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Biosensors Based on Micro-algae for Ecological Monitoring of the Aquatic Environment

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Additional information is available at the end of the chapter

Abstract

This chapter is devoted to research possibilities of using micro-algae as sensor elements for biological sensors of aquatic pollutants. The characteristics of the fluorescence of micro-algae pigments caused by laser light, called laser-induced fluorescence (LIF), were used as informative parameters. It is shown that the functioning of biological sensors is determined by the consistency and concentration of micro-algal cells as well as their internal state, which depends on the temperature, illumination, and chemical composition of the water. The results indicate a significant dependence of the LIF intensities of micro-algae from exposure of heavy metal ions, temperature, and illumination. In text is demonstrated specificity of the reaction of various micro-algae, belonging to different phylum. This fact can be used for identify the phylum of micro-algae in situ, and for the creation the biosensors of ecological monitoring aquatic environment.

Keywords: laser-induced fluorescence, micro algae phylum, ions of heavy metals, depending on the lighting and temperature, biosensors

1. Introduction

Monitoring of aquatic environments requires obtained information in real time, long before the appearance of visible signs of pollution, far exceeding the norms of maximum permissible concentrations (MPC). In recent years, organisms, which have high sensitivity to adverse factors, are widely studied as bio-indicators for water monitoring. Single-celled organisms including

micro-algae of phytoplankton can quickly respond to changes in their habitat due to their small size and high rate of intracellular metabolic processes [1].

Unicellular microorganisms violation of life appears to change their functioning—reversibly or irreversibly. In the latter case usually occur damage cells, typically leading to its destruction. The initial, specific impact of the damaging environmental factor is aimed at very specific molecular structure of the cells. The composition of unicellular organisms mainly includes nucleic acids, proteins, lipids, and polysaccharides. All these components may be the target for of the damaging action of factors of the environment surrounding the cell. Violation of the functions of these structures causes a cascade of events that ends with response of the cell as a whole system. Thus, it is possible to distinguish several stages cellular response to external adverse impact. As a rule, at first, there is a nonspecific reaction, typical for any irritations. For most of the cells, there is a change of cell membrane permeability during the action of metal ions [2] and subsequent activation of intracellular systems aimed at the suppression of stress reactions and stabilization of intracellular metabolism [3]. For example, in the presence of calcium ions at the initial stage is an activation of different intracellular systems: protein kinases, phospholipases, protein biosynthesis systems, phosphodiesterase, cyclic nucleotides, adenylatecyclase, a contractile apparatus of the cell, etc. This is the first, reversible stage when the cell tries to compensate the effect of damaging factors. When the damaging factor is greater or more prolonged exposure, violation of cellular functions occurs. By direct action is the damaging effect of poisons, aimed directly at the cell, such as potassium cyanide, which inhibits cellular respiration. Direct violation of cell activity and its damage can be caused by lack of oxygen, excessively low pH, and low osmotic pressure of the substances that are necessary for the life of cells, ultraviolet or ionizing radiation, and so on.

These properties of unicellular organisms determined the increased interest in using them as a part of sensory systems. Analysis of publications demonstrates the sensitivity of the micro-algae of the phytoplankton to rather a wide spectrum of aquatic pollutants [4], including metal ions [5, 6], herbicides, [7, 8], pesticides, cyanide, methyl parathion (MPt), N′(3,4-dichloro-phenyl)-N, N-dimethyl-urea (DCMU), toxic agents of chemical weapons [9, 10]. For example, in article [11] shows the results of experiments in which the presence of DCMU was detected in a concentration of 6×10^{-7} M, which is less than the MRL for a given substance in the seven times (4.3×10^{-6} M), and also the presence of mercury ions at a concentration of 3×10^{-6} M.

Reduction of photochemical quantum yield of algae under influence of some toxic substances noted in [12]. **Table 1** shows the analysis of the influence of potassium cyanide (KCN), MPt, and DCMU on photochemical quantum yield for certain water area. Here, $A = F_v/F_m$ shows the efficiency of energy use of light during photosynthesis, F_m—the maximal fluorescence, which is caused by intense flashes of inducing light, F_0 is the value of the minimum of chlorophyll fluorescence intensity in the absence of constant illumination, $F_v = F_m - F_0$—variable of fluorescence.

Methods of the phytoplankton investigations are regularly improved. Their development began by capture of the phytoplankton samples with special nets and investigations them under an ordinary microscope, to *in situ* measurements of the phytoplankton condition using

lasers or satellite systems. The electrochemical, optical, laser, radiation, statistical, and other methods of studying the state of the phytoplankton exist are developing.

Sample site	Toxic agent [A ± probable error (%)]		
	KCN	MPT	DCMU
1 Clark Center Recreation Park	22.78 ± 1.63	8.32 ± 0.21	17.71 ± 1.32
2 Melton Hill Hydroelectric Dam	29.85 ± 4.17	7.66 ± 0.90	23.45 ± 4.77
3 Oak Ridge Marina	25.88 ± 0.90	8.58 ± 0.27	12.81 ± 0.81
4 Tennessee River	21.89 ± 0.76	3.28 ± 0.18	14.77 ± 1.81

Table 1. Decrease in photochemical yields of naturally occurring algae in primary-source drinking waters from the Clinch and Tennessee Rivers following exposure to toxic agents.

The fluorescence of pigments contained in the cell caused by the laser light, is one of the most important properties of the single-celled algae, and is called laser-induced fluorescence (LIF). Methods LIF measurements are characterized by high sensitivity, rapidity, and the possibility of analysis of fluorescent signal nonlinear parameters [13, 14]. Furthermore, the fluorescent response of micro-algae cells depends not only on the type and concentration of the culture, but also on the conditions in which they were during the measurement: temperature and light, the presence of dissolved organic and inorganic matter [15]. At present, LIF is one of the main methods used in modern techniques and instruments for the study of condition of the phytoplankton and measuring the concentration of micro-algae [16, 17]. An important feature is the possibility of LIF measurements directly into the environment without damaging the cells.

2. LIF spectra analysis as a method of research of phytoplankton internal conditions

A LIF spectrum depends on the composition of pigments in micro-algae cells, and external environmental conditions which affect occurring therein biochemical reaction. **Table 2** shows the list of the main pigments in algae belonging to different phylum [18].

Phylum	Chlorophylls	Phycobilins	Main carotinoids	Main xanthophylls
Cryptophyta	*a*, *c*2	B-phycoerythrin (545), R-phycocyanin. allophycocyanin	α-, β-, ε-carotin	Alloksantin
Ochrophyta	*a*, *c*1, *c*2, *c*3	Missing	α-, β-, ε- carotin	Fucoxanthin, violaxanthin
Haptophyta	*a*, *c*1, *c*2	Missing	α-, β-carotin	Fucoxanthin
Chlorophyta	*a*, *b*	Missing	α-, β-, ε- carotin	Lutein, violaxanthin

Table 2. Main pigments in algae belonging to different phylum.

Possibility of measurement techniques LIF micro-algae is that under the influence of the exciting laser radiation photosynthetic pigments absorb energy inducing light. Part of the energy emitted as fluorescence, the other part is converted into heat energy and a part is used in photosynthesis. The basic idea of using LIF for investigating the internal state of phytoplankton is that the reduction transferring or storage of light energy in photosynthesis, leads to a change in fluorescence intensity [19]. Competition between the processes of photosynthesis, the heat scattering and fluorescence allows estimating a condition of cells of micro-algae using LIF. **Figure 1** shows a typically diagram of the electronic levels, which shows the main competing processes in the cell of phytoplankton.
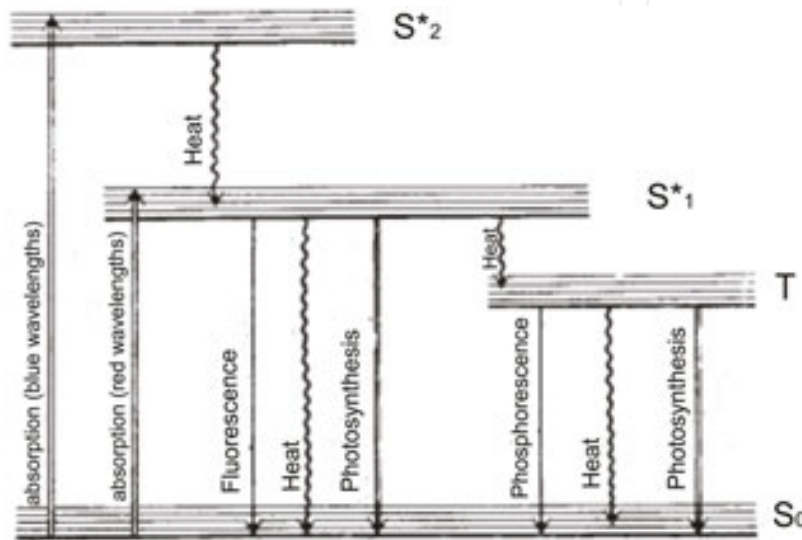
Figure 1. A diagram of the electronic levels in the cell of phytoplankton.

Multiple studies LIF spectra of phytoplankton cells demonstrate that chlorophyll-*a* is the predominant pigment whose the spectral density of the fluorescence is much higher than that of other pigments. So chlorophyll-*b* fluoresces very weakly, due to the fact that transfers the absorbed energy of the exciting light to chlorophyll-*a* with almost 100% efficiency. Other pigments such as phycobilins may have different intensity of LIF, depending on what part of the absorbed energy of the exciting light they transmit to chlorophyll-*a*. The difference among pigments of LIF spectral maxima and the maximum value of the absorption spectra allows using LIF for the study of specific pigments and reactions in which they participate. The level of fluorescence intensity depends on the photochemical reactions that occur in reaction centers (RCs), and especially in the second photosystem (PS2). In essence, PS2 is not only responsible for the absorption of light and the forming of oxygen, but also provide the main part of the fluorescence spectrum of phytoplankton cells. Methods of collect and process data, obtained by measurement of the fluorescence spectra, may be different. Are widely used methods as follows:

- assessing the effectiveness of the photosynthetic apparatus of phytoplankton cells [19];

- analysis of the chlorophyll-*a* fluorescence induction [20];

- calculating of variable fluorescence of chlorophyll-*a* [21];

- pump-and-probe [22, 23];

- studying the fast repetition rate fluorescence [24, 25].

The spectral density of phytoplankton fluorescence is a weighted average, depending on the relative amount of PS2 systems, inducing of light intensity and condition of chlorophyll-*a* in these complexes [21]. To study the composition of the fluorescent, pigments are exploring wideband fluorescence spectra with high spectral resolution. A monochromatic high-density light is used to increase the resolution of the measuring devices. The LIF spectra of phytoplankton contain spectral components from different pigments that are part of both photosystems, but the largest part of the observed fluorescence is generated by chlorophyll-*a* in PS2 [26]. Therefore, only the measurement of a wide spectrum of LIF allows take account the contribution of all pigments in the spectral density of fluorescence at the wavelength of 680 nm [27, 28].

The dependence of the fluorescence intensity by lighting changes over time is called kinetics of fluorescence. The parameters of chlorophyll-*a* fluorescence kinetics are very informative to characterize the state of the photosynthetic apparatus. This is due to the fact that changes in the state of the photosynthetic apparatus are accompanied by a change in the probability of electronic excitation energy quenching of chlorophyll-*a*, which appears to change intensity of LIF at the light. The study of photosynthesis using the kinetics of fluorescence allows the detection of damage under the influence of anthropogenic pollution, increasing the intensity of the sun and UV radiation, a lack of mineral nutrients, temperature, well before they will find their external manifestation, for example, in reducing the number of cells [26, 29, 30].
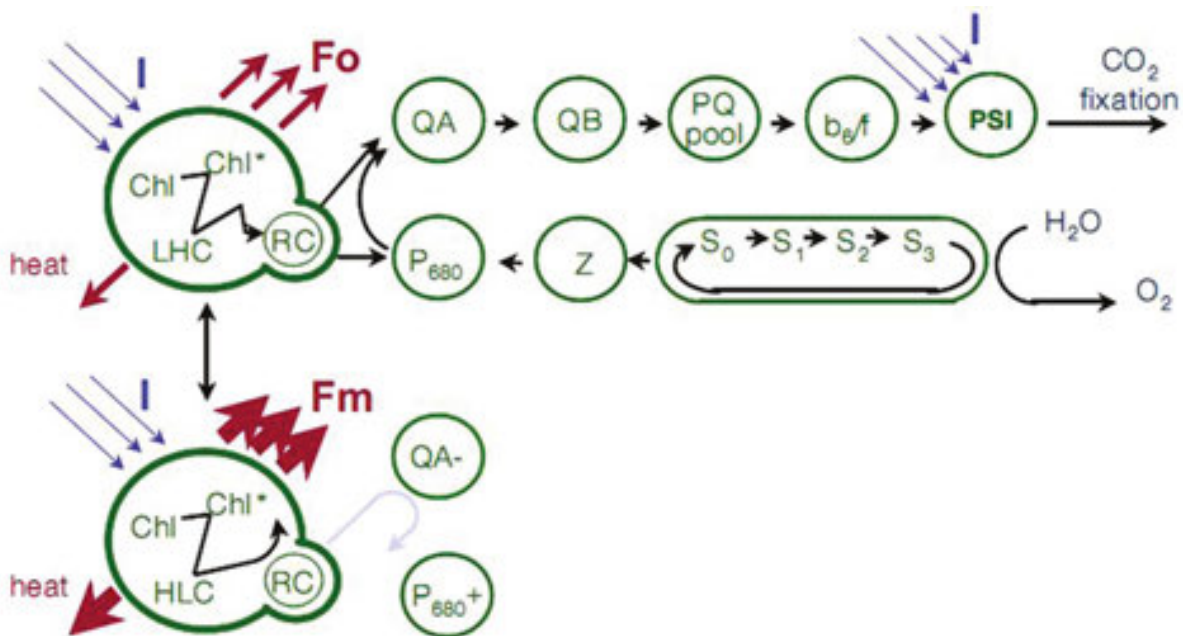

Figure 2. Simplified diagram of energy migration in photosynthesis.

In the state, where the RC of phytoplankton cells are "closed" (quinone acceptor Q_A is in a reduced state), the fluorescence quantum yield of chlorophyll-*a* in the PS2 is considerably greater than for Q_A in the oxidized state. This phenomenon is shown, schematically, in **Figure 2** [28]. Here, in the upper part of the figure shows a diagram of the energy migration in the normal state of PS2, the bottom diagram shows the migration of energy in closed RC with the restored Q_A.

The closing of the centers can be caused by a powerful flash of light, at which quickly restored quinone acceptors Q_A, and the intensity of LIF reaches the maximum level F_m.

The actual increase in illumination conditions or an increase in ambient temperature leads to a reduction of the fluorescence intensity to a value F_m' ($F_m' < F_m$). This can be caused by the processes of adaptation of the photosynthetic apparatus of the cell and damage its internal structures. If the reduction in fluorescence intensity is due to the redistribution of the absorbed light energy in a result of photosynthesis, there is a photochemical quenching. If the reduction in fluorescence intensity caused by an increase in heat transfer or damage cell structures, there is a nonphotochemical quenching.

If measurements are made in real conditions, the difference between the values fluorescence intensity of maximum F_m' and the level of initial F shows the share of PS2 complexes in which the acceptor Q_A was in the oxidized form (up to flashes of light). LIF intensity F_m' is registered under irradiation of phytoplankton by weak flashes of light. In this case, the RC of phytoplankton cells not closed. The difference between the values of F_m' and F_0' shows the share of PS2 complexes in which the acceptor Q_A was in the reduced form.

Changes in fluorescence due to the reaction of the photosynthetic apparatus of the cell are characterized by a coefficient of photochemical quenching (qP) [19] as follows:

$$qP = \frac{F_m' - F}{F_m' - F_0'} \tag{1}$$

The degree of nonphotochemical quenching is characterized by coefficient of nonphotochemical quenching (NPQ) [19] as follows:

$$NPQ = \frac{F_m - F_m'}{F_m'} \tag{2}$$

Quenching coefficients (1) and (2) can be successfully used for determining the presence of chemical substances in the aqueous environment using the cells of micro-algae. Disadvantage of the use of these coefficients is the impossibility during their calculations to separate the contributions of illumination, temperature, and dissolved substances.

Since the F_m is measured with closed RCs, it is best suited for noninvasive study of the characteristics of cells under changing environmental conditions. Therefore, given below

results of experiments on analysis of the fluorescence spectra of algae, obtained in a saturating laser exposure.

3. Realization of biosensors based on measurement of phytoplankton LIF parameters

At the present time, there are many different methods for determining the concentration of chlorophyll-*a*. Most of them do not allow studies of living phytoplankton cells since include mechanical action on the cells, as well as the use of cytocidal chemical reagents. Bathometric water sampling and subsequent laboratory tests are used at great depths. The procedures for such measurements take much time, and the accuracy is highly dependent on the concentration of phytoplankton and qualification of the personnel. Laboratory tests require the use of chemical reagents, and consequently, quite expensive, moreover, such studies are making changes in the state of phytoplankton cells and usually lead to the death of the samples. Large areas of the oceans and seas are investigated by the color analysis from satellites, but these methods do not work for measurements in coastal waters, rivers, and lakes, the most important in terms of their use in economic activities [16].

Currently, there are several major realizations of biosensors based on measurement of parameters of phytoplankton LIF. Are widely used biosensors, whose action is based on the use of green micro-algae cell suspensions [6]. In the presence of toxicants, fluorescent characteristics of chlorophyll-*a*, which is a part of PS2 photosynthetic apparatus of these algae are changed. The level of micro-algae fluorescence is registered using a fluorometer in the wavelength range 680–690 nm. From the change in fluorescence intensity relative to its initial value (before toxic effects) determine fact of the presence and the concentration of toxicant in water. Using the biosensors based on of micro-algae cell suspensions in research practice are require the search of micro-algae species is most sensitive to the effects of various matters. However, the choice of species is determined not only by the initial fluorescence intensity of cells and its changes in the presence of dissolved substances, but also their availability, and the possibility of long-term preservation in a viable form.

A perspective direction is the development of biological sensors based on immobilized cells of micro-algae placed into a porous, water permeable matrix. Small pore sizes prevent the movement of the cells and their division. Immobilization of cells algae in a porous matrix allows to give to them spatial and temporal stability, to provide convenient storage and use the sensors based on these not only for discrete analysis of toxicants in the samples taken from the water, but also to analyze the presence of toxicants *in situ* using a flow-through systems. Biosensor configurations realized on the basis of the immobilized cells are distinguished of micro-algae culture and the matrix in which they are immobilized.

There are implementation of sensors in which are used as templates a various materials, for example, porous glass [31], silica gel [11], filter paper, coated for hardening Ca-alginate gel [32], PVA cryo gel [33]. These biosensors can be used in flow systems to determine the presence

of heavy metals and herbicides at a flow rate of 60, 120, and 360 ml/h. **Table 3** shows the data from [34] which confirms the prospectivity of such directions of biosensors realization.

Toxicants	Detection limit, g/l (*Chlorella autotrophica*)	Detection limit, g/l (*Thalassiosira weissflogii*)	MPC, g/l
Zn^{2+}	$(6.5 \pm 0.2) \times 10^{-5}$	$(5.2 \pm 0.3) \times 10^{-6}$	5.0×10^{-5}
Hg^{2+}	$(1.4 \pm 0.1) \times 10^{-5}$	$(1.0 \pm 0.1) \times 10^{-5}$	3.4×10^{-5}
Cu^{2+}	$(6.4 \pm 0.1) \times 10^{-6}$	$(1.3 \pm 0.1) \times 10^{-5}$	1.9×10^{-3}
Atrazine $(C_8H_{14}ClN_5)$	$(6.5 \pm 0.2) \times 10^{-5}$	$(1.3 \pm 0.1) \times 10^{-5}$	1.0×10^{-4}
DCMU $(C_9H_{10}Cl_2N_2O)$	$(7.0 \pm 0.3) \times 10^{-6}$	$(7.0 \pm 0.3) \times 10^{-6}$	1.0×10^{-3}
Paraquat $(C_{12}H_{14}N_2)$	$(7.7 \pm 0.4) \times 10^{-5}$	$(7.7 \pm 0.2) \times 10^{-6}$	1.0×10^{-4}

Table 3. The lower detection limit of toxicants when using biosensor in the form of immobilized cells of micro-algae in the flow system.

It should be noted that for the biosensors based on immobilized of micro-algae cells is necessary to provide optimal storage conditions under which a high level of fluorescence signal of immobilized cells is maintained.

4. Influence of environmental conditions on the parameters of micro-algae LIF

Stable operation of biological sensors is determined by the constancy of composition and concentration of micro-algae cells, as well as the stability of their internal state, which depends on the chemical composition of the environment, temperature, illumination, salinity. Under real-life conditions (*in situ*), the current changes in environmental parameters can lead to significant measurement errors. In the [35, 36] indicates that an increase in temperature leads to a nonphotochemical quenching of chlorophyll-*a* LIF. The appearance of temperature quenching processes caused by increased frequency of molecular collisions, which is accompanied by deactivation of the excited levels by nonradiative vibrational relaxation of molecules and decrease in the quantum yield of LIF. The increase in temperature should lead to a change in the spectrum and to decrease the spectral density of chlorophyll-*a* LIF and, consequently, should be taken into account when calculating the concentration of chlorophyll-*a*. This is important in case of monitoring the state of phytoplankton, when the water temperature varies during the day, and the measurements of the vertical distribution profile of phytoplankton, when the difference in temperature between the surface and the depth may reach tens of degrees Celsius.

The article [15] describes the results of experiments obtained by study of the dependence of chlorophyll fluorescence on the temperature and concentration of algae. The experiment demonstrates that by measuring intensity of chlorophyll-*a* LIF, the correct determination of the concentration is only possible in cases where the ambient temperature does not change

and is equal to the calibration temperature. Otherwise, the error may be very large. For example, **Figure 3** [15] showed that the intensity of the LIF at a temperature of 17°C for a sample of the culture at a concentration of 106 cells/ml (upper curve in **Figure 3**) differs little from the LIF intensity at a temperature of 3°C for a sample at a concentration of 2×10^5 cells/ml (second curve in **Figure 3**). In their turn, the intensity of LIF at 17°C for a sample with a concentration about 2×10^5 cells/ml differs little from that obtained at a temperature of 3°C for a sample with a concentration of 105 cells/ml. Consequently, the relative error in determination of the chlorophyll-*a* concentration, excluding the temperature can reach 2–5 times.
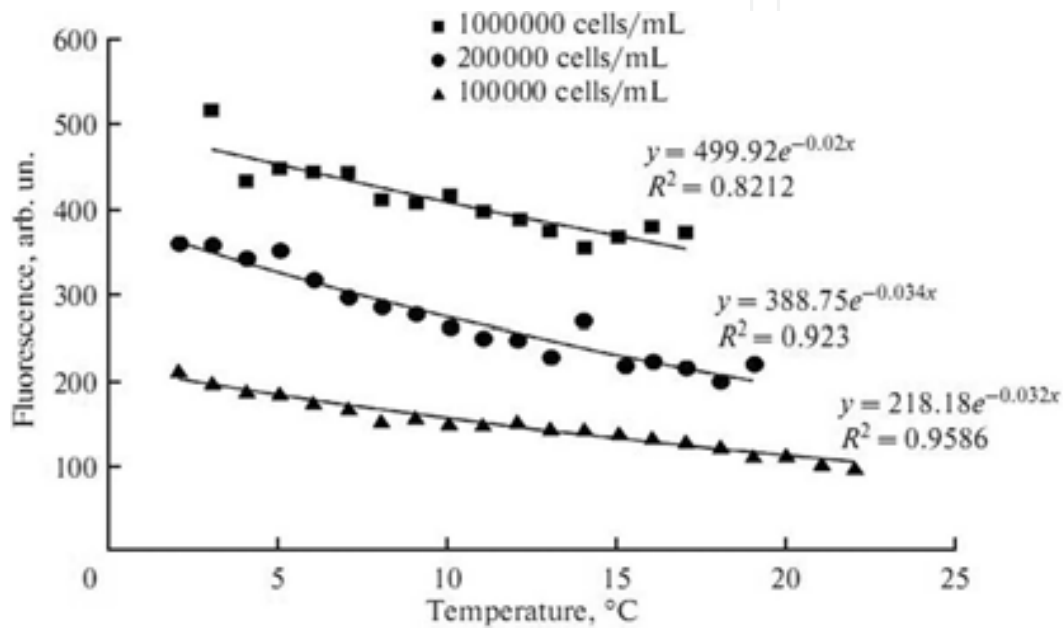
Figure 3. The appearance of the error in determining of the micro-algae concentration.

Increasing light exposure leads to a reduction of fluorescence of phytoplankton cells for two reasons: firstly, due to the increase of energy expended in the cells on photosynthesis, which consequently causes a photochemical quenching [37–39], secondly, due to changes in the structure of chloroplasts containing chlorophyll-*a* [40]. In the article [41], also shows that if a critical value of light exposure saturation occurs, the fluorescence reaches its maximum or minimum level.

To determine the fluorescence intensity dependence on illumination and temperature, it was carried out a series of experiments with the culture of micro-algae from the collection of the Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences. The experimental values of fluorescence intensity dependence on intensity of ambient illumination for the culture of micro-algae *Tisochrysis lutea* are shown in **Figure 4** [15]. The obtained data are well approximated by a straight line whose equation is shown in graph field. Calculated correlation coefficient between the experimental data and approximation line confirms the high degree of coincidence.
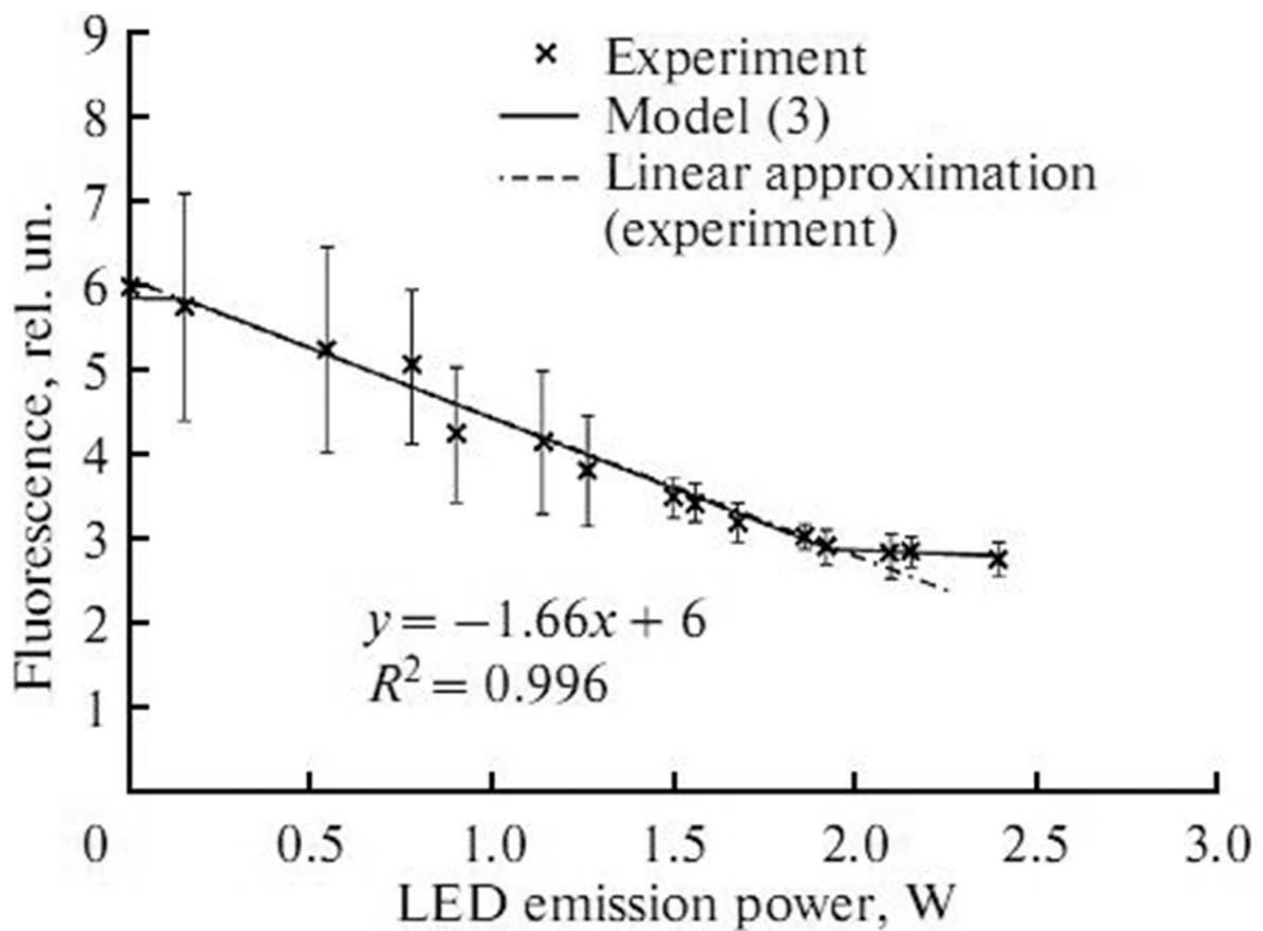
Figure 4. The experimental values of fluorescence intensity dependence on intensity of ambient illumination for the culture of micro-algae *Tisochrysis lutea*.

Since the F_m, for measurements *in vivo* depends on the parameters of the environment, such as illumination $F_m(Q)$ and the temperature $F_m(T)$, it is necessary to carry out the adjustment of the measured value F_m taking into account these modifications. **Figure 4** is clearly seen the presence of two areas of fluorescence saturation: at low and at high illumination, which confirms the conclusions drawn in [40, 42]. Taking into account, these nonlinear effects of saturation of fluorescence, fluorescence dependence on the illumination can be represented by the following system of Eq. (3):

$$F_m(Q) = \begin{cases} f_{max}, & if\ Q \le Q_1 \\ \left(f_{max} + k(Q - Q_1)\right), & if\ Q_1 < Q < Q_2 \\ f_{min}, & if\ Q_2 \le Q \end{cases} \tag{3}$$

where f_{max}—fluorescence intensity measured at a saturating stimulating radiation at a minimum illumination;

f_{min}—fluorescence intensity measured at a saturating stimulating radiation at maximum illumination;

Q_1—the value of the light level below which the fluorescence intensity is stabilized at the maximum level f_{max};

Q_2—the value of the light level above which the fluorescence intensity is stabilized at the minimum level f_{min};

k—coefficient of proportionality, which is for the linear section of the chart can be calculated by the formula:

$$k = \frac{f_{max} - f_{min}}{Q_1 - Q_2} = \frac{\Delta f}{\Delta Q} \qquad (4)$$

Representation of the coefficient k as 4 allows interpreting it as a rate of fluorescence variation of light levels. Determination of the coefficient k can be easily fulfilled by sequential measurements of fluorescence in a sample of selected cultures of micro-algae under different levels of ambient illumination, for each species of phytoplankton. Depending on the species of phytoplankton, values of the critical illuminations Q_1 and Q_2, and the corresponding fluorescence intensity f_{max} and f_{min} will be different. This can be used to identify the species composition of micro-algae in the water samples.

For the graph in **Figure 4**, the coefficient $k = -1.66$; $f_{max} = 5.8$; $f_{min} = 2.7$; $Q_1 = 0.15$ W; $Q_2 = 1.9$ W. The correlation coefficient between the experimental data and approximation line is 0.996, which confirms the high degree of conformity.

To account for the dependence fluorescence intensity by changes in illumination, we introduce a function $s_1(Q)$ as the coupling coefficient between the maximal value of fluorescence intensity the dark, and the value obtained during the lighting changes:

$$s_1(Q) = \frac{f_{max}}{F_m(Q)} \qquad (5)$$

By substituting (3)–(5), we obtain an expression for $F_m(Q)$ as a piecewise linear function (6), which determines dependence of the intensity of fluorescence of micro-algae by change in the intensity of illumination:

$$s_1(Q) = \begin{cases} 1, & if \ Q \leq Q_1 \\ f_{max} / (f_{max} + k(Q - Q_1)), & if \ Q_1 < Q < Q_2 \\ f_{max} / f_{min}, & if Q_2 \leq Q \end{cases} \qquad (6)$$

In [43], it was shown that dependence of intensity LIF of chlorophyll-*a* by changes of temperature is well approximated by an exponential function (7):

$$F_m(T) = f_0 \cdot e^{a \cdot T} \tag{7}$$

where f_0—the fluorescence intensity, measured at saturated excitation without the temperature quenching (including any normalization);

a—temperature coefficient of the fluorescence;

T—temperature of the environment.

To account for the dependence of fluorescence intensity by change in ambient temperature, we introduce a function $F_m(T)$ as the coupling coefficient between the maximal fluorescence values (at the optimal temperature for the specific culture of micro-algae) and the observed at change of temperature as follows:

$$s_2(T) = \frac{f_0}{F_m(T)} \tag{8}$$

Having substituted (7) at (8), we obtain an expression for $F_m(T)$ as a function (9), which determines dependence of fluorescence intensity of micro-algae from ambient temperature as follows:

$$s_2(T) = e^{a \cdot T} \tag{9}$$

Traditionally [43], when determining the concentration of chlorophyll-*a* by the fluorescence method, assume a linear dependence of fluorescence intensity by chlorophyll concentration. Calculation of concentration may be performed by the formula (10) as follows:

$$C = K \cdot F_m \tag{10}$$

where C—the concentration of chlorophyll-*a*;

K—coefficient of proportionality, determined by the design of the measuring instrument;

F_m—the measured fluorescence intensity.

From [43] implies that the coefficient K has various values depending on the type of micro-algae present in the water sample. In [15] presented data, confirming the dependence fluorescence intensity and the coefficient K from light intensity and temperature. Assuming their influence independent, then the coefficient K can be represented by the function (11) [15]:

$$K = s_0 \cdot s_1(Q) \cdot s_2(T) \tag{11}$$

where s_0—coefficient of proportionality, depending on the design of a measuring instrument and phytoplankton species;

$s_1(Q)$—function, depending on the illumination;

$s_2(T)$—function, depending on the temperature.

Coefficient K can be interpreted as a function, which characterizes the efficiency of fluorescence of micro-algae cell under a changing of environment parameters. The greater the numerical value of K is, the smaller the fluorescence intensity of cells.

Derived analytical expressions are aimed at accounting of the influence of external factors on the intensity of LIF of micro-algae for reducing the measurement error. Below are the results of studies of the temperature influence and the presence of certain metals ions on the intensity of LIF for the several cultures of micro-algae that belong to different phylum.

5. The dependence of the intensities of micro-algae LIF by temperature stress

Biology, Far Eastern Branch of the Russian Academy of Sciences, allocated into the culture from the Japan Sea, which have a single cells and are characterized by high mobility. Also, the choice of micro-algae has been associated with a variety of their pigment composition (**Table 2**).

Plagioselmis prolonga. Butch (Cryptophyta) strain is PP-02. The average size of the cells is 7.0 ± 0.5 microns in length and 3.5 ± 0.4 microns in width.

Chroomonas salina (Wisl.) Butch (Cryptophyta) strain is CS-92. Cells were 10–14 microns in length, 5–8 microns in width, oval, or ellipsoidal.

Heterosigma akashiwo Hada (Ochrophyta) strain is HA-ZR 11. Cells size 9.7–15.3 microns.

Tisochrysis lutea Bendif Probert (Haptophyta) strain is TL-V 08. Cells size 5.7–7.5 microns, ellipsoidal.

Nannochloris maculata (Chlorophyta) strain is NM-86. The cells are small 2–3 microns in diameter, spherical, rarely—elliptical.

LIF excitation was performed by laser radiation with a wavelength at 442 nm. Time of action of laser radiation on the sample in all cases was 2 s. LIF spectra of phytoplankton at a different temperature were measured by a spectrometer Shamrock 303i company [Andor Technology (USA)] input slit of the monochromator was 100 microns, range of wavelengths was 500–770 nm, the resolution of the diffraction grating was 0.167 nm. Measurement of spectra was carried in the mode of accumulation: the exposure time of the intermediate spectrum was 0.2 s, the

number of accumulations—10, the frequency of measurement of intermediate spectrum was 5 Hz.

Samples of micro-algae cultures were placed in a thermostatic cuvette holder QPOD 2e [Quantum (USA)], which provides the rate of change of temperature 1°C/min. Accuracy of digital meter with an immersed thermocouple was ±0.15°C. Uniform distribution of temperature of the sample was ensured by a magnetic stirrer with rotation speed 300 rev/min.

A sample of micro-algae culture in volume of 1.5 ml by dose was placed into a quartz cuvette in volume 3.5 ml. LIF spectra measurements were performed in increments of changes of temperature 1°C in the range of 20–80°C. For each culture, the measurement procedure was repeated at least three times.

For graphs forming data derived in the experiments were processed as follows:

1. From the LIF spectra were subtracted dark current of the spectrometer.

2. To reduce the noise signal obtained data were subjected to filtration (using a fast Fourier transform) for frequencies above 0.5 Hz.

3. After filtration, the chlorophyll fluorescence intensity in the wavelength range 680–690 nm and Raman scattering (RS) of water were determined. The obtained results were normalized according to the formula (12) as follows:

$$F_S(T) = \frac{I_{max}(T)}{I_{RS}} \qquad (12)$$

where $F_S(T)$—LIF intensity of chlorophyll-*a*, determined from the fluorescence spectrum at a predetermined temperature;

$I_{max}(T)$—maximum LIF intensity in the wavelength range 680–690 nm at a predetermined temperature;

I_{RS}—water Raman intensity at a predetermined temperature.

1. The relative LIF intensity obtained by rationing $F_S(T)$ by formula (13) as follows:

$$F_m(T)_N = \frac{F_S(T)}{F_{max}} \qquad (13)$$

where $F_m(T)_N$—LIF relative intensity of chlorophyll-*a* at a predetermined temperature;

F_{max}—maximum LIF intensity of chlorophyll-*a* in the whole temperature range.

Figures 5–7 present the results of measurements of the fluorescence of investigated micro-algae culture in the temperature range 20–80°C. On the graph, each point is the result of averaging $F_m(T)_N$ of three independent experiments. In all this figures, the results of experiments, each performed in triplicate. Error bars on the graph show the standard deviation.
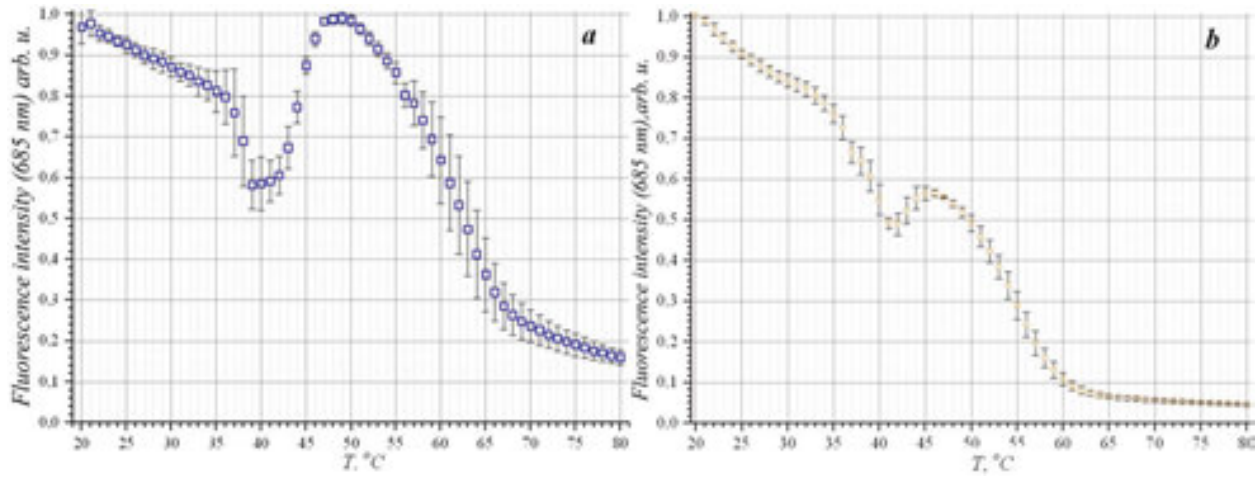
Figure 5. The temperature dependence of the LIF intensity for Cryptomonad: (a) *Chroomonas salina*; (b) *Plagioselmis pro-longa*.
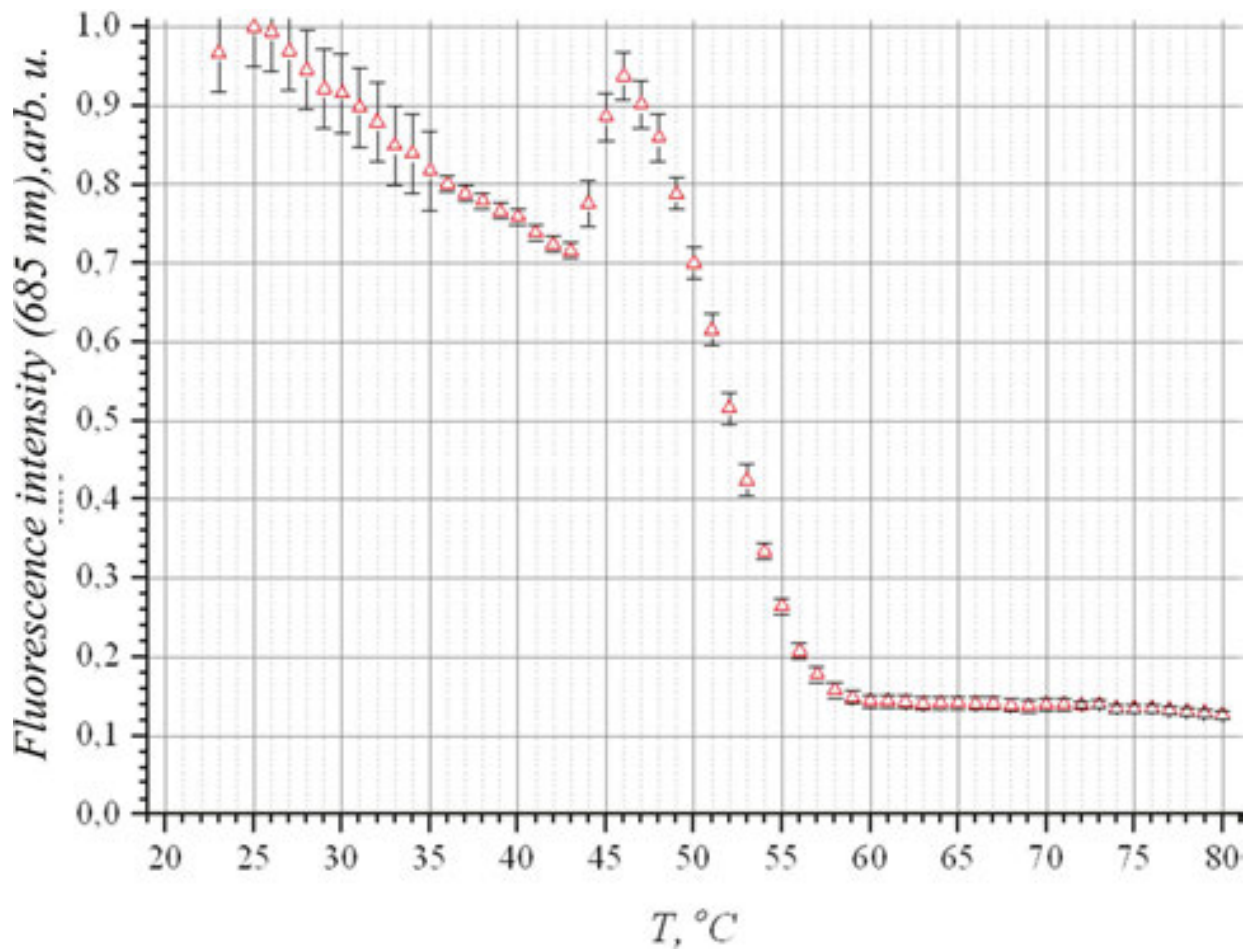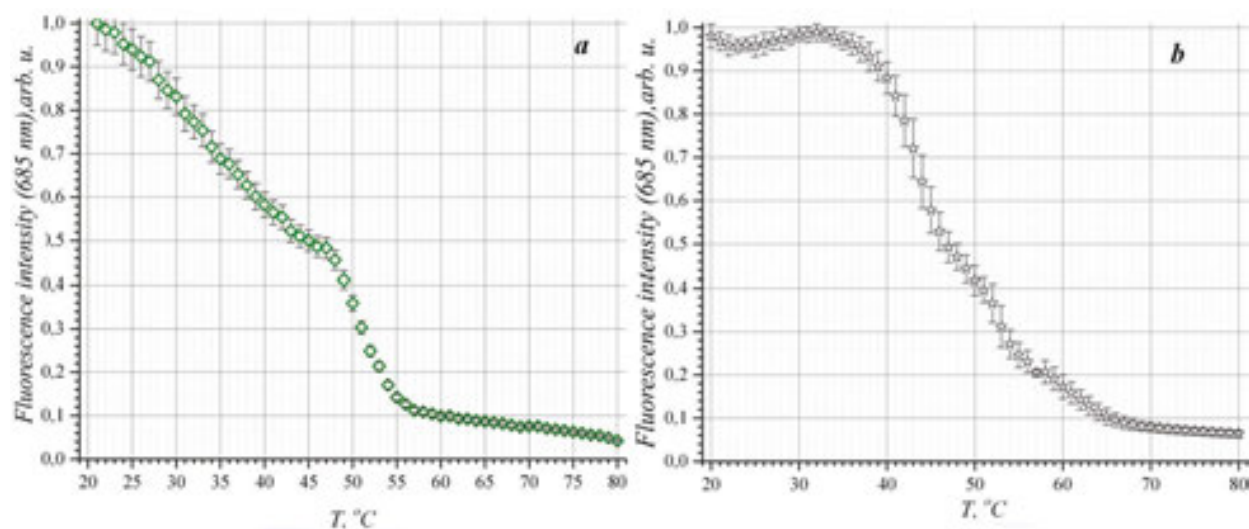

Figure 6. The temperature dependence of the LIF intensity for *Heterosigma akashiwo*.

Figure 7. The temperature dependence of the LIF intensity for micro-algae containing no of phycoerythrin: (a) *Tisochrysis lutea*; (b) *Nannochloris maculata*.

Analysis of charts allows selecting specific areas of the LIF intensity, having a different degree of expressiveness for each culture:

1. The range of 20–35°C—there is a monotonic decrease in the fluorescence intensity.

2. The range of 35–42°C—is typical for Cryptomonad which distinctive feature is the presence of phycobilins. For them, in this range, there is accelerate of decrease in fluorescence intensity, whereas for other algae of this effect is not observed.

3. The range of 42–50°C—a rapid increase in fluorescence intensity to the maximum.

4. The range of 50–80°C—monotonic decrease in fluorescence intensity followed by stabilization at the minimum level.

The values of the maximum and minimum temperatures and the corresponding amplitudes of the LIF intensities for the characteristic points of their temperature dependence for the investigated cultures of micro-algae are shown in **Table 4**.

Cultures of micro-algae	T_{min}, °C	$F_n(T_{min})$	T_{max}, °C	$F_n(T_{max})$
Chroomonas salina	40	0.5	49	1.0
Heterosigma akashiwo	43	0.7	46	0.92
Plagioselmis prolonga	41	0.5	46	0.56
Tisochrysis lutea	–	–	–	–
Nannochloris maculata	–	–	–	–

Table 4. The values of the maximum and minimum temperatures and the corresponding amplitudes of the LIF intensities for the characteristic points of their temperature dependence for the investigated cultures of micro-algae.

The absence of characteristic points for cultures *T. lutea* and *N. maculata*, in our opinion, related to the lack of phycobilins.

According to [35, 36], the temperature quenching of fluorescence caused by increase of molecular vibrational energy which results in the deactivation of excited levels and, consequently, to a decrease in the fluorescence quantum yield. In our case, there is in the range of 20–33°C decrease in the fluorescence intensity has the character of nonphotochemical quenching and is caused by the influence of temperature on the chlorophyll-*a* molecules which is as a part of cells of micro-algae. In [41], it is shown that in this temperature range, the experimental data are well approximated by an exponential function (7). The values for the temperature coefficient of the fluorescence (a) for the investigated cultures of micro-algae and the maximum deviation of the experimental data from calculated data by the least squares method (S) are presented in **Table 5**.

Cultures of micro-algae	a	S
Chroomonas salina	−0.0073	0.07
Heterosigma akashiwo	−0.0035	0.12
Plagioselmis prolonga	−0.017	0.015
Tisochrysis lutea	−0.0080	0.09
Nannochloris maculata	−0.01	0.13

Table 5. The temperature coefficient of the fluorescence of micro-algae cultures.

Expressed minimum intensities of fluorescence in the temperature range 35–42°C, probably associated with the beginning of denaturation of protein structure in micro-algae cells, and their subsequent destruction. Thus, for Cryptomonad, there is an infringement in the electron transport chain, because of which is stopped transmission to chlorophyll-*a* the energy absorbed by other pigments. This is well illustrated in **Figure 8** by an increase in phycobilins fluorescence intensity for Cryptomonad cells in the temperature range 35–42°C.

Increase of temperature to 46–50°C leads to intensive degradation membranes and internal components of cells. As a result, a chlorophyll-*a* is released, which, as is known, has fluorescence intensity always higher than that for chlorophyll-*a*, which is associated in the chloroplasts of cells. Therefore, in this temperature range, there is a sufficiently intense of fluorescence increase to a maximum value. Further temperature increase leads to a decrease of fluorescence due to the increase of nonradiative relaxation and chlorophyll-*a* destruction.

Data obtained by us demonstrate a significant depending of LIF intensities of micro-algae on the temperature and its distinction for different cultures of micro-algae. Furthermore, the temperature coefficient, which is introduced by us, determines the change of LIF intensities in the range of actual temperatures of an aqueous environment that can be used as a distinguishing feature for identifying of micro-algae.
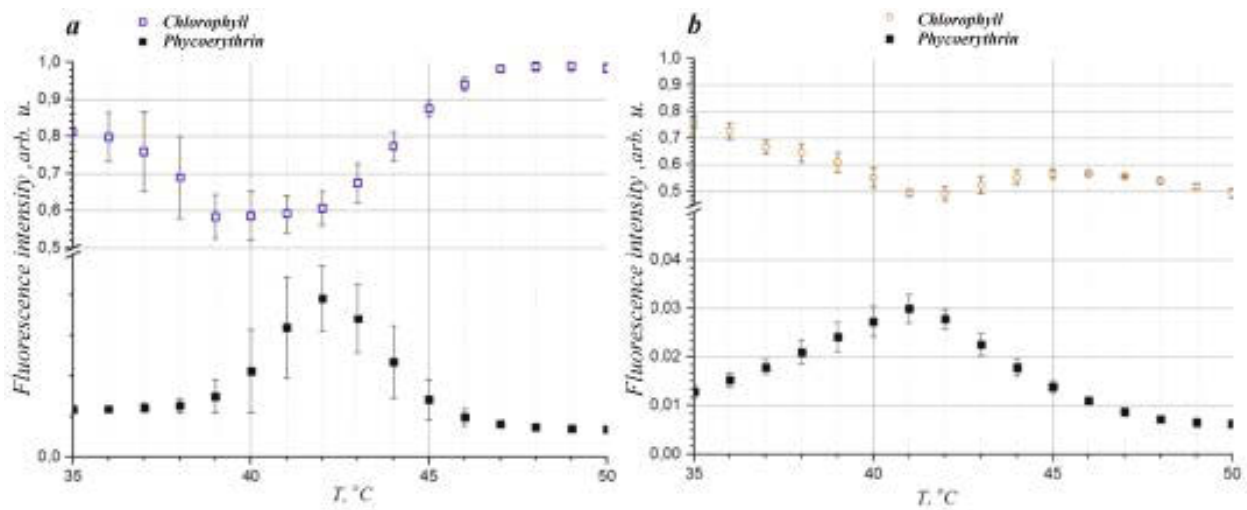
Figure 8. Simultaneous LIF intensities changes of chlorophyll-*a* and phycoerythrin for Cryptomonad cells: (a) *Chroomonas salina*; (b) *Plagioselmis prolonga*.

On the other hand, such researches are subsidiary in solving the main task—the development of biological sensors of aquatic pollutants based on the analysis of micro-algae LIF. Particular interest is the group of parameters characterizing the sensitivity of each species of micro-algae to the presence of specific substances in the environment, such as heavy metal ions. Such investigations allow identifying the suitable species of micro-algae for use as biological indicators of the presence of certain types of pollutants or hazardous organic and inorganic substances.

6. Influence of heavy metal ions on intensities of micro-algae LIF

For the experiments were selected cultures of micro-algae *N. maculata* and *T. lutea* and salts of copper, zinc, and iron(II).

N. maculata is representative of the green algae, which in their turn, is the most extensive department, which incorporates more than 13,000 species. The presence such a great of species diversity suggests that the representatives of green algae can be found in different waters and, therefore, can become convenient indicators of their pollution.

T. lutea is Haptophyte, which has a high resistance to adverse environmental conditions, in particular, to a lack of nutrients. Due to its structure, this type of phytoplankton can be kept in a hostile environment over many months and can be used as a potential basis for creation of chemo sensors [34, 35].

Figure 9 illustrate the temporal variations of fluorescence intensity of chlorophyll-*a* for *N. maculata* at a 680-nm wavelength at the presence in salt solution ions of copper, zinc, and iron(II) at a concentration equal to the MPC.
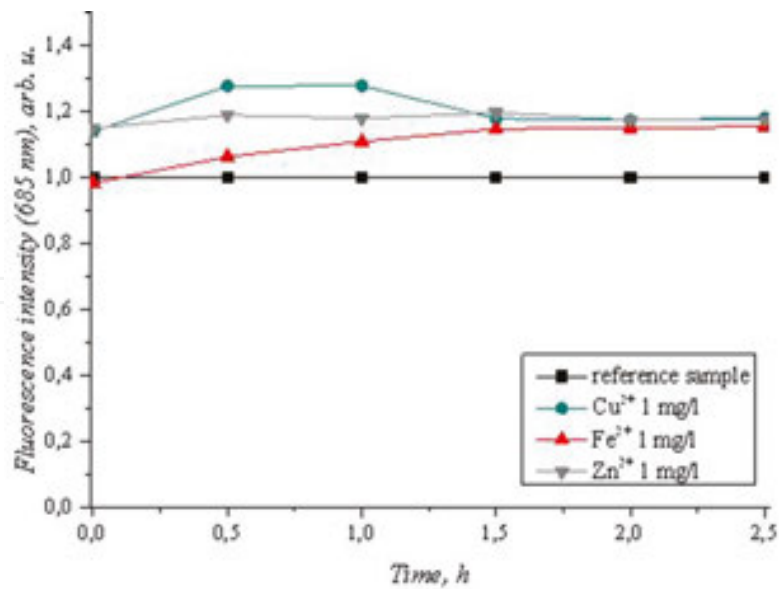
Figure 9. Change of the *Nannochloris maculata* chlorophyll fluorescence intensity under the influence of salts of copper, iron, and zinc at a concentration equal to MPC.

It can be seen that adding of metal salts increases the fluorescence intensity of the micro-algae culture in the first half hour after the addition of salt, and then stabilizes at a constant value. Iron in the time of addition is not strongly influence, unlike of copper and zinc, from which it can be concluded that zinc and copper ions are more toxic for the species of micro-algae.

To define the minimal concentration of metal ions, which influence the photosynthetic apparatus of micro-algae, we have carried out measurements with salt concentrations below
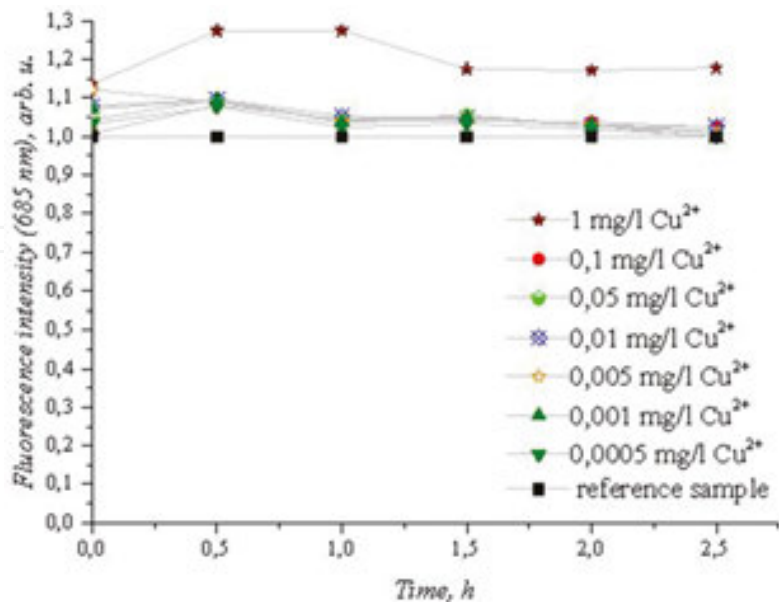
Figure 10. Changing the fluorescence intensity of micro-algae *Nannochloropsis maculata* under the influence of Cu^{2+} at concentrations of copper ions in the solution below the MPC.

the MPC. The **Figures 10** and **11** show the results of the influence of salts of copper and zinc, respectively, at the green micro-algae *N. maculata*.
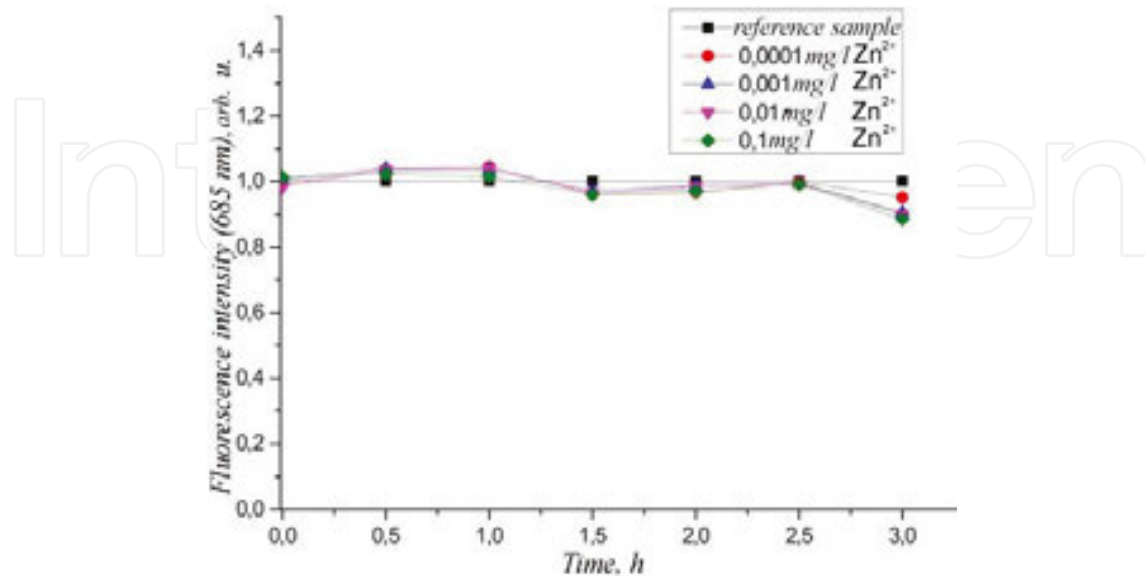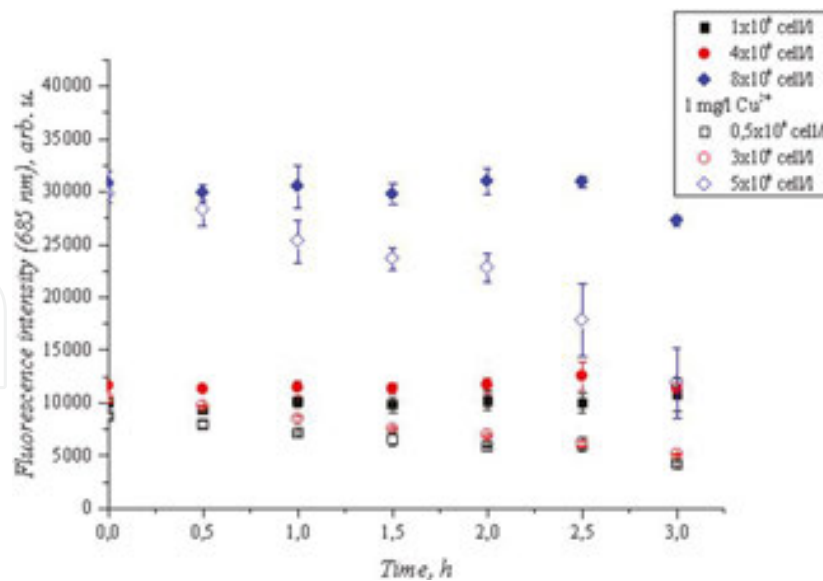

Figure 11. Changing the fluorescence intensity of micro-algae *Nannochloropsis maculata* under the influence of Zn^{2+} at concentrations of zinc ions in the solution below the MPC.

The resulting data from these experiments showed that, after the series of measurements, there is change of concentration of phytoplankton cells in the sample. Increasing concentrations of phytoplankton cells C_{Php} leads to that the relative concentration of the metal ions C_{MI} becomes very small and the effect of exposure of metal ions on phytoplankton not observed. The ratio of metal ion concentration to the concentration of phytoplankton cells can be called the coefficient of sensitivity of phytoplankton to metals (14):

$$s_3(MI) = \frac{C_{MI}}{C_{Php}} \tag{14}$$

From this expression, it follows that the increasing the concentration of phytoplankton in biosensor, for a constant concentration of the metal salt, will reduce the sensor sensitivity, at the same time, reducing the concentration of phytoplankton, on the contrary, increases the sensitivity of the sensor.

For confirmation of that conclusion of experiments were repeated with the culture of micro-algae *T. lutea*, which are resistant to changes in nutrient composition of the environment, therefore for a long time, culture can be regarded as stable and use it for long-term experiments without changing the concentration of each sample. **Figure 12** shows the results of measuring fluorescence intensity of samples with different concentrations of micro-algae *T. lutea* at the fixed copper ion concentration equal to 1 mg/l. The hatched symbols refer to the control sample without the shaded symbols to the sample under the influence of copper ions.

Figure 12. The results of measuring fluorescence intensity of samples with different concentrations of micro-algae *Tisochrysis lutea* at the fixed copper ion concentration equal to 1 mg/l.

Figure 13 shows the results of measuring the intensity of fluorescence of micro-algae *T. lutea* with a combination of variations in the concentrations of copper and phytoplankton in the investigated samples. For convenience of representation, fluorescence intensity of cultures by copper ions is given in the meanings of normed on fluorescence intensity of control cultures
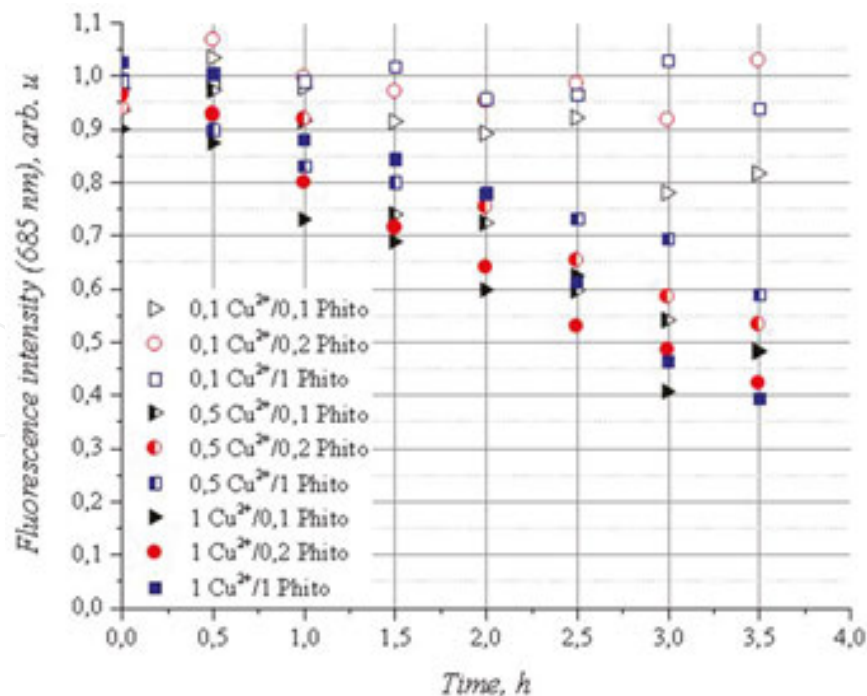
Figure 13. The fluorescence intensity of micro-algae *Tisochrysis lutea* with a combination of variations in the concentrations of copper and phytoplankton in the investigated samples.

In approximating of the data by the equation $y = ux + b$, the u factor in the equation will be equal to the rate of decrease in the fluorescence of the investigated interval of time. The equations for the approximation of all graphs in **Figure 13** are shown in **Table 6**.

$C_{Php}\backslash C_{MI}$	0.1	0.5	1
1	$1 - 0.007\times$	$1 - 0.1\times$	$1 - 0.18\times$
0.2	$1 - 0.01\times$	$1 - 0.13\times$	$0.98 - 0.17\times$
0.1	$1 - 0.05\times$	$1 - 0.15\times$	$0.92 - 0.15\times$

Table 6. The rate of change fluorescence intensity micro-algae *Tisochrysis lutea* with a combination of variations in the concentrations of copper and phytoplankton.

Figure 13 shows that within the first 3 h of exposure copper ions on micro-algae culture, the fluorescence intensity decreases uniformly. If the concentration of phytoplankton is high, the influence of copper ions is small. However, if the copper concentration reaches high values, its effect does not depend on the concentration of micro-algae. This allows us to make an important conclusion that for sensitivity to low concentrations of copper, well below the MPC, are needed culture of micro-algae with a low concentration of cells. This reaction may be among micro-algae, which have both individual and collective metabolism, which increases resistance to stress. However, if the stress reaches a critical value (copper concentration of 1 mg/l in our experiment), the rate of change of intensity fluorescence of chlorophyll increases with increasing concentration of phytoplankton.

In addition to changes in the intensity of fluorescence of algae under the influence of heavy metal salts having spectral changes of fluorescence intensities in the wavelength range from 645 to 740 (**Figure 14**). Peak fluorescence at 740 nm is caused by the presence of chlorophyll belonging to PS1, which, in turn, is not involved in the primary reactions of photosynthesis.
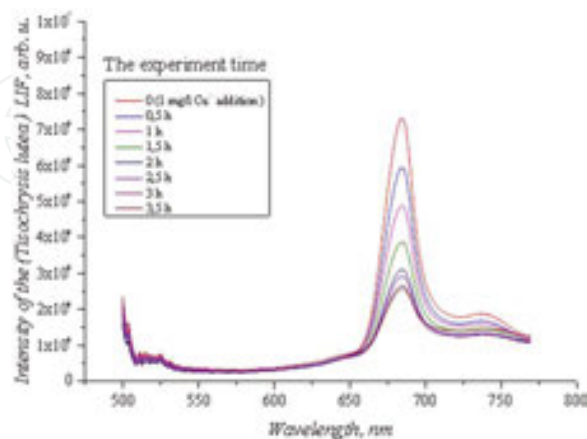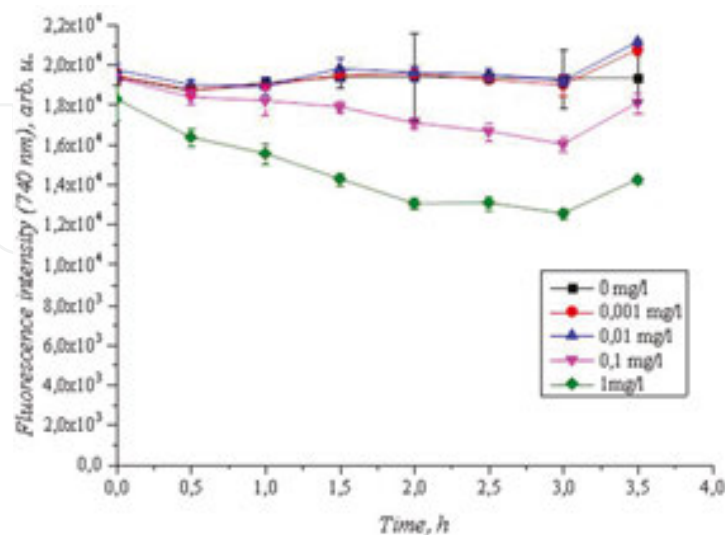

Figure 14. The fluorescence spectra of micro-algae *Tisochrysis lutea* under the influence of copper ions at a concentration of 1 MPC.

Nevertheless, this photosystem also exposed to heavy metals as reflected on the spectral picture, shown in **Figure 14**.

Figure 14 shows that the change of fluorescence intensity at a wavelength of 685 nm and is significantly faster than the fluorescence at wavelength of 740 nm. This is due to the fact that the PS2 is involved in the primary stage of photosynthesis and the metal ions primarily impact on its fluorescence. **Figures 15** and **16** shows the intensity of fluorescence at wavelengths 685 and 740 nm for the culture of *T. lutea* at a concentration 27.25×10^4 cells/ml.
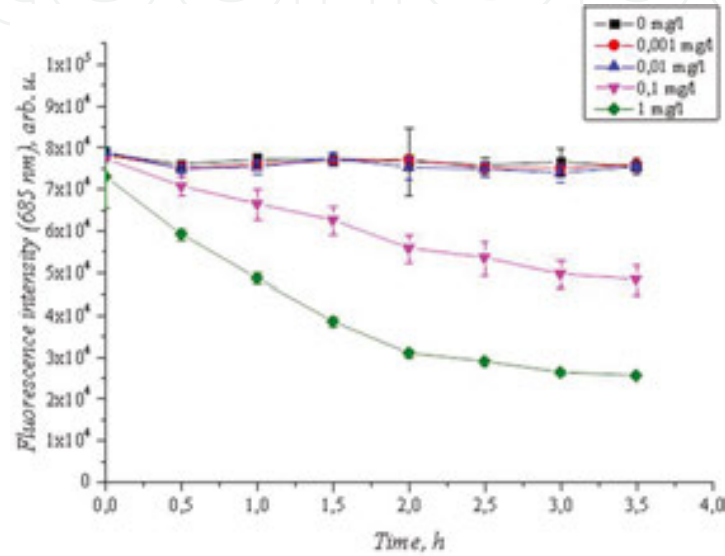
Figure 15. Dependence of the fluorescence intensity of micro-algae *Tisochrysis lutea* at a wavelength of 685 nm on the concentration of copper ions.

Figure 16. The fluorescence intensity of micro-algae *Tisochrysis lutea* at a wavelength of 740 nm on the concentration of copper ions.

Stabilization of the intensity of fluorescence of chlorophyll-*a* at a wavelength 685 nm in **Figure 15**, with the copper ion concentration of 1 mg/l, occurs earlier than in **Figure 13** because of the difference in the concentration of micro-algae. Increasing the concentration of phyto-plankton at high concentrations of copper (1 MPC) accelerates the fall of the fluorescence intensity. It is easily seen that the concentration of micro-algae 27.25 × 10⁴ cells/ml and a copper ion concentration of 1 mg/l, the fluorescence intensity decreases according to the equation $y = 0.93 - 0.28u$, where 0.28 is the rate of change. From this, it can be concluded that for reducing the fluorescence to a stationary state in the given conditions, less time is required (1.5 h) than in the experiments shown in **Figures 12** and **13**.

The ratio of fluorescence intensities at a wavelength 740 nm to the fluorescence intensity at a wavelength 685 nm is a spectral characteristic, reflecting the impact of ions of heavy metals on the whole photosynthetic apparatus (15).

$$S_R = \frac{F_{740}}{F_{685}} \tag{15}$$

where S_R—spectral feature (of the right shoulder of chlorophyll fluorescence);

F_{740}—the chlorophyll fluorescence intensity at a wavelength 740 nm;

F_{685}—the chlorophyll fluorescence intensity at a wavelength 685 nm.

Figure 17 shows the change in S_R under the influence of copper ions within 3 h. The increase in spectral feature, as mentioned above, reflects the fact that the PS2 is more sensitive to metal ions than PS1. Moreover, after the second hour of the experiment, if copper concentrations 0.01 and 0.001 mg/l, also begins to increase spectral feature. This phenomenon requires a thorough and detailed study.
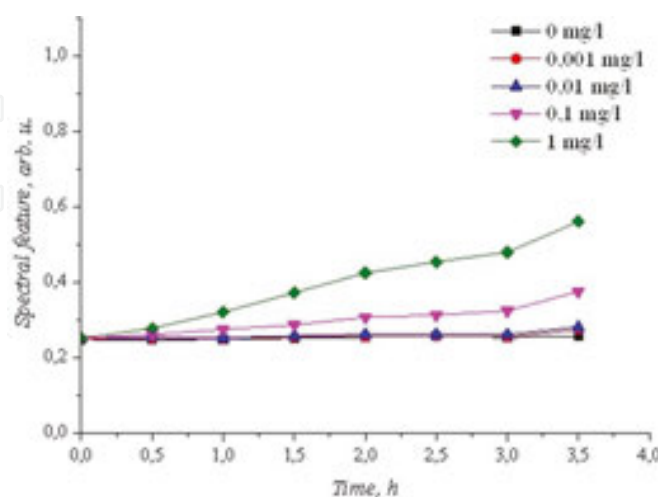
Figure 17. Dependence of the spectral feature of micro-algae *Tisochrysis lutea* from time under the influence of copper ions.

7. Conclusions

Analysis of the bibliography and the experimental data obtained for the four phyla of micro-algae confirms the dependence of their LIF from the environment parameters such as temperature, light and the presence of metal ions. It demonstrates the importance of analysis of the mutual influence of environment parameters to minimize the error in obtaining the final result. The possibility of practical application of the concept of the proposed measurements and calculation of the chlorophyll-*a* concentration in the composition of phytoplankton cells in view of the impact of environmental parameters. Error of determination of the chlorophyll-*a* concentration at calculation by the proposed method can be reduced by three times as compared to the error of determination of a concentration by classical method.

Obtained experimental data demonstrate the fundamental possibility of micro-algae cultures identification according to the parameters of LIF spectra. In cultures of micro-algae studied wavelength, maximum LIF has a different value in the wavelength range 680–690 nm at 22°C. This allows to using it as informative features for species identification by their fluorescence spectra. In addition, for all the studied cultures of micro-algae, dependence intensity of the chlorophyll-*a* LIF on the environmental temperature is well approximated by an exponential function. At the same time, the value of a temperature coefficient of fluorescence different for the diverse cultures, which also allows to use it as an informative feature to identify species of micro-algae. The obtained reactions of micro-algae for the presence of metal ions demonstrate their specificity and can be used at creation of biosensors ecological monitoring of water environments.

Acknowledgements

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