Research article

Omri Nahor, Cristina F. Morales-Reyes, Gianmaria Califano, Thomas Wichard*, Alexander Golberg and Álvaro Israel

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; Jönsson et al. 1985; Kessler et al. 2018; Nordby and Hoxmark 1972; Stratmann et al.

Omri Nahor and Cristina F. Morales-Reyes contributed equally to this work.

*Corresponding author: Thomas Wichard, Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Lessingstr. 8, 07743 Jena, Germany, E-mail: Thomas.Wichard@uni-jena.de. https://orcid.org/0000-0003-0061-4160

Omri Nahor, Israel Oceanographic and Limnological Research, The National Institute of Oceanography, Haifa, Israel; and Porter School of the Environment and Earth Sciences, Tel Aviv University, Tel Aviv, Israel

Cristina F. Morales-Reyes and Gianmaria Califano, Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Lessingstr. 8, 07743 Jena, Germany. https://orcid.org/0000-0003-0923-4500 (C.F. Morales-Reyes)

Alexander Golberg, Porter School of the Environment and Earth Sciences, Tel Aviv University, Tel Aviv, Israel

Álvaro Israel, Israel Oceanographic and Limnological Research, The National Institute of Oceanography, Haifa, Israel
sporulation events 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 mm² 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 et 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* (Fyn 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; Kruptnik 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.
NaH₂PO₄ and 0.59 mM NH₄Cl. The outdoor tanks included an aerating system to optimise gas exchange (O₂ and CO₂) and maintained the continuous movement of the thalli.

Haploid gametophytes from the fast-growing, naturally occurring developmental mutant “slender” of *U. mutabilis* (Layville 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-cm² 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 mm² 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-cm² 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_FCM) 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 mm² 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 agitation 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-multwell 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 SkanIt™ 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-cm² piece of Ulva, which was weighed and photographed prior the incubation:

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

where \( S \) is the area of the examined thallus (μm²), \( N \) is the number of thallus cells counted under the microscope in the area \( S \) multiplied by two for both layers of the thallus, \( W_{\text{sample}} \) is the weight of the sample used in the experiment, and \( W_{\text{square}} \) is the weight (g) of the 1-cm² thallus, which was cut from the same thallus as the sample. The value 10,000² was needed to calculate how many cells were present in 1 cm² to convert μm² to cm².

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 cm² Ulva) was determined to calculate the ratio of the thallus cells per gram of Ulva. The weight of 1 cm² 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):

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

Flow cytometry was applied for counting the gametes and zooids.

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
numbers in culture medium (cubation number of gametes discharged with number of thallus cells in 1 cm², number of thallus cells in each specimen measured. (iii): (h) Using cells per unit area and weight of 1 cm², number of thallus cells in each flask calculated. SPI combined number of gametes discharged with number of thallus cells in incubation flask.

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 cm² 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 (Vtotal), defined volume (Vcell) of well-mixed culture medium (Vtotal) fixed with 2% glutaraldehyde before measuring number of gametes using flow cytometer and calculating total numbers in Vtotal. (iii): (h) Using cells per unit area and weight of 1 cm², number of thallus cells in each flask calculated. SPI combined number of gametes discharged with number of thallus cells in incubation flask.

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 zooids 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 Tytlivanov 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,

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 swarvers (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
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...
observed (e.g., Dan et al. 2002; Gao et al. 2017; Kalita and Tytljanov 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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**Bionotes**

Omri Nahor received his MSc (2019) from Porter School of Environmental Studies, Tel Aviv University in cooperation with the Israel Oceanographic and Limnological Research (IOLR) institute. His thesis topic was entitled “Improving the domestication of the marine macroalga Ulva as a sustainable feedstock for biofuel production”. He focused on the life cycle and the starch content of Ulva species. Omri Nahor is a PhD student in the department of Marine Biology, Haifa University, Israel. His research focuses on the metabolic and ecological aspect of the red macroalgae Asparagopsis and the ecological aspect of the Asparagopsis species of the Israeli coastline.

Cristina F. Morales-Reyes
Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Lessingstr. 8, 07743 Jena, Germany
https://orcid.org/0000-0003-0923-4500

Gianmaria Califano
Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Lessingstr. 8, 07743 Jena, Germany

Omri Nahor is a PhD student in the department of Marine Biology, Haifa University, Israel. His research focuses on the metabolic and ecological aspect of the red macroalgae Asparagopsis and the ecological aspect of the Asparagopsis species of the Israeli coastline.

Cristina F. Morales-Reyes is a PhD student at the Institute for Inorganic and Analytical Chemistry of the Friedrich Schiller University Jena. She obtained her Licenciatura degree in Applied Engineering and vocational qualification as a chemical engineer, University of Santiago of Chile, Chile. Cristina Morales is interested in macroalgal-bacterial interactions along with trace metal recruitment and metal homeostasis in the chemosphere of Ulva (Chlorophyta) using various methodologies in analytical chemistry and chemical ecology.

Gianmaria Califano is a marine biologist running for a PhD at the Institute for Inorganic and Analytical Chemistry of the Friedrich Schiller University Jena.
Schiller University Jena supported by Marie Skłodowska-Curie Action grant. His current scientific interest aims to explore the interactions among Ulva and associated bacterial community envisioning the knowledge transfer to commercial applications. With tools like flow cytometry, next-generation sequencing and analytical chemistry techniques, he addresses the diversity and function of the microbial community and molecular mechanisms at the origin of algal physiological changes.

Alexander Golberg
Porter School of the Environment and Earth Sciences, Tel Aviv University, Tel Aviv, Israel

Alexander Golberg is a faculty member at the Porter School of Environment and Earth Sciences, Tel Aviv University, Israel. His interests are in biological systems engineering, bioenergy, macroalgae biorefineries, new sources for food, chemicals and fuels, exergy and applied thermodynamics, and electroporation. He received his PhD in bioengineering at the Hebrew University of Jerusalem.

Álvaro Israel
Israel Oceanographic and Limnological Research, The National Institute of Oceanography, Haifa, Israel

Álvaro Israel is a senior scientist at Israel Oceanographic & Limnological Research in Haifa, Israel. During his career, he engaged in studying photosynthesis, carbon fixation and ecology of marine macroalgae, and has a vast experience in seaweed aquaculture and strain selection intended to produce food and valuable molecules. Seaweed taxonomy using molecular tools, seaweed invasions and issues related to global change are also among his current research activities.