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ARTICLES

Influence of Carbon Nanotubes on Chlorophyll Fluorescence Parameters of Green Algae *Chlamydomonas reinhardtii*

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Abstract—It has been shown that carbon nanotubes are capable of decreasing the development speed of *Chlamydomonas reinhardtii* algae culture and significantly changing the parameters of chlorophyll fluorescence that characterize the prime processes of light energy storage throughout photosynthesis. The quantum yield reduction of photochemical light energy transformation during photosynthesis and the relative speed of noncycle electron transfer calculated using the fluorescence parameters have been observed. The inhibition of the electrochemical proton gradient involved in ATP synthesis has been determined using delayed fluorescence. A conclusion on the prospects of implementing highly sensitive fluorescent methods for evaluating the toxic effect of modern nonmaterials on water objects is made.

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INTRODUCTION

New carbon-containing materials (nanotubes and fullerenes) are in widespread use in industry [10, 24]. Carbon nanotubes and similar materials have been known since 1985, when the first fullerene, a hollow sphere consisting of 600 carbon atoms, was obtained by graphite evaporation [17]. In further research, cylindrical fullerene forms—nanotubes with controlled sizes and diameters—were obtained. The first products were multiwalled carbon nanotubes with concentric cylinders reaching 10 μm in length and 5–40 nm in diameter. Later, the possibility of obtaining single-walled nanotubes in the presence of a cobalt–nickel catalyst was shown. These fullerene structures possessed high electro- and heat conductivity. Single-walled nanotubes possess a specific tensile strength 460 times higher than steel [18].

Fullerenes and nanotubes can appear completely differently in the environment. In terms of their impact on human health, nanotubes are grouped with other particles with prolate forms due to the awareness that they can act as asbestos [32]. In water systems, carbon nanoparticles can be disposed to precipitation and aggregation due to their hydrophobic properties. This constrains their application in water systems and biomedicine. However, modifying the surfaces of nanotubes can increase the stability of their water suspensions. Covalently joining polyethylene glycol and the noncovalent modification of carbon nanoparticles with phospholipids makes it possible to create stable suspensions of carbon nanoparticles [27, 28]. This modification can also affect their behavior and presence in the environment.

It is expected that, by 2011, the world's production of multiwalled nanotubes will be over 1000 tons [32]. Carbon nanotubes and their derivatives have been used for the production of plastics, catalysts, accumulators and electrodes, fuel elements, water purification systems, orthopedic implants, conductive coatings, and components for electronics. An increase in production will result in the growth of emissions into the environment. Therefore, it is important to evaluate the ecological consequences of the impact that new carbon materials have on living systems in ecological conditions.

Microalgae are the main producers in water reservoirs and are specific ecological targets for various anthropogenic pollutions flowing into water ecosystems [1, 2]. Algae have also been recommended as the one of the most important objects for biotests [5]. It is necessary to choose the most sensitive processes in organisms to see their reaction to toxic effects during biotests. For algae, this is photosynthesis. The advantages of using photosynthesis as a test function are due to its high sensitivity to the effect of many pollutants and the opportunity to use it in biomonitoring methods for measuring chlorophyll fluorescence [8, 30]. The basis of fluorescence methods lies in the ability of chlorophyll located in photosynthetic membranes to serve as a natural detector for the state of the algae cells [4]. Measuring the chlorophyll fluorescence intensity ratio upon photosynthesis saturating the exciting light (Fm) and conditions which do not cause changes in the state of the photosynthetic apparatus (Fo) makes it possible to determine the efficiency of the prime processes of photosynthesis, which equal (Fm – Fo)/Fm =

Fv/Fm. Recently, while working with leaves and algae cultures, methods for quickly measuring the light dependences (light curves) of various fluorescence parameters (which indicate the development of photochemical and nonphotochemical decay under light), have made it possible to register changes in the functionality of a photosynthetic apparatus in light and the effect of environmental factors [31, 34].

One important advantage of fluorescence methods is their expressive and high sensitivity, which makes it possible to quickly diagnose the microalgae cell state, which is affected by toxicants both in cultures and in native phytoplankton directly in the inhabitation environment in situ in real time [4, 23]. Efficiently measuring fluorescence indexes is important for the early identification of pollutants in the environment [1, 3, 8, 30].

There are currently some studies on the influence that nanoparticles have on algae cultures [14, 25]. However, the effect of carbon-containing nanoparticles on the fluorescence parameters of algae has not been studied.

In this work, the alterations of the fluorescence parameters in the cells of *Chlamydomonas reinhardtii* in the presence of carbon nanotubes have been studied. It has been shown that using the light dependences of fluorescence makes it possible to register early changes in the energy-storage photosynthetic processes of algae cells in the presence of these modern materials.

MATERIALS AND METHODS

A culture of *Chlamydomonas reinhardtii* Dang photoheterotrophically grown at 25°C on a tris-acetate-phosphate medium, pH 7.0, [12] and under luminescent 30 μE/(m^2 s) lamps served as the study object. The duration of the light and dark period was 14 and 10 h, respectively. In the initial growth phase, the culture in a concentration of 300000 cells/ml was poured (50 ml) in sterile conditions and added into the substances under study.

Single-walled carbon nanotubes (SW) (average diameter 1.2–1.4 nm) produced by NanoCarbLab company (Moscow) with a purification rate of 80% and multiwalled carbon nanotubes (MW) (average diameter 60–80 nm), kindly provided by S.M. Shebanov from the Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, were used in toxicological tests. No additional purification was carried out. Before the experiment, a water solution of nanotubes in a concentration of 2 mg/ml was dispersed on a UZDN-2T ultrasound disintegrator with a frequency of 22 kHz and maximal power for an hour. Algae were exposed with supplements of nanotubes in conditions similar to cultivation. The quantity of algae was determined by direct calculation in a Goryaev camera using an Axioplan 2 imaging light microscope (Carl Zeiss). An iodine alcohol solution was used for the fixation of cells during their calculation.

The measurements of fluorescence indexes of algae were carried out using a WaterPAM impulse fluorimeter (Walz, Germany). The minimal Fo and maximal (Fm) fluorescence values were registered in dark-adapted samples as well as the maximum quantum yield (Fv/Fm), which characterizes the potential quantum efficiency of photosystem 2 (PS 2). The fast light dependences of various fluorescence parameters on the light were measured at a subsequent increase in intensity from 0 to 400 μE/(m^2 s) [34]. The time of illumination for each intensity was 50 s. It is shown that, in these periods of illumination, the transient processes associated with changes in intensity end and the light dependences are close to those registered during prolonged steady-state illumination. At the end of each period of illumination, the fluorescence yield of chlorophyll F(t) [29] and parameters Fm' were measured using saturating flash (0.8 s, 2000 μE/(m^2 s). Based on all parameters, the quantum yield of photochemical transformations of absorbed light energy in PS 2 was determined as the ratio $Y = (Fm' - Ft)/Fm'$ and the relative speed of noncyclic electron transport in certain light intensity (rETR). The speed of electron transport was calculated according to the formula $rETR = YE_i$, where E_i is the illumination, μE/(m^2 s) [19]. On the basis of the obtained light curves, the following photosynthetic parameters were evaluated: the coefficient of maximal light energy utilization (slope angle of light curve, α), the maximal relative speed of electrons along electron transfer chain ($rETR_{max}$), and the saturation intensity of light (E_H). Parameter α was calculated as the coefficient of linear regression built by points located in the light-limited region of the light curve and $rETR_{max}$ as the average of rETR values located in the light-saturated region [15]. E_H was calculated according to formula $E_H = rETR_{max}/\alpha$ [22, 26]. Abbreviations and the explanation of the photosynthetic parameters are referred to according to standard nomenclature [29].

Induction curves of delayed fluorescence (DF) were measured on a rotor-disk phosphoroscope with light and dark intervals of 16 and 4 s, respectively. Fluorescence was registered for 3.2 ms in 0.4 ms after the termination of red-light illumination (300 μE/(m^2 s) [2].

RESULTS AND DISCUSSION

Algae *Chlamydomonas reinhardtii* are motile cells with flagella. It was observed in a light microscope that, after colliding with tubes, algae cells slowed down; changed trajectory; or, after few turns, lost motility, creating aggregates which consisted of cells and tubes. The accumulation of cells on the eighth day of growth in the region of tubes after their fixation can be seen in Fig. 1.

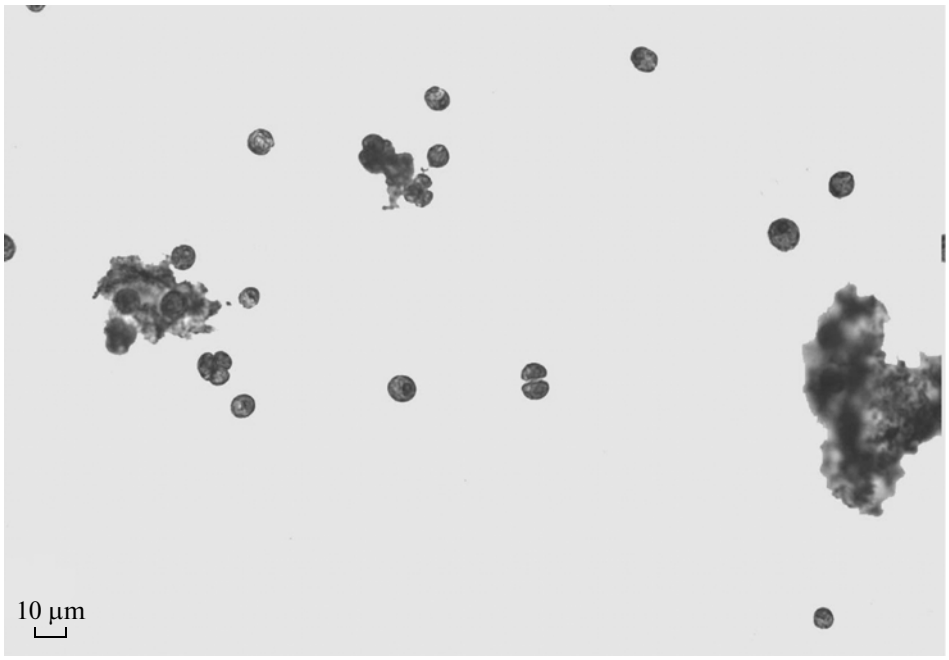

Fig. 1. Aggregates created from *C. reinhardtii* cells and SW carbon tubes in a concentration of 2 μg/ml on day 8 of growth.

Alterations in the concentration of *Chlamydomonas reinhardtii* cells during growth in control conditions and after the addition of nanotubes have been shown in Fig. 2. The growth in the cell amount was observed in a control culture. The effect from tubes reduced the growth speed, especially for SW tubes. A diminished growth speed of *Chlamydomonas reinhardtii* cells, which was affected by carbon-fullerene nanoparticles, was found in research [20].

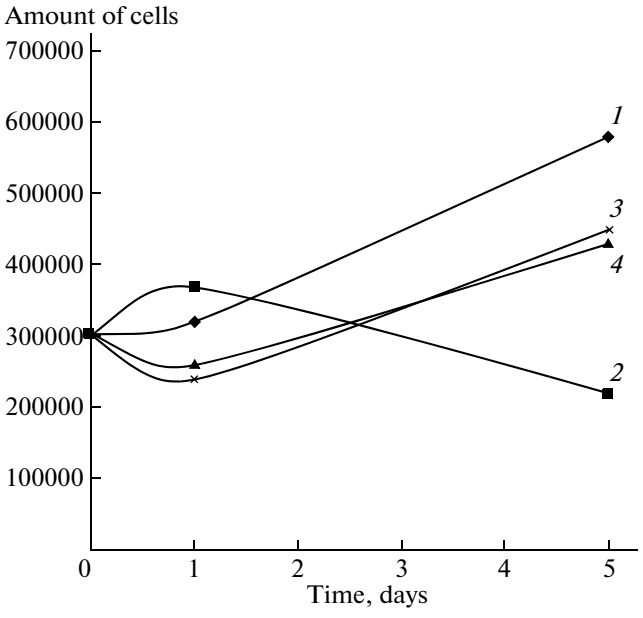

Fig. 2. Alterations of concentrations of *C. reinhardtii* cells during growth. (*1*) Control; (*2*) after the addition of SW tubes in a concentration of 2 μg/ml; and (*3*, *4*) MW carbon nanotubes in concentrations of 1 and 2 μg/ml, respectively.

Similar dependences were observed when the level of constant fluorescence Fo was altered, which, with a high correlation, corresponds to the total amount of pigments in algae cells [23] that collect light energy (data not shown).

The almost total algae disappearance was observed after incubation for a month in flasks containing cells with carbon nanotubes. The records of absorption specters for these suspensions are shown in Fig. 3. It can be seen that there is no absorption maximums for chlorophyll at 670–690 nm in samples with nanotubes.

It has been found on *Chlamydomonas reinhardtii* algae that nanotubes also influence the photosynthetic apparatus. It is known that the value Fv/Fm characterizes the content of photochemically active centers of PS 2 and indicates the current balance between processes of light destruction and the reparation of PS 2 [8]. PS 2 is related with the processes of water destruction and oxygen release. A high constant value of Fv/Fm (0.73) was kept for 8 days. In contrast to growth processes, the value of Fv/Fm was considerably affected by MW tubes. At a concentration of 2 μg/ml, they caused the decrease of the Fv/Fm value from 0.73 to 0.57 within 24 h of incubation (table). SW tubes insignificantly reduced Fv/Fm.

It is important to note that, during the prolonged incubation of algae with tubes, the dynamics of Fv/Fm was phase-dependent. The first activity decay of Fv/Fm terminated usually 2–3 days into the exper-

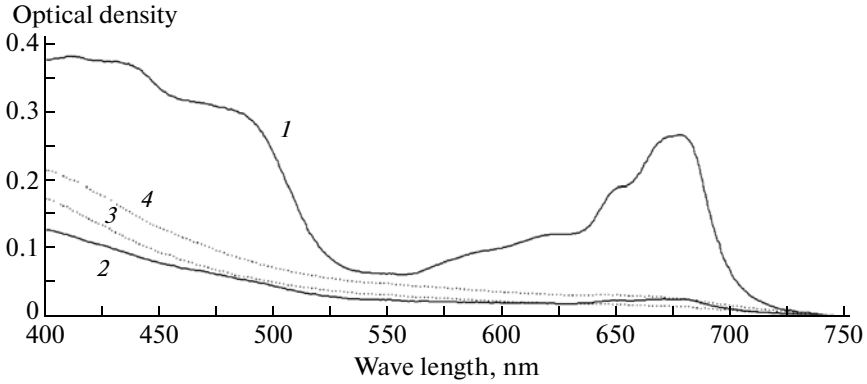

Fig. 3. Absorption specters of suspension after the 28-day-incubation of *C. reinhardtii* cells with carbon nanotubes. (*1*) Control; (*2*) after the addition of SW tubes in a concentration of 2 μg/ml; and after the addition of (*3*, *4*) MW carbon nanotubes in concentrations of 1 and 2 μg/ml, respectively.

iment, which was followed by its reduction on day 8 and further decay. This complies with early established common regularities of the development of the toxic effect, which include the phase interchange of suppression and reduction phases of the physiological-state indexes of various organisms during toxicological experiments [6]. A similar effect was found early in our experiments with methyl mercury. Methyl mercury in a concentration of 10^{-7} M caused a decrease in the Fv/Fm value within 24 h of incubation, followed by total recovery until the initial level showed that the cell had adapted to the toxicant [1].

Since the greatest changes in fluorescence parameters Fv/Fm affected by nanotubes were observed after exposition for 1 day, when the adaptation processes had still not developed, studies on the light dependences of fluorescence parameters were carried out (for the conditions of increasing the light loading, see the protocol). Currently, measuring light-curves is widely used in ecological–physiological investigations of photosynthesis [13, 31]. A quantum yield of photochemical fluorescence transformations of absorbed light energy in PS 2 on the light is determined as the equation Y = (Fm'/Ft)/Fm' [11]. The values of this parameter vary from 0.75 (the object adapted to the light) to values close to zero (in the case of photosynthesis failure). In an object adapted to the dark, the value of Y is Fv/Fm, which is the potential quantum yield of PS 2 photochemistry. It was shown previously that, when the illumination of plants and algae increases, the photochemical quenching and quantum yield decrease in PS 2 (Y) due to the total inhibition of electron transport as a result of limitation by the quick dark photosynthesis processes and a rise in nonphotochemical quenching [29]. In our experiments, an increase in the illumination of algae cells in the control resulted in a decrease in the quantum yield of photochemistry in PS 2 (Y) (Fig. 4). The decrease in this parameter on light intensity significantly accelerated upon incubation with nanotubes, especially in experiments with MW tubes. Light intensity, when the value of Y reduced by half ($E_{Y1/2}$, μE/(m^2 s), was 166 μE/(m^2 s) for cells in the control, while, for the cells incubated with MW tubes, this value decreased to 128 μE/(m^2 s) (table).

Alterations of the light dependences of fluorescence parameters of *C. reinhardtii* cells in control and after the addition of nanotubes (1 day incubation)

Fluorescence parameters	Control	Tubes SW (2 μg/ml)	Tubes MW (1 μg/ml)	Tubes MW (2 μg/ml)
Fv/Fm	0.73 ± 0.001	0.70 ± 0.01	0.68 ± 0.01	0.57 ± 0.03
Y (at 400 μE/(m^2 s))	0.17 ± 0.02	0.15 ± 0.07	0.12 ± 0.04	0.11 ± 0.03
$E_{Y1/2}$, μE/(m^2 s)	166 ± 8.5	161 ± 9.1	121 ± 7.6	128 ± 6.2
$rETR_{(max.)}$, relative units	23.8 ± 1.7	22.7 ± 1.3	20.6 ± 1.5	17.9 ± 0.9
α	0.23 ± 0.02	0.22 ± 0.01	0.168 ± 0.01	0.149 ± 0.03
E_n, μE/(m^2 s)	120.2 ± 14.2	124.1 ± 13.5	137.9 ± 10.5	136.3 ± 14.5

Note: Fv/Fm are parameters of samples in the dark, Y = (Fm' – Ft)/Fm' is the photochemical activity of PS 2, and $E_{Y1/2}$ is the light intensity of half-recession for value Y. Parameters that describe the dependence of electron transfer (rETR) on illumination (light curves) are the coefficient of maximal light energy utilization, the slope angle of light curve (α), the maximal relative speed of noncyclic electron transfer ($rETR_{max}$), and saturating light intensity (E_H).

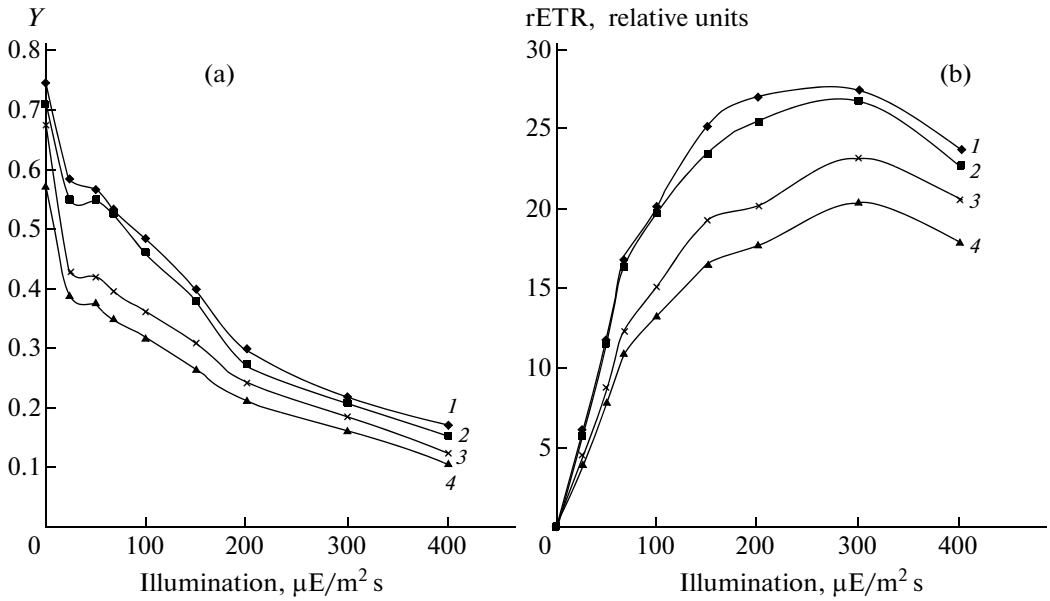

Fig. 4. Alterations of fluorescence parameters depending on light intensity in *C. reinhardtii* cell suspension affected by SW and MW carbon nanotubes. Incubation period is 1 day. (a) Quantum yield of photochemical transformation of absorbed light energy in PS 2 as the equation Y = (Fm' – Ft)/Fm' and (b) relative speed of noncyclic electron transport. (*1*) Control; after the addition of (*2*) SW and (*3*, *4*) MW carbon nanotubes in concentrations of 1 and 2 μg/ml, respectively.

Y is the parameter that characterizes the effective quantum yield of energy transformation in PS 2 and can serve to evaluate the value of the real quantum yield of electron transport on the light. A linear correlation between Y and the quantum yield of carbonic gas fixation was shown on leaves [11]. Based on Y, the relative speed of noncyclic electron transport (rETR) can be calculated by multiplying the illumination intensity on coefficient Y. In order to calculate noncyclic electron transport in absolute values, it is necessary to determine the amount of absorbed quanta that was not undertaken in the present research. The light dependences for algae rETR in the presence of nanotubes are shown in Fig. 4b. The table indicates the parameters describing these dependences of photosynthetic activity on illumination (light curves): the coefficient of maximal light energy utilization, the slope angle on the linear region of light curve (α), the maximal relative speed of electrons along the electron transport chain ($rETR_{max}$), and the saturating light intensity (E_H). The maximal relative speed of electrons along the electron transport chain ($rETR_{max}$) was greatest for the algae in the control. The parameters of P/E curves changed insignificantly after incubation with SW tubes for 1 day. Deviations were observed in light curves after treatment by MW tubes. The value of $rETR_{max}$ decreased after treatment with MW tubes. The coefficient of maximal light energy utilization (α) was higher in the control algae and decreased after treatment with MW tubes. Saturating light intensity (E_H) for algae in the control was 120 μE/(m² s). For cells treated by MW tubes in concentrations of 1 and 2 μg/ml, these values were 137 and 136 μE/(m² s), respectively.

Nanotubes can affect the ability of photosynthetic membranes to create electrochemical gradient of protons. Induction curves of delayed fluorescence (DF) serve as the information source describing this process [7]. This phenomenon is as follows: after light excitation in photosynthetic cells, the weak long-term fading luminescence emitted by chlorophyll is observed. This luminescence appears after the termination of fluorescence due to energy being emitted during dark reactions upon the reverse electron transfer in RC and their recombination with chlorophyll and the regeneration of exciting state. As a result, delayed luminescence in the cells is emitted with a time delay. It has been shown that the intension of DF increases due to the energy of the transmembrane electro-chemical proton gradient on the photosynthetic membranes necessary for the synthesis of ATP molecules [2]. Phosphorylation uncoupling agents, which decrease the proton gradient, thus decrease DF intension [2, 7]. This fact makes it possible to use the DF method to evaluate the failure of energy processes in photosynthetic membranes affected by various toxicants. A study of the alteration of the millisecond-delayed fluorescence parameters of chlorophyll has shown that, in the presence of nanotubes, the amplitude of the rapid phase of the DF induction curve is related to the electric potential on a membrane; then the slow phase, which is due to the creation of an electrochemical proton gradient on a membrane, decreased (Fig. 5). These data indicate the ability of carbon tubes to dam-

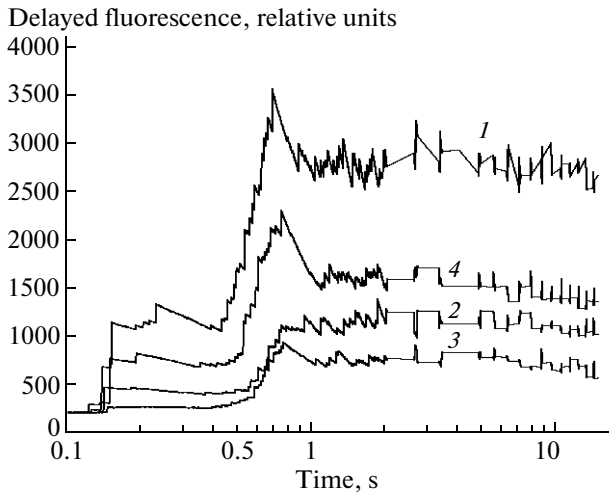

Fig. 5. Alterations of induction curves of delayed fluorescence of *C. reinhardtii* cells affected by SW and MW carbon nanotubes. Incubation period is 1 day. (*1*) Control; after the addition of (*2*) SW and (*3*, *4*) MW carbon nanotubes in concentrations of 1 and 2 μg/ml, respectively.

age membranes and thus decrease energy-storing processes in cell membranes.

CONCLUSIONS

These studies have shown that carbon nanotubes can negatively affect microalgae *C. reinhardtii*. These data agree with the research data in [6], where the influence of carbon fullerene nanoparticles on these algae (which is related to a decrease in cell growth) has been demonstrated. In our experiments, carbon nanotubes also have a decreased speed of cell growth in the diapason, reaching 5 days. In the case of incubation for 1 month, no algae cells in probes containing nanotubes were found.

Studies have indicated the effect of nanotubes, especially MW tubes, on photosynthetic processes in *C. reinhardtii* algae. After 1 day of incubation, MW tubes decreased the quantum yield of the photochemical transformation of absorbed light energy in PS 2 and the relative speed of noncyclic electron transport. Nanotubes affected photosynthetic membranes and thus decreased the electrochemical proton gradient on the membrane involved in the process of ATP synthesis.

A study of the light dependences of fluorescence parameters has shown that the greatest inhibition of the PS2 quantum-yield efficiency under the effect of MW tubes was observed at 30–100 $\mu E/(m^2\ s)$. These data show the influence of the light factor on the development of the toxic effect supported by the effect of nanotubes. Similar effects for the light sensitivity of algae-cell enhancement have been shown earlier for other pollutants [1].

It is difficult to make conclusions on the ways nanotubes influence photosynthesis processes. Perhaps the nanotubes result in cell-wall damage, as well other cell membranes, which leads to the failure of algae cell metabolism. Particularly, fluorescent and microscope analyses have shown that the direct contact of carbon nanotubes with *E. coli* cells results in their death; their external membranes break and their intercellular content flows out in to the environment [16]. The cytotoxic and antibacterial effect of spherical carbon nanoclusters of C_{60} fullerenes found in some works probably come from the physical damage of the cell membrane, and it is not the generation of active oxygen species that causes cell death [21].

Some researchers suppose that the negative effect does not come from carbon nanotubes, but from the addition of the catalysts that are used in their production process [33]. It should be noted that the effect of MW tubes on algae in our experiments is probably because insufficiently purified commercial nanotubes were used. At the same time, it was demonstrated in experiments with bacteria that highly refined nanotubes can show bactericide properties [16].

It also should be noted that highly refined carbon tubes are very expensive. Therefore, cheap and insufficiently refined carbon materials are widely used in practice. Thus, it is important to evaluate the influence of such nanomaterials on the environment. Microalgae, which use the sun's energy for the synthesis of organic compounds, are the main energy source in water systems. They are also nutrients for other organisms, like zooplankton, which is food for larger water organisms. Thus, phytoplankton can be the first stage of nanoparticle accumulation in the food chains of water ecosystems. Therefore, it is necessary to study their influence on microalgae for the bioindication of nanomaterials.

There is the opinion that, in order to evaluate the risk related to nanoparticles, the existing normative tests of the ecotoxicity of chemical compounds can be applied. In Russia, there are some approved methods to test pollutions using microalgae which are collected and described in special handbooks and manuals [3, 5]. These methods of biotests are used to evaluate water pollution by various agents or the norms of the permitted loadings in water systems.

However these methods are based on an analysis of the growth-speed alteration of algae affected by toxicants. The data obtained in this study have shown the advantage of registering chlorophyll fluorescence for the identification of the early toxic effect of various carbon nanotubes. These methods can be used to evaluate photosynthesis, which is the most important process in the algae cell. Registering fluorescence with various light intensities makes it possible to increase the method sensitivity in order to learn the damaging effect of nanomaterials. It should be noted that fluorescence equipment for bioindication can be included in the system of automated operative control for the appearance of toxic nanomaterials in a water environment.

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