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EXPERIMENTAL ARTICLES

Increased Toxic Effect of Methylmercury on *Chlorella vulgaris* under High Light and Cold Stress Conditions

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Abstract—The toxic effect of methylmercury on the photosynthetic activity of *Chlorella vulgaris* was shown to increase under high illumination and unfavorable low temperature. Increased toxic action of methylmercury resulted from the decreased capacity of photosystem II (PS II) for reparation. It was proposed that mild stress conditions might be used to enhance the detection limit of toxicants by microalgae used as test objects.

Key words: Chlorella vulgaris, methylmercury, chlorophyll fluorescence, biological testing, ecology.

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For the tasks of monitoring aquatic environments, the methods are required enabling diagnostics at early stages of anthropogenic influences, before visible abnormality appear in the system. Biotesting as a method of water toxicology is usually used for regulating the toxic pollution of the aquatic environments or for developing the norms of permissible loads on aquatic ecosystems [1]. Fertility or survivability of various organisms in chronic tests are the main criteria of toxicity [2]. The long duration and technical complexity of chronic biotests make them possible only on the basis of specialized toxicological laboratories. Biotesting is acquiring great importance due to the necessity for organizing the systems of operative control of the quality of natural waters and sewage toxicity at enterprises. The development of methods using organisms as universal indicators of the quality of aquatic environments and enabling us to reveal the fact of toxic pollution is presently an independent line of research [1–5].

One of the main requirements for operative biotests is their high sensitivity to toxicants of different nature. In order to increase the biotest sensitivity, firstly, the organisms least resistant to toxicants are searched for. These are primarily small plant organisms, microalgae, which are the main producers in reservoirs and unique ecological targets for toxicants ending up in the aquatic ecosystems [6, 7]. The second step is to determine the processes which are most sensitive to the toxic effect. For algae, it is primarily photosynthesis. The advantages of using photosynthesis as a test function are determined by its high sensitivity to the action of pollutants, because, when metabolism is inhibited by toxicants, excessive light energy not used in the photosynthetic reactions results in the formation of reactive oxygen species with the resultant photodestruction of chlorophyll and cell structures [8, 9].

The third important direction, though not yet properly developed, may be the study of the effect of incubation conditions, including the application, along with the toxic load, of an additional physiological load not exceeding the tolerance limits of the test object. The importance of the studies of the effect of toxicants under additional physiological loads is also determined by the fact that, under natural conditions, the organisms almost always experience stress caused by various environmental factors [2, 4].

The methods based on the measurement of chlorophyll fluorescence are promising for biomonitoring the effect of toxicants on algae [6, 7, 10–13]. These methods are based on the fact that chlorophyll (located in the photosynthetic membranes) serves as a kind of a natural sensor of the state of algal cells [14]. The energy of light quanta absorbed by the light-harvesting complex may either be converted to the energy of separated charges to be used in further photosynthetic reactions or lost by emitting fluorescence quanta or by dissipation in the form of heat [15]. The measurement of the ratio of the intensity of chlorophyll fluorescence under photosynthesis-saturating excitation light (*Fm*) and the conditions not causing any changes in the state of the photosynthetic apparatus (*Fo*) allows determination of the effectiveness of the primary photosynthetic processes equal to $(Fm–Fo)/Fm = Fv/Fm$. The effectiveness of the primary photosynthetic processes (Fv/Fm) is a dimensionless energy characteristic of photosynthesis similar to the coefficient of efficiency and independent on the species specifics of the organism. The maximal, theoretically possible light utilization effectiveness value in photosynthesis (Fv/Fm) is 0.8; the minimal value is 0. The close-to-zero utilization efficiency indi-

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cates inhibition of the processes of photosynthesis. Importantly, the fluorescence methods are time-saving and highly sensitive, which permits rapid diagnostics of the state of microalgal cells influenced by toxicants directly in the environment in situ in the real-time operation mode [14, 16]. Efficient measurement of the fluorescence parameters is of special importance for early detection of the presence of pollutants in the medium and for predicting the processes of intense algal development (blooming).

Due to their high toxicity, as well as the ability to accumulate in the organisms and to be transferred along the trophic chain, the salts of heavy metals occupy a special position among environmental pollutants [4]. Among heavy metals, mercury compounds, especially organic ones, are most toxic, methylmercury chloride in particular [17]. Algae were shown to be highly sensitive to the action of methylmercury manifesting itself in changes in the quick fluorescence parameters [17, 18].

The goal of this work was to apply the fluorescence methods to the study of the effect of methylmercury on the microalga *Chlorella vulgaris* depending on the cultivation conditions. It was shown that resistance of the alga to the action of a toxicant sharply decreases under additional high light and low temperature stress. It was proposed that additional stress conditions could be used for algal test systems to increase the sensitivity of biotesting methods.

MATERIALS AND METHODS

The experiments were carried out using the unicellular green alga *Chlorella vulgaris* (Beijer) S-39/64688 grown in Uspenskii medium [11] in cultivators at 28°C in the case of high light stress and at 35°C in the case of low temperature stress. The cultures were illuminated with daylight fluorescent lamps at 30 μE m^{-2} s^{-1} in the region of photosynthetically active radiation (PAR). Before the experiments, the alga was cultivated for 24 h. The initial culture density was 3×10^5 cells/ml. For the experiments, the exponential-phase *C. vulgaris* cells were collected by centrifugation (5000 *g*, 15 min). In the low temperature stress experiments, the alga was grown at 35°C, with gradually lowering it to 15°C.

The chlorophyll fluorescence parameters in the algal suspension were measured on a pulsed fluorometer developed at the Department of Biophysics, the Faculty of Biology, Moscow State University, and intended for measuring highly diluted microalgal suspensions. Some experiments were made using a ToksiRAM laboratory fluorometer (Walz GmbH, Germany) [12]. The relative variable fluorescence yield characterizing the efficiency of photosynthetic light reactions was calculated as *Fv*/*Fm*.

For the photoinhibition of photosynthesis, the algal cells were illuminated by a KGM 150/24 halogen lamp. Irradiation was carried out in a 6-mm thick, 5-ml quarts cuvette. The quantum flow density in the PAR region was measured with the Walz quantometer (Germany).

The photosystem II (PS II) photoinhibition (inactivation) rate and the PS II recovery (reactivation) rate constants (K_i and K_r, respectively) were calculated by nonlinear regression analysis from the changes in the fluorescence *Fv* using the equations given in [20]. Prior to determination of the PS II constant of the photoinhibition rate (K_i), the synthesis of chloroplast proteins was blocked with chloramphenicol (1 g/l); on addition of methylmercury, the algae were incubated at the illumination intensity of 90 μE m^{-2} s^{-1}. In the course of incubation, samples for *Fv* measurement were taken. Based on the data obtained, the K_i value was determined using the following equation:

$$Fv(t) = Fv(0) \exp(-K_i \cdot t), \quad (1)$$

where $Fv(t)$ and $Fv(0)$ are variable fluorescence intensities at the time moments t and $t = 0$.

To determine the PS II constant of the reactivation rate (K_r)), the alga were incubated for 4 h in the presence of different methylmercury concentrations, illuminated for 5 min with intense white light (900 μE m^{-2} s^{-1}), and then incubated in the shade at low illumination (2 μE m^{-2} s^{-1}). Such illumination is required for the normal process of recovery [7, 9, 20]. In the process of incubation, the fluorescence parameters were periodically recorded. Based on the values obtained, K_r and Fv_{max} were calculated using the following equation:

$$Fv(t) = Fv_{max} - (Fv_{max} - Fv(0)) \exp[-(K_r + K_i) \cdot t], \quad (2)$$

where $Fv(t)$, $Fv(0)$, and Fv_{max} are the values of variable fluorescence immediately after illuminating with light at 900 μE m^{-2} s^{-1}, at the time moments t and $t \longrightarrow \infty$, respectively. The values calculated earlier according to the formula (1) for each specific toxicant concentration and a light intensity of 2 μE m^{-2} s^{-1} were substituted for K_i. For the dark reactivation conditions, the zero K_i value was accepted. The K_i and K_r calculation error did not exceed 5%.

In the toxicological experiments, the methylmercury preparation (Aldrich Chemical Company Inc., United States) was used. Methylmercury is formed as a result of mercuration of organic compounds and is an extremely toxic substance [2]. The algae were incubated from several hours to 3 days at different methylmercury concentrations under the same conditions as those for culture growing. The cell concentration before the addition of the preparations was 3×10^5 cells/ml.

The experiments were made in two or three replicates. The results were processed using the statistical methods according to Fisher.

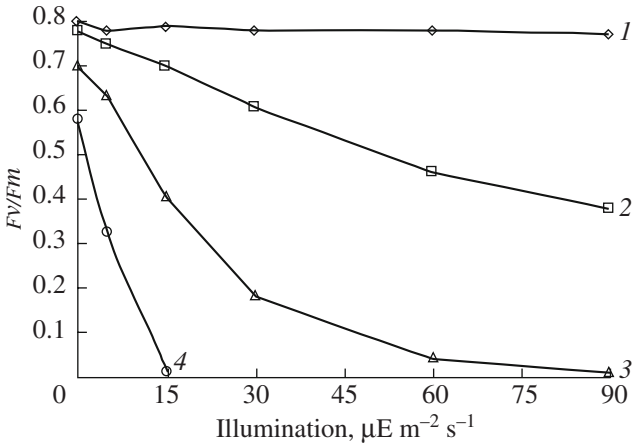

Fig. 1. Inhibition of relative variable fluorescence *Fv/Fm* in *C. vulgaris* depending on illumination at the following methylmercury concentrations: 0 (*1*); 5 μg/l (*2*); 15 μg/l (*3*); 25 μg/l (*4*). The time of incubation with the toxicant is 4 h.

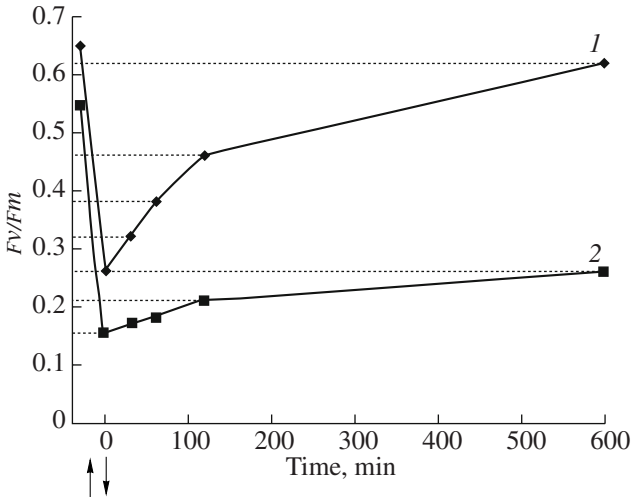

Fig. 2. Changes in the fluorescence parameters (*Fv/Fm*) of *C. vulgaris* after 30-min photoinhibition by intense light (800 μE m^{-2} s^{-1}) with the subsequent recovery upon darkening (2 μE m^{-2} s^{-1}). Control (*1*); methylmercury (15 μg/l) (*2*). The arrows directed up and down show the moments when intense illumination was switched on and off. The light was switched on 4 h after the addition of the methylmercury salts to the exponential-phase culture.

RESULTS

The composition of the nutrient medium determines the form in which many toxicants, especially heavy metals, are present in solution and, hence, their toxicity; it is therefore of vital importance for testing. The studies of many authors showed that in complex media with chelating agents and phosphate buffers, the heavy metal ions are converted to inactive forms of lower toxicity [19]. *C. vulgaris* was therefore grown in cultivators (100 ml) in the medium without phosphates and EDTA; these compounds are capable of binding the salts of heavy metals. We showed in the preliminary experiments that the cultivation of *C. vulgaris* in a similar medium for several days does not result in significant changes in the physiological state of the cells. The advantages of using the *C. vulgaris* culture in toxicological experiments are due to its high growth rate, which makes it possible to obtain large amounts of the biological material and to determine the effect of toxicants on the cells of different physiological age at the main growth phases.

The energy of light determines the rate of photosynthesis, the growth and development of the algae. At the same time, the action of high light intensity may trigger long-lasting and serious rearrangements of the photosynthetic apparatus, which reflect the development of the process of photosynthesis photoinhibition and the actuation of the protective processes of radiation-free dissipation of light energy [9, 22]. So far, the molecular mechanisms of the initial stages of photoinhibition have not been completely understood [23]. Formation of the twice reduced quinone *Qa* [24], which leaves its binding site in PS II, is believed to be the primary act of PS II impairment in the course of photoinhibition. Under aerobic conditions, proteolytic breakdown of the D1 32 kDa protein participating in the PS II reaction center and encoded by the chloroplast gene *psb A* is the next stage. For PS II reactivation, the resynthesis of protein D1 and its incorporation into the PS are necessary [9, 25, 26]. Since the processes of photoinhibition and reactivation in the light proceed simultaneously [20], the total PS II activity may be determined as the ratio of the constants of photoinhibition and activity recovery (reparation). At present, numerous data indicate that unfavorable factors, such as low or high temperatures and the deficiency of mineral nutrients, may considerably increase photoinhibition which is revealed as the *Fv*/*Fm* decrease [23, 27]. It was noted that the sensitivity of the microalgae to copper salts varies significantly, depending on the intensity of illumination [7, 28]. It was shown on the marine diatom *Thalassiosira weissflogii* that the processes of recovery after photoinhibition are inhibited by the action of the mercury compounds [29].

In our experiments, when algae were incubated in the dark, low methylmercury concentrations (1–15 μg/l) had almost no effect on the *Fv*/*Fm* value after 4-h exposure to a toxicant. However, increased illumination during the incubation period resulted in a significantly enhanced effect of the toxicants (Fig. 1). In the light, a decrease in the relative yield of variable fluorescence in the toxicant-treated algae resulted from *Fm* decrease with the slightly changed *Fo* yield, as is normally observed in photoinhibition under aerobic conditions [9].

Figure 2 shows changes in the *Fv*/*Fm* value in the *C. vulgaris* culture under intense illumination

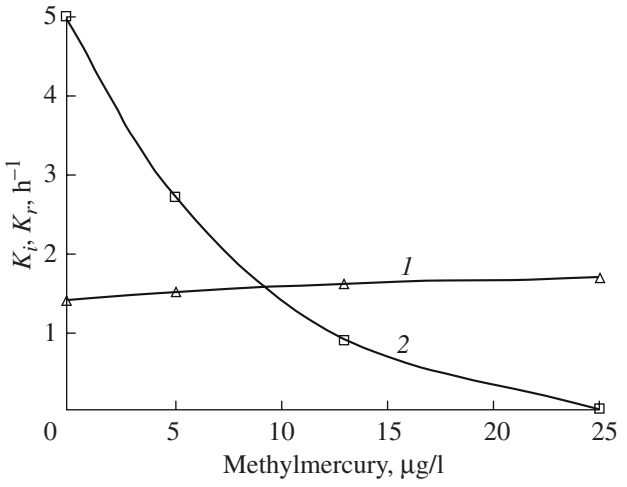

Fig. 3. Photoinhibition rate constant (K_i, *1*) and the reparation rate constant (K_r, *2*) in *C. vulgaris* depending on the methylmercury concentration. The time of incubation with toxicant is 4 h.

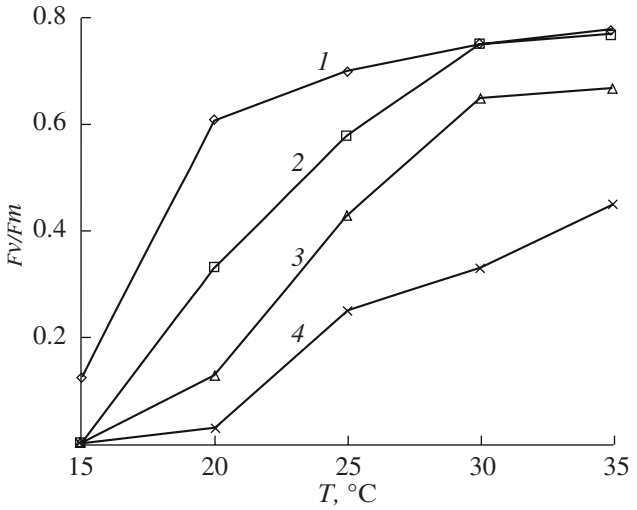

Fig. 4. Relationship between the effect of methylmercury salts on the fluorescence parameter (*Fv/Fm*) of *C. vulgaris* and the incubation temperature. The *Fv/Fm* parameter values were recorded after 4 h of incubation with methylmercury at the preassigned temperature: control (*1*); 2.5 μg/l (*2*); 5 νg/l (*3*); 15 μg/l (*4*). The intensity of illumination was 30 μE m^{-2} s^{-1}.

(800 μE m^{-2} s^{-1}) followed by the incubation under shading conditions (2 μE m^{-2} s^{-1}). As seen from the figure, the recovery rate of the *Fv/Fm* value in the dark slowed down significantly in the methylmercury-treated samples compared to the control sample (without toxicants). This result gives evidence of the inhibition by mercury of the process of reparation of the light-impaired PS II centers in *C. vulgaris* cells.

The influence of mercury salts on the photoinhibition constant K_i and the reparation reaction constant K_r was analyzed (Fig. 3) according to the scheme described in the Materials and Methods section. It is seen from the figure that under intense illumination, methylmercury had an insignificant effect on the photoinhibition constant, although it inhibited the processes of activity recovery after photoinhibition (K_r) when the algae were placed in the dark. The data shown indicate the role of light as an active damaging factor causing PS II inactivation in the toxicant-treated microalga. The inhibitory effect of low toxicant concentrations of reparation of the light-impaired PS II centers related to resynthesis of the reaction center (RC) proteins [9] possibly shifts the balance between the RC protein synthesis and destruction towards the latter, which is reflected in a decreasing *Fv/Fm* ratio, indicating impairment of the primary photosynthetic reactions. The rate of alteration due to intoxication increases with illumination, which is determined by the K_i increase.

The effect of increased sensitivity of the algae under additional high-illumination stress may be used for reliable detection of relatively low toxicant concentrations in the medium. The light intensities to which the algae were preliminarily adapted should be taken into account. Our results demonstrated that under increased illumination intensity, a much more pronounced effect of methylmercury was obtained in the algae preliminarily grown under low illumination, than in the algae already adapted to a highly light intensity.

The effect of increased sensitivity of the algae to toxicants was also observed under unfavorable temperature conditions. A temperature decrease is known to slow down the processes of reparation of the RC proteins in thermophilic plants, which results in enhanced photoinhibition [20]. At decreased temperatures, the photoinhibition of photosynthetic reactions in plants may be observed even under moderate illumination [27].

We studied the sensitivity of the algae to methylmercury at decreased temperatures from 35°C (optimum) to 15°C under normal illumination conditions (30 μE m^{-2} s^{-1}). The low temperature stress was created by a rapid decrease in temperature (for 10–15 min); different concentrations of the toxicant were then applied. The parameters of chlorophyll fluorescence were measured after 4-h incubation with methylmercury at the preassigned temperature. The effect of methylmercury on the algae (4-h exposure) at different temperatures is shown in Fig. 4. The temperatures up to 20°C were within the limits of tolerance for this *C. vulgaris* strain and did not induce considerable inhibition of the *Fv/Fm* ratio in the control without the toxicant. However, the tolerance of the algae to methylmercury decreased sharply at low temperatures. Under these conditions, the presence of even relatively low methylmercury concentrations (2.5–5 μg/l) led to a *Fv/Fm* decrease. The probit analysis [3] showed that the methylmercury concentrations semi-inhibiting the algal activity at 35 (the

optimum temperature) and 15°C (stress) were 15 and 5 μg/l, respectively.

It was previously noted that the K_i/K_r value in chlorella cultures increased at decreased growth temperatures due to the K_r decrease with no considerable changes in K_i [30]. Our experiments (the data are not given) showed that addition of mercury salts to such algae further decreased the reparation processes, i.e., synergically decreased K_r, as was evident from a significant inhibition of the *Fv*/*Fm* value. This seems to be a possible cause of an increased degree of *Fv*/*Fm* inhibition by methylmercury under the conditions of low temperature stress.

DISCUSSION

The results of this work show that photoinhibition of the PS II activity plays a major part in the changes of the sensitivity of microalgal photosynthesis to low methylmercury concentrations under illumination. PS II operates under extremely stringent conditions, with a high oxidation potential and production of oxygen radicals due to water oxidation [26]; it certainly requires a system of activity regulation. The process of photoinhibition is one of the ways to achieve such regulation aimed at rapid replacement of the failing elements under unfavorable conditions, including, apparently, the action of toxic substances. A change in the performance of the PS II reaction center during photoinhibition is caused by the changes in the structural organization, its photosynthetic activity, and relevant changes. The latter is achieved by changes in the rate of biosynthesis of the chloroplast proteins involved in this center. The data available in the literature show that this possibly applies to the D1 protein of the reaction center which has a high rate of renewal in the light [9, 25]. The PS II inactivation upon photoinhibition is known to be accompanied by protein D1 disassembly, and the recovery of photosynthetic activity after photoinhibition results from protein D1 resynthesis and incorporation of the new protein at the site of the disassembled one [9, 22]. Our results showed that a decrease in the activity of PS II in microalgae at low methylmercury concentrations is determined by a decrease in the rate of reparation of the photosynthetic system, supposedly due to the inhibition of protein synthesis in the chloroplasts. A decrease in the amount of the PS II reaction center protein (protein D1) in the presence of chromium salts in the light was shown in [31]. A change in the ratio of the rate constants of the processes of light-induced damage and recovery of the PS II RC activity observed in the presence of toxicants may cause a decrease in the PS II activity even at physiological intensities of illumination. This is the reason for a sharp increase in the light sensitivity of microalgae in the presence of pollutants. The ability of microalgae to get adapted to unfavorable conditions, including low toxicant concentrations, also depends substantially on the activity of biosynthetic reactions. Thus, the intensity of damage to the PS II RC under inhibition by toxins increases with illumination. Hence, sufficiently intense illumination, which does not cause photoinhibition by itself (in the control variant) is a necessary factor for detecting the effect of pollutants on the algae.

Our data showed that the intensity of damage to the PS II RC by methylmercury increases under cold stress at low temperatures. The difference in the influence of high light and temperature stress on the microalgal PS II is due to the fact that, under cold stress, the K_i/K_r ratio increases at the expense of the K_r decrease; in high light stress, at the expense of the K_i increase [30]. The effect of methylmercury on K_r increases still further the K_i/K_r ratio against the background of these unfavorable factors. Accordingly, a decrease in the activity of PS II is revealed at lower pollutant concentrations.

The specific features of the action of methylmercury under stress conditions indicate an increasing danger of pollution in natural ecosystems, in which rapid changes in environmental factors usually occur, primarily of illumination level in surface waters. Preliminary studies of the Lake Baikal vernal phytoplankton supported this conclusion. Thus, the average copper concentrations resulting in semi-inhibition of the phytoplankton *Fv*/*Fm* value (in 6-h experiments) under the optimum (9 μE m^{-2} s^{-1}) and stress (50 μE m^{-2} s^{-1}) illumination were 20 and 6.3 μg/l, respectively.

The experiments also show that for the results of the toxicological experiments to be standardized, strict control is necessary over all the conditions of cultivation and test-incubating the algae, because they determine, to a considerable degree, the sensitivity of the objects to toxicants. For rapid and reliable detection of the toxic action of heavy metals at low concentrations, it is expedient to use an additional stress (light, cold), which does not exceed the limits of tolerance of the test object but significantly increases the sensitivity of the algae to toxicants. The use of highly sensitive fluorescence methods providing quick and efficient information about the state of the algal photosynthetic apparatus in the real-time mode combined with the use of an additional physiological load on the test object allows us not only to shorten the duration of biotesting to detect intoxication but also to reduce the concentration limits for reliable detection of toxicants. Summing up, it should be noted that high light stress may easily be realized in the fluorescence apparatus for biotesting and included in the system of automated operative control of the toxicity of natural waters and sewage.

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