Non-linear fluorimetry as diagnostics method for natural organic complexes

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Abstract

This article summarizes the results of research on fluorescence saturation in natural organic complexes conducted by the author and his group at Moscow State University. The problems to be solved for establishing non-linear fluorimetry (saturation fluorimetry) as a principally new method of spectroscopy are analyzed which allow to determine molecular photophysical parameters of complicated organic compounds and complexes in vivo and in situ, are analyzed. Two approaches to the solution of inverse problems in saturation fluorimetry are discussed. The first one is the variation method with the quasi-Newtonian technique of residual functional minimisation; the second one is Artificial Neural Networks. The results of computer simulations and real experiments are presented, which illustrate the capabilities of non-linear laser fluorimetry in diagnostics of natural organic complexes. The photophysical parameters – effective cross section $\sigma^*$ of fluorescing molecules excitation and the rate constant of singlet-singlet annihilation $\gamma$ – of phytoplankton, aquatic humic substances, and oil films, are measured in vivo and in situ for the first time using the method of non-linear fluorimetry.

Introduction

The central problems in laser remote sensing of natural organic complexes (NOC) in water and on land, are the identification and determination of the state of such complexes. This is important not only for its own sake, but is an essential condition for quantitatively characterising an object using optical methods, in particular, the method of fluorimetry, which is used most widely for the diagnostics of NOC.

There are at least three obstacles in the way of solving this problem. They are:

(a) the large width of the fluorescence bands of NOC and their overlapping in natural environments, particularly in natural water. Even if we pump the object at a wavelength of 266 nm (the fourth harmonic of a Nd:YAG laser), which is the most favourable situation, we cannot completely get rid of this drawback [1];

(b) the identity or similarity of the fluorescence bands of organic complexes belonging to a single class, because, as a rule, the fluorescing centers in such complexes are the same: chlorophyll-a in most algae [2]; evidently, one or two fluorophores [3] in aquatic humic substances (AHS); two – three aminoacids in protein-like compounds [1]; and, most likely, a single fluorophore in a definite class of petroleum and oil products [4]. Due to the large width of fluorescence bands and variable polarity of the micro-environment, changes in shape and position of these bands are hardly noticeable [2,4,5];

(c) basically, changes in the state of NOC have little effect on the shape or position of the fluorescence band, but influence its intensity. Such an influence is most clearly pronounced in the case of phytoplankton [2].

Our twenty-year experience in the field of fluorimetry of NOC makes us believe that it is impossible to solve the NOC identification problem fully, employing only the phenomenological approach, that is only dealing with spectra – emission and excitation fluorescence spectra, and even total luminescence spectra. Although the spectra provide a large volume of information, it is necessary to penetrate downward, to the molecular level, and to supplement spectral data with molecular photophysical parameters. These parameters can include the absorption and fluorescence cross sections, the rates of intramolecular transitions, and the rates of intermolecular excitation-energy transfer. Furthermore, it is necessary to measure these parameters in vivo and in situ under conditions of lacking accurate a priori data on the local concentrations of the molecules within a complex, and the rates of radiation and radiationless relaxation.

At present, the nonlinear laser fluorimetry (or saturation fluorimetry) seems to be the most promising method of measuring these photophysical parameters. It is important to note that proceeding this way, one can solve not only the applied problems of the diagnostics of NOC, but also a number of fundamental problems: to establish the mechanisms of photophysical processes (on the molecular level) resulting from the interaction of laser radiation with NOC; to reveal the nature of the fluorescence bands; to study the processes of genesis and transformation of complexes in water (for example, the studies of AHS can imply the measurements of the parameters of organic compounds of the meloidine.
type, which are involved in the Maillard reaction), or to study the processes of the transformation of petroleum and oil products in water (the processes of aging).

Thus, nonlinear fluorimetry is principally a new approach in fluorescence spectroscopy and the importance of its development extends far beyond the scope of the diagnostics of NOC.

In this article, we summarize the results of work that has been conducted episodically since 1975 and, purposefully, since 1990 with permanently increasing intensity by the author and his group in the Moscow University.

Note that nonlinear effects in NOC fluorescence have been pointed out in the papers of other groups. This especially refers to the fluorescence of photosynthesizing organisms (see, for example, [6, 7]). However, we do not know any publications that would apply the above statement of the problem of investigating fluorescence saturation as the basis of the method of determination of photophysical parameters by using strict procedures of inverse problems solution.

**Laser saturation fluorimetry: principles, first steps toward its application in diagnostics of NOC**

In 1975 – 1977, our first experiments have revealed saturation of the phytoplankton fluorescence. The spectra displayed in Figure 1 clearly illustrate this phenomenon. For the first time, these spectra were published in [8]. The most intriguing fact is that a noticeable saturation is observed for phytoplankton fluorescence at very low pulse lasers photon flux densities: $F \approx 10^{22} \text{cm}^{-2} \cdot \text{s}^{-1}$. This value corresponds to the laser power density of approximately 1 kW/cm$^2$ [9]. For complicated organic compounds, like dyes or pigments in photosynthetic organisms, a typical value of the absorption cross section is $\sigma_{\text{abs}} \approx 10^{-14} \text{cm}^2$, and the value of the lifetime of the excited singlet state is $\tau_\text{S} \approx 10^{-8} \ldots 10^{-9}$ s. For such values, such a photon flux density produces the quasi-stationary population of the excited state $n_\text{S} \approx (0.01 \ldots 0.001)n_\text{g}$. Hence, the trivial mechanism of saturation, i.e. the dynamic depletion of the ground state, does not make a noticeable contribution to the saturation effect [10]. Now, we know the reason for such a strong fluorescence saturation of phytoplankton-like organic complexes at low levels of the photon flux density: this is the singlet-singlet annihilation. This process manifests itself in fluorescence saturation when the local concentrations of fluorescing molecules are very high ($C \geq 10^3$ M). In pigment-protein complexes of photosynthetic organisms, the concentration of the fluorescing molecules (chlorophyll-a) exceeds this limit by two-three orders of magnitude: $C_{\text{chl-a}}^{\text{loc}} \approx 0.1 \ldots 1$ M. There is some evidence that it is also high in the other NOC. Thus, by 1976, we realized that there is a need to make corrections of the measured values of the fluorescent parameter $\Phi = I_s/I_{gs}$, which is equal to the ratio of the fluorescence intensity to the intensity of the Raman signal from water [11], for fluorescence saturation. Such a correction is necessary to obtain the value $\Phi_0$, which must be used in determinations of concentration of fluorescing molecules. We worked out the algorithms for the correction [10], which have been permanently improved [2, 9].

Soon, it became clear that the effect of fluorescence saturation can be used to determine the photophysical characteristics of complicated organic compounds and complexes, because the parameters of the saturation curve are dependent on them. For the first time, we formulated this problem in 1979 [12], and then, further improved in [10, 13, 14]. At this first stage of research, that was summarized in [15], we employed the simplest approach based on the analytical formulas for the saturation factor. These formulas are the consequences of the solutions to the set of the rate equations for the populations of molecular levels in the monomolecular approximation, that is, without regard to the effect of intermolecular interactions on the saturation curve.

Such an approach gives brilliant results for dye dilute solutions. In particular, we demonstrated that the employment of this approach can result in a determination of the absorption cross section without a priori information on the dye concentration. Using pumping laser pulses of a duration much longer than the lifetime of the excited singlet level, we can also measure this lifetime. Such determinations are impossible within the framework of linear spectroscopy.

In [15] we applied this technique to one of the most intriguing natural complexes, AHS. Doing so, we realized that such a generalization is not absolutely correct, because in AHS, we can expect intermolecular interactions not to be negligibly weak. However, we evaluated the absorption cross section (in general, the cross section of the fluorescing-center excitation) and, what is even more important, compared this parameter for different weight fractions. These results have played some role in formulating the hypothesis on the nature of the fluorescence band of aquatic humic substances (in [5, 16] this hypothesis was further developed).

It is natural that the approach based on the application of analytical expressions written out within the monomolecular approximation under very rigid conditions, could not be regarded as satisfactory. So, in 1992 [17, 18], we began to develop a strict method of nonlinear fluorimetry, which involves a canonical procedure of solving inverse problems. This procedure includes the elaboration of an adequate physico-mathematical model, the solution of the direct problem within the model assumptions (in general, by numerical methods), and the search for an optimal algorithm for solving the
inverse problem. This algorithm should ensure the solution stability and minimization of possible errors in calculating the required parameters, provided the experimental errors are given.

**Physico-mathematical models of photophysical processes in NOC and general procedure of calculation and representation of the saturation curves (direct problem)**

The general view is that the saturation curve is the fluorescence intensity \( I_0 \) or the number of the fluorescence photons \( N_0 \) as a function of the flux density \( F \) of pumping radiation. It can be calculated by solving (either analytically or numerically) the system of kinetic equations for the populations of energy states of fluorescing molecules.

The focal point of this procedure is an optimal choice of the physical model describing the interaction of laser radiation (with its specific parameters) with an ensemble of fluorescing organic molecules or complexes. In its mathematical representation, this choice of the model defines the number of unknown parameters that should be determined [19].

In the case of a monomolecular solution of non-interactive organic compounds (like dyes), we should give priority to the following parameters defining the saturation curve (see Fig. 2, [20]):

- the absorption cross section \( \sigma_{abs} = \sigma_{1,3} \), which determines the probability of the transition of a molecule from the ground singlet state \( S_0 \) (level 1) to the first excited singlet state \( S_1 \) (level 3) stimulated by the photon flux with the density \( F \);
- the lifetime of the molecule in the \( S_1 \) state, that is on the level 3 \( \tau_3 \);
- the quantum yield of the process of the molecule transition to the lower triplet state \( T_1 \) (level 2) due to the intercombination conversion \( S_1 \to T_1 \); \( \eta_1 = k_{32}/k_3 \), where \( k_3 = k_{31} + k_{32} + k_{33} \) and \( k_{31} \) and \( k_{32} \) are the rates of radiative and radiationless transitions from \( S_1 \) to \( S_0 \); \( k_{32} \) is the rate of the \( S_1 \to T_1 \) transition; \( k_{3}^{-1} = \tau_3 \).

In some cases, this list of parameters can be enlarged with the cross sections of absorption of pumping radiation at excited states \( S_1 \) and \( T_1 \). Note that this process proceeds differently in optically thin and thick layers. Its effect on the saturation curve will be essentially different for the molecules whereupon the transition \( S_1 \to S_0 \), the molecule rapidly returns back to the \( S_1 \) state, and the molecules featuring a high probability of transition from any \( S_n \) state directly to the ground state \( S_0 \).

When the objects under study were dyes, we restricted this list to the first three parameters, providing the model governed by the following set of equations (1):

\[
\begin{align*}
\frac{\partial \bar{n}_1(t, r, z)}{\partial t} &= k_3 n_3(t, r, z) - F(t, r, z) \sigma_{1,3} n_1(t, r, z) \\
\frac{\partial \bar{n}_2(t, r, z)}{\partial t} &= k_{32} n_3(t, r, z) \\
\frac{\partial \bar{n}_3(t, r, z)}{\partial t} &= F(t, r, z) \sigma_{1,3} n_1(t, r, z) - k_3 n_3(t, r, z) \\
\bar{n}_1 + \bar{n}_2 + \bar{n}_3 &= \bar{n}_0
\end{align*}
\]

Here \( z \) is the coordinate along the direction of laser radiation propagation, \( \vec{r} = \{x, y\} \) is the coordinate across the beam. Here, we neglect \( T_1 \to S_0 \) transitions (and, hence, we neglect the \( k_3 \) parameter). This assumption is valid if the pulse duration is much less than the lifetime of the triplet level: \( \tau_p \ll \tau_3 \). For pulse lasers commonly used in remote laser diagnostics, the pulse duration is \( \tau_p = 10 \text{ ns} \), and this condition is fulfilled.

For the NOC discussed here: phytoplankton, AHS, protein-like compounds, and oil films – the local concentrations of fluorescing molecules are high (\( C \geq 10^3 \) M). Then, we should take additional parameters into our consideration. These parameters:

- the rate of the singlet-singlet annihilation \( k_{ss} = \gamma \);
- the rate of the energy transfer from the "subsidiary" ("AM") to the fluorescing molecules \( k_{AM,6} \);
- the rate of the energy transfer from the fluorescing molecules to the "reaction centers" ("RC") \( k_{IL,RC} \).

In this case, the set of rate equations become more complicated (2):

\[
\begin{align*}
\frac{\partial \bar{n}_1}{\partial t} &= -\sigma_{1,3} F \bar{n}_1 + k_3 \bar{n}_3 + \frac{\gamma}{2} \bar{n}_5 - k_{AM,6} \bar{n}_5 \bar{n}_1 + k_{IL,RC} \bar{n}_5 \bar{n}_4^{RC} \\
\frac{\partial \bar{n}_5}{\partial t} &= k_{32} \bar{n}_3 \\
\frac{\partial \bar{n}_1}{\partial t} &= \sigma_{1,3} F \bar{n}_1 - k_3 \bar{n}_3 - \frac{\gamma}{2} \bar{n}_5 + k_{AM,6} \bar{n}_5 \bar{n}_1 - k_{IL,RC} \bar{n}_5 \bar{n}_4^{RC} \\
\bar{n}_1 + \bar{n}_2 + \bar{n}_3 &= \bar{n}_0
\end{align*}
\]

The equations for energy levels populations of "subsidiary molecules" \( \bar{n}_5^{AM} \) and of "reaction centers" \( \bar{n}_5^{RC} \) must be added to this set. For pigment-protein complexes of algae (the photosynthetic units), all three mechanisms are established quite reliably, and parameters \( \gamma, k_{AM,6} \) and \( k_{IL,RC} \) are specified. For the
other natural organic complexes. This remains the subject of further investigation.

Under certain presumptions, one can perform a substitution of parameters. In particular, one can introduce the effective cross section \( \sigma^* \) of fluorescing molecules excitation. This cross section is determined by absorption of exciting radiation by fluorescing molecules themselves (cross section \( \sigma_{13} \)), by energy transfer to these from "subsidiary molecules" (for phytoplankton, energy transfer occurs from subsidiary pigments). Introducing the cross section \( \sigma^* \) and including energy transfer from fluorescing molecules to "reaction centers" into constant \( k_3 \) (denote it as \( k_3^* \)), one can simplify the system (2):

\[
\begin{align*}
\frac{\partial n_1}{\partial t} &= -\sigma^* F n_1 + k_3^* n_3 + \gamma n_3^2 \\
\frac{\partial n_2}{\partial t} &= k_1 n_3 \\
\frac{\partial n_3}{\partial t} &= \sigma^* F n_1 - k_3^* n_3 - \gamma n_3^2 \\
n_1 + n_2 + n_3 &= n_0
\end{align*}
\]

Having found the population of the first excited level \( n_3 \) from the set of kinetic equations, we can proceed with the calculation of the number of fluorescence photons born in the volume \( V=S-L \) (S is the cross section of the beam, \( L \) is the thickness of layer, from which the media response is recorded).

\[
N_{\Phi} = C k_3 \int_0^l \int_S dT \int_S dF \cdot n_3(t, r, z) ,
\]

Here, \( C \) is a coefficient which is dependent on the distribution instability of fluorescing molecules. We remind that \( n_0 \) and, hence, \( n_1 \) are the local concentrations of fluorescing molecules in the complex.

In most calculations, we used the approximation of an optically thin layer, that is \( \sigma_{abs} n_0 L \ll 1 \), where \( F(z) \) is nearly a constant and, hence, \( n_3(z) \) is also a constant. This approximation is valid for our laboratory experiments, but may not be fulfilled in the case of remote sensing of a whole water bulk (we shall come back to this point again).

Various photophysical parameters have different effects on the fluorescence saturation curve. This is illustrated by Figure 3. This figure demonstrates that the S-S-annihilation manifests itself at lower values of \( F \) as compared to the transulence of an optically thin layer, when the local concentrations are high. However, comparing experimental saturation curves obtained for phytoplankton and for a low-concentration solution of chlorophyll "a" (chl-a), which is the main pigment of photosynthesizing organisms (Fig.4), to the curves of Fig.3 shows that the contribution of S-S-annihilation to the photophysical processes in phytoplankton is high, and that there is no such contribution in chlorophyll solution (as it is in general in low-concentration solutions of dyes and related organic compounds).

Note that distinguishing between the mechanisms of saturation is relative. They are all related to each other, in particular, by their influence on the time of return of molecules back to the ground state. For example, the S-S-annihilation leads not only to the vanishing of one of two excited states (excitons), but also to a decrease in return time of a molecule from the \( S_1 \) to the \( S_0 \) state, thus working against the mechanism of its depletion.

In all the algorithms of solving inverse problems of saturation fluorimetry it is very important to optimally choose the form of representation of the saturation curve. The best information-containing representation is the \( (N_0, F) \) coordinates, where \( N_0 \) is the number of fluorescence photons emitted by the unit volume, and \( F \) is the photon flux density of pumping radiation, both parameters being expressed in absolute units. However, to measure the absolute number of photons is a very difficult task; it is practically impossible under conditions of real experiments. This difficulty is almost completely overcome in diagnostics of NOC in an aqueous medium by using the Raman signal from water as the internal standard. This corresponds to the representation of the saturation curve in coordinates \( (\Phi, F) \), where \( \Phi \) is the ratio of the number of fluorescence photons to the number of Raman photons from water \( (\dot{O}=N_0/N_{R}) \). One can represent the saturation curve in coordinates \( (A, F) \), where \( A = N_0/N_R = \dot{O}_0/\dot{O} \) is the saturation factor; \( \dot{O}_0 = \lim F \rightarrow 0 \dot{O} \). This representation describes the form of the saturation curve, but it does not give the information about the absolute number of fluorescence photons. To find \( \dot{I} \) from experimental data means to find the value of \( \dot{O}_0 \). This can be done by extrapolating the measured section of curve \( \dot{I}(F) \) to \( F = 0 \). In the case of dye dilute solutions this is a simple task, but for NOC, it is a far more complicated problem due to S-S-annihilation (Fig.3). For phytoplankton, where the S-S-annihilation is noticeably pronounced (it is also possible for other NOC), the parameter \( \dot{O}_0 \) can be determined from the experimental saturation curve which should be represented in the coordinates \( (\dot{O}, F) \). Representing the saturation curve in such a way, we ensure a strict enough fulfillment of the following approximations:

\[
\dot{O} = \dot{O}_0 + A - N_0 \quad \text{for} \quad F \leq 5 \times 10^{24} \text{ cm}^{-2}\text{s}^{-1}
\]

\[
\dot{I} = \dot{O}_0 + N_1 N_0 + N_2 N_0^2 + N_3 N_0^3 \quad \text{for} \quad F \leq 5 \times 10^{25} \text{ cm}^{-2}\text{s}^{-1}
\]

However, using the saturation curve representation \( (\dot{I}, F) \) is not always efficient. In particular, in the case of
dye dilute solutions, the dependence $\Gamma(F)$ is nearly linear, $\Gamma = 1 + \beta F$, up to $F = 10^{35} \text{cm}^{-1} \cdot \text{s}^{-1}$. Therefore, we can reduce our problem to the single-parameter problem of finding the factor $\beta$. In each particular case and for each particular algorithm for solving an inverse problem, it is necessary to find out a specific optimal representation form for the saturation curve. In a number of cases, one can simultaneously use several representations.

**Stability and uniqueness of the solutions to inverse problems of the saturation fluorimetry.**

In general, an inverse problem of the saturation fluorimetry is incorrect by Hadamard [21]. However, involving a priori information about photophysical processes forming the fluorescence of complicated organic compounds and NOC, one can reduce the number of required parameters in a model and the ranges of their variations, so that the problem is upgraded to the class of conditionally correct or simply correct problems by A.N. Tikhonov [22]. In the work [23] performed by our group in cooperation with Professor A.V. Tikhonravov and K.V. Popov from the Research Computing Center of Moscow University, the solution of the three-parameter inverse problem for monomeric solution of non-interactive molecules (dyes solution) was shown to be unique and stable. We can expect that taking into account the S-S-annihilation would not lead to the downgrade of the problem, although a special proof for this statement may be needed.

We approved two methods of solving the inverse problem of the saturation fluorimetry. It has been consistently done for three solutions of dyes with known photophysical parameters $\sigma_{\text{abs}}$, $\tau_3$, and $\tau_1$ determining the saturation curve; values of these parameters for dyes used for solving the inverse problem in saturation fluorimetry are presented in the first lines of Tables 1 and 2. One of these methods was employed for solving this problem for the representatives of three NOC classes: phytoplankton, aquatic humic substances, and oil films. Saturation of AHS fluorescence was studied also in [3], however, without using procedures of the inverse problems solution.

**Solution of an inverse problem by the variation method with the quasi-Newtonian technique of the residual functional minimization.**

The first of the chosen methods of solution of saturation fluorimetry inverse problems belongs to the class of variation methods [23]. These methods are based on the minimization of residual between experimental and theoretical data. Using variation methods encounters technical problems, connected with multi-extremity of the residual of functional and with its complex "valley" structure. An effective method of overcoming these difficulties is the quasi-Newtonian technique [24]. It was this method that was used for the calculation of parameters $\sigma_{13}$ and $\tau_3$ in the two-parameter problem without accounting for intermolecular interactions. Use of the set of equations (1) made it possible to perform a numerical experiment, in which the parameters $\sigma_{13}$ and $\tau_3$ were determined for three dyes, which have essentially different values $\eta_1$ (see Table 2). Modeled experimental saturation curves were distorted by the addition of artificial noise with amplitudes from 0% to 10%. The mean values and dispersions of parameters were determined [23]. It turned out, that the mean values of parameters resulting from the solution of the inverse problem differ from the true ones by no more than 2-3% (with the exception of value $<\tau_3>$ for dye Bengalish rose, for this dye the deviation from true value $\tau_3$ made from 10 to 40% under noise levels 3...10%, see Table 1), and the dispersion of the restored parameters did not exceed the noise level in “experimental” saturation curves. Such surprisingly good results are connected to the fact the problem was solved for the simplest model. Within this model, one can obtain an analytical expression for the dependence $N_0(\sigma_{13}, \tau_3)$. So, there are no errors that would emerge from a numerical solution of the direct problem. In a certain sense, this case represents the best possible results of the method. Our calculations showed that the precision of parameter determination drops when the model becomes more complicated (taking into account the distribution of photon flux density $F$ across the beam and in time, simultaneous determination of three parameters etc.). These difficulties stimulated the use of artificial neural networks.

**Solution of inverse problems of saturation fluorimetry using the method of artificial neural networks.**

Artificial neural networks (ANN) are a powerful method used for the effective solution of different problems in image recognition, classification, and forecasting. The ability of neural networks to learn by examples is their unique property. Neural networks do not simply memorize, they can also generalize information, revealing hidden dependencies and classifying the presented data [25]. There are single publications about the use of neural networks for recognition of spectral images of organic complexes in water [26] and in problems of remote sensing [27].

The ability of neural networks to extract important characteristics from the input data and to reconstruct the parameters, even in those cases when the influence of such parameters on a signal is unknown or distorted by noise, makes it possible to use the neural network technique in solving incorrectly posed problems.

Below, our first results on the use of the neural network technique in solving inverse problems of the saturation...
fluorimetry are presented. For more details, please refer to the papers [28-31].

Two- and three-parametric inverse problems (determination of $\sigma_{\text{ref}}$ and $\tau_2$ or determination of $\sigma_{\text{ref}}, \tau_3$ and $\eta_\tau$) were solved for saturation curves modeled according to the set of equations (1) for the parameters of the three dyes, presented in Tables 1 and 2. With several exceptions (parameters $\eta_\tau$ for Bengalis rose and $\eta_\tau$ for Rhodamin 6G), the dispersions of the restored parameters and their deviations from their true values, being larger than in the case of a two-parametric problem (see Table 1), are still satisfactory.

The capacity to solve inverse problems with a precision sometimes higher than the precision of input data, is a unique property of artificial neural networks determined by the specific way they work.

The method of ANN was used to determine the parameters $\sigma^*$ and $\gamma$ from experimental saturation curves for three species of algae, for optically thin films of oil and Diesel fuel, and for AHS in natural waters of different types. The saturation curves are presented in Figures 5-7, while the results of the solution of the inverse problem are given in Table 3 ($n_0$ is the local concentration of fluorescing molecules in NOC, therefore, $\gamma n_0$ is the maximal value of the S-S annihilation rate at a given value of $\gamma$). In curve modeling, we assumed the photon flux density of the exciting radiation $F(t, \vec{r})$ to be spatially rectangular and temporarily Gaussian, which was a very rough approximation of real distributions $F(t, \vec{r})$ in the laser beams used in experiments.

Unfortunately, the volume of statistical material was insufficient to determine the accuracy of determination of $\sigma$ and $\gamma n_0$ values in Table 3. However, based on the results of a numerical experiment conducted using the system of equations (3), one may suppose that the accuracy of determination of $\sigma$ and $\gamma n_0$ values is approximately equal to the precision of initial experimental data (while this precision is higher than 10%).

The obtained values of $\sigma^*$ and $\gamma n_0$ must be considered preliminary: they will be improved along with the method enhancements and the development of other models. However, the comparison of $\sigma^*$ and $\gamma n_0$ for different classes of NOC and for different complexes within the single class, leads to a number of conclusions. The first of these is that the obtained values of $\sigma^*$ and $\gamma n_0$ for phytoplankton agree qualitatively well with expected values of $\sigma^*$ (the values of this parameter were obtained in situ for the first time) and with known values of $\gamma$, obtained by the method of picosecond laser spectroscopy. These results provide evidence that energy migration plays an important role in the formation of the fluorescent response of phytoplankton to optical pumping.

Concerning the other two classes of NOC, there is no data available to compare the obtained values of $\sigma^*$ and $\gamma n_0$. However, we can conclude that the local concentration of fluorescing molecules $n_0$ in complexes is much lower than in phytoplankton, and, hence, the contribution of the energy transfer process to the fluorescence saturation is also smaller. This conclusion must also be regarded as preliminary, as it was obtained for a model disregarding the singlet-triplet conversion ($\eta_\tau = 0$) and probably other processes that may have an effect on fluorescence.

On the possibility of using NOC saturation fluorimetry for remote sensing

There are a number of obstacles, which have to be overcome on the way of applying the method of saturation fluorimetry for remote sensing, in investigation and diagnostics of NOC in aqueous media and on land.

The first obstacle originates from the necessity to operate with accurate absolute values of the photon flux density of laser radiation $F_0$, at which the numbers of fluorescence photons $N_{\text{ph}}$ or the fluorescent parameters $\Phi_i(F_0)=N_{\text{ph}}/N_{\text{ph0}}$ are measured. This is an extremely difficult task even in laboratory experiments with samples. In remote diagnostics, it is much more difficult, especially if we have to receive the fluorescence signal from the surface layer (as in the case of sensing oil films), but from the whole water depth or from a deeper layer. The most effective way to measure $F_0$ is the use of “standard” dye solution, which was proposed in [15]. In experiments with samples, such a standard dye could be any dye with known values of $\sigma_{\text{abs}}, \tau_3$, and $\eta_\tau$ (preferably, with $\eta_\tau = 0$). In remote sensing, there is a sole candidate to the role of the standard compound. It is aquatic humic substance. The applicability of this approach depends on the extent to which theophysical processes in humic substances are studied, and on their stability, at least within a given area.

The second problem is the violation of the thin-layer condition in the case where the durations of the laser pulse $\tau_\text{p}$ and of the gate-pulse of the receiver $\tau_\text{g}$ do not satisfy the conditions

$$ct_\tau \leq \varepsilon^{-1}, \quad c\tau_g \leq \varepsilon^{-1}$$

where $\varepsilon^{-1}$ is the factor of the overall attenuation of water at the laser wavelength. In the case of sensing the layer of an arbitrary optical thickness, it is necessary not only to integrate the response signal over the whole path of light (such a procedure was already performed in [32]), but also to take into account other saturation mechanisms discussed above (in particular, the absorption of laser photons by excited states). This matter is the subject of further theoretical and experimental studies aimed at the choice of the optimal regime of sensing.
Conclusion

The analysis of the results of research into fluorescence saturation of NOC that our group has been engaged in since 1975, and the analysis of the approaches to solving inverse problems of NOC saturation fluorimetry, have revealed the following: (a) it is necessary to take this effect into account when interpreting the results of laser sensing of aqueous media; (b) it is possible to measure photophysical parameters of NOC with acceptable accuracy by means of saturation fluorimetry using at least two algorithms for solving inverse problems.

The photophysical parameters of phytoplankton, AHS, and oil films have been measured in vivo and in situ for the first time.

We regard these studies only as a demonstration of the abilities of laser saturation fluorimetry, which stimulate large-scale investigations in this field and their applications to diagnostics of NOC. To do this, it will be necessary to overcome a number of difficulties discussed in this paper. However, the effort here seems to be reasonable in view of the perspectives that lie ahead using this method, whose importance as a principally new approach in fluorimetry extends far beyond the framework of laser diagnostics of NOC.

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References


Table 1. Parameters of dyes determined from fluorescence saturation curves with the help of least squares fit (LSF) and artificial neural networks (ANN) (results of numerical experiment)

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<th>ANN</th>
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Table 2. Simultaneous determination of three photophysical parameters of dyes by use of ANN (numerical experiment).

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<th>Noise level</th>
<th>$&lt;\sigma_{11}&gt;$, cm$^2$</th>
<th>$&lt;\tau_1&gt;$, ns</th>
<th>$&lt;\eta_1&gt;$</th>
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Table 3. Photophysical parameters of natural organic complexes determined from experimental saturation curves.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$\lambda_{ex}$, nm</th>
<th>$\sigma$, cm$^2$</th>
<th>$\gamma\eta_0$, s$^{-1}$</th>
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<tbody>
<tr>
<td>Diatomite algae</td>
<td>532</td>
<td>2.3×10$^{-15}$</td>
<td>1.2×10$^{11}$</td>
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<tr>
<td>Piridinium algae</td>
<td>532</td>
<td>4.2×10$^{-15}$</td>
<td>2.4×10$^{11}$</td>
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<tr>
<td>Green algae</td>
<td>532</td>
<td>2.2×10$^{-15}$</td>
<td>11.8×10$^{11}$</td>
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<tr>
<td>Diesel fuel</td>
<td>355</td>
<td>1.0×10$^{-16}$</td>
<td>8.8×10$^8$</td>
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<tr>
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<td>8.0×10$^{-17}$</td>
<td>5.3×10$^8$</td>
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<tr>
<td>AHS from Baltic Sea</td>
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<td>2.9×10$^{-17}$</td>
<td>3.0×10$^8$</td>
</tr>
<tr>
<td>AHS from r. Moscow</td>
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<td>6.0×10$^{-17}$</td>
<td>2.6×10$^8$</td>
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<td>AHS from Mexican Bay</td>
<td>308</td>
<td>5.7×10$^{-17}$</td>
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