SOME PECULIARITIES OF FLUORESCENCE DIAGNOSTICS OF PHYTOPLANKTON IN COASTAL WATERS OF THE BLACK SEA

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ABSTRACT

Some peculiarities of phytoplankton fluorescence which were observed in coastal waters of the Black Sea (near Gelendzhik) in Aug-Sept 1997, 1998 and 1999 are discussed. Possibilities for the development of a method of water quality bio-indication based on phytoplankton photophysical parameter measurements are reported. A 3-parametric model describing the process of phytoplankton fluorescence formation is considered. Theoretical approximate expressions for generalised parameters are obtained. These expressions indicate the possibility of using generalised parameters for water quality bio-indication.

INTRODUCTION

Phytoplankton is one of the basic organic compounds of natural waters. The diagnostics of phytoplankton is important for evaluation of the ecological status of coastal seawater areas. The traditional interest of marine biologists is concerned with the quantification of ocean primary production (1). For this, the concentration of chlorophyll-*a* (Chl) as the main pigment of photosynthesising organisms and the photosynthetic activity of algae should be determined. Different methods including fluorimetry are used for these purposes.

Phytoplankton was the first substance in seawater where the possibilities of ocean laser remote sensing were demonstrated (see (2) and bibliography given there). In a first approach, chlorophyll-*a* concentrations were derived from the measured chlorophyll fluorescence intensity. A normalisation of this signal to the water Raman scattering band intensity (Figure 1) allowed to express it in units of the fluorescence parameter $F_o = N_{fl}^o \big/ N_{RS}$ (3), where N_{fl}^0 and N_{RS} are photon numbers of phytoplankton fluorescence and water Raman scattering, and the index 0 denotes the absence of phytoplankton fluorescence saturation (see below).

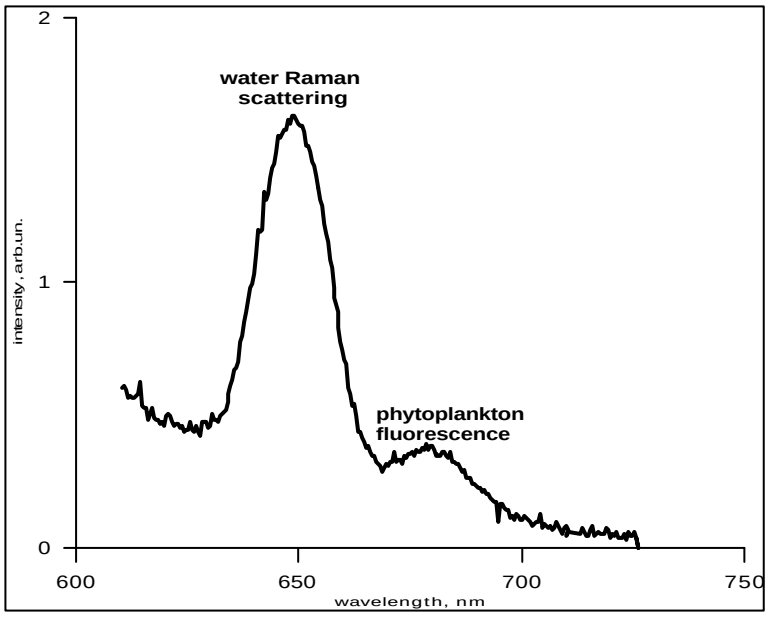


Figure 1. Emission spectrum of seawater with 532 nm excitation wavelength obtained with the shore-based lidar in the Blue Bay (Black Sea); measurement distance 50 meters; September 10, 1999.

The *in-situ* investigation of features of phytoplankton fluorescence (by means of a submergible fluorimeter or a remotely operated lidar) has shown that measurements of F_0 do not provide a unique value of the chlorophyll-*a* concentration: in addition to the Chl concetration, the parameter F_0 can vary due to other phytoplankton parameters and environmental conditions (4). Therefore, simultaneously with measurements of F_0, the parameters influencing the Chl fluorescence cross-section should be determined. These are the taxonomic composition of algae, the photosynthetic activity and, under laser excitation, the value of the fluorescence saturation factor, which, in turn, depends on photophysical parameters of the photosynthetic units (4). Apparently, all these parameters characterising the phytoplankton status are needed not only for correct measurements of F_0 and the Chl concentration, but are also interesting for studying the primary stages of photosynthesis (5) and for establishing fluorescence methods of phytoplankton diagnostics (4). Moreover, it is the phytoplankton status, which characterises the status of the marine ecosystem, in particular the presence of pollutants in seawater. Pollution can change the taxonomic composition of algae and their photosynthetic activity, lead to conformational changes, which, in turn, change the photophysical parameters of the photosynthetic units: excitation and absorption cross-section, constants of intramolecular relaxations and pigment interactions, rates of singlet-singlet annihilations, and others. The development of an *in situ* method for analysing the marine ecosystem (in particular, water quality) using phytoplankton as a bio-indicator must include a knowledge of these parameters.

A method for the *in-situ* determination of a parameter characterising phytoplankton status, i.e. photosynthetic activity, which is determined as quantum yield of charges separation in reaction centres of the photosynthetic unit, is the pump-and-probe technique, which can be realised by means of a submergible fluorimeter (6) and with laser remote sensing (7). To measure molecular photophysical parameters, the use of the non-linear fluorimetry method is proposed (4). Its implementation for an operational use is a very difficult problem. Choosing the best approach for solving this problem is strongly influenced by features of phytoplankton fluorescence in real conditions of marine coastal areas. In this paper we investigated features of phytoplankton fluorescence in coastal areas of the Black Sea (in the regions of Novorossiisk and Gelendzhik). The results of these investigations are presented below. The second direction of our work is the development of a model of phytoplankton fluorescence under pulse laser excitation. Based on the inverse model algorithm for the determination of photophysical parameters a field verification of the method is anticipated, to make use of the revealed features of phytoplankton fluorescence in future.

RESULTS OF FIELD EXPERIMENTS

Field experiments on phytoplankton diagnostics were carried out in coastal waters of the Black Sea near Novorossiisk and Gelendzhik (including the Blue Bay) in August-September 1997-1999. In these expeditions the following tools were used for phytoplankton diagnostics:

- a Perkin Elmer model LS50 luminescence spectrometer;
- a dual-pulse submersible filter-fluorimeter;
- a laser spectrometer for samples analysis;
- a shore-based LIDAR.

The Chl concentration and taxonomic composition of the phytoplankton of some samples were analysed by means of standard methods of marine biology.

YAG:Nd lasers with frequency multipliers were used as laser devices; their characteristics are given in Table 1. The choice of the excitation wavelength l_{exc} = 532 nm was made for the following reasons:

- the YAG-laser with frequency doubling is a reliable device that also provides enough average power for remote sensing;

- water Raman scattering is well positioned in the spectrum (see Figure 1): it is close to phytoplankton fluorescence band so that dispersive effects of the water attenuation coefficient can be neglected when normalising the phytoplankton fluorescence band to the water Raman scatter band (3), however both bands still can be resolved.

Table 1. Parameters of the laser devices.

device	wavelength, nm	pulse energy, mJ	pulse duration, ns	repetition rate, Hz	divergence, mrad
laser spectrometer	532	10	10	10	5
LIDAR	532	80	10	10	5

Chlorophyll-*a* molecules are also excited by energy transfer from accessory pigments (5). On the one hand this makes a fluorescence analysis sensitive to the type of algae, however, it causes some difficulties for the quantitative measurement of chlorophyll-*a* concentrations.

It is known (4,8) that a main feature of phytoplankton fluorescence under laser excitation with the parameters listed above is fluorescence saturation. This occurs if the laser photon density F reaches levels above $10^{22}…10^{23}$ $cm^{-2}s^{-1}$. This fact causes a strong variability of the fluorescence parameter $F = N_{fl} / N_{RS}$, where N_{fl} is the photon number of phytoplankton fluorescence taking into account the saturation effect, under different sensing modes. Excitation by the high laser emission of the laser spectrometer, where the photon density was about 10^{25} $cm^{-2}s^{-1}$ (see Table 2), lead to $F \leq 0.5$, that is, phytoplankton fluorescence is small and close to the noise level compared with the water Raman scattering band intensity (Figure 2). In this case the saturation factor

$$G(F) = \frac{N_{fl}^{0}}{N_{fl}(F)} = \frac{F_0}{F(F)} \geq 6 \ .$$

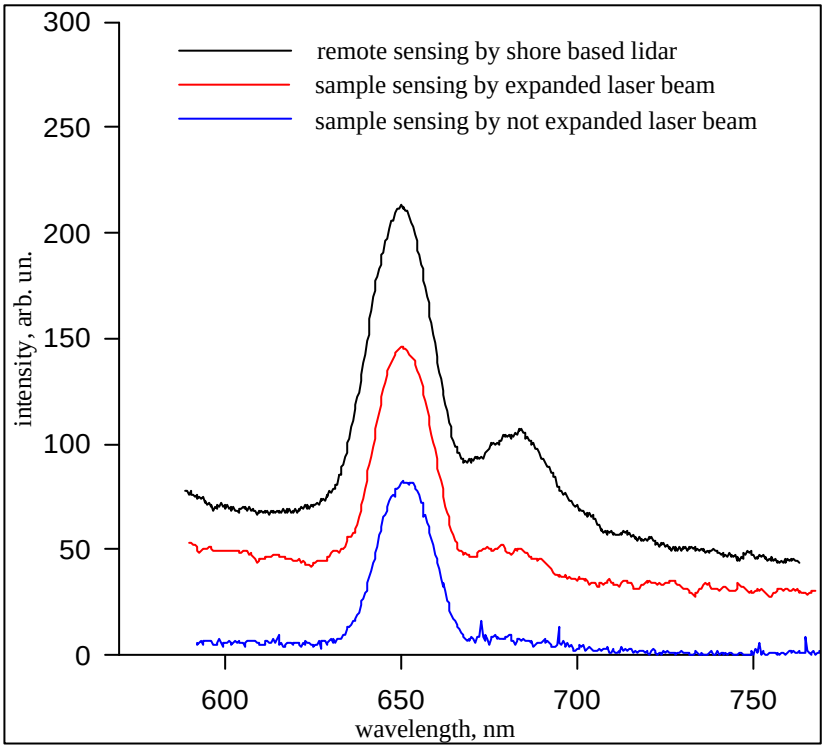


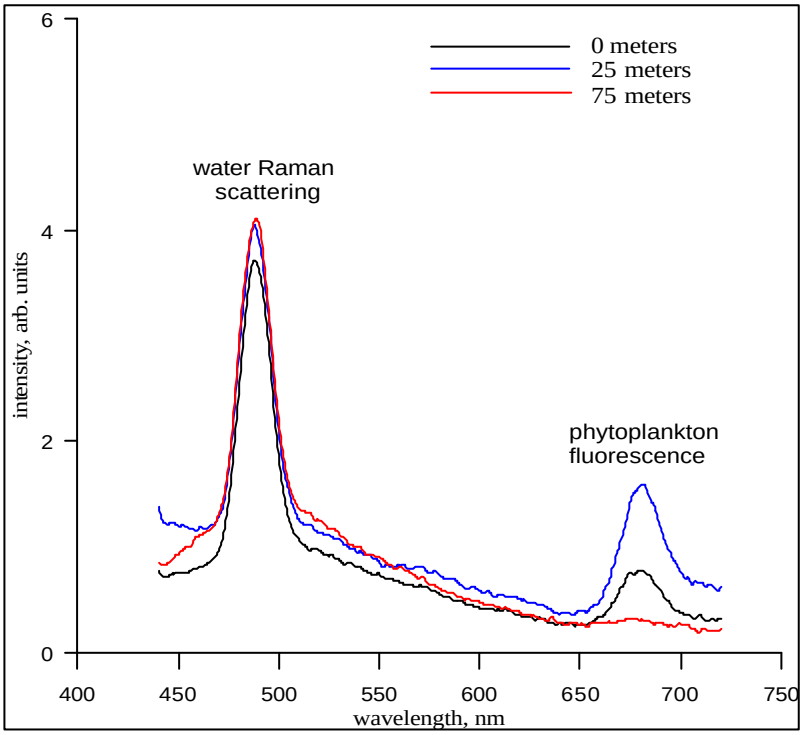
Figure 2. Emission spectra of the seawater for 532 nm excitation wavelength obtained under different sensing modes.

To verify whether the low fluorescence signal is really caused by a saturation effect, we expanded the cross-section of the laser beam by using a telescope and thus reduced the photon flux density by two orders (to $3 \cdot 10^{23}$ $cm^{-2}s^{-1}$, Table 2). In this sensing mode, phytoplankton fluorescence clearly shows itself on the background of the water Raman scattering signal (Figure 2). When remote sensing was used and when photons density on the water surface is $5 \cdot 10^{22}$ $cm^{-2}s^{-1}$ (Table 2), the parameter Φ reached its maximal value 0.3. In this case the saturation effect was practically absent (and

therefore the saturation factor was $\Gamma = 1$, and $\Phi=\Phi_0=0.3$, Table 2), since an integral echo-signal from 2-3 m deep water layer we detected, in which the average value is even less.

Table 2. Characteristics of the different sensing modes.

Sensing mode	Photon density *F*, $cm^{-2}s^{-1}$	Φ	saturation factor *G*
Remote sensing (sensing distance ≅ 50 m, cross-section of the laser beam near the water surface ≅ 400 cm^2)	~$5*10^{22}$ (on the water surface)	0.3	1
Sensing by the laser spectrometer with expanded laser beam (laser beam cross-section ≅ 10 cm^2)	~$3*10^{23}$	0.15	2
Sensing with the laser spectrometer with unexpanded laser beam (laser beam cross-section ≅ 0.3 cm^2)	≥10^{25}	≤0.05	≥6



A saturation effect does not occur when the spectrofluorimeter Perkin Elmer LS50 and the submergible filter-fluorimeter are utilised. During our field researches we used the excitation wavelength λ_{exc} = 420 nm in the Perkin Elmer fluorimeter. Typical spectra are shown in Figure 3.

Figure 3: Emission spectra of seawater with 420 nm excitation wavelength obtained with the spectrofluorometer Perkin Elmer LS 50 (spectral resolution 7 nm) from different water depths. September 9, 1998, Blue Bay.

The dependence of the fluorescence intensity versus the water depth is in good correlation with the vertical profile measured with the *in situ* filter-fluorimeter (see Figure 4), with a range of excitation wavelengths from 400 to 480 nm and an emission wavelength λ>660 nm. Different excitation and detection wavelengths of the used instruments do not allow a quantitative comparison of their data. The fluorescence maximum corresponds to the thermocline, which is at about 20…25 m depth in these coastal areas. The fluorescence intensity at the surface is lower, presumably due to sunlight-induced photo-inhibition.

The submersible filter-fluorimeter has a pump-and-probe mode (6,7) for the determination of photosynthetic activity of algae. The behaviour of the photosynthetic activity parameter $h = I_n / I_m$ (where $I_n = I_m - I_o$, and I_m and I_o are the response intensities to the probe pulse with and without pump pulse, respectively) can be described by the simultaneous influence of several factors, including photoinhibition.

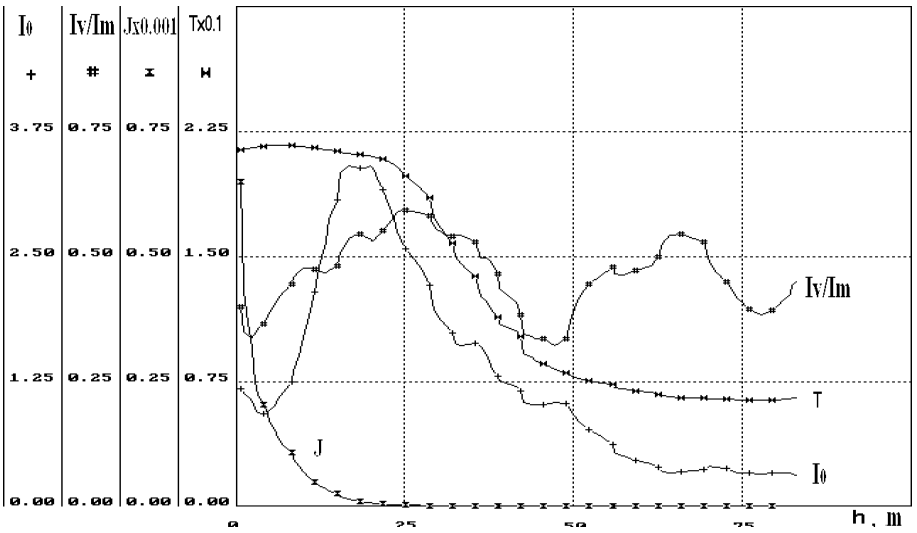


Figure 4. Depth profiles of temperature T (°C), irradiance J (mE·m^{-2}·s^{-1}), chl fluorescence (arb. units) and photosynthetic activity I_v/I_m, measured with the submergible filter-fluorimeter, September 9, 1998, Blue Bay.

The vertical and horizontal distribution of the phytoplankton fluorescence obtained in the experiments will not be further discussed here. Instead, some interesting features of this parameter are outlined:

- only the 685-nm band is represented in the phytoplankton fluorescence spectra (Figures 1 and 3). There were no blue-green algae, which would show additional bands corresponding to the auxiliary pigments phycobilins.
- Phytoplankton fluorescence excitation spectra for the coastal areas of the Black Sea show a much higher variability than for the open sea. This might be caused by the variety of the taxonomic composition of the phytoplankton. A rough selective analyse has shown that diatomic, peridinae and green algae dominate in the research area in early autumn (end of August – begin of September); their relative content (on biomass) changes in the following limits: diatomic from 5 to 78 %, peridinae from 16 to 56 %, green algae from 0 to 52 %.

The average chlorophyll-*a* concentration for the region is about 1 µg/l. This corresponds to a value of $F_0 \cong 1$ at l_{exc} = 420 nm and $F_0 \cong 0.3$ at l_{exc} = 532 nm. Excitation at l_{exc} = 420 nm (Perkin Elmer) and especially with wideband excitation at 400-480 nm (submersible filter-fluorimeter) provides a closer connection between fluorescence intensity and chlorophyll-*a* concentration than laser excitation at l_{exc} = 532 nm. This is due to two reasons:

- at l_{exc} = 420 nm (and excitation in the 400-480 nm wavelength range) chlorophyll-*a* molecules are excited mainly by direct absorption by these molecules, but at l_{exc} = 532 nm the energy transfer from the auxiliary pigments is much more efficient than the direct absorption of the light by chlorophyll-*a*. Therefore, the fluorescence intensity with 533 nm excitation depends more on the taxonomic composition of the algae than with 420 nm excitation.
- fluorescence saturation can occur with remote sensing if the photon flux near the water surface is about $10^{23}…10^{24}$ cm^{-2}s^{-1}, which can easily be reached with a laser beam divergence of 2-3 mrad. For phyto-

plankton present during the expeditions the saturation reduced the parameter Φ to values of 0.05, when the samples were analysed with the laser spectrometer without laser beam expansion (Table 2).

Consequently, excitation in the range l_{exc} = 420…440 nm and photon flux densities of about $F = 10^{21}$ cm^{-2}s^{-1} should be used for measuring chlorophyll-*a* concentrations. These wavelengths are inside the blue chlorophyll-*a* absorption band. However, in this case not only chlorophyll-*a* but also degradation products such as pheophytin-*a* are detected. Their fluorescence bands are close to that of chlorophyll-*a*. It is also possible to use the excitation wavelength $l_{exc} \cong$ 660 nm that lies inside the red absorption band of chlorophyll-*a*. Then the water Raman scattering band will have its maximum around wavelength 850 nm, which makes a quantitative samples analysis difficult and gives rise to significant problems in remote sensing due to high the water absorption coefficients in this spectral range. However, for some tasks the last feature may even be useful.

A THREE-PARAMETRIC MODEL OF PHYTOPLANKTON FLUORESCENCE WITH PULSE LASER EXCITATION

As noted in the introduction, a more relevant task in the ecological analyses of seawater areas than measuring chlorophyll-*a* concentrations is the determination of the phytoplankton status, i.e. the taxonomic composition of the algae and their functional status. Pump-and-probe (6,7) and Fast Repetition Rate (FRR) (9) methods that can be applied with laser remote sensing allow the determination of some parameters that indicate the functional status of phytoplankton.

However, the wish to develop remote methods of bio-indication of water quality requires more information on the functional status of the phytoplankton. It is possible to obtain this by analysing photophysical parameters of the phytoplankton. According to the most popular model of primary stages of photosynthesis where the fluorescence response of phytoplankton cells on the excitation laser pulse is formed, information on the following parameters is required (4):

- chlorophyll-*a* and auxiliary pigment concentrations in the light-harvesting antenna;
- the rates of singlet-singlet annihilation of the excited states of the pigments;
- the energy transfer rates between pigments;
- the absorption cross-section of pigments and the lifetime of their excited states;
- the rates of the energy transfer to the reaction centres, that can exist in four different states (at τ_p=10 ns, ν=10 Hz) – open, closed, and in two intermediate ones;
- the time of charge recombination in the reaction centre, and the probability of the following exciton return to the light-harvesting antenna.

Non-linear fluorimetry (5,9,10) allows the determination of these parameter. However, at the moment this method does not allow to determine more than three parameters with satisfactory precision, in future the number of measurable parameters will hardly exceed five. This restriction is explained by features of the saturation curves $N_{fl}(F)$, which are initial data for solving the inverse problem. Thus the problem of cutting down of the number of defined parameters without loss of quality of describing the fluorescence formation becomes relevant.

In the following, a three-parametric model is outlined, based on the kinetic equation for the concentration of excited chlorophyll-*a* molecules

$$\frac{dn}{dt} = s^* F(n^o_{Chl} - n) - \frac{n}{t^*} - n^2 \qquad (1)$$

and the equation for the photon number detected from a volume V:

$$N_{fl} = K^{fl} \int_{V} d^3\vec{r} \int_{0}^{\infty} dt\ n(\vec{r},z,t) \quad , \tag{2}$$

where:

- k^{fl} is the radiation rate of deactivation of excited states of the Chl molecules;
- F is the density of the exciting photons;
- n^0_{Chl} is the concentration of chlorophyll-a molecules;
- n is the concentration of excited chlorophyll-a molecules;
- s^* and t^* are generalised parameters with the following physics sense:
- s^* is the effective excitation cross-section of chlorophyll-a molecules; it takes into account both light absorption by chlorophyll-a and energy transfer to chlorophyll-a from accessory pigments;
- $(t^*)^{-1}$ is the rate of linear deactivation of the excited chlorophyll-a molecules; it is the sum of the rates of the intramolecular deactivation and the energy transfer to the reaction centres;
– g is the rate constant of singlet-singlet annihilation of the excited molecules of the chlorophyll-a.

The following approximated expressions hold for the generalised parameters s^* and t^* as functions of photophysical parameters of the initial model (4):

$$s^* = s_{Chl-a} + \frac{s_{AP}(n^o_{AP}/n^o_{Chl})}{\left(t_{AP} k_{12} n^o_{Chl}\right)^{-1} + 1} \tag{3}$$

$$\frac{1}{t^*} = \frac{1}{t} + \frac{N^o_3}{N_o} p_4 \left(1 + \frac{a-1}{b\, s^* F\, t^* + 1} \right) + \frac{N^o_1}{N_o} p_1$$

with

$$a = \frac{1-x}{p_4/p_3};\ b = p_3 \frac{n_o/N_o}{p_r};\ N_o = N^o_1 + N^o_3 \tag{4}$$

and where

- s_{Chl} and s_{AP} are chlorophyll-a and auxiliary pigment absorption cross-sections respectively;
- n^0_{Chl} and n^0_{AP} are chlorophyll-a and auxiliary pigment's concentrations respectively;
- $(t_{AP})^{-1}$ is the rate of intramolecular deactivation of the auxiliary pigment molecules;
- k_{12} is the constant of the rate of energy transfer from auxiliary pigment to chlorophyll-a molecules;
- t^{-1} is the rate of intramolecular deactivation of the excited states of the chlorophyll-a molecules;
- p_1 and p_3 are the rates of the energy transfer to the reaction centres in open and closed states;
- p_2 and p_4 are the same for two intermediate states (it is supposed that $p_1 = p_2$);
- N_0 is the concentration of the reaction centres;
- N_1^0 and N_3^0 are the concentrations of the initially open and closed reaction centres respectively;
- p_r is the charge recombination rate in closed reaction centres;
- x is the probability of an exciton to return to the antenna from the closed reaction centre.

Figure 6 shows the dependencies of s^* and t^* upon F. These curves are numerically obtained from the initial equations. They differ from curves obtained from approximate expressions (3) and (4) not more than 15%. It is shown that the parameter s^* is almost independent from F. The dependence of $t^*(F)$ has the following peculiarities. If all reaction centres are open before the laser pulse ($N_1^0 = N_0$, $N_3^0 = 0$), then $1/t^* = 1/t + p_1$ which does not depend upon F. If all reaction centres are closed before the laser pulse then the behaviour of $t^*(F)$ significantly changes for different sets of photophysical parameters. This fact can be used for verification of the model of primary stages of the photosynthesis.

For a diagnostics of the phytoplankton status it is important to develop a method of simultaneous determination of the parameters s^*, t^*, and g, and secondly, to relate them to the physiological status and its changes under various toxicants appearing in the water. The parameters s^*, t^*, and g can be found as a solution of a 3-parametric problem of non-linear fluorimetry (5,9,10) for those areas of the parameter F, where they are constant. The fact that they carry information on the status of the algae follows from (3) and (4), as all the parameters of the model depend on the geometrical parameters of the cell, the status of the reaction centres, the correlation between chlorophyll-a and auxiliary pigment concentrations and the taxonomic composition of these pigments. All of them are sensitive to the quality of the water environment. The determination of the direct correlation between the parameters s^*, t^*, and g and the status of the algae is the task for our research in future.

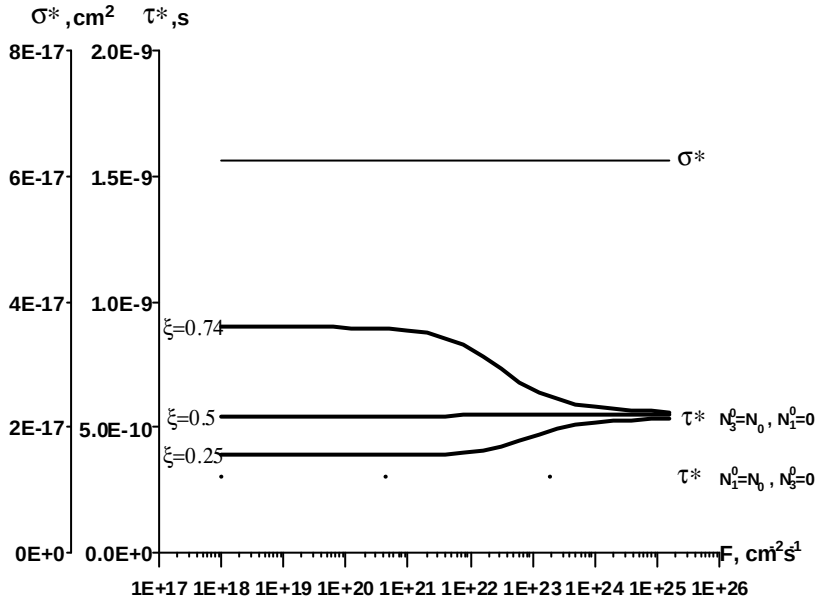


Figure 6. Dependence of the parameters s^* and t^* versus photon flux density of the exciting emission.

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