Research article

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Flow cytometric measurements as a proxy for sporulation intensity in the cultured macroalga *Ulva* (Chlorophyta)

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Abstract: Controlling the life cycle of the green macroalga *Ulva* (Chlorophyta) is essential to maintain its efficient aquaculture. A fundamental shift in cultivation occurs by transforming the thallus cells into gametangia and sporangia (sporulation), with the subsequent release of gametes and zoids. Sporulation occurrence depends on algal age and abiotic stimuli and is controlled by sporulation inhibitors. Thus, quantification of sporulation intensity is critical for identifying the biotic and abiotic factors that influence the transition to reproductive growth. Here, we propose to determine the sporulation index by measuring the number of released gametes using flow cytometry, in proportion to the total number of thallus cells present before the occurrence of the sporulation event. The flow cytometric measurements were validated by manually counting the number of released gametes. We observed a variation in the autofluorescence levels of the gametes which were released from the gametangia. High autofluorescence level correlated to phototactically active behaviour of the gametes. As autofluorescence levels varied between different groups of gametes related to their mobility, flow cytometry can also determine the physiological status of the gametes used as feedstock in seaweed cultivation.

Keywords: aquaculture; flow cytometry; gametogenesis; seaweed; sporulation.

1 Introduction

The green macroalgal genus *Ulva* (Linnaeus, 1753; Chlorophyta) comprises around 130 recognised species, most of which are distributed worldwide. *Ulva* species are edible and are often used as a raw material in various industries (Meghanath et al. 2019; Nikolaisen et al. 2011). For example, they have bioremediation applications and can act as biofilters in integrated multi-trophic aquaculture systems (Neori et al. 2003; Shpigel et al. 2017). The inherently high growth rates of *Ulva* species make them promising candidates to produce sustainably high biomass yields. In general, marine macroalgae (seaweeds) can be beneficial to humans by providing valuable chemicals such as carbohydrates, proteins, vitamins, and minerals (Charrier et al. 2017; Ito and Hori 2009; Li et al. 2018).

Ulva undergoes alternate sexual and asexual reproduction throughout generations, forming isomorphic sporophytes and gametophytes (Hiraoka and Yoshida 2010). In both reproduction stages during sporulation, *Ulva* releases haploid swarmers (i.e., zoids and gametes), which differ by the number of flagella present and their phototactic behaviour (Hiraoka et al. 2003; Kuwano et al. 2012; Løvlie et al. 1964). Sporulation, the transformation of a thallus cell into a gametangium, is a highly regulated process. Specific intra- and extra-cellular sporulation inhibitors control the transformation of thallus cells into either a gametangium or sporangium (Hoxmark 1975; Jónsson et al. 1985; Kessler et al. 2018; Nordby and Hoxmark 1972; Stratmann et al.

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1996; Vesty et al. 2015). In *Ulva compressa*, *Ulva mutabilis*, and *Ulva linza*, upon the removal of the sporulation inhibitors, the release of swarmers occurs after approximately 72 h (Vesty et al. 2015; Wichard and Oertel 2010). Nonetheless, during the first 24–46 h sporulation is reversible, after which the cells are "committed" to the differentiation and the swarmers are finally developed (Kessler et al. 2017; Stratmann et al. 1996). If an additional swarming inhibitor is removed, the swarmers will leave the cell through an individual pore in the cell wall (Katsaros et al. 2017; Wichard and Oertel 2010). Sporulation in *Ulva* is also seasonal as it is influenced by external factors such as temperature, salinity, irradiance, photoperiod, and pH (Balar and Mantri 2020; Dan et al. 2002; Kalita and Titlyanov 2003).

In *Ulva* cultures, cutting mature thalli into 1–2 mm^2 fragments is one of the most practical methods employed for sporulation induction (Alsufyani et al. 2017; Hiraoka and Enomoto 1998; Stratmann et al. 1996). The fragments are then immersed in seawater which is changed several times to wash away any sporulation inhibitors. As thalli mature, sporulation events can occur spontaneously due to the lack of sporulation inhibitor production or its reception (Alsufyani et al. 2017; Stratmann et al. 1996). In addition, larger thalli may break down into fragments, triggering sporulation and the release of swarmers, which can contribute to the formation of green tides (Gao et al. 2017). Therefore, thallus fragmentation can also have negative implications for the maintenance of *Ulva* cultivars. Some efforts have been made to predict spontaneous sporulation using metabolomic markers (He et al. 2019; Kessler et al. 2017). However, further knowledge of the life cycle and sporulation patterns of *Ulva* is required to achieve a sustainable *Ulva* aquaculture, reduce and accelerate sporulation, and facilitate strain selection.

So far, the understanding of the sporulation events is limited to descriptive observations and we cannot accurately determine the strength of sporulation events in *Ulva*. To the best of our knowledge, no studies have focused on using flow cytometry to help to determine the strength of sporulation events. Therefore, in this study, we suggest a workflow to semi-quantify the strength of sporulation events (i.e., gametogenesis and sporogenesis) by counting the number of swarmers using flow cytometry.

Currently, there are three main approaches to assess the intensity of sporulation: (i) by monitoring the colour change of the thallus, from dark green to brown, during the transformation of thallus cells (Dan et al. 2002); (ii) by counting the empty gametangia and sporangia after the swarmers have been discharged; and (iii) by estimating the number of discharged swarmers in a counting chamber as a proxy for the strength of the sporulation event. The ratio of empty to non-empty gametangia is often used to determine sporulation intensity (Gao et al. 2017; Kalita and Titlyanov 2003; Nilsen and Nordby 1975); thus, staining empty gametangia with Evans Blue dye improves the reliable identification of these cells (Lee et al. 2019). However, an issue arises with the application of Evans Blue since the dye does not distinguish between discharged and dead cells. This approach may overestimate sporulation intensity in the case of high cell mortality. Further, previous experiments have revealed only a weak correlation between thallus colour and sporulation intensity in *Ulva* (Stratmann et al. 1996), as changes in salinity, temperature, irradiance, and nutrient supply can also cause colour changes (Gao et al. 2016; Pinchetti et al. 1998).

In this context, fluorescence detection can be a powerful tool to increase the sensitivity and selectivity of liquid chromatography (LC) analysis for fluorescent compounds. Thus, fluorescence microplate readers are widely used in high-throughput screenings of fluorescent cells (Petersen et al. 2014). Flow cytometry facilitates the counting of cell numbers through fluorescence or light-scattering (Hogg et al. 2015; Franklin at al. 2004) and the surveying of those cells (Krutzik et al. 2008). Indeed, cells can be characterised by the scattered or fluorescent light pulses [side scatter (SSC) and forward scatter (FSC), respectively] created when the particles pass through a focused light beam (Hoffman 2008; Olson et al. 1989). Methods have been established for monitoring of contamination, succession, and overall growth in both unicellular algae and microbial communities (Peniuk et al. 2016).

Here, we suggest a method for quantifying sporulation intensity and estimating gamete mobility by measuring the number of discharged gametes of *U. mutabilis* (model system) and *U. rigida* (aquaculture) using flow cytometry.

2 Materials and methods

2.1 Algal material and cultivation

Ulva rigida (C. Agardh 1823; foliose morphotype) and *U. mutabilis* (Føyn 1958; tubular morphotype) were studied under controlled conditions. The algal identities were confirmed by molecular approaches and the microscopic observation of the thallus (Brodie et al. 2007; Krupnik et al. 2018). *Ulva rigida* thalli were cultivated in outdoor tanks (V = 750 l), irrigated by a continuous flow of surface seawater pumped from the Mediterranean Sea adjacent to the Israel Oceanographic and Limnological Research (IOLR) facility in Haifa. Nutrients were added once a week at concentrations of 0.057 mM

NaH_2PO_4 and 0.59 mM NH_4Cl. The outdoor tanks included an aerating system to optimise gas exchange (O_2 and CO_2) and maintained the continuous movement of the thalli.

Haploid gametophytes from the fast-growing, naturally occurring developmental mutant "slender" of *U. mutabilis* (Løvlie 1964) were cultivated in an *Ulva* culture medium (UCM) in a 17:7 (L:D) regime at 18 °C with an illumination of 80–120 µmol photons m^{-2} s^{-1} (50% GroLux, 50% daylight fluorescent tubes; OSRAM, München, Germany) without aeration (Stratmann et al. 1996; Wichard and Oertel. 2010).

2.2 Induction of the sporulation (gametogenesis) of *Ulva rigida* and *Ulva mutabilis*

Fresh weights of approximately 1–5 g of *U. rigida* were cleaned from the epiphytes and debris in the tanks and acclimated for one week in a growth room (set at 20 °C, 100 µmol photons m^{-2} s^{-1}, 17 L:7 D). After acclimation, the *U. rigida* thalli were washed with UCM and incubated at 20 °C for 1 h to evaporate the water prior to cutting the thallus. Three 1-cm^2 pieces of *U. rigida* were then cut from each specimen and left to dry with the thallus under the same conditions. Each specimen was photographed using a light microscope (Olympus Optical Co., Ltd., Japan). The area (S) of a known number of cells (N) was measured using ImageJ software (v. 1.53c) (Schindelin et al. 2012).

The partly dry *U. rigida* thalli were then chopped into 2–3 mm^2 fragments to induce the transformation from vegetative cells into reproductive cells. The fragments were weighed (W_{sample}), and 0.5 g of the chopped subsamples were inserted into a 150-ml Erlenmeyer flask containing UCM.

The 1-cm^2 pieces were also weighed (W_{square}) at the same time as the samples to ensure the same moisture content (fresh weight). The algal flasks were then incubated for 72 h in a growth chamber with a 17 L:7 D photoperiod.

To test the effect of temperature, algal flasks were placed on three different trays set at 15 °C, 20 °C, and 25 °C using an aquarium heater and placed at the same distance from the light source under 70–100 µmol photons m^{-2} s^{-1}. For the irradiance experiments, the environmental temperature was set to 20 °C and the algal flasks were placed at three different irradiance levels: 10, 80, and 120 µmol photons m^{-2} s^{-1}. The lowest irradiance treatment samples were held inside a black net, while for the high irradiance treatment, a regular table lamp was used. Irradiance was measured with a LI-250 light metre (LI-COR®, Nebraska, Canada). Each treatment was carried out in triplicate.

After 72 h, the release of the gametes was induced through a change of UCM (V_{total}): a defined volume (V_{FCM}) of the well-mixed culture medium (V_{total}) was fixed with 2% glutaraldehyde before measuring the number of gametes by flow cytometry. By knowing the concentration of the gametes in the samples and the volume of medium (V_{total}), the total number of gametes in each sample was calculated.

Approximately 2–3 g of *U. mutabilis* fresh weight was manually separated into 2–4 mm^2 fragments using a chopper (Zyliss, Zürich, Switzerland) (Califano and Wichard 2018; Wichard and Oertel 2010). The fragments were washed three times with UCM. Then, they were cultivated for three days, as described above. On the morning of the third day, gamete release was initiated by changing the UCM. The gametes were collected either from the brightest spot closest to the light source or obtained after mixing the UCM.

2.3 Flow cytometry counting

The discharged gametes were diluted into a series of densities using UCM and then fixed with 2% glutaraldehyde for 2 h at 20 °C ± 1 °C (2% [v:v] final concentration; Merck, Darmstadt, Germany). The glutaraldehyde fixation prevented the gametes from settling on surfaces. The flow cytometer (BD Accuri C6, Heidelberg, Germany) was calibrated with six- and eight-peak validation beads (Spherotech 3 µm, BD Accuri) to identify the gametes. The dilution series of gametes was measured in triplicate. Alignment beads with known size were excited at a wavelength of 488 nm, and fluorescence was measured at FL3 > 670 nm to obtain the optimal gamete count (Califano and Wichard 2018).

For quantification, the samples were measured under the following conditions: sample volume = 50 µl; flow rate = 35 µl min^{-1}; threshold of FSC-H = 800. Wash and agitate cycles were performed between each measurement of the samples. By interpolating FSC-H, SSC-H, and FL3-H, it was possible to display defined clusters of gametes and thus determine the number of gametes per microliter using the flow cytometry software (BDC Sampler software, Heidelberg, Germany).

2.4 Manual counting and autofluorescence measurement

Manual counting was carried out under a Leica DM 2000 microscope (Leica, Wetzlar Germany) using a Neubauer chamber (chamber of 0.100 mm depth, Lo-Laboroptik, UK). The larger squares at the four corners were used for the counting of the gametes. Aliquots of each independent sample were transferred to 96-multiwell plates (Sarstedt, Nümbrecht, Germany) to measure the fluorescence intensity (FI) using a Varioskan Flash plate reader (Thermo Fisher Scientific, Waltham, MA, USA) for three technical replicates. The same excitation wavelength (λ = 488 nm) was used as that applied for flow cytometry, and the emission was recorded between 500 and 700 nm. The shaker was operated for 10 s at 300 rpm with a break of 30 s after each measurement. For data processing, the fluorescence intensity between 678 and 682 nm was obtained using the Skanlt™ Software for microplate readers (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 Chlorophyll removal

Chlorophyll was bleached in the gametes using the protocol outlined by Li et al. (2016) to identify cells in the flow cytometry image that showed low fluorescence due to a reduced amount of chlorophyll. In brief, the sample of gametes was incubated with 100% methanol and 1% NaOH (1:4, v:v) at 70 °C for 30 min. After incubation, the sample tubes were centrifuged for 3 min at 3000 × g. The supernatant was removed and replaced with sterile-filtered UCM and measured by flow cytometry.

2.6 Statistical analysis

To compare the various sporulation indices (SPI) for the gametophytes of *U. rigida* upon induction of gametogenesis at different temperatures and irradiances, a one-way ANOVA was performed along with

the Tukey's *post-hoc* range test using Rstudio (2020) (v. 1.1.456, RStudio, Inc).

3 Results and discussion

3.1 Determination of the sporulation index

To determine sporulation intensity, the number of discharged gametes needs to be related to the number of thallus cells involved. We thus defined an SPI to estimate sporulation strength that was based on the ratio between the overall number of gametes that were released and the number of thallus cells in each sample before treatment. The SPI allowed us to easily compare the results from different experiments and treatments within an established workflow (Figure 1). The number of thallus cells before the incubation or treatments was calculated according to the following equation, using the 1-cm^2 piece of *Ulva*, which was weighed and photographed prior the incubation:

$$\text{Number of thallus cells in the sample} = \frac{10,000^2}{S} N \cdot 2 \cdot \frac{W_{\text{sample}}}{W_{\text{square}}} N \cdot 2 \cdot \frac{W_{\text{sample}}}{W_{\text{square}}} \quad \text{(Eq. 1)}$$

where S is the area of the examined thallus (μm^2), N is the number of thallus cells counted under the microscope in the area S multiplied by two for both layers of the thallus, W_{sample} is the weight of the sample used in the experiment, and W_{square} is the weight (g) of the 1-cm^2 thallus, which was cut from the same thallus as the sample. The value 10,000^2 was needed to calculate how many cells were present in 1 cm^2 to convert μm^2 to cm^2.

This equation was used to calculate the number of *Ulva* thallus cells per unit weight of thallus. The area of the thallus cells was measured under the microscope, then the weight of the known area (the weight of 1 cm^2 *Ulva*) was determined to calculate the ratio of the thallus cells per gram of *Ulva*. The weight of 1 cm^2 *Ulva*, originating from the same thallus, was determined, with a relative standard deviation (SD) ranging from 2.9 to 16.6%. The high SD resulted from changes in the moisture of the different samples and the size of the selected thallus. A disc cutter with a known diameter should be used to minimise the relative SD to approximately 2%. The SPI combined the number of gametes discharged with the number of thallus cells in the incubation flask. Based on the determined cell numbers, the SPI can be calculated to estimate sporulation intensity under controlled conditions (Eq. 2):

Flow cytometry was applied for counting the gametes and zoids.

3.2 Autofluorescence and mobility of gametes

We compared the autofluorescence of gametes harvested at the brightest light spot in the culture flask (Figure 2A, i and ii) with those from a well-mixed culture (Figure 2A, iii and iv). First, FSC (Forward scatter) versus SSC (Side scatter) was plotted to identify and separate the population of gametes (Figure 2A, i and iii) from the debris. Plotting fluorescence versus FSC revealed that autofluorescence level depended on the status of the gametes (Figure 2A, ii and iv). Actively phototactic gametes harvested at the brightest light spot in the culture flask exhibited a high level of autofluorescence (Figure 2A, i and ii), whilst gametes collected from the well-mixed culture medium revealed a broader range of fluorescence emission values, including that of inactive (i.e., low mobility) gametes (Figure 2A, iii and iv). Therefore, the loss of fluorescence may indicate the low mobility of the gametes which can vary from experiment to experiment (Figure 2B). In general, there was no homogeneous distribution in fluorescence emission of discharged gametes when gametes were collected from well-mixed culture medium.

To prove whether lower levels of autofluorescence were due to the reduction in active chlorophyll content, the chlorophyll from the phototactic gametes (Figure 3, i and ii) was removed using the methanol incubation approach (Li et al. 2016) (Figure 3, iii and iv). As reported by Li et al. (2016), the cells without chlorophyll remained intact and spherical and were detected by flow cytometry in high yields (Figure 3, i and iii), but their intracellular constituents changed significantly, resulting in a low level of autofluorescence (Figure 3, iv). The methanol-treated gametes (Figure 3, iii) showed low autofluorescence, similar to that of the collected gametes that exhibited low mobility (Figure 2). Differences in the chlorophyll content have been already reported between + and – mating type

$$\text{SPI} = \frac{\text{Counts of gametes}}{\text{Number of thallus cells in the sample before the incubation}} \quad \text{(Eq. 2)}$$

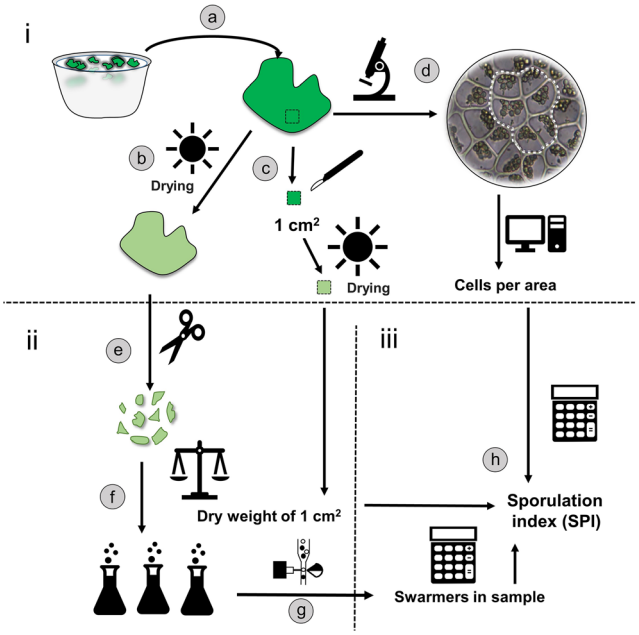

Figure 1: Workflow to determine sporulation index (SPI). (i): (a) *Ulva rigida* thalli collected from cultivation tank. (b) Thalli dried at 20 °C for 1 h. (c) Three pieces of 1 cm^2 *U. rigida* cut from each specimen used in experiment and left to dry together with thallus. (d) Each specimen photographed using light microscope (and area of known number of cells measured. (ii): (e) Fresh thalli chopped to induce sporulation. (f) Fragments weighed, washed with seawater, and inoculated into incubation flask. (g) After differentiation of thallus cells into gametangia, release of gametes induced through change of culture medium (V_{total}), defined volume (V_{FCM}) of well-mixed culture medium (V_{total}) fixed with 2% glutaraldehyde before measuring number of gametes using flow cytometer and calculating total numbers in V_{total}. (iii): (h) Using cells per unit area and weight of 1 cm^2, number of thallus cells in each flask calculated. SPI combined number of gametes discharged with number of thallus cells in incubation flask.

gametes (Hiraoka et al. 1998). However, in our study, we observed that gametes of the same mating type possessed various fluorescence levels, which were correlated with reduced swimming ability or phototactic behaviour. Interestingly, previous studies showed that the flagellar autofluorescent substance of the brown alga *Scytosiphon lomentaria* is probably involved in the photoreception of the phototaxis of the swarmers (Yamano et al. 1996). Moreover, autofluorescent compounds have been used to enumerate dinoflagellate cysts in marine and estuarine sediments in the context of anticipating and monitoring harmful algal blooms (Tang and Dobbs 2007). In any case, the reasons for the differences in autofluorescence that have been observed remain unexplained. Thus, when determining flow cytometric measurements and calculating the SPI, we recommend mixing the culture medium before sampling the gametes. Interestingly, mobile gametes identified by their high autofluorescence may possess a higher viability and work as an efficient feedstock. The reasons for variation in the fluorescent rates of the discharged gametes must be identified through future studies. The photosynthetic activity of the released gametes should be measured by pulse amplitude modulation fluorometry.

3.3 Counting gametes and method comparison

Fluorescence measurements and flow cytometry were applied for counting the discharged gametes (Figure 4). Autofluorescence measurements corroborated that more gametes caused increasing levels of fluorescence measured by the plate reader (Figure 4A) (Pearson correlation: $r = 0.99$, $p < 0.01$). However, fluorescent emission is subject to strong fluctuations depending on the physiological state of the cells (Figures 2 and 3; Tang and Dobbs 2007). Therefore, the counts obtained by the flow cytometric technique (FSC-H versus SSC-H) were compared with the counts obtained by the Neubauer-counting chamber (Figure 4B). The comparison demonstrated that gamete concentrations could be accurately measured by flow cytometry (Pearson correlation: $r = 0.97$, $p < 0.05$). Consequently, this methodology was further applied to determine the counts of gametes for the SPI of *U. rigida* in culture. It was noted that gametes or zoids may occasionally remain in the parental cell and germinate into "false-branches". However, such occurrences were not observed in our study.

3.4 Temperature and light dependency

Changes in the culture temperature affected the sporulation of *Ulva*. SPI was higher in the aquaculture of *U. rigida* ($p = 0.001$) at 15 °C and 25 °C than at 20 °C (Figure 5). Our results are consistent with previous findings that a higher level of sporulation occurs at 15 °C than at 20 °C for e.g. *Ulva fenestrata* (Kalita and Tytlianov 2003).

In our study, only a small portion of the used thallus cells was transformed into gametangia, indicated by a SPI of 0.3 (Figure 5A). However, the proposed SPI approach showed differences in sporulation intensity resulting from the different treatments, indicating the high sensitivity of this approach to even very small differences in sporulation. The same was true for the incubation of *U. rigida* at different irradiances during sporulation (Figure 5B). If all cells of an examined thallus differentiated in gametangia,

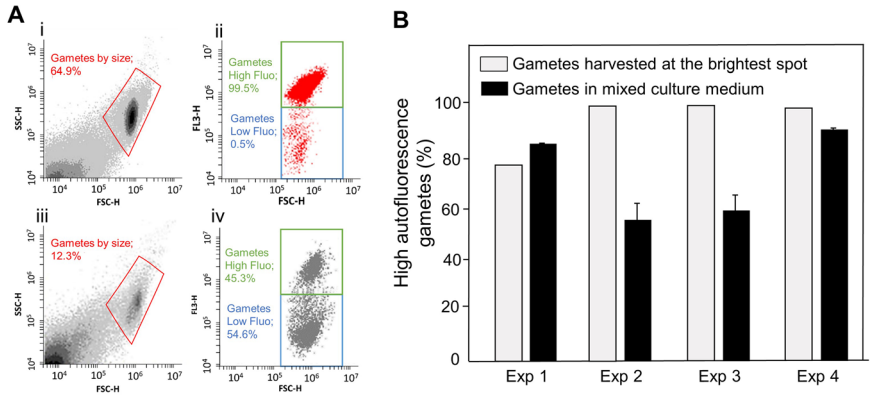

Figure 2: Distinction of gametes of *Ulva mutabilis* according to their autofluorescence using flow cytometric measurements. (A: i, ii) Gametes released by *U. mutabilis* were collected from the green layer in the spotlight (i.e., phototactically active gametes). (A: iii, iv) Gametes were collected after the culture medium was well-mixed. Plots present populations of gametes separated by their expected size (i, iii, % of the total counting events is given) and by the measured chlorophyll autofluorescence (ii, iv). The autofluorescence measurements correspond to the gametes framed by the red gates in (i, iii). (B) Percentages of high-level autofluorescence. Error bars represent mean ± standard deviation (n = 3); FSC-H, forward scatter height; SSC-H, side scatter height; FL, fluorescence (Fluo).

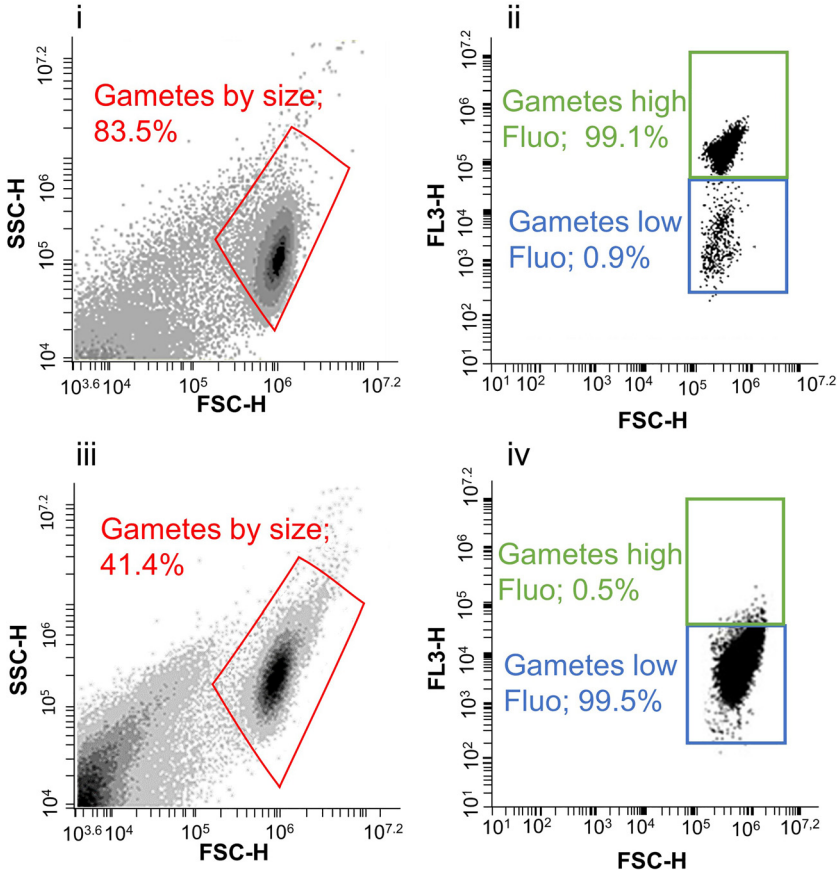

Figure 3: Flow cytometric measurements used to compare active with inactivate gametes of *Ulva mutabilis*. (i, ii) Mobile gametes were collected at the brightest spot and prepared for flow cytometric measurements. (iii, iv) Gametes were collected at the brightest spot as well. After chlorophyll removal, they were prepared for flow cytometric measurements. Plots present populations of gametes separated by their expected size (i, iii, % of the total counting events is given) and by the measured chlorophyll autofluorescence (ii, iv). The fluorescence measurements correspond to the gametes framed by the red gates in (i, iii). FSC-H, forward scatter height; SSC-H, side scatter height; FL, fluorescence (Fluo).

the maximum SPI of 16 would be reached, assuming a gametangium usually forms 16 gametes (Løvlie 1964). However, this high yield has only been observed in *Enteromorpha*-like tubular *Ulva* species, while foliose species, such as *U. rigida* or *U. lactuca*, which was recently redesignated to *U. fenestrata* (Hughey et al. 2019), often only show sporulation at the thallus margins (Wichard and Oertel 2010).

These findings confirmed that temperature and light intensity have an impact on sporulation, as previously

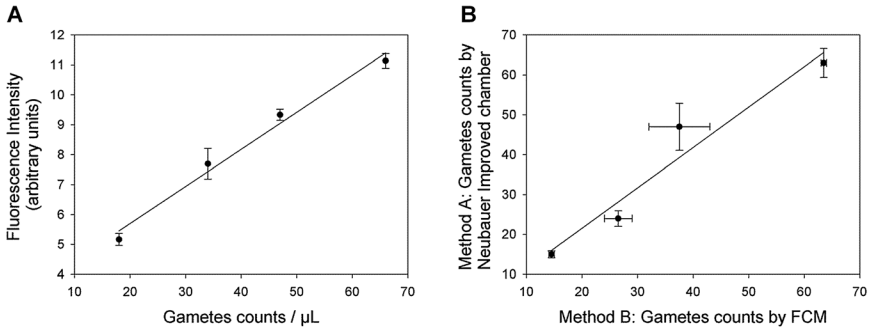

Figure 4: Gamete counts of *Ulva mutabilis* collected from well-mixed culture medium. (A) Fluorescence of a dilution series of gametes measured using a plate reader. (B) For method validation, number of gametes measured by light-scattering flow cytometry (FCM) was compared with number of gametes determined by the Neubauer improved chamber. Error bars represent the mean ± standard deviation (n = 3).

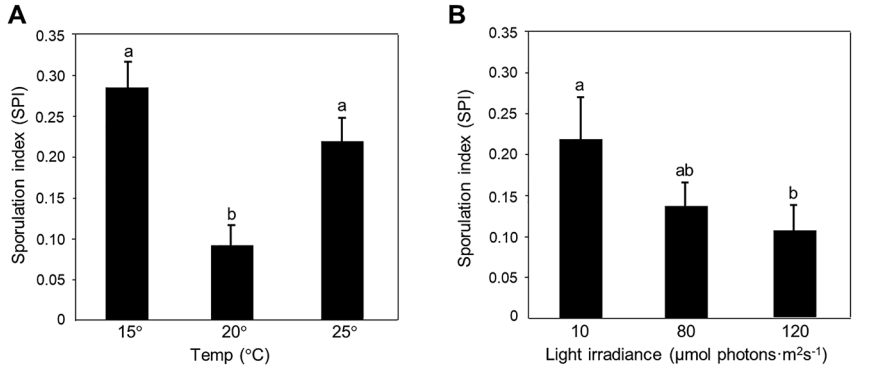

Figure 5: Sporulation index (SPI) for gametophytes of *Ulva rigida* after induction of gametogenesis at different temperatures (A) and irradiances (B). Experiment performed according workflow shown in Figure 1. Significant differences among means are indicated by different letters. Error bars represent mean ± standard deviation (n = 3).

observed (e.g., Dan et al. 2002; Gao et al. 2017; Kalita and Tytlianov 2003). As a result, it will be interesting to study whether these stresses also influence fluorescence intensity and mobility of the discharged gametes.

4 Conclusion

Our research is of interest and use to those who seek to enhance their understanding of the life cycle and sporulation patterns of *Ulva* species. Assessing sporulation intensity provides valuable information for preparing a feedstock and monitoring the status of the algal propagation process in *Ulva* aquaculture. Flow cytometry is a rapid method to quantify gametes and can indicate cell characteristics such as the mobility of discharged gametes. The SPI is a useful parameter to measure the level of production and release of gametes upon a given specific (abiotic) stimulus, upon removal of the sporulation inhibitors, or after spontaneous sporulation due to maturation. The simplicity of the test also ensures that the SPI can be applied to zoids or a mixture of gametes and zoids. The SPI would also be a suitable reference for determining survival rates of the released gametes and zoids. Overall, flow cytometry provides an accessible tool for the rapid prognostic assessment of sporulation processes in seaweed cultivation.

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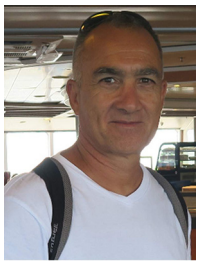

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