EFFECT OF VARIATIONS IN SALINITY AND NITROGEN CONCENTRATION ON PHOTOPHYSICAL PARAMETERS OF PHYTOPLANKTON OBTAINED WITH FLUORESCENCE SPECTROSCOPY

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ABSTRACT

Variations in salinity and nitrogen concentration in the aquatic environment are among the observed effects of global climate change. They affect the structure of phytoplankton communities and the physiological state of algae and cyanobacteria. Results of laboratory studies of these effects are presented. A combination of Nonlinear Laser Fluorimetry (NLF) and Fluorescence Induction and Relaxation (FIRe) fluorimetry is used to evaluate the photophysical parameters of photosystem II and Chlorophyll *a* in native samples of the diatom algae *Thalassiosira weissflogii,* the zooxanthellae *Symbiodium sp. CCMP 2467*, and the cyanobacteria *Synechococcus sp. CCMP 1379,* grown under different salinity (40, 18, and 5 psu) and nitrogen concentration (normal, ×0.5, ×2).

Cyanobacteria are shown to be most resistant to these variations, while zooxanthellae are the most sensitive species. This suggests that an effect of global climate change on the phytoplankton community might be the transformation of its structure towards an increasing role of cyanobacteria. Another alarming outlook is the negative impact of climate change on the physiological state of corals, which live in symbiotic relationship with zooxanthellae. This suggests that the reasons for the degradation of coral reefs are not entirely anthropogenic. It is suggested that coral reef monitoring of variations in the photophysical characteristics of zooxanthellae might be one of the most effective ways for detecting the influence of global climate change on marine biota in early stages. It is advisable to monitor coral reefs in the oceanic areas with lowest anthropogenic impact as "background stations" for climate change monitoring.

INTRODUCTION

Aquatic photosynthetic organisms (PSOs) - mainly phytoplankton and cyanobacteria - play a key role in the global ecosystem (1): they represent a primary link in the food chain; they supply of a significant portion of oxygen on Earth and pump down carbon dioxide from the atmosphere; and some species of PSOs (particularly, cyanobacteria) are capable of fixing atmospheric nitrogen (2), making it available for other organisms. This explains the particular importance of fundamental and applied research aimed at developing new methods for studying and monitoring the physiological state of PSOs in their habitat, i. e., *in situ.*

Recently, this importance has increased due to the necessity to control the impact of global climate change on biota, for example, on the taxonomic structure of populations of aquatic PSOs and on the physiological state of coral reefs (3).

Optical methods for solving this problem largely prevail among other techniques, as they allow for fast and remote collection of the required information. Most of them are based on the optical excitation of the main pigment of a photosynthetic unit, Chlorophyll *a* (Chl *a*), and consequent registration of fluorescence response of the pigment (4). Most of the light absorbed by the photosynthetic cell is transformed during the primary stages of photosynthesis into the energy of

chemical bonds, which is further used for the synthesis of organic substances (5), while some of the Chl *a* excitation energy is radiationally dissipated in the form of fluorescence (6). Fluorescence methods for studying and monitoring PSOs can be divided into two major classes:

a) methods that allow one to obtain photophysical parameters of the photosynthetic apparatus (particularly, parameters of photosystem 2) as a whole (7), the most advanced and informative of which is the Fluorescence Induction and Relaxation method (FIRe) (8)

b) methods that allow one to evaluate molecular photophysical parameters of individual fluorophores (e. g., the Chl *a* molecules) (9) in native photosynthetic cells, i. e., *in vivo.* One of the most advanced methods among them is the Nonlinear Laser Fluorimetry (NLF) method (10).

In this paper we present an approach based on an integrated application of both FIRe and NLF techniques for a complete diagnostics of the functional state of PSOs. The paper describes the basic principles of these techniques, particularly, an algorithm for solving the inverse problem of Nonlinear Laser Fluorimetry, which allows to evaluate up to four molecular photophysical parameters of Chl *a* molecules (11). Simultaneous measurement of a large number of parameters that characterise the photophysical processes in photosynthetic organisms and exhibit differences in the sensitivity to various environmental factors can increase the selectivity of fluorescence monitoring of photosynthetic organisms under the influence of a combination of several factors at once.

The possibilities provided by the proposed approach are illustrated by the example of fluorescence diagnostics of the physiological state of various classes of aquatic PSOs (algae and cyanobacteria) grown under various salinity and nitrogen concentration conditions. These effects are observed in the natural aquatic environment, particularly, in the North Atlantic region, and are largely associated with global climate change (12). Thus, the presented experimental results are not only useful as an illustration of the proposed techniques, but also provide valuable applied information.

METHODS

The NLF technique is based on registration of the nonlinear dependence of fluorescence photons count N_{fl} on the laser excitation photon flux density *F, i.e.,* the fluorescence saturation curve (10). Due to high local concentration n_0 of the fluorophore molecules (Chl *a*) in the pigment-protein complexes of a photosynthetic unit ($n_0 = 10^{19}\dots10^{21}$ cm^{-3}), fluorescence saturation in case of nanosecond pulse laser excitation already develops at relatively low photon flux densities of $F \approx 10^{20}$ cm^{-2}s^{-1} and is associated primarily with the process of singlet-singlet annihilation of the excited fluorophore states. The second mechanism of fluorescence saturation, i.e., the dynamic depletion of the fluorophores ground state, develops at higher photon flux densities of $F \approx 10^{24}$ cm^{-2}s^{-1}. Basically, these two processes determine the shape of the fluorescence saturation curve of PSOs (10). Thus the parameters which describe the saturation processes can be determined from the experimental saturation curve by solving the corresponding inverse problem using a mathematical model of formation of the fluorescence response of fluorophores to pulsed laser excitation.

In general, the number of photophysical parameters, which determine the two mentioned photo-physical processes in microalgae, is greater than ten, as there is a whole number of paths of excitation and relaxation of the Chl *a* excited state in the photosynthetic apparatus. The inverse problem of Nonlinear Laser Fluorimetry with such a high number of variable parameters is generally incorrect.

A reduced mathematical model was proposed in (13,14,15), which describes the fluorescence saturation curve of objects with high local concentration of fluorophores and features three photophysical parameters, each describing a set of photophysical processes: the excitation cross section σ of Chl *a* molecules, which takes into account both the direct absorption of light by these molecules and energy transfer from the auxiliary pigments; the effective lifetime τ of the Chl *a* excited states, which takes into account all the processes of deactivation of the excited states other

than singlet-singlet annihilation; and the maximum rate γn_0 of singlet–singlet annihilation (where γ is the rate constant of singlet-singlet annihilation, and n_0 is the local concentration of Chl *a* molecules in a photosynthetic unit).

The population *n* of the first excited state of Chl *a* molecules is given in the framework of the mathematical model by the following expression:

$$\frac{\mathrm{d}n(t,\vec{r})}{\mathrm{d}t} = F(t,\vec{r}) \cdot \sigma \cdot \left[n_0 - n(t,\vec{r})\right] - \frac{n(t,\vec{r})}{\tau} - \gamma \cdot n^2(t,\vec{r}) \quad (1)$$

The total count of fluorescence photons, emitted from the excited volume *V* can be determined using the following expression:

$$N_{fl} = k_{fl} \cdot \iiint_V \mathrm{d}^2\vec{r}\ \mathrm{d}z \int_{-\infty}^{\infty} n(\vec{r},z,t;F)\ \mathrm{d}t \quad (2)$$

where k_{fl} is the radiative decay rate of the Chl *a* excited state, $n(\vec{r},z,t;F) = n(\vec{r},z,t;F(\vec{r},t),\sigma,\tau,\gamma,n_0)$ is the solution of Eq. (1), $\vec{r}$ is the radius vector in the cross section of the laser beam, and *z* is the coordinate along the direction of the laser pulse propagation.

The dependence of the relative number of fluorescence photons N_{fl} on the laser excitation photon flux density *F* is usually measured in the experiment. In order to obtain quantities that can be measured in absolute terms, it is necessary to normalise N_{fl} on a reference signal N_r, which depends linearly on the photon flux density *F* of exciting radiation. In case of a remote application of the NLF method, the signal of the Raman scattering of the laser radiation used for fluorescence excitation can be taken as a reference (16). The ratio $\Phi = N_{fl}/N_r$ is called fluorescence parameter, and the experimental fluorescence saturation curve is described as $\Phi^{-1}(F)$. In addition to three photophysical parameters of Chl *a* (σ, τ and γn_0), the so called unsaturated fluorescence parameter $\Phi_0 \underset{F\to 0}{=} \lim \Phi(F)$ is obtained from the experimental data; Φ_0 describes the normalised fluorescence intensity in the absence of the saturation processes, and in case of a known fluorescence quantum yield it can be used to evaluate the concentration of Chl *a* molecules in the sampled media.

The latest implementation of the NLF method, and particularly the algorithm for solving the four–parameter inverse problem (the evaluated parameters being (σ, τ, γn_0 and Φ_0) requires that the experimental saturation curve is measured in a wide range of intensities of laser radiation (i. e., $F = 10^{21} - 10^{25}$ $cm^{-2} \cdot s^{-1}$) with an experimental error of 5% or less (11). This makes it possible to evaluate the photophysical parameters of Chl *a* with an error of less than 20%, which is sufficient for most applications.

In our laboratory setup a custom-built laser spectrometer is used, based on a two–stage solid-state Nd:YAG laser emitting pulses with 25 ns pulse duration. A diagram of the setup is shown in Figure 1. The output radiation at the fundamental wavelength of 1064 nm is converted to the second harmonic (i.e., 532 nm) while the output pulse energy can be smoothly varied from zero to the maximum value of 12 mJ using an integrated Pockels cell.

Fluorescence is detected with a H5784-20 photomultiplier module (Hamamatsu) with enhanced sensitivity in the red spectral region; the spectral selection is implemented with a narrow-band filter with a transmission maximum at 685 nm, which corresponds to the maximum of the Chl *a* fluorescence band. The concentration of algae used in the experiments is such that the contribution of the water Raman scattering band (with a maximum at 651 nm and a full width at half–maximum of 20 nm) to the acquired optical signal can be neglected.

The fluorescence signal is normalised using the reference channel signal, proportional to the intensity of laser radiation. The detection in the reference channel is implemented using a PIN photodiode with built-in preamplifier S8746-01 (Hamamatsu). A similar detector is used in the ADC triggering system.

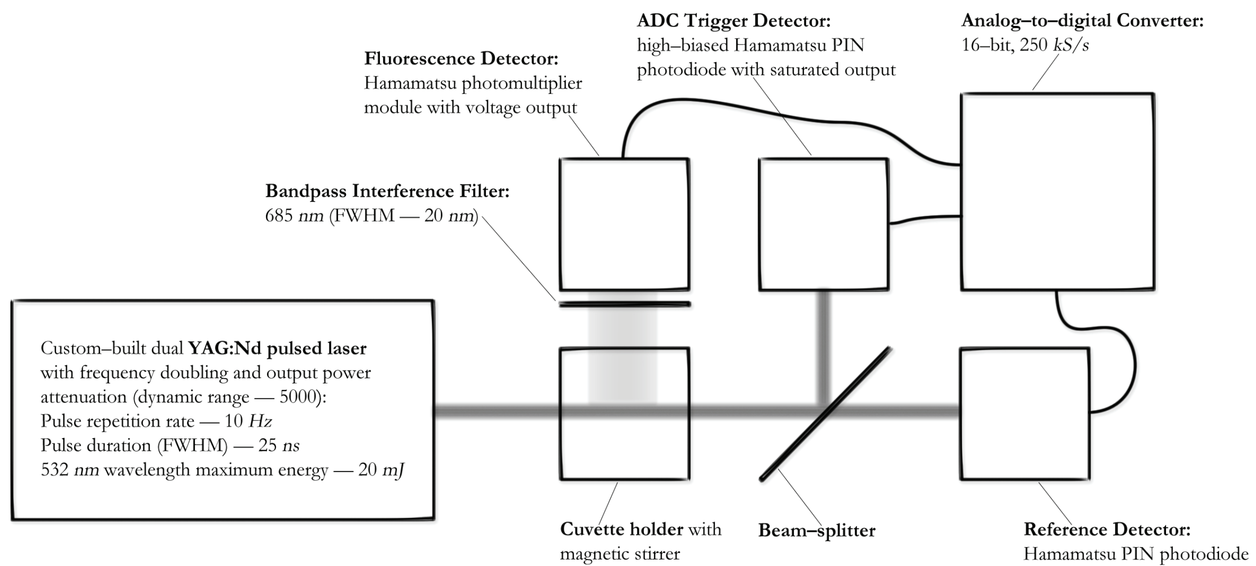

Figure 1: Block diagram of the laser fluorimeter for Nonlinear Laser Fluorimetry of photosynthetic organisms.

Two multifunctional devices USB-6211 (National Instruments) with 16-bit ADCs are used to digitise the signals coming from detectors. With the noise and the detector nonlinearity taken into account, the registration system provides an effective dynamical range of ~ 5000, which allows registration of the fluorescence saturation curve of the PSOs in the range of the exciting radiation photon flux densities $F = 6{\cdot}10^{21}\ 3{\cdot}10^{25}$ $cm^{-2}{\cdot}s^{-1}$.

Typical experimental fluorescence saturation curves are presented in Figure 2.

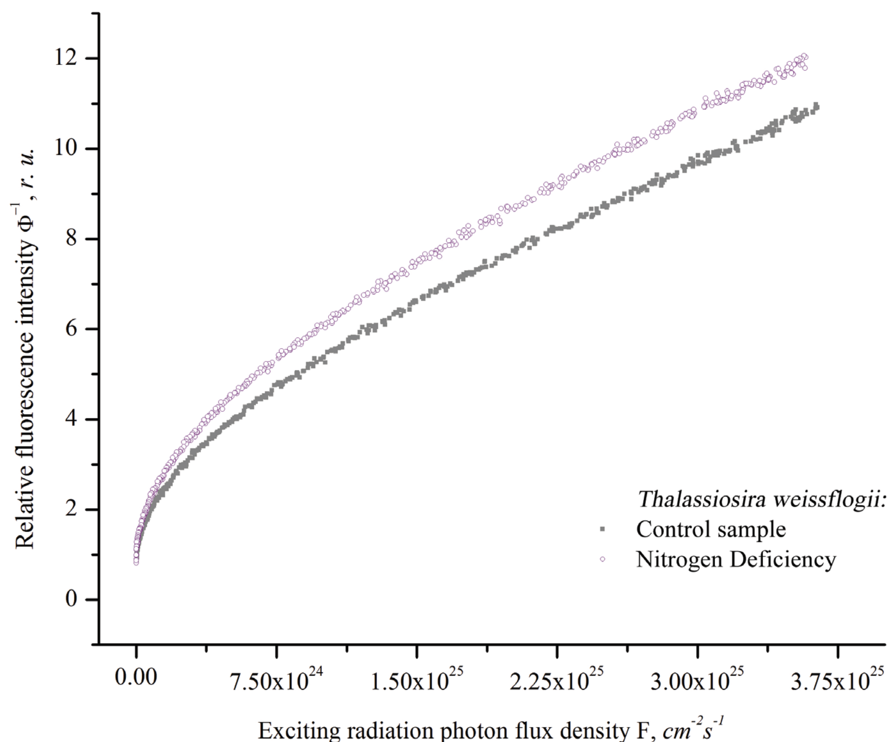

Figure 2: Typical fluorescence saturation curves obtained using the Nonlinear Laser Fluorimetry technique with samples of alga Thalassiosira weissflogii grown under differing conditions.

The FIRe method is based on obtaining Chl *a* fluorescence induction and the subsequent relaxation curve with high temporal resolution (up to 1 μs) (8). Chl *a* molecules are excited with pulses having a duration of 0.5 μs…100 ms and a 1 W/cm^2 pulse peak power, and with variable duty factor. A typical FIRe measurement protocol consists of four phases (Figure 3).

In the first phase a single powerful pulse of light with a duration of 100 ms is used for the fluorescence excitation, rendering all of the reaction centres (the pigment–protein complexes which utilise the absorbed solar energy for primary charge separation) of the photosynthetic apparatus to the so called closed state, when the energy transfer from the Chl *a* molecules within a light-harvesting antenna to the reaction centre drops significantly. During that excitation pulse the fluorescence intensity increases from its minimum value F_0 (when all reaction centres are in the open state and most of the absorbed light energy is used for primary charge separation) to its maximum value F_M (for the closed reaction centres).

In the second phase with 500 ms duration the fluorescence relaxation is recorded. It is generally characterised by the gradual opening of the reaction centres. A special sequence of short light pulses with a low duty factor is used during this phase. It has an almost negligible effect on the state of photosynthetic apparatus, which is taken into account during the consequent mathematical processing of the relaxation curves.

In the third phase a long light pulse (with a duration of about 50 ms) with high peak power is used for the fluorescence excitation, which not only renders all of the reaction centres to the closed state, but also affects other parts of the energy transfer path in the photosynthetic apparatus. The maximum fluorescence intensity reaches the value of F'_M during this phase, which differs, in general, from the F_M value.

During the fourth phase the fluorescence relaxation curve is registered, with the fluorescence intensity achieving the value of F'_0.

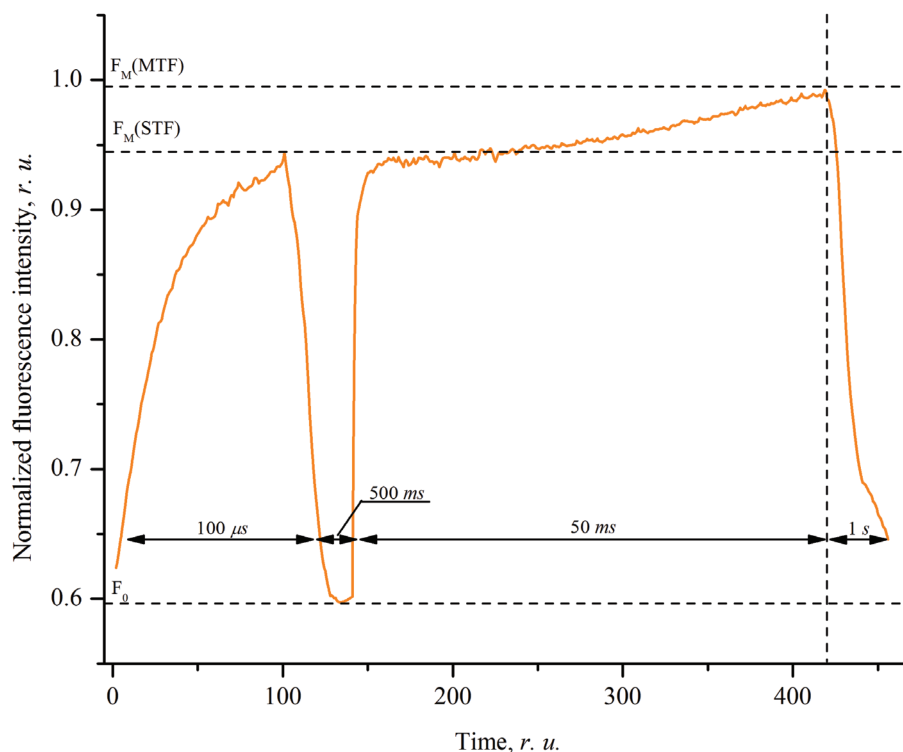

Figure 3: Typical measurement protocol of photophysical parameters of photosynthetic organisms using the Fluorescence Induction and Relaxation method.

The obtained fluorescence intensity, as well as the time constants of the excitation and relaxation phases are used to calculate a number of photophysical parameters that characterise the functional state of the photosynthetic apparatus. These parameters include:

- the variable fluorescence $F_V = F_M - F_0$;
- the photosynthetic activity rate F_V/F_M, which characterises the relative amount of initially open (photosynthetically active) reaction centres involved in charge separation;

- the cross section σ_{PS2} of the photochemical charge separation, which characterises the probability of a single act of absorption of one photon of light in the light-harvesting antenna and consequent charge separation in the reaction centre;
- the fluorescence relaxation rate τ^{-1}_{Qa} during the second phase, which describes the recovery of the reaction centres to the open state (particularly, the time constant of the electron transfer between primary and secondary acceptors in the electron transport chain);
- the coefficient n_q of non–photochemical quenching (NPQ), which characterises the efficiency of the photoprotective mechanisms in the photosynthetic cell (17).

High–power LEDs are used as the excitation light source in the FIRe fluorimeter, with wavelengths of 470 nm and 590 nm (20 nm full width at half maximum of the emission band), which fall in the absorption band of Chl *a* molecules and/or other light-harvesting pigments. The fluorescence signal is registered with an avalanche photodiode, and digitised with a 12 bit ADC at frequencies of up to several MHz. A narrowband filter with a maximum bandwidth at the wavelength of 685 nm is used for the spectral selection of the fluorescence signal. A small part of the exciting radiation is sent to the reference channel, which is used for normalising the radiation output power.

MATERIALS

In order to assess the proposed approach based on the integrated application of both FIRe and NLF methods for the diagnostics of PSOs, a set of photophysical parameters of various classes of aquatic PSOs grown under different conditions was obtained. Three pure cultures of the aquatic PSOs were chosen as sample objects: the diatom microalgae *Thalassiosira weissflogii,* the zooxanthellae *Symbiodium sp. CCMP 2467* and the cyanobacteria *Synechococcus sp. CCMP 1379.* Each of the selected samples was divided into several groups: a control group, grown under standard conditions for a given sample, and groups grown at different levels of salinity (40, 18, and 5 psu) and nitrogen concentration (normal 1N; twice higher than normal 2N; two times lower than normal 0.5N).

RESULTS

As an example, the photophysical parameter data of the diatom algae *Thalassiosira weissflogii,* grown under different conditions, are presented in Table 1, with parameters of the Chl *a* molecules determined with Nonlinear Laser Fluorimetry (Table 1a) and parameters of the photosynthetic apparatus obtained with Fluorescence Induction and Relaxation (Table 1b).

The results presented in Table 1a show that the variations in the nitrogen concentration and salinity lead to noticeable alterations of all three photophysical parameters of the Chl *a* molecules. The time constant of the linear deactivation of the Chl *a* molecules excited state τ is particularly sensitive to variations of the growing conditions: the doubling in the nitrogen concentration, as well as variations in salinity lead to an increase in the τ almost by the factor of ten. The increase in the nitrogen concentration also leads to a drastic alteration in the remaining photophysical parameters. This may be due to the decrease in the rate of energy transfer to the reaction centre (the probability of photochemical quenching). In (18) this is associated with a decrease in the proportion of photosynthetically active reaction centres with increasing concentration of nitrogen in the environment.

The significant increase in the linear deactivation time τ in case of deviations of the salinity from the normal value of the media indicates a serious inhibition of the primary reactions of photosynthesis. Furthermore, the described changes in the environment lead to a slight decrease in the maximum rate of singlet-singlet annihilation γn_o, which may be due to the influence of these factors on the conformational characteristics of the light-harvesting complexes which affect the local concentration of Chl *a* molecules.

The photophysical parameters of the photosynthetic apparatus of diatoms, obtained by using the FIRe technique, are presented in Table 1b. They reveal only a slight dependence on variations in

salinity and nitrogen concentration. The only parameter being sensitive is the rate constant τ^{-1}_{Qa}, which characterises the electron transfer between primary and secondary electron acceptor of the reaction centre (and thus the dynamics of the open reaction centres). There is an increase in the value of this parameter for the culture grown under conditions of high nitrogen concentration. This suggests that there are irregularities in the electron transport chain of the studied sample and it is consistent with the data obtained with NLF technique and complements it in the same time.

Table 1a: Photophysical parameters of samples of the diatom alga Thalassiosira weissflogii grown in different conditions, obtained with the NLF technique. Grey fill denotes samples grown under normal conditions.

Growing Conditions	σ, 10^{-17} cm^2	τ, 10^{-10} s	γn_o, 10^{12} s^{-1}
1N, 18 psu	5.26	3.73	2.06
2N, 18 psu	3.53	21.7	1.42
0.5N, 18 psu	5.12	4.71	1.73
1N, 40 psu	3.02	23.4	1.72
1N, 5 psu	3.62	22.9	1.67

Table 1b: Photophysical parameters of samples of the diatom alga Thalassiosira weissflogii grown under different conditions obtained with the FIRe technique. Grey fill denotes samples grown under normal conditions.

Growing Conditions	F_V/F_M, r. u.	σ_{PS2}, 10^{-15} cm^2	τ_{Qa}, 10^{-3} s
1N, 18 psu	0.54	36.0	280
2N, 18 psu	0.53	36.0	340
0.5N, 18 psu	0.52	36.0	295
1N, 40 psu	0.52	36.3	315
1N, 5 psu	0.52	35.8	310

Another set of complementary parameters of the photosynthetic apparatus consists of the parameter σ_{PS2}, the effective cross section of the photosystem 2 evaluated with FIRe and the parameter σ, the excitation cross section of the Chl *a* molecules, obtained with NLF. The effective cross section of photosystem 2 is the product of the total absorption cross section of the light-harvesting antenna of photosystem 2, the efficiency of the energy transfer to reaction centres and the quantum yield of charge separation in the reaction centres (the product of the latter two describes the *maximum* quantum yield of charge separation). The first multiplier can be roughly approximated as a product of the excitation cross section σ and the number of Chl *a* molecules in the antenna (the size of the light-harvesting antenna). We emphasise that the effective cross section of the photochemical quenching σ_{PS2} remains constant for all samples, while parameter σ exhibit slight variations. Assuming a conservation of the maximum quantum yield of charge separation for the described samples this suggests variations in size of the light-harvesting antenna, which correlates well with the results described in (18).

Thereby, in case of the diatoms the parameters of Chl *a* molecules obtained with the NLF technique exhibit a significantly higher sensitivity to variations in salinity and nitrogen concentration of the growth medium than the photosynthetic apparatus parameters in general obtained with the FIRe technique. Therefore the complementary application of both techniques for studying the PSOs considerably extends the obtainable information on the physiological state of photosynthetic apparatus (particularly, the state of reaction centres).

The photophysical parameter values of the other studied objects determined with FIRe are presented in Table 2. Even higher sensitivity to environmental variations exhibit the zooxanthellae, i.e., algae living in the tissues of coral polyps. The difference in the photophysical parameters obtained with FIRe were mainly observed with the sample grown at lowest salinity: there is a

significant decrease in the data of photosynthetic activity F_V/F_M and σ_{PS2}, while the rate of electron transport τ^{-1}_{Qa} increases. This suggests a major inhibition of the studied sample due to the inability to adapt to an environment with such a low salinity. For other samples we note the following changes: a slight decrease in the rate of photosynthetic activity F_V/F_M when grown at low salinity (18 psu) and with increasing concentration of nitrogen in the environment; a decrease in the non-photochemical quenching parameter n_q of the sample with a double nitrogen content.

Table 2: Photophysical parameters of zooxanthellae Symbiodium sp. CCMP 2467 and cyanobacteria Synechococcus sp. CCMP 1379 grown in different conditions obtained using FIRe technique. Grey fill denotes samples grown under normal conditions.

Sample	Growing Conditions	σ_{PS2}, 10^{-15} cm^2	τ_{Qa}, 10^{-3} s	n_q, r. u.	F_V/F_M, r. u.
Symbiodium sp. CCMP 2467	1N, 40 psu	36.0	180	2.45	0.48
	1N, 18 psu	35.0	202	2.43	0.43
	1N, 5 psu	15.0	560	0.12	0.07
	2N, 40 psu	35.5	165	1.83	0.43
	0.5N, 40 psu	35.5	200	2.25	0.47
Synechococcus sp. CCMP 1379	1N, 40 psu	14.5	723	0.77	0.40
	1N, 18 psu	14.8	710	0.46	0.44
	1N, 5 psu	15.1	704	0.27	0.42
	2N, 40 psu	14.5	656	0.73	0.42
	0.5N, 40 psu	14.7	603	0.74	0.42

For cyanobacteria (where the normal salinity is 40 psu) salinity variations affect primarily the NPQ rate. Lower salinity reduces the effectiveness of NPQ, while the other parameters do not undergo significant changes. It can be assumed that a change in salinity in the medium leads to changes in intracellular characteristics (e. g., viscosity), which alters the processes associated with the dissipation of the excess energy.

Thus, variations in the mineral nutrition are reflected primarily in the electron transport rate between the primary and secondary electron acceptor τ^{-1}_{Qa}: there is a slight decrease in this parameter.

CONCLUSIONS

The possibility of using a set of photophysical parameters of aquatic PSOs (algae and cyanobacteria), i.e., the parameters of photosystem 2 as a cellular unit (obtained with the Fluorescence Induction and Relaxation technique) and of individual molecules of Chl *a in vivo* (obtained using the Nonlinear laser Fluorimetry technique) for the study and diagnostics of their physiological state and dependence on various environmental factors (particularly, variations in salinity and nitrogen concentration) is presented in the paper. The analysis of experimental results shows that different parameters obtained with both methods respond to environmental factors in a similar, although not identical way. It is therefore suggested to use this complementary set of parameters for a complete diagnostics of PSOs.

The presented results of an experimental evaluation of photophysical parameters of various species of PSOs grown in aquatic media with varying salinity and nitrogen concentration suggest that there are drastic differences in the level of influence of such variations on the physiological state of different classes of PSO. Cyanobacteria are shown to be the most resistant to these variations, while zooxanthellae are the most sensitive ones. This suggests that an effect of global climate change on the phytoplankton community might be the transformation of its structure towards an increasing role of cyanobacteria, whereby the toxicity of some species of cyanobacteria must be considered.

Another eventually alarming outlook is the negative impact of climate change on the physiological state of corals, which are in symbiotic relationship with zooxanthellae. This suggests that the reasons for the observed degradation of coral reefs are not entirely anthropogenic. We suggest that coral reef monitoring for the variations in the photophysical characteristics of the zooxanthellae might be one of the most effective ways for detecting the influence of global climate change on marine biota in the early stages. It is advisable to monitor coral reefs in oceanic areas with lowest anthropogenic impact as "background stations" for climate change monitoring.

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