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# Optical Assessment of *Dunaliella salina* Microalgae Viability upon Toxicological Testing

**D. S. Chumakov, A. A. Golubev, L. A. Dykman, N. G. Khlebtsov, and V. A. Bogatyrev***

*Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, 410049 Russia*

**e-mail: bogatyrev_v@ibppm.ru*

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**Abstract**—A method has been developed for monitoring the dynamics of the number and viability of *Dunaliella salina* microalgae taking into account the spectral contribution from aggregates of metal particles tested as a toxic agent in a microplate assay system. The method is based on the in vivo determination of the chlorophyll content from the intensity of an absorption peak at 680 nm corrected for the nonselective extinction as calculated using the values of nearest local minima at 640 and 740 nm.

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## INTRODUCTION

The determination of chlorophyll content in suspensions of microalgae (MAs) has a great diagnostic importance for the ecology monitoring and biotechnology. Under normal conditions of the balanced growth of MAs, the relations between the contents of basic plant pigments, the number of cells, biomass, total protein, and other parameters determined by analytical methods remain almost unchanged. On the contrary, a change in these relations may indicate that a system exists outside the normal physiological limits. Attempts at nondestructive determination of chlorophyll in suspensions of MAs have been made repeatedly. The complexity of the problem is associated with the two following main factors: first, the hard-to-control contribution of light scattering on MAs to an extinction spectrum and, second, different extinction coefficients of chlorophyll being determined in vitro and in vivo. The measurement of the contents of chlorophyll and other plant pigments extracted with organic solvents is the main method for determining the viability of green plants [1]. Traditionally, they are measured from the absorption and fluorescence spectra of acetone and alcohol extracts. Light absorption by pigment complexes of green plants depends fundamentally on the physiological state, primarily, illumination [2] (the ratio between the oxidized and reduced forms of chlorophylls); the molecular organization of photosystems in thylakoid membranes; and the structural organization of chloroplasts, cells, and cellular systems as a whole [3, 4]. Collimated light transmission is rather sensitive to light scattering, which is inherent in suspensions of microorganisms [5, 6].

At present, efficient microplate assay systems and corresponding fluorometric and spectrophotometric techniques are widely used [7]. Microplate scanning spectrophotometers (readers) are advantageous in having a horizontal position of a measuring cell (plate well), which makes the measurements less sensitive to dilution or sedimentation of suspensions than are the standard measurements in cells of spectrophotometers and spectrofluorometers. Moreover, the optical effects that accompany the sedimentation of suspensions and are recorded with microplate readers may be determined in preliminary calibration experiments.

Spectrophotometry of acetone extracts is considered a standard method for determining the content of chlorophyll [8]. As we have previously shown [9, 10], photometric determination of variations in the growth rate of *Dunaliella salina* MAs using a microplate reader equipped with a filter monochromatizer (690 nm) enables one to determine the toxic effect of different pollutants. Such an assessment is, at least in principle, possible even in the presence of disturbances caused by an optical contribution to an extinction spectrum from aggregates of metal particles, such as colloidal silver (CS) or colloidal gold (CG). It is known [11] that the aggregation of such particles transforms their plasmon-resonance spectra into "gray" spectra without distinct spectral peaks. However, the uncontrolled contribution of light scattering by MAs substantially limits the application of this approach.

The goal of this work was to develop a method for the in vivo determination of chlorophyll content by the spectrophotometric method.

## EXPERIMENTAL

### *Materials and Reagents*

*D. salina* microalgae culture Teod. IPPAS D-294 was taken from the microalgae collection of the Institute of Physiology of Plants, Russian Academy of Sciences. Ben-Amotz medium [12] with a NaCl content of 1.5 M was used to support MAs and grow the biomass under continuous illumination with luminescent daylight lamps emitting photosynthetic active radiation (PAR) of 80–100 μmol $m^{-2}$ $s^{-1}$. To support the MA culture, its cells were reseeded every week. A 3- to 5-day MA culture occurring at an early stage of the stationary growth was used in the experiments.

Citrate gold nanoparticles nearly 15 nm in diameter (CG-15) were obtained via the Frens method [13] by reducing $HAuCl_4$ (Sigma-Aldrich, United States) with sodium citrate (Fluka, Switzerland) under boiling. The obtained particles were stabilized with poly(vinylpyrrolidone) (PVP). An initial sol with a concentration of 57.9 mg Au/L was concentrated by centrifugation (15000 *g*, 10 min) and redispersion of the sediment in the supernatant (9.6% of the initial liquid volume).

Phosphine colloidal gold with a particle diameter of nearly 2–3 nm (PCG-3) was obtained via the Duff method [14] by reducing $HAuCl_4$ with tetrakis(hydroxymethyl)phosphonium chloride (Fluka, Switzerland) in an alkaline medium (final concentration of 2 g Au/L). PVP was also used as a stabilizer. To separate dispersed phase particles from the dispersion medium, an aliquot of the obtained suspension was filtered through an Amicon centrifuge ultrafilter (Millipore, United States) with a molecular mass cutoff threshold of 3 kDa by means of centrifugation (7500 *g*, 15 min). The concentrate of the particles was redispersed in an aqueous 20-mg/mL PVP solution.

### *Toxicological Experiments*

Test experiments were performed in flat-bottom 96-well plates (Greiner Bio-One, Austria) by placing 200 μL of a suspension into each well in triplicate rows (three analytical replications). This scheme for creating series of concentrations of toxicants and introducing MA inoculum was presented in [10].

Extraction was performed in spin-columns made from 0.7-mL Eppendorf tubes (SSI, United States), the bottoms and covers of which had been transfixed with a preparation needle. GFF Whatman microfiber glass filters (United Kingdom) 9 mm in diameter were placed onto the bottoms of the separation tubes, and uncovered 2-mL collector tubes were inserted into them. Cellular suspensions (200 μL) were quantitatively transferred into the columns and precipitated by centrifugation at 50 *g* for 5 min. Residual medium was "squeezed out" by centrifugation at 10000 *g*. The filters were removed, and 80% ethanol (100 μL) was added to each separation column. The procedure was repeated three times under the same centrifugation conditions. The extracts were transferred (each into its well) and stored in the dark until the measurements were made.

The spectrophotometric measurements were carried out with a Spark 10M microplate reader (Tecan, Switzerland) by recording absorption spectra in a range of 400–800 nm with a step of 1 nm in a cycle of three measurements with an interval of 3 min after a 7-min dark adaptation pause. The peak intensities were calculated using the values of reference points (640, 680, and 740 nm) averaged over five neighboring wavelengths and three measurements. The peak height was calculated by the following equation:

$$\tilde{A}_{680} = (E_{680} - E_{740}) - 0.6(E_{640} - E_{740}).$$

The spectra of the same samples were recorded in cells with different thicknesses from 1 to 10 mm employing Specord S250, S300, and S600 spectrophotometers using analogous programs.

Alternatively, a Spark 10M reader was used to measure the absorption at the wavelengths of the same reference points in the mode of scanning a well (5 × 5 points, distance from well edge of 2500-μm, shaking for 10 s) for 30 min with an interval of 3 min in accordance with [7].

Chlorophyll peak heights in the spectra of the extracts were determined as the differences between the absorptions at 665 and 700 nm:

$$\tilde{E}_{665} = E_{665} - E_{700}.$$

## RESULTS AND DISCUSSION

### *Extinction Spectra of Suspensions*

The resulting light-extinction spectrum of an MA suspension is a superposition of light absorption and scattering. In contrast to absorption, light scattering depends to a large extent on the aperture angle at which transmitted light is detected, and, other conditions being equal, the recorded scattered light intensities are different for spectrophotometers of different types.

Figure 1 shows the light-extinction spectra measured for a *D. salina* suspension in a 1-mm cell using different spectrophotometers.

The structural features of microplate readers imply a rather large aperture angle of detection, which minimizes the contribution of light scattering to the measured extinction. This fact is illustrated by the similarity of the spectra measured for the same suspension with a microplate reader and a Specord S250 spectrophotometer (Fig. 1). On the contrary, the spectra recorded with Specord S600 and S300 instruments, which have a fundamentally different optical scheme (monochromator is located behind the cell), are substantially different. In the spectra measured with the high-speed spectrophotometers, the contribution of

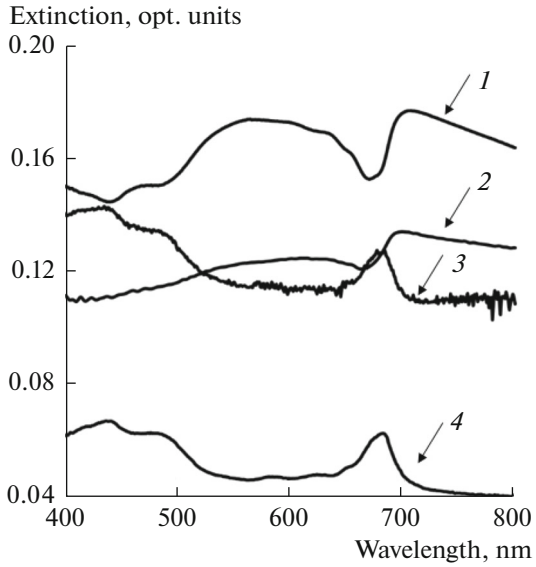


**Fig. 1.** Light extinction spectra measured for *D. salina* suspension in a 1-mm cell with (*1*) S600, (*2*) S300, and (*4*) S250 spectrophotometers and (*3*) Spark 10M microplate reader.

light scattering to the measured extinction is significantly larger because of the smaller aperture of detection. Specord S600 and S300 spectrophotometers have analogous optical schemes of the measuring compartments and differ from each other only in the width of the recorded optical range, i.e., the UV-Vis and visual range, respectively.

*Dependence of Measured Extinction on Dilution*

Figure 2a presents the dependences of light absorption by *D. salina* suspensions and their alcohol extracts on the degree of dilution and corresponding light extinction spectra (Fig. 2b). The extrapolation straight lines in Fig 2a attest to the existences of linear dependences of the optical parameters of the suspensions and extracts on the degree of dilution throughout the range of the measured values. It should be noted that the growth rate of MAs is nearly one doubling per day ($\mu \approx 1$). The growth rate was calculated by the following equation:

$$\mu = 3.3(\log(N_2/N_1)/t,$$

where $N_2$ and $N_1$ are the optical estimates of the final and initial concentrations of the cells, respectively, in a suspension at the balanced growth (in a reference) and $t$ is the time (days).

Therefore, a dilution coefficient equal to unity corresponds to a suspension with a cell concentration the same as that in reference wells after 48 h of the experiment, while a coefficient of 0.25 corresponds to the initial cellular suspension. The light-extinction spectra are presented for a suspension and an extract, and the corresponding degrees of dilution are equal to unity. The peak maxima are located at 680 and 665 nm for the suspensions and extracts, respectively.

*Interference with Colloidal Gold*

Figure 3 illustrates variations in the extinction spectra in vivo and in the spectra of alcohol extracts during a toxicological experiment with PCG-3. The

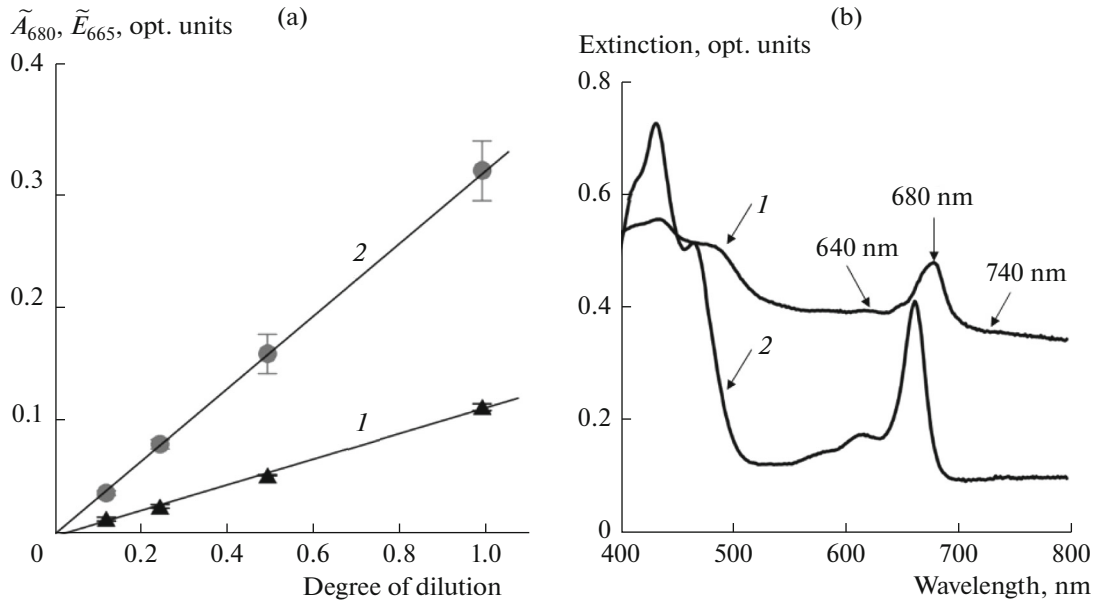


**Fig. 2.** Panel (a): Experimental dependences of chlorophyll light absorption (*1*) in vivo and (*2*) in extracts on the degree of dilution (points) and their linear extrapolations and panel (b) extinction spectra of (*1*) *D. salina* suspension and (*2*) its alcohol extract.

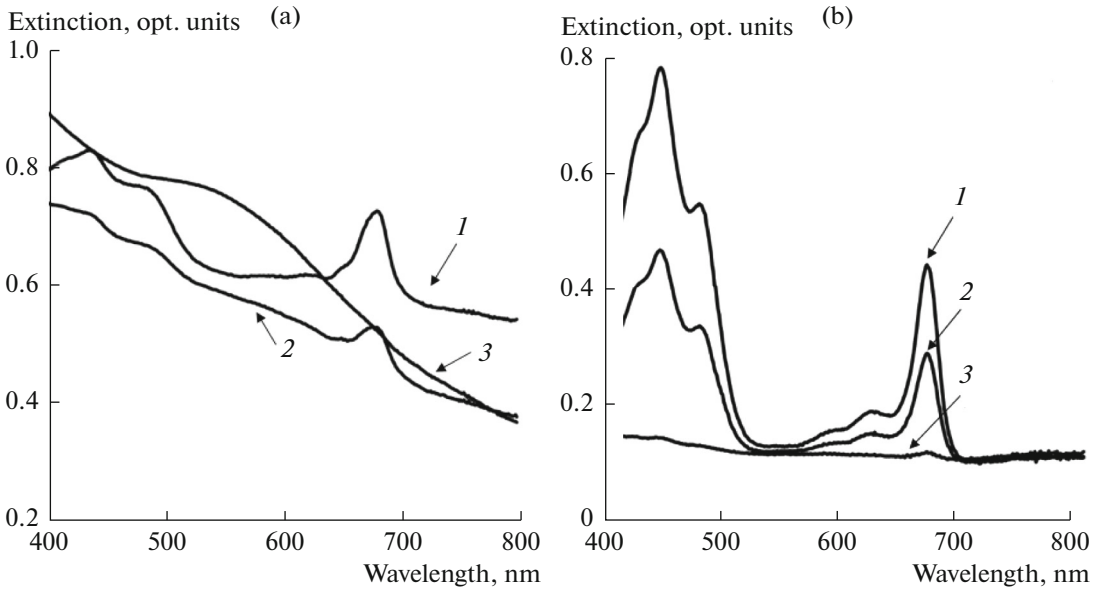


**Fig. 3.** Extinction spectra recorded for (a) *D. salina* suspensions and (b) their alcohol extracts 48 h after mixing with PCG-3 taken in concentrations of (*1*) 0, (*2*) 40, and (*3*) 100 mg Au/L.

presence of gold in a concentration of 100 mg Au/mL causes the complete death of the cells after 48-h exposure with PCG-3, which is evident from the absence of the chlorophyll peak both in vivo and in the extracts. At a concentration of 40 mg Au/mL, the chlorophyll peak intensities in vivo and in the extracts are $50 \pm 7.5$ and $59 \pm 9.7\%$ of the reference, respectively.

A completely different situation is observed for the preparations used in the toxicological experiment with CG-15, the results of which are presented in Fig. 4. CG-15 has no toxic effect. Moreover, as gold concentration increases, a certain stimulation of the growth (hormesis) is observed. When determining the chlorophyll peak height by the proposed protocol in vivo, this effect is substantially stronger than that for the extracts. This is because the spectrum of aggregated CG in the examined region has a nonmonotonic (bell-shaped) character. For the extracts, the peak heights at

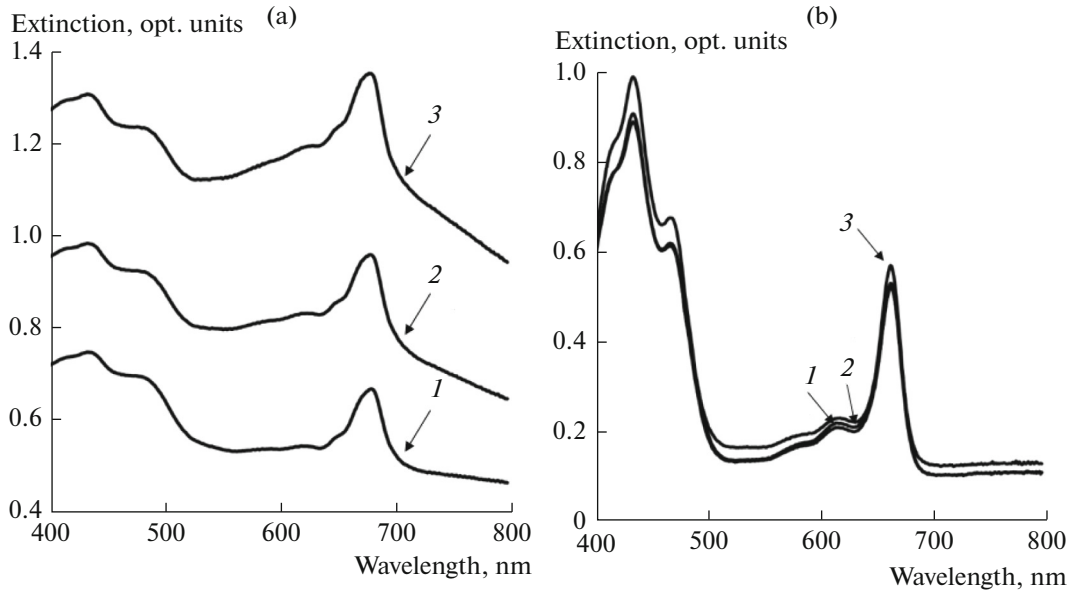


**Fig. 4.** Extinction spectra recorded for (a) *D. salina* suspensions and (b) their alcohol extracts after 48-h exposure with CG-15 taken in concentrations of (*1*) 0, (*2*) 40, and (*3*) 100 mg Au/L.

CG-15 concentrations of 40 and 100 mg Au/mL are $110 \pm 5.4$ and $121 \pm 3.8\%$ of the reference, respectively. Being determined in vivo, the corresponding values are $118 \pm 2.0$ and $142 \pm 5.6\%$ respectively.

## CONCLUSIONS

Changes in the viability of the population in a suspension of microalgae culture are most adequately determined from the height of the peak corresponding to chlorophyll light absorption in the red region $\tilde{A}_{680}$ corrected for the value of nonselective extinction relative to the values of $A_{640}$ and $A_{740}$ nm. The results of the spectrophotometric measurements performed for suspensions of live microalgae with a Spark 10M microplate scanning spectrophotometer have a high correlation (about 0.98) with the spectrophotometric data on the chlorophyll content in alcohol extracts of the same cultures.

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