# ON THE NOVEL POSSIBILITIES OF USING PHYTOPLANKTON AS A BIOINDICATOR OF TOXICANTS IN NATURAL WATERS

*Timofey Gostev[1], Fyodor Kouzminov[2], Vladimir Paschenko[3] and Victor Fadeev[4]*

1. Moscow State University, Department of Physics, Moscow, Russia; onewhowaits@mail.ru
2. Moscow State University, Department of Physics, Moscow, Russia; kouzz@list.ru
3. Moscow State University, Department of Biology, Moscow, Russia; paschenko@yandex.ru
4. Moscow State University, Department of Physics, Moscow, Russia; victor_fadeev@mail.ru

**ABSTRACT**

The novel algorithms of evaluating four molecular photophysical parameters of the photosynthetic apparatus of photosynthetic organisms (by the example of phytoplankton) were developed. These algorithms open up new possibilities for using photosynthetic organisms as natural bioindicators of the state of ecosystem and particularly for using phytoplankton as an indicator of presence of various pollutants in natural waters. Proposed approaches were tested during evaluation of the photophysical parameters of alga Chlorella pyrenoidosa under various stress factors, including presence of DMCU and $Cu^{2+}$ ions.

**INTRODUCTION**

Using natural components of ecosystem as indicators of its status is state of the art environmental monitoring strategy. Photosynthetic organisms provide vast opportunities for bioindication (1) — this is due to their high sensitivity to the changes in the environment, such as illumination regime variations, climatic changes and particularly presence of various toxicants. Photosynthetic organisms are complex objects, and determination of photophysical parameters of photosynthetic apparatus that are the most sensitive to the variations of its physiological state is a difficult task.

Conventional spectroscopic techniques (such as pump and probe, pulse amplitude modulation fluorimetry or absorption spectroscopy) play a key role in studying photosynthetic organisms (2), various biophysical processes in them and assessing the state of the photosynthetic apparatus. Still, their applications are sometimes very limited, especially in the absence of the *a priori* information on the studied object. There is an increasing necessity in using optical methods that allow evaluating the parameters of the photosynthetic organisms on the molecular level.

We develop one of such methods — nonlinear laser fluorimetry, or saturation fluorimetry (3). The major difference from the conventional methods based on the measurement of the chlorophyll *a* or other pigments fluorescence consists in the application of high energy pulsed laser radiation for fluorescence excitation which allows to assessing processes on a molecular level and obtaining the photophysical parameters of fluorophores. Our previous studies revealed these parameters to be very sensitive to the state of the photosynthetic apparatus, and consequently to the state of the environment of the studied object (4).

In this paper, we report a newest approach in the saturation fluorimetry of photosynthetic organisms by the example of phytoplankton. The approach allows obtaining up to four molecular photophysical parameters of chlorophyll *a* pigment. The theoretical model and the inverse problem of the saturation fluorimetry are discussed. The experimental setup is described and the results of evaluation of molecular photophysical parameters of alga *Chlorella pyrenoidosa* under various stress factors, such as presence of DCMU herbicide and $Cu^{2+}$ ions are presented. The correlation between these molecular parameters of the photosynthetic apparatus and its status gives way to the ecological monitoring applications with laser sensors, which use photosynthetic organisms as fluorescent bioindicators.

**METHODS**

The technique of saturation fluorimetry lies in the registration of the nonlinear dependence of the fluorescence photons count $N_{fl}$ emitted by the fluorophores (chlorophyll *a* molecules) on the excitation radiation photon flux density $F$ — the saturation curve. This saturation is due to various physical processes in the pigment molecules, such as singlet–singlet annihilation and the dynamic decrease of the molecule's ground state population (5). These processes take place when the fluorescence is excited using laser radiation pulses with high photon flux density $\left(F > 10^{21} cm^{-2} s^{-1}\right)$ and are defined by the molecular photophysical parameters of the fluorophores. The rates of these processes depend significantly on the state of the photosynthetic apparatus. Photophysical parameters define the experimental saturation curve and could be evaluated through the solving of the saturation fluorimetry inverse problem.

Our current approach to the saturation fluorimetry application is based on the theoretical model of the fluorescent response of the photosynthetic cell under pulse laser radiation that uses four photophysical parameters: $\sigma$ — the excitation cross–section of the chlorophyll *a* that defines both the direct absorption of excitation radiation and the energy transfer from other pigments; $\tau$ — the chlorophyll *a* excited state lifetime that defines all the paths of its relaxation with the exception of singlet–singlet annihilation; $\gamma n_0$ — the maximum rate of singlet–singlet annihilation (here $n_0$ — local concentration of the chlorophyll *a* molecules in the photosynthetic cells) and $\Phi_0$ — normalized fluorescence intensity in the absence of the saturation effects.

Previous implementations (6) of the method only allowed obtaining two parameters from the experimental data: $\Phi_0$ and $A = \sigma\tau^2\gamma n_0$ — saturation factor which is a product of the three parameters of the fluorescent response model. It was shown that the saturation factor is very sensitive to the state of the photosynthetic apparatus, as well as to the taxonomic group of the photosynthetic organisms. Still, it wasn't possible to separately assess each of the photophysical parameter. This is due to the fact that the saturation curve is smooth, with no particular features, and the four parametric inverse problem becomes unstable even in the case of low input data noise levels.

In this paper we propose an algorithm for separate evaluation of the molecular photophysical parameters of the chlorophyll *a* molecules. It is based on the fact that these parameters define the saturation curve in the different regions of $F$, and thus it is possible to divide the four parametric inverse problem in two parametric problems, solved in the different sections of the single experimental curve. This requires that the experimental excitation photon density flux varies in a very wide range $\left(F \approx 10^{21} \sim 10^{25} cm^{-2} s^{-1}\right)$.

Measuring the absolute number of the fluorescence photons $N_{fl}$ is prone to various errors. One of the major aspects of the saturation fluorimetry technique is that the fluorescence signal is normalized using reference signal $N_r$ that is linearly dependent on the excitation radiation photon flux density — such as water Raman scattering signal. The experimental data is usually presented as $N_r(F)/N_{fl}(F)$.

In order to show the principal possibility of using phytoplankton as a fluorescent bioindicator of the state of the aqueous system, laboratory experiments were conducted with the alga *Chlorella pyrenoidosa*, and molecular photophysical parameters were evaluated using the proposed algorithm, for the intact phytoplankton, as well as for the samples with the presence of DCMU herbicide and heavy metals ions ($Cu^{2+}$). Phytoplankton cells were dark adapted for 30 minutes before the experiments, the concentrations of pollutants were chosen in order to be 10 times below the maximum permissible concentrations for each pollutant respectively.

The experimental setup is presented in the *Figure 1.* The parameters of the excitation laser radiation are the following: pulse duration — 20 ns (quasistationary regime of *chlorophyll a* molecules excitation), pulse repetition rate — 10 Hz, maximum pulse energy — 12 mJ, radiation wavelength — 532 nm. The excitation radiation flux density can be varied in the range of $F \approx 5 \cdot 10^{21} \sim 2 \cdot 10^{25} cm^{-2} s^{-1}$. The excitation beam passes the cuvette with the sample and the beam splitter. One part of the beam is used as a reference normalization signal, and the other triggers the ADC board (National Instruments).

The fluorescence signal is registered using photomultiplier module (Hamamatsu) with the 690 nm interference filter, the reference signal is measured using PIN photodiode (Texas Instruments).

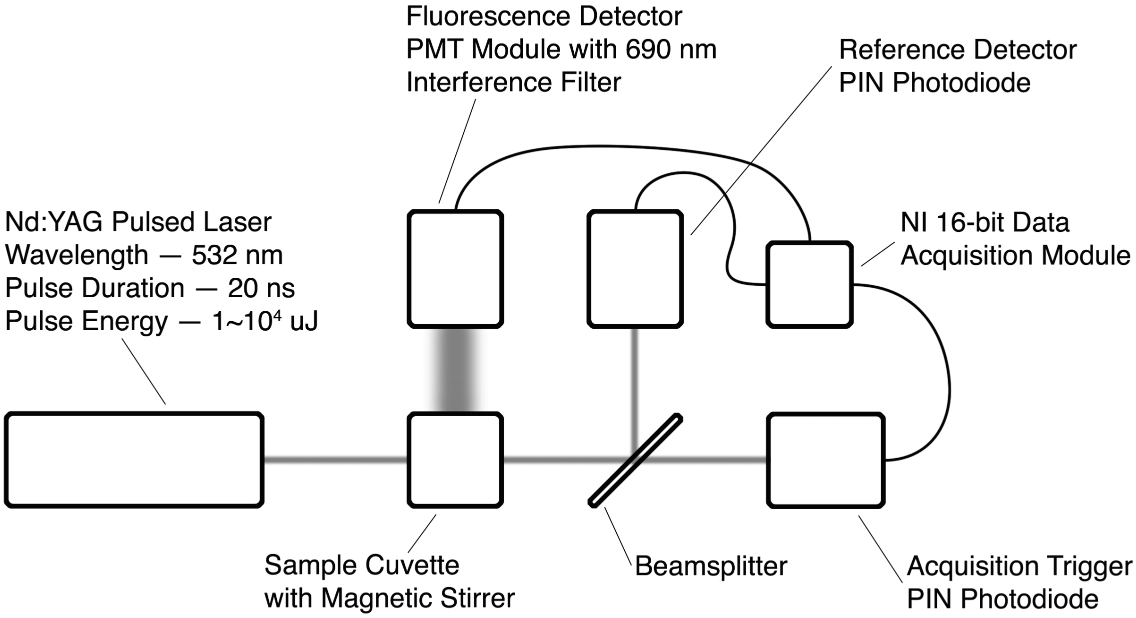


*Figure 1: The experimental setup diagram.*

## RESULTS

Typical experimental curves of fluorescence saturation are presented in the *Figure 2.*

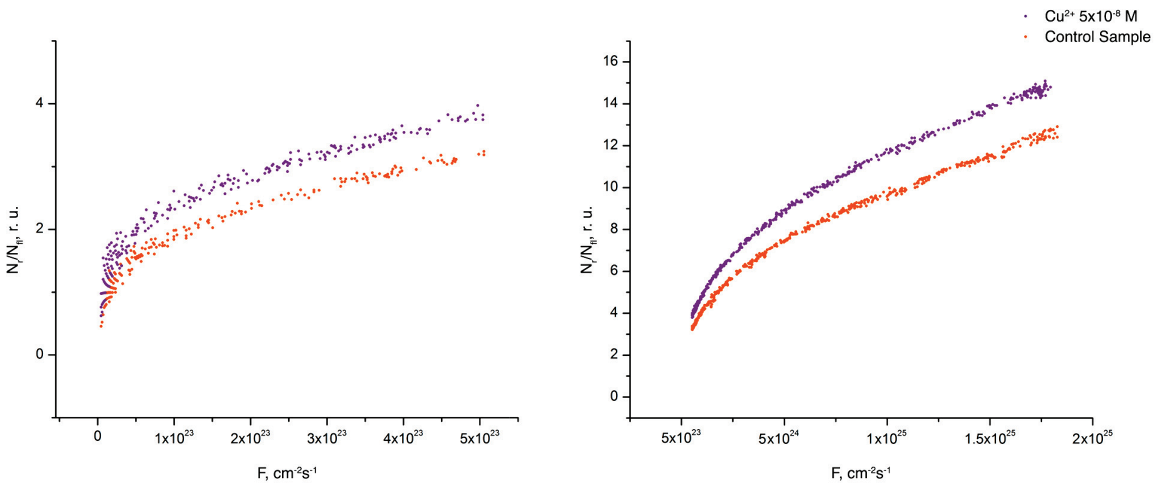


*Figure 2: Experimental curves of fluorescence saturation of two samples divided in two sections:* $F \approx 5\cdot 10^{21} \sim 5\cdot 10^{23} cm^{-2}s^{-1}$ *and* $F \approx 5\cdot 10^{23} \sim 2\cdot 10^{25} cm^{-2}s^{-1}$*.*

The results of evaluation of photophysical parameters of phytoplankton from the experimental data during solving the inverse problem are presented in the Table 1.

*Table 1: Molecular photophysical parameters of the alga Chlorella pyrenoidosa in three different states: Intact (Control Sample), in the presence of the $Cu^{2+}$ ions and DCMU herbicide.*

| *Sample* | $\tau, ns$ | $\sigma, 10^{-16} cm^2$ | $\gamma n_0, 10^{12} s^{-1}$ |
|---|---|---|---|
| Control Sample | 0.18 | 2.2 | 2.6 |
| $Cu^{2+}$ $10^{-9}$ M | 0.21 | 2.1 | 1.6 |
| DCMU $10^{-7}$ M | 0.46 | 1.8 | 0.8 |

The results show that the molecular photophysical parameters of the chlorophyll *a* molecules depend on the physiological state of the phytoplankton, and their variations correlate with the presence of various pollutants. The rate of singlet–singlet annihilation changes drastically in the presence of $Cu^{2+}$ ions, while variations of other parameters are almost negligible; presence of DCMU herbicide produces variations of both the singlet–singlet annihilation rate and the excited state lifetime. Investigation of the mechanisms by which heavy metals ions and herbicides alter the rate of singlet–singlet annihilation is essential for studying photosynthesis biophysics and for developing the described bioindication approach.

## CONCLUSIONS

The prospects of using phytoplankton as a fluorescent bioidicator of presence of various toxicants in natural waters in the concentrations below the MPC presented. This information is derived from the molecular photophysical parameters of the phytoplankton, while at the indicated concentrations of toxicants variations of photophysical parameters are beyond the errors caused by experimental data noise factors, inaccuracies in theoretical model and in solving the inverse problem. Further development of the proposed technique of bioindication lies in the application of matrix method of laser fluorimetry (9) and in developing of an integrated approach that includes methods of laser fluorimetry and conventional optical spectroscopy, such as PAM and FIRe (8) fluorimetry. It should be noted that laser bioindication using phytoplankton fluorescence can be implemented in remote mode — non–contact (LIDAR (10, 11)) and contact (laser spectrometer with receiving-transmitting fiber–optic cable probe).

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