to 7 days of LDC,  $F_v/F_m$  increased significantly but not  $F_v$  and  $\sigma_{PSII}$  indicating a revival of cells without division.

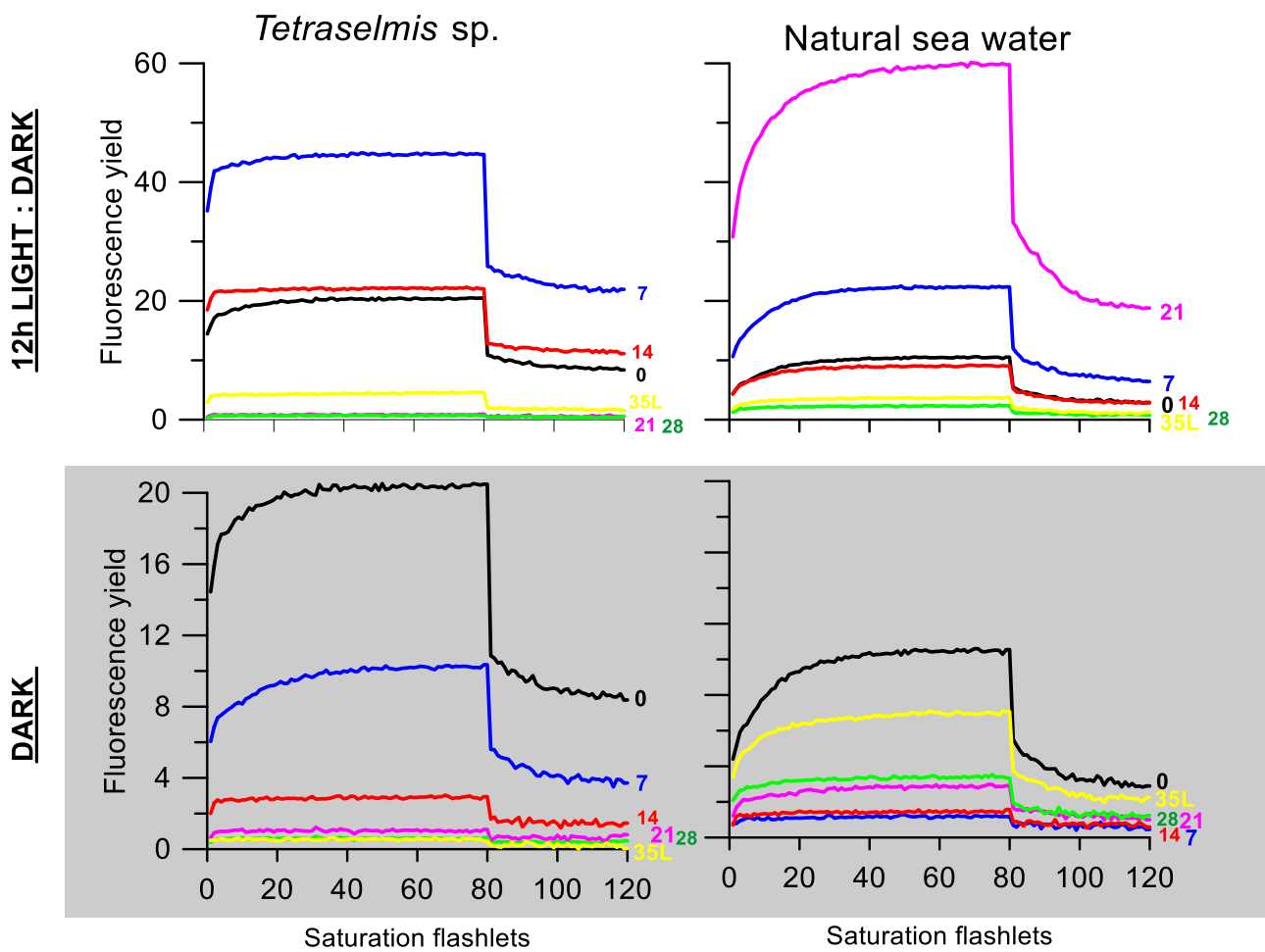
*3.1.2. Natural sea water as samples:* Similar to *Tetraselmis*, the curve patterns and trends in fluorescence parameters with the days of the incubation for 28 days were different in the dark and LDC (Figs. 2 and 3). However, the trend pattern observed with natural phytoplankton was distinctly different from that observed for *Tetraselmis*. Here also both  $F_0$  and  $F_v$  showed a similar trend under the respective treatments. In light-dark condition, both  $F_0$  and  $F_v$  showed an increasing trend up to 17 days and after that showed a decreasing trend. Whereas in dark condition both  $F_0$  and  $F_v$  showed a downward trend up to 28 days. However, upon re-exposure of these 28 days, dark-adapted cells to light-dark condition a significant increase in the  $F_0$  and  $F_v$  values were evident indicating an increase in biomass. However, similar trends were not evident with  $F_v/F_m$  and  $\sigma_{PSII}$ . The trends of  $F_v/F_m$  and  $\sigma_{PSII}$  concerning incubation days also varied according to the treatments, but the pattern was different compared to that observed for *Tetraselmis*. In LDC,  $F_v/F_m$  remained high (0.7) throughout the incubation period whereas  $\sigma_{PSII}$  showed an increasing trend and reached a maximum value of 800 on day 28 and maintained the same up to 35 days. Under dark conditions, both  $F_v/F_m$  and  $\sigma_{PSII}$  showed considerable variations.  $F_v/F_m$  showed a decreasing trend from day 0 (0.7) to day 14 (0.2), and after that, a drastic increase in the values was observed on day 17 (0.5) which further rose to 0.7 on day



28.  $\sigma_{PSII}$  also followed a similar trend, and the only exception was the increase in values from day 0 (500) to day 4 (650). Upon re-exposure to 7 days of LDC, distinct increase in  $F_v$  and  $F_v/F_m$  was evident but not  $\sigma_{PSII}$  indicating a revival of cells with some division.



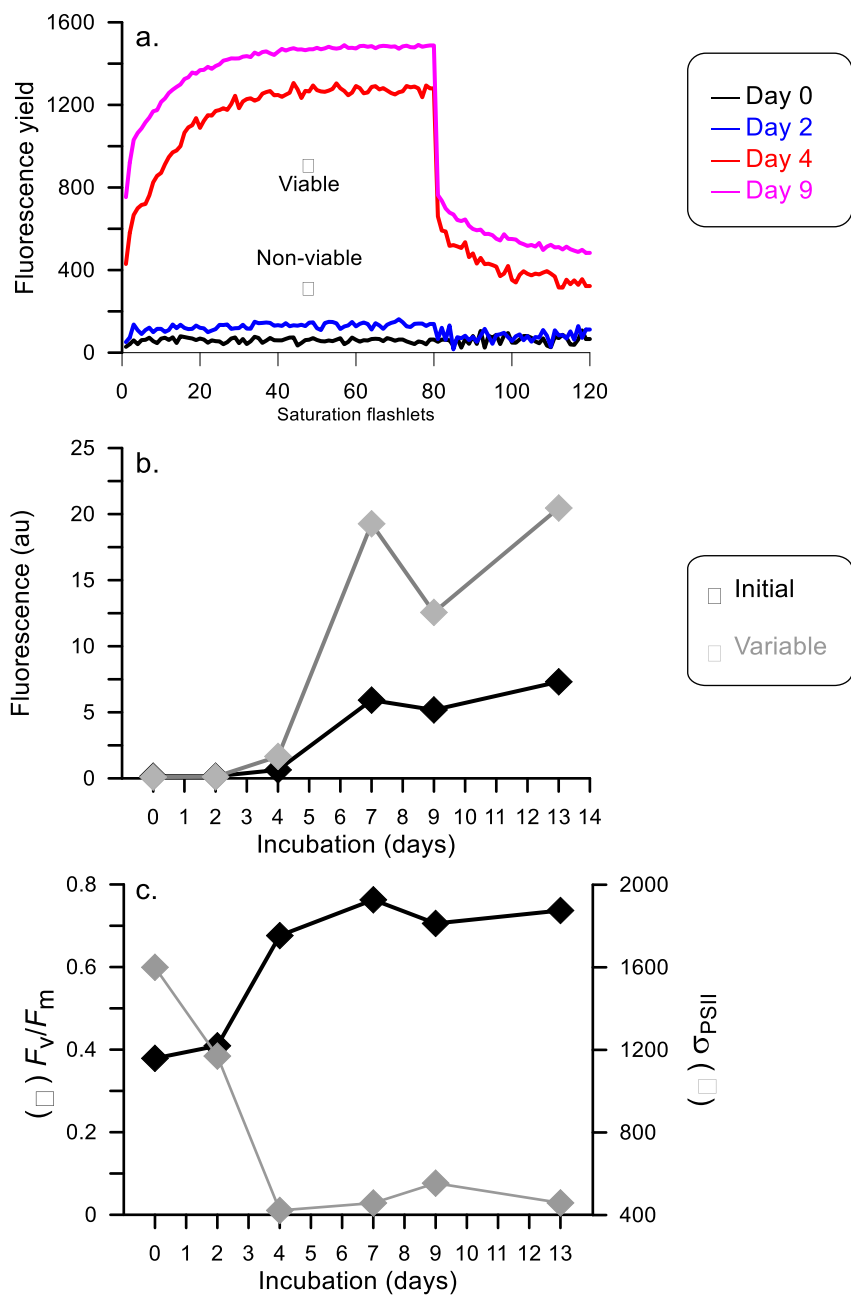
**Figure 2.** Variations in the fluorescence parameters ( $F_0$ ,  $F_v$ , and  $F_v/F_m$ ) and  $\sigma_{PSII}$  of *Tetraselmis* sp. and natural phytoplankton assemblage grown under 12h light:dark (L:D) photocycle, continuous darkness and continuous darkness to light:dark photocycle (12h L:D).



**Figure 3.** Variations in the fluorescence yield of *Tetraselmis* sp. and natural phytoplankton assemblage grown under 12h light:darkphotocycle,continuous darkness and from continuous darkness to 12h light:dark photocycle (35L)

### 3.2. Fluorescence measurements of sediment samples

The curve fitting of the fluorescence measurements made on sediment samples suspended in the f/2 media indicated an almost flat curve rather than an induction curve typically observed for the samples with live organisms (Fig. 4a). The fluorescence values of the flat curve revealed insignificant variations between  $F_0$  and  $F_m$  thereby leading to positive but near zero  $F_v$  value and a very low  $F_v/F_m$  (Fig. 4b-c). These initial values suggested that the sediment used for incubation was harboring very low viable chlorophyll (at the undetectable range) with reduced quantum efficiency ( $F_v/F_m = <0.2$ ). However, the same when incubated under a LDC at room temperature a significant improvement in the induction curve was obtained from day four onwards. The same was also reflected in the fluorescence properties, i.e., a distinct increase and improvement in the fluorescence values ( $F_0$  and  $F_v$ ), and photosynthetic efficiency ( $F_v/F_m$ ) respectively was evident on the 4<sup>th</sup> day of incubation, and these positive changes were significant with further incubation of 7, 9 and 13 days. On the other hand  $\sigma_{PSII}$  was high initially ( $>1000$ ) and after that decreased drastically to  $\sim 400$  (day 4) and remained at the same level till day 13 (Fig. 4c). Such positive changes were mainly due to the growth of the photosynthetic organisms.

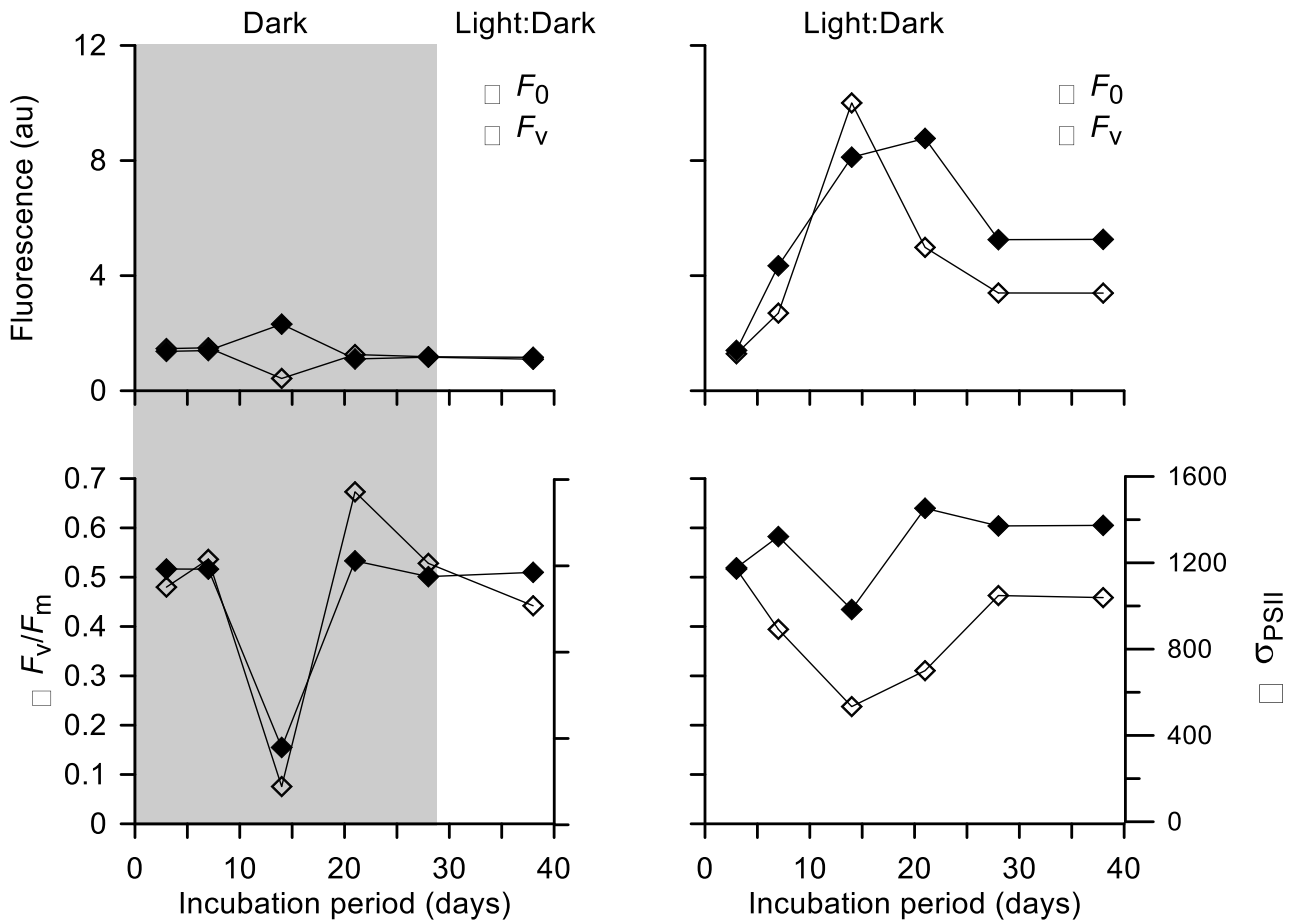


**Figure 4.** Variations in the (a) fluorescence yield, (b) initial -  $F_0$  and variable -  $F_v$  fluorescence, and (c)  $F_v/F_m$  and  $\sigma_{PSII}$  of sediment samples incubated under photocycle (12h light:dark).

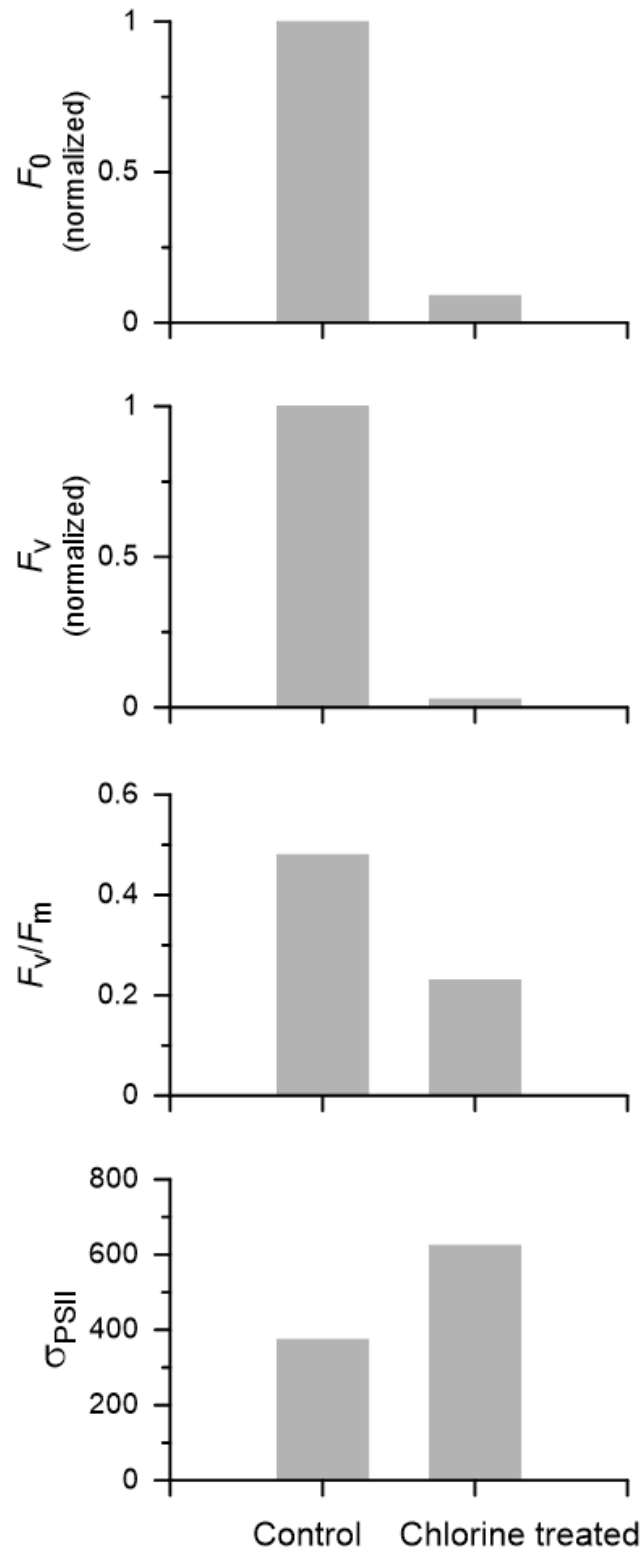
### 3.3. Fluorescence measurements of natural biofilms (NB) exposed to continuous darkness, light-dark conditions (LDC) and biocides

Fluorescence measurements of the NB showed a similar trend to that of water samples when exposed to continuous darkness and LDC (Fig. 5). However, the magnitude of variations was relatively weaker in the former than the later. In LDC, initial  $F_0$ ,  $F_v$  and  $F_v/F_m$  of NB increased significantly on incubations whereas  $\sigma_{PSII}$  decreased (Fig 5). However, such an increase (in fluorescence parameters) and decrease (in  $\sigma_{PSII}$ ) was not observed when incubated under continuous dark conditions. In dark conditions,  $F_0$  and  $F_v$  was relatively lower than that of the NB incubated under LDC, and the reverse was observed for  $F_v/F_m$ . Unlike observed for light-dark treated NB,  $\sigma_{PSII}$  of continuous dark treated NB remained constant (except on day 14 and 21). Upon re-exposure of 35 days dark adapted NB to 10 days in LDC, a marginal improvement in  $F_v/F_m$  and slight decrease in  $\sigma_{PSII}$  was evident indicating revival of cells.

The utility of the FIRE fluorometer as a potential tool to study biofilms was further confirmed with the measurements made on the diatom biofilms treated with and without biocide, i.e., chlorine. The fluorescence parameters ( $F_0$ ,  $F_v$  and  $F_v/F_m$ ) of the diatom biofilms reduced significantly and  $\sigma_{PSII}$  increased after biocide treatment compared to that of the untreated biofilms (Fig. 6). The curve fitting of the fluorescence measurements made on diatom treated with biocide indicated an almost flat curve rather than an induction curve typically observed for the untreated biofilms. Such significant reduction was due to the loss of viability in diatoms.



**Figure 5.** Variations in the fluorescence parameters ( $F_0$ ,  $F_v$  and  $F_v/F_m$ ) and  $\sigma_{PSII}$  of microalgal biofilms grown under photocycle (12h light:dark), continuous darkness and from continuous darkness to 12h light:dark photocycle.



**Figure 6.** Variations in the fluorescence parameters ( $F_0$ ,  $F_v$ , and  $F_v/F_m$ ) and  $\sigma_{PSII}$  of biofilms treated with and without chlorine.

## 4. Discussion

### 4.1. Importance of type of fluorometer or saturation protocol for microalgal assessment

Currently, the two most important techniques based on saturation protocol (i.e., single (ST) and multiple (MT) turnover flashes) are in use to measure variable fluorescence. The application of the different approaches to an algal sample will result in differing  $F_m$  values and, as a result, different values for the photochemical efficiency of PSII, with the MT method giving higher values than ST. Given this, Kromkamp and Forster (2003) suggested the use of different terminology (for example  $F^{ST}$  and  $F^{MT}$ ) to avoid confusion, until the underlying physiological differences are resolved. So far the claims on the potential usage of variable fluorescence ( $F_0$  and  $F_v/F_m$ ) for ballast water assessment were based only on the active fluorometers with MT protocols. Here for the first time, FIRE fluorometer measuring the variable fluorescence using both ST and MT protocol was used for the assessment of the samples representing different compartments (water, sediments, and biofilms) of the ballast tank. It is to be noted that the initial fluorescence ( $F_0$ ) will remain same irrespective of the protocol used. The rest of the fluorescence parameters ( $F_m$ ,  $F_v$ , and  $F_v/F_m$ ) did not show significant difference between MT than ST indicating that irrespective of the type of the fluorimeter or saturation protocol used the outcome of the results will remain same.

### 4.2. Importance of correct estimation of $F_0$

$F_0$ , a proxy for chlorophyll or biomass, has been suggested as a potential parameter for the ballast water assessment (First et al. 2018). However, for accurate  $F_0$  estimation background signal corrections (i.e., dissolved fluorescence matter) for the sample of interest is a prerequisite. As the uncorrected readings will result in overestimation of  $F_0$  and underestimation of the  $F_v/F_m$  but  $\sigma_{PSII}$  is not affected drastically (Suggett et al. 2006). In general, the background signal correction can be executed by taking the blank readings and utilizing the same readings for corrections. However, the background corrections for water samples is simple and straight forward but not with the sediment and biofilm samples. In the case of water samples, blank corrections can be achieved by taking the readings of the water sample and its filtrate (i.e., obtained by gently passing the sample of interest through, preferably using GF/F Whatman filter paper). After that, the readings of the latter was used to correct the readings of the former either by re-running the curve fitting by incorporating the blank  $F_0$  value in the provision made by the software or by manually subtracting the blank  $F_0$  value from the sample  $F_0$  and  $F_m$  ( $F^{corrected} = F^{sample} - F^{blank}$ ). So far, no direct methods are available for performing blank corrections for sediments and biofilm samples. In the case of sediment samples, the only way to carry out the corrections is by suspending the sediment in a known volume of filtered seawater,



and utilizing the same for the blank corrections as measured for water samples. Whereas in the case of biofilms, blank corrections can be performed either by removing (using the cuvette-based fluorometer) as well as without removing the biofilms (using the fiber optic based fluorometer) from the substratum surfaces depending on the type of the fluorometer available. The former way of correction is merely converting the biofilms into liquid samples (i.e., scraping the biofilms from a known surface area with a known volume of filtered seawater) and do corrections similarly to that mentioned for the water samples. The later way of corrections involves the measurements done without disturbing the biofilms developed on a substratum of interest using fiber optic probe and in this case the measurements were done on a clean substratum of interest without the biofilms was considered as blank. It is to be noted that  $F_0$  in both the cases need not be or near accurate because of the following: Firstly there is a chance of cell loss and breakage during scraping and secondly not accounting the signals from the attached biofilm organic matter and results of another biological process during direct measurements. In this study, the approach of direct measurements was adopted to assess the accumulation of phototrophic biofilm buildup on clean glass slides used in the experiments on the effect of continuous darkness and biocide treatments.

#### *4.3. Importance of $F_v$ in the microalgal assessment*

It is to be noted that both live and dead chlorophyll contributes to  $F_0$ . However, in ballast water assessment the quantification of the live chlorophyll, i.e., viable cell is desirable. Typically the live or healthy microalgae, upon induction of saturation flashes and the curve fitting of the fluorescence yield for each flash, results in a fluorescence transient curve (resembling Kautsky curve). In this case,  $F_0$  is always less than  $F_m$ , and  $F_v (=F_m - F_0)$  will always be positive. However, the magnitude is directly proportional to the density of the healthy biomass. On the other hand, when saturation flashes applied to a dead cell or non-viable chlorophyll the curve fitting of the fluorescence yield results into a flat curve (absence of typical fluorescent transient curve). In this case,  $F_0$  and  $F_m$  are very close to each other and  $F_v$  will be near to zero or very small. This was also confirmed by the measurements made on the samples (water, sediment, and biofilms) subjected to stress (continuous darkness and biocide treatment) during the experiments. The difference between  $F_0$  and  $F_v$  was negligible for the samples exposed to continuous darkness (up to 28 days), chlorination and sediments (before the start of the incubation, i.e., Day 0). If biocide treatment has damaged chl *a* or detached it from the PSII reaction center, then initial fluorescence ( $F_0$ ) is affected, and no fluorescence transient takes place. In this case,  $F_v$  will be very low. However, upon re-exposure of stressed samples (except chlorine treated samples) to normal growth conditions or NPC appearance of the typical curve was evident and also the magnitude of the difference between  $F_0$  and  $F_m$  was

increasing with the incubation period (days). Recovery was observed after seven days of incubation for water, sediment and biofilm samples. In case of chlorine treated samples, such a recovery was not observed due to the stress-induced death of microalgae. These results indicated that the  $F_v$  and pattern of the curve are directionally proportional to the viable status of the microalgae and also its biomass/density. Given this, it is suggested that the  $F_v$  can be used as a potential proxy for viability and will also offer the required, but most relevant, information on viable cells along with the  $F_0$  (total biomass) from the perspective of ballast water assessment as well as for any water testing or assays related to microalgae. Further,  $F_v$  will also serve the purpose of the assessment of samples (e.g., sediment and biofilms) where accurate  $F_0$  determinations is an issue.

#### 4.4. The advantage of active fluorometer with single turnover (ST) protocol

The additional benefit of fluorometers with ST protocol over MT protocol is the computation of the  $\sigma_{PSII}$  using the rate of rise from  $F_0$  to  $F_m$ .  $\sigma_{PSII}$  is a key photosynthetic parameter essential for understanding the physiological process (e.g., non-photochemical quenching) as well as in the estimation of the primary productivity. Literature suggests the existence of strong variability in  $\sigma_{PSII}$  and the main responsible factors are light and the phytoplankton composition, in particular, size groups (Suggett et al. 2009). Generally,  $\sigma_{PSII}$  is very low when the  $F_m$  is close to  $F_0$  (i.e., the fluorescent transient curve is almost flat), and possible reasons could be the excess light and other stress effect on microalgae. In this study,  $\sigma_{PSII}$  of *Tetraselmis* was low when exposed for a more extended period of darkness. On the other hand, for natural waters,  $\sigma_{PSII}$  was high even when the  $F_m$  is close to  $F_0$  or  $F_v$  is  $<1$  (as evident on day 4 and day 7 of dark experiment with natural water) which is unrealistic. Unlike in *Tetraselmis*,  $\sigma_{PSII}$  of natural microalgae and  $F_v$  was relatively higher even after prolonged dark incubation of days 14 to 28. Such contrasting findings suggest the existence of species which not only can survive under prolonged continuous darkness but also thrive well when re-exposed to normal growth conditions. These results confirm the following: (1)  $\sigma_{PSII}$  and  $F_v$  combo provides useful information on cell viability, (2) prolonged darkness need not always yield very low  $\sigma_{PSII}$  in natural microalgal assemblage and (3) microalgae unable to survive in continuous darkness yield meager  $F_v$  and  $\sigma_{PSII}$ . Overall, the fluorescence-based investigations of natural populations must account for potential variations in phytoplankton community structure before making interpretations of physiological status as both  $F_v/F_m$  and  $\sigma_{PSII}$  will inevitably consist of a physiological signal superimposed upon a taxonomic signal (Suggett et al. 2009). For instance, the cells with larger fractions exhibited high  $F_v/F_m$  and low  $\sigma_{PSII}$  values whereas the cells with smaller cell sizes exhibited lower  $F_v/F_m$  and higher  $\sigma_{PSII}$  values. The cell size or algal group within the mixed community that contributes the most considerable proportion of the total chl *a* fluorescence appears to dominate the

mixed community value measured for  $F_v/F_m$  and  $\sigma_{PSII}$  (Suggett et al. 2004). This evidence suggests that the  $F_v/F_m$  and  $\sigma_{PSII}$  combo will also provide a potential proxy for the relative determination of the dominant algal group/size. Further, with the incorporation of different wavelength illumination sources for saturation protocol in the new generation active fluorometers, the determination of algal groups in routine assessment will be possible shortly. Though the two instruments, PhytoPAM (based on MT flash) and FastOcean Fluorometer (based on ST by Chelsea technologies), with multiwavelength illumination sources for saturation protocol are available but, its potential in routine assessment is in infancy.

## 5. Conclusion

This study concludes that irrespective of the saturation flash protocol (ST or MT) used to any samples (water, sediments, and biofilms) the outcome of the results (concerning  $F_0$ ,  $F_m$ ,  $F_v$  and  $F_v/F_m$  from viable and non-viable micro-algae) will not alter substantially. However, accurate  $F_0$  determinations is a prerequisite which is straightforward for water samples but not for sediment and biofilms samples. This study proposes procedures for correcting  $F_0$  values from sediment and biofilms. Stress experiments (continuous darkness and biocide treatments) confirm that  $F_v$  can be used as a potential proxy for viable cells as the values were negligible for nonviable cells compared to viable cells. Further  $F_v$ ,  $F_v/F_m$  and  $\sigma_{PSII}$  obtained with ST protocol, will provide additional information on cell viability and the dominant algal size group respectively along with physiological status during the assessment. This study demonstrates the importance of  $F_v$  and  $\sigma_{PSII}$  in addition to  $F_0$  and  $F_v/F_m$  during the evaluation of ballast water, testing/assays (e.g., toxicity, water treatments, and antifouling) and ecological studies involving microalgae. Further, the FIRE fluorometer along with fiber optic probe makes a useful package for microalgal assessment from water, sediments, and biofilms.

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