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Chlorophyll-*a* Fluorescence in Ecology: Theoretical Considerations and Examples around Marine Macroalgae

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Abstract: Over the last decades, the usage of PAM (pulse amplitude modulated) fluorometers for assessment of chlorophyll-*a* fluorescence variations became widely applied on marine macroalgae physiology and ecophysiology researches. Due to the increased use of these methods, a large number of studies, mainly relating to macroalgae ecology and physiology were worldwide reported. In this context, it was also created a mismatch of concepts about fluorescence of the chlorophyll-*a* and its application. Under this background, this study compile and summarize the state of the art knowledge regarding to the chlorophyll fluorescence, contextualizing the use of the PAM method with the main factors regulating photosynthesis (light, temperature, salinity nitrogen and phosphorus) in marine macroalgae. Moreover, this study also references the most used terms and shows some examples found in literature about the applicability of fluorescence parameters. The herein findings and the discussed examples, helps to emphasize the importance of fluorescence usage, that highlights the understanding of photosynthetic responses in macroalgal physiology and ecology.

Key words: Fluorometry, PAM, quenching analysis, stress.

1. Introduction

Photosynthesis is the biological reaction which converts the light energy into chemical energy, this is the main way in which the carbon fixation and storage is guided into organic compounds [1]. During the photosynthesis, the energy absorbed by the chlorophyll molecules is subjected to three dissipative pathways: (1) the photochemical pathway, whereas the photosynthesis itself occurs; (2) non-photochemical pathway, in which the energy is dissipated as heat; (3) or via a non-photochemical fluorescence emission, wherein the excess of energy is dissipated through the emission of light in wavelengths from red to far red [2, 3]. This dissipation through fluorescence emitting represents only 0.5% to 5% of the total absorbed light, which under natural conditions, is totally and exclusively derived from the excitement of chlorophyll-*a* [2, 4].

On the last decades, the usage of PAM (pulse amplitude modulated) fluorometers to chlorophyll-*a* fluorescence evaluation, became widely applied on marine macroalgae physiology and ecophysiology studies [5-9]. The increasing number of studies using fluorometers with modulated light (1-200 KHz), mainly occurred due to the fact that several fluorescence-derived measurements can be obtained under the presence of ambient light and under field

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conditions [2, 10]. In addition to this, the usage of fluorometers are notable, once it is a non-intrusive method which also provides a real time result, of the main aspects of the light fluorescence emitted by the photosynthetic system [6]. Another highlighted point is that, theoretically, this method does also establish direct association with the physiological state and the photosynthetic primary production of the organism [11-14]. Where, for example, it was showed the straight association between the absolute electron transport rate both to the gross oxygen production [15-18], as to photosynthetic CO_2 fixation rates [19]. However, studies carried out by Beer *et al.* [20] and Beer and Axelsson [21] reported that, in particularly situations, the measurements of fluorescence parameters do not correspond with primary production, particularly oxygen evolution.

Considering the various applications of fluorescence analysis of chlorophyll-*a*, there are in literature several books [22, 23] that evaluate the abiotic factors effects on the fluorescence aspects of macroalgae [24, 25]. From this point of view, this study compile and summarize some concepts about chlorophyll-*a* fluorescence applied on phycology, discussing some regulating factors (light, temperature, salinity and nutrients) on fluorescence parameters, referencing to the most used terms in literature and with example studies from it, involving marine macroalgae. Moreover, considering that most of the knowledge about fluorescence-derived techniques on macroalgae are found on books, and general reviews on fluorescence-derived technique [2, 6, 9], it is timely for a review and discussion of chlorophyll fluorescence techniques applied on macroalgae ecological studies.

2. Background

The first experiments showing the relationship between the photosynthetic reactions and the quantum efficiency of photosystem (ϕ_{PS}), were carried out by Kaustsky and Hirsch [26] when a fluorescence profile was obtained following the transfer of darkened leaves to light (the fluorescence decay became known as "Kautsky effect"). Subsequently, Duysens, et al. [27] demonstrated the biochemical redox process of plastoquinones proteins (electron acceptor) of the photochemistry pathway, which occurs on the thylakoid membrane, introducing the term "Q" as an energy quencher (redox substance). Butler and Kitajima [28] detailed the charge separation of the plastoquinone and established the terms on its ground state (Q_A), denoting this as 'opening' of the photosystem reaction centers, and as 'closing' when on the plastoquinone is on its reduced state (Q_B). Throughout the plastoquinone electronic state variation, it was possible to understand the photochemical and non-photochemical quenching ways [29]. After that, it has been demonstrated that a relationship between the fluorescence of chlorophyll-*a* and oxygen evolution [16, 22] and the carbon assimilation [19, 30] during the photosynthetic process. This relationship emphasized the chlorophyll fluorescence technique as an important tool on investigations around the physiological states and stress conditions in macroalgae [14, 31-34].

3. Quenching Analysis

The understanding of the quenching process allowed the estimation of several parameters related to the photosynthetic performance [22, 35]. Thereafter, it has been elaborated diverse and quite distinct parameters around the induction of chlorophyll-*a* fluorescence kinetics, mainly after the advances of PAM fluorometry techniques [2, 36, 37].

The PAM fluorometry techniques generally are used to demonstrate responses of effective quantum yield from the photosystem II (ϕPSII), obtained before and/or after the application of the saturating light pulse [19]. This fluorescence response can be interpreted through the formation of distinct parameters, which can be better explained by the experimental fluorescence curve of variation (*see* 38,

39]. Herein, fluorescence parameter will be shown, using terminology in agreement with numerous papers in phycology. However, other terms for the same parameters certainly can be found in the literature.

4. Photochemical Quenching Parameters

Parameters based on photochemical quenching are related to the redox state of plastoquinone, in which are attributed the differences between the maximum and minimum fluorescence yield values [6, 40]. These parameters indicate the fraction of photosystem II (PS II) reaction centers that are open and able to get started to the photochemical process [41]. Among the applicable parameters on macroalgae, the most used are:

4.1 Maximum Quantum Yield of PSII Photochemistry (Fv/Fm)

This parameter represents the PSII quantum yield in samples after dark acclimation [28, 42]. This is related to the plastoquinone reduction (Q_A), and quantifies the maximum photochemical efficiency. In an ecological context, this parameter helps to indicate the metabolism photosynthetic stress, such as photoinhibition [43]. In general, stressed macroalgae shows lower value than healthy ones [10].

$$Fv/Fm = \frac{Fm-Fo}{Fm} \quad (1)$$

where, *Fm* is maximal fluorescence after saturation flashes in dark-acclimatized sample and; *Fo* is minimal fluorescence after dark acclimatation [38].

4.2 Effective Quantum yield of PSII (ϕ_{PSII})

This is found on literature as "quantum yield" or “Y”, referrers to the PSII quantum yield, in light acclimated samples [19]. It quantifies the proportion of photons utilized in the PSII photochemistry. Based on saturation pulse method, it has been showed as a quite useful tool for the analysis of photosynthetic performance of plants [3].

$$\Phi PSII = \frac{(Fm'-Ft)}{Fm'} \quad (2)$$

where, *Fm'* is maximal fluorescence after saturation flashes in light-acclimatized sample; *Ft* is the minimal fluorescence in light-acclimatized sample [38].

4.3 Photochemical Quenching of Variable Chlorophyll-a Fluorescence (qP)

This parameter indicates the fraction of PSII reaction centers that are on its open state during the photosynthetic process. Thus, when $qP = 0$, the redox state of plastoquinones are completely reduced and when $qP = 1$, the plastoquinonas are completely oxidized [44].

$$qP = \frac{(Fm'-Ft)}{Fm'-Fo'} \quad (3)$$

where, *Fo'* is the minimal fluorescence after saturation flashes [38].

4.4 ETR (Electron Transport Rate)

Parameter which relates the effective quantum yield of PSII to light absorption ($\mu mol \cdot photons \cdot m^{-2} \cdot s^{-1}$), considering that 1 mol of photons causes excitation of electrons on 1 mol of chlorophyll-*a* [22, 45]. Although this fluorescence parameter is securely used to estimate macroalgae primary production [2, 17, 19], it is needed an accurate measurement of light absorptance in PAR range [46]. Moreover, it is also important to highlight that the correlation of ETR to O_2 evolution have been shown only for a few species [21]. Moreover, it was demonstrated for macroalgae that this clear positive correlation is only observed at low irradiances [21]. It is important to note, that there are clearly and fundamental differences in the nature of measurement made using PAM fluorometer compared to those obtained using an O_2 exchange chamber [16]. PAM fluorometers provide an instantaneous measure of ETR from a spot on the surface of the thallus [6], while the O_2 exchange techniques provide an integrated measure of oxygen evolved under a complex light environment [16, 47].

$$ETR = \Phi\text{PSII} \bullet \text{PAR} \bullet \text{AL(PAR)} \bullet 0.5 \quad (4)$$

where, A_L·(PAR) refers to the light absorptance values

by the plant's tissue, the fraction of incident PAR absorbed by a plant [45]; *PAR* is the ambient photosynthetically-active radiation (400-700 nm) that is received by the plant surface; and 0.5 assumes equal sharing of electrons between PSII and PSI [48].

Theoretically, the association between ETR (electron transport rates) and gross production of oxygen (O_2) should be 4 to 1, where four electrons are expected to be driven through PSII for each molecule of oxygen produced [17, 18]. Examples of this relationship were found by Longstaff *et al.* [16], where was observed a significant correlation between O_2 evolution rates and electron transport rates on the marine macroalgae *Ulva fasciata* (Chlorophyta).

4.5 rETR (Relative Electron Transport rates)

Parameter more often used than ETR, once does not consider A_L (PAR) factor. It is also related to the effective quantum yield of PSII and light absorption. However, this parameter should not be used on comparison among different species and between different experiments [22, 49].

$$rETR = \Phi\text{PSII} \bullet \text{PAR} \bullet 0.5 \quad (5)$$

In general, studies after Beer *et al.* [15], that investigate ecological photosynthetic parameters associated to the primary production, assume the existence of a linear relationship between the electron transfer and oxygen production or carbon dioxide consumption [16, 50].

5. Non-Photochemical Quenching Parameters

Non-photochemical dissipation is a mechanism used by the photosynthetic organisms to protect themselves against the adverse effects of high irradiance through heat dissipation [51]. It is characterized as the chlorophyll-*a* conversion in its excited state to the fundamental state, mainly induced by the transthylakoid proton gradient (ΔpH) and xanthophyll cycle [52, 53]. In general, this process occurs *via* thermal dissipation to regulate and to protect photosynthetic structures on environments where the light absorption exceeds the energy utilization capacity of on photochemical processes [54]. The non-photochemical quenching processes very often can be measurable by Stern-Volmer equation, which measures a fraction of the maximum fluorescence [2, 55]. Among the photochemical dissipation parameters applicable to most macroalgae, there are:

5.1. NPQ (Non-photochemical Fluorescence Quenching)

This parameter indicates the dissipation of energy throughout heat emission, ranging on a scale from zero to infinity. On algae, it is expected to find values between 0.5 and 3.5 when submitted to the saturating light [2]. In ecological terms, there are specific evidenced differences between the species and organisms depending on the acclimation conditions [56]. This is an indicator of the excess-radiant energy into thermal dissipation.

$$NPQ = \frac{Fm-Fm'}{Fm'} or \frac{Fv'}{Fm'} - 1 \quad (6)$$

where, *Fm* is maximal fluorescence after saturation flashes in dark-acclimatized sample and *Fm'* is maximal fluorescence after saturation flashes in light-acclimatized sample[38].

Non-photochemical quenching of variable fluorescence (qN)—It also shows the energy decay by emission of heat, which ranges from zero to one [57]. This parameter reflects the non-photochemical process (regulation of ATP-synthesis, pH gradient build up, photoinactivation of reaction centers of PSII) during light period [58]. The usage of this parameter is associated to the photoinhibition in response to environmental changes [59].

$$qN = \frac{Fm-Fm'}{Fm'-Fo} \quad (7)$$

In this context, photosynthetic organisms under stress condition exhibit *qN* values underestimated due to the reduction of *Fm* and then, the *Fv* value [22, 60, 61].

6. Abiotic Factors Analysis

Many studies were found in literature reporting applications of fluorescence parameters in macroalgae based on researches which evaluates the influence of abiotic parameters on the photosynthetic process. In particular, there are many studies that evaluate the ability of macroalgaes to tolerate environmental stress factors, to cope with an environmental stress and to quantify damages caused to the macroalgae photosynthetic apparatus [24, 34, 62].

Some examples of the applicability of fluorescence parameters will be presented and discussed, relating them to the main factors regulating the macroalgae.

6.1 Light

It is well known that, macroalgae can maintain and preserve their photosynthetic mechanism under large range of light intensity and quality [63]. On the other hand, it is also known that extremes values may lead to temporary damage (photoinhibition) or permanent (photodesnaturation) on PSII [64]. Inevitably, the PSII fluorescence parameters will reflect the photosynthetic performance and light conditions [65-67]. Many studies involving macroalgae apply the fluorescence parameters in evaluation of the best and the worst light condition [10, 13, 16, 21, 68-74]. In general, these works quantifies the relationship between light and the physiology of macroalgae trough the fluorescence parameters range or variations of photosynthetic electron transport rates. Thereby, it was demonstrated a specificity of fluorescence parameters in relation to each macroalgae specie and its environmental condition.

Moreover, issues involving UV radiation has been highlighted in literature due to its permanent effects on the genetic material and proteins of plant cells [75]. Studies around the genera *Ulva* and *Chaetomorpha* (Chlorophyta) demonstrated that excessive ultraviolet radiation, in synergy with the photosynthetically active radiation, acts directly on the photosynthesis inhibition (reduction of *Fv/Fm*); consequently, it leads to a reduced pigments production for photoprotection [24, 76, 77]. Under this scenario, UV radiation has also been linked to the stimulus on the synthesis of photoprotection compounds [78].

6.2 Temperature

The effects of temperature on chemical reactions and molecular structures in algae is well documented, mainly related to fluorescence parameters through enzymatic activities, processes of phosphorylation, electron transport chain and diffusion of plastoquinone [79-81]. However, until recent days, the specific effect of temperature on the photosynthetic process, particularly around fluorescence parameters, has not been fully elucidated in macroalgae. This is mostly attributed to the great difficulty in isolating the factor temperature in terms of the natural environment. In addition, on specific scale, several biochemical processes are linked to this factor [82].

There are several applications of fluorescence parameters on macralgae. Among the first works with an ecological involving the temperature factor, Henley *et al.* [83] report the photosynthetic response of *Ulva rotundata* (Chlorophyta) during on an intertidal sand flat. We can also cite other examples: Necchi [84] in a study involving *Cladophora glomerata* (Chlorophyta) on tropical lotic macroalga; Pang *et al.* [85] in laboratory experiment evaluated *Fv/Fm* variation of sporophytes from distinct populations of *Laminaria japonica* (Phaeophyta), showing distinct tolerances to temperature through the potential quantum yield; [86] in an evaluation the interactive effects of radiation, temperature and salinity on Arctic kelp *Alaria esculenta* (Phaeophyta) and [87], that showed the importance of physiological acclimation of floating on *Macrocystis pyrifera* (Phaeophyta) against variation of temperature and irradiance.

6.3 Salinity

Similarly to temperature, salinity variations entail structural changes on the photosynthetic apparatus due to cellular osmotic balance and cellular ionic level

[88-92]. The specific influences of this factor upon fluorescence parameters are observed [86, 93-95].

An exploratory study around the stress caused by salinity on *Ulva lactuca* (Chlorophyta) was carried out by [34]; in which reduction on photosynthesis was recorded when the salinity raise over 48 PSU. Consequently, there was a decrease on quantum yield in proportion to the salt concentration increase. These results were associated to an inactivation of the reaction centers of photosynthesis and to the inhibition of ETR (electron transport rates) on PSII.

6.4 Nitrogen

Nitrogen is very important on photosynthesis mainly on the composition of the pigment and on the nitratereductase activity [96]. In this context, changes in nitrogen concentrations are invariably reflected on the photosynthetic processes in biophysical terms [23], which allow the assessment of macroalgae by fluorescence analysis.

A study realized by Davison *et al.* [97], exemplifies the application of fluorescence to elucidate the effects of nitrogen input, where was evaluated the effects of a long-term exposure of *Laminaria saccharina* (Phaeophyta) to high concentrations of nitrogen. In this case, the authors observed higher effective quantum yields (~0.8) compared to treatments without nitrogen enrichment (~0.6). The addiction of nitrogen was also responsible for an incensement of the electron transport rates.

Malta, et al. [98] carried out experiments on *Caulerpa prolifera* (Chlorophyta) and found that limitations by nitrogen did not affect the maximum quantum yield (*Fv/Fm*). These same authors, using mass spectrometry, found that this species, when exposed to high nitrogen concentrations associated with the low intensities of light, composed thallus with 75% more nitrogen than thallus in natural environments. Others examples based on nitrate can be found in: Refs. [13, 14, 73].

6.5 Phosphorus

In a photosynthetic aspect, the deficiency of phosphorus affects the energy conversion by reducing the synthesis of proteins at the photosynthetic apparatus and the substrates of Calvin's cycle, such as ribulose-1,5 bisphosphate [99]. On the other hand, small input of orthophosphate can increase the uptake and fixation in *Ulva lactuca* (Chlorophyta), as demonstrated by Waite and Mitchell [100].

Until date, there are quite a few studies in literature demonstrating the implications of phosphorus in the fluorescence parameters. In this scenario [13] assessed the physiological status of *Cladophora* sp. (Chlorophyta) in relation to the maximum quantum efficiency (*Fv/Fm*) in a gradient of light and nutrients in which the phosphate was included. Their results showed a nonlinear relationship between photosynthetic efficiency and a limitation by phosphorus.

7. Conclusions

The marine macroalgae are known due to its great importance on primary production and its ecological function at the benthic environment. The knowledge about the regulation of photosynthetic processes and their relationship with several environmental parameters has opened new perspectives on ecophysiological studies. Therefore, the use of chlorophyll fluorescence parameters, throughout the usage of the PAM methodology has emerged as an important tool that helps to answer many questions regarding to macroalgae metabolism.

The herein findings help to emphasize the consolidation of fluorescence techniques and measurements of chlorophyll-*a* parameters of its understanding of photosynthetic responses in algal physiology and ecology. However, it should be noted that the lack of an effective study that properly links the biochemistry knowledge (the knowledge of the photosynthetic system itself), the biochemical processes and the ecology make it harder to generate an effective comparison of this biological system. In

addition to this, the lack of a standard method for the analysis based on fluorescence is one of the last boundaries that trammel the advance of fluorescence analysis.

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