

ECOPHYSIOLOGICAL STUDIES BASED ON CHLOROPHYLL FLUORESCENCE IN ALGAL CELL CULTURES

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Abstract: The different parameters of short-term and long-term chlorophyll fluorescence induced upon illumination of dark-adapted algal cell suspensions indicate the action site of water-polluting herbicides and heavy metals in the photosynthetic apparatus which performs the light energy conversion and thus ensures the primary production in the aquatic ecosystems. The influence of the herbicides diuron and methylviologen and of two heavy metals (cadmium and nickel) on the ground fluorescence, on the maximal fluorescence yield, on the variable short-term chlorophyll fluorescence and on the potential and effective quantum efficiency of photosynthesis are discussed.

Introduction

Chlorophyll fluorescence measurements provide a very useful non-destructive tool to detect early symptoms of a large scale of stressful conditions that alter the normal photosynthetic processes of the light reactions. A part of the light absorbed by green plants is re-emitted in form of chlorophyll fluorescence, which competes with the useful photochemical reactions for the same incident light energy. Thus, the intensity of fluorescence emission from the photosynthetic apparatus is directly related to the light reactions that occur in thylakoids upon illumination. Chlorophyll fluorescence constitutes an optical signal that can be precisely detected if the wavelength of irradiation is chosen so as not to overlap the wavelength of light emission, which is between 670 and 770 nm [1,9,16].

Upon a dark-light transition the induced chlorophyll fluorescence intensity of green plants undergoes characteristic variations with time, known as the Kautsky effect. There can be distinguished rapid transients, completed within about one second, and slow transients, extended to several minutes. The rapid transients reflect processes close to the primary photoreactions (charge separation between the photoexcited P₆₈₀ reaction centre and its acceptors in photosystem II), whereas the slower transients are related to secondary photosynthetic processes upon light energy conversion, involving ion fluxes through the thylakoid membrane, formation of pH gradients, heat dissipation, activation of Calvin cycle enzymes etc. [3,4,12].

At room temperature, chlorophyll fluorescence originates almost exclusively from photosystem II (PSII), and the fluorescence yields registered immediately after the onset of illumination is determined primarily by the redox state of the quinone

acceptors of PSII. The light energy absorbed by the antenna pigments of PSII is transferred by inductive resonance to the reaction centre chlorophyll-a dimer (P_{680}), which transfers an energized electron to the acceptors located on the stromal side of PSII. Fluorescence is at a minimum level (F_0) when all of the reaction centres are open (the quinone acceptors are completely oxidized and they can take up the electrons), while maximum fluorescence emission (F_m) occurs upon the complete reduction of the quinone acceptor pool, which cannot deal with any more electrons, so all the reaction centres become closed. The difference between F_m and F_0 is the variable fluorescence (F_v). The main factors influencing the kinetics of the induced chlorophyll fluorescence are light intensity, temperature, O_2 and CO_2 concentrations, and the state of dark-adaptation. E.g. pronounced changes of the dark-state initial fluorescence (F_0 , also known as ground fluorescence) can be induced by heat stress, anaerobic conditions, ATP-induced reverse coupling reactions and the binding of specific inhibitors [2,7,10,20].

Ecophysiologically oriented photosynthesis research uses chlorophyll fluorescence measurements not only to evaluate *in vivo* the overall quantum yield and bioproductive capacity, but also to allow insights into the biochemical partial reactions and the partitioning of excitation energy in the autotrophic plant metabolism [8,11,17].

Two major factors cause changes in the chlorophyll fluorescence yield: the rate of photochemical energy conversion and the rate of nonradiative energy dissipation. Although fluorescence changes reflect primarily the functional state of PSII, variations in energy distribution between the two photosystems will also influence fluorescence yields. As photosystem I (PSI) is essentially nonfluorescent, any increase in energy transfer from PSII to PSI may be considered equivalent to nonradiative dissipation. Fluorescence emission competes with photochemical reactions and heat dissipation, therefore there are two basic types of chlorophyll fluorescence quenching: a photochemical and a nonphotochemical one. Provided that changes in heat dissipation are relatively slow, the rapid fluorescence induction transients reflect changes in photochemical quenching only, which is determined by the amount of open reaction centres [13,18,19].

When upon illumination a ΔpH is formed between the stromal and the lumenal sides of the thylakoids, this not only leads to ATP formation and induction of assimilatory electron flow, but also to conformational changes in the pigment-protein complexes and reaction centres, as well as to the de-epoxidation of violaxanthin to form zeaxanthin in the xanthophyll cycle. All of these events are closely linked with an increase of nonradiative energy dissipation and the development of the nonphotochemical quenching of the slow fluorescence. Hence, the secondary fluorescence decline and the low steady-state fluorescence yield (F_{ss}) are determined by two overlapping, different quenching components: a photochemical one, due to assimilatory and nonassimilatory electron flow (where the ascorbate peroxidase activity plays an essential role), and the nonphotochemical one, due to increased heat formation. These two parameters

have to be separated in order to correctly interpret the fluorescence information. This separation and the quantification of the two types of chlorophyll fluorescence quenching is achieved by the saturation pulse method, when upon application of a sufficiently strong light pulse the primary quinone acceptor Q_A pool is fully reduced, and hence the photochemical component of the fluorescence quenching becomes suppressed [3,15,19].

Material and methods

Axenic monoalgal cultures of *Scenedesmus opoliensis* P. Richter [14], obtained from the culture collection of the Biological Research Institute in Cluj [5], were grown in Kuhl-Lorenzen (KL) nutrient media supplemented, according to the different experimental variants, with 50 micromole diuron or DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl-urea), 50 micromole paraquat or methylviologen (1,1'-dimethyl-4,4'-bipyridinium cation), 50 micromole cadmium chloride, or 50 micromole nickel chloride. The pH of all the culture media was adjusted to 6.5 and the cell suspensions were illuminated 14 hours per day with fluorescent lamps at a photon flux density of 80 micromoles $m^{-2}s^{-1}$ on the surface of the cultures [7].

While the algal cell suspensions of the different experimental variants are still in the dark-adapted state, the minimal and maximal fluorescence yields are determined. The ratio $(F_m - F_0)/F_m$ or F_v/F_m reflects the potential maximal quantum efficiency of photochemical energy conversion of PSII under the given conditions. During illumination, the fluorescence yield (F) undergoes complex changes. With the help of saturation light pulses the changed levels of maximal fluorescence (F_m') can be determined, and $F_m' - F_m$ represents the nonphotochemically quenched fluorescence. Because not only F_m , but also F_0 becomes lowered by ΔpH -dependent nonphotochemical quenching, the correct calculations require the previous determination of this modified minimal fluorescence F_0' . This can be achieved upon sample darkening and application of weak far-red background light for PSI-driven Q_A oxidation. On the other hand, nonradiative energy dissipation can be also assessed by the expression $F_m' - F_m/F_m'$, which does not involve F_0' .

The variations of the *in vivo* chlorophyll fluorescence of the differently treated (chemically stressed) algal cell suspensions were measured in samples which were dark-adapted for 15 minutes. F_0 was determined upon excitation by a weak red light beam (650 nm) modulated at 1.6 kHz. F_m was induced by a white saturating flash which provided a photon flux density of 4000 $\mu mol m^{-2} s^{-1}$ for 0.8 seconds. After a lag phase of 90 s, a fluorescence transient of 15 minutes duration was induced by continuous actinic light of 100 $\mu mol m^{-2} s^{-1}$. In order to evaluate the fluorescence quenching mechanisms, saturation pulses were triggered every 10 s. The optimal quantum yield or potential maximal photochemical efficiency is reflected by the value of F_v/F_m , while the effective quantum efficiency of the linear photosynthetic electron transport under the given conditions is expressed by the ratio $(F_m' - F)/F_m'$ or $\Delta F/F_m'$ [6,19].

Results and discussion

The different environmental factors that interfere with the photosynthetic processes induce characteristic modifications in the value of the different parameters of the induced chlorophyll fluorescence upon the illumination of the dark-adapted samples. Because the photosynthetic light energy conversion is a primary step in the long sequence of physiological processes that end up with the net primary biomass production of the algal populations, the detection of early symptoms of the disturbances caused by different unfavorable environmental parameters on the dynamics of chlorophyll fluorescence enables us to predict the consequences of water pollution in a very early stage of the damage that can be caused to the entire aquatic ecosystem.

It is known that among the widely used herbicides that may finally accumulate in the water ponds, the DCMU or diuron exerts a strong inhibition on the function of photosystem II by replacing the second quione acceptor Q_B in its binding site on the D1 polipeptide of the core complex. Another herbicide, called paraquat or methylviologen, acts on the acceptor side of PSI by taking up electrons from the mobile ferredoxin and generating very dangerous superoxide radicals [4]. Among the heavy metals that can accumulate in anthropically polluted water, nickel induces, besides many other effects, a drastical decrease in the amount of thylakoidal plastoquinone molecules. Cadmium inhibits the carboxylase activity of Rubisco, and impairs the formation of ferredoxins and of light-harvesting chlorophyll-protein complexes [18]. It is presumable that because their specific actions the above mentioned water-polluting agents influence the chlorophyll fluorescence parameters of the algal cells exposed to chemical stresses [7].

In dark-adapted algal cell suspensions, the minimal or ground fluorescence that arises immediately after the onset of illumination, gives information about the level of organization of the light-harvesting pigment complexes and about the overall capacity to absorb the incident photons. F_0 is related only to the capture of light, but not to subsequent processes of energetic transformations. This parameter was highly increased by the presence of DCMU, and modified in a smaller extent by cadmium, methylviologen and nickel (Fig. 1). It means that because the light energy conversion is severely impaired in the PSII by DCMU, the algal cells try to compensate this defective photochemistry by increasing the cross-section of the pigment antennae which are able to provide much more light energy than it would be needed for a normally functioning photosynthetic apparatus. However, the increased amount of absorbed energy will be dissipated and lost in the subsequent steps because the herbicide does not allow an efficient electron transport between the two types of photosystems.

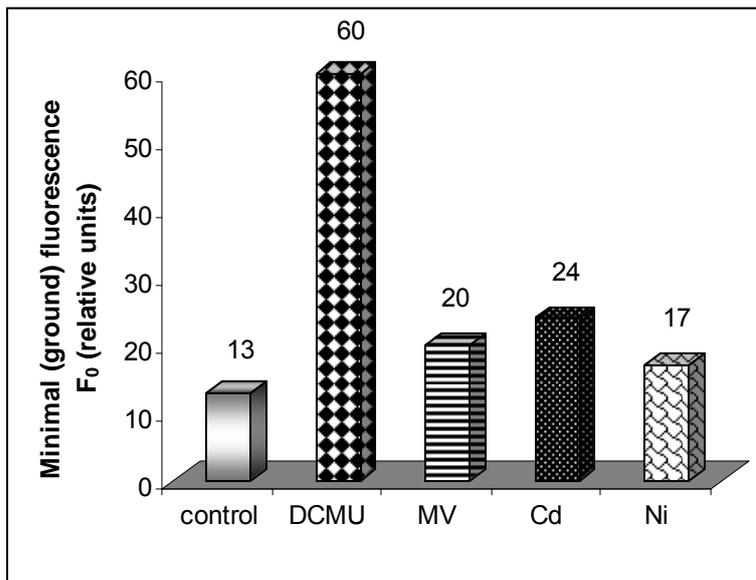


Fig. 1: Influence of different water-polluting agents (herbicides and heavy metals) on the minimal chlorophyll fluorescence in cultures of *Scenedesmus opoliensis* (DCMU – diuron; MV – methylviologen)

The transitory maximal fluorescence, which reflects the degree of photoreduction of the primary quinone acceptor pool of PSII upon primary charge separation leading to the conversion of light into chemical energy, exhibits decreased values in the presence of cadmium and is higher than in the control when the algal populations are exposed to herbicides and nickel. The highest value is shown in the presence of DCMU, suggesting that this substance maintains the Q_A pool in an overreduced state because the energized electrons cannot be transferred to the secondary quinone acceptor (Fig. 2).

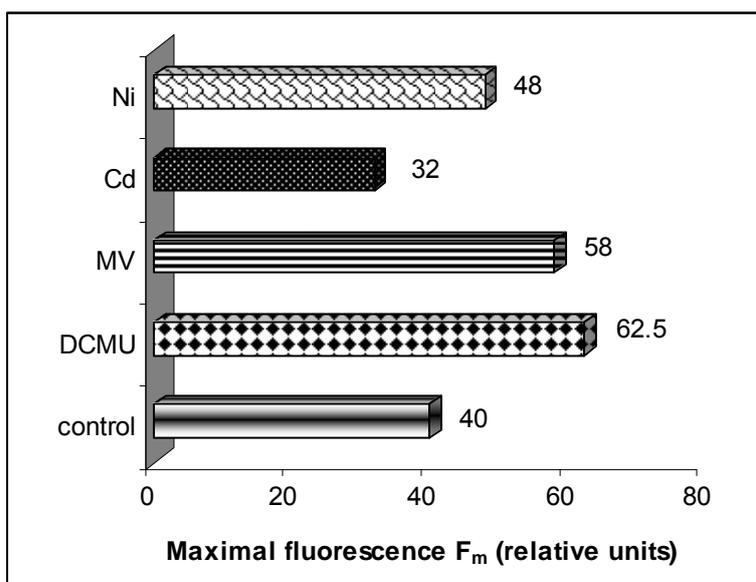


Fig. 2: Influence of two herbicides and two heavy metals on the maximal chlorophyll fluorescence yield in cell suspensions of *Scenedesmus opoliensis*. For concentrations and culture conditions see the Material and methods section

The variable part of the short-term chlorophyll fluorescence reflects the overall dynamics of the primary photosynthetic events that take place upon illumination in the acceptor region of photosystem II. Its value is higher when the light-absorbing pigment antenna is less developed and the reduction state of the Q_A pool is more pronounced. F_V is significantly decreased by DCMU (because in the presence of this herbicide light absorption is intensified but light energy conversion is inhibited) and by cadmium, which increases F_0 and decreases F_m , meaning that the photochemical conversion became inefficient. Methylviologen and nickel increase the value of F_m without influencing significantly the F_0 , which directly results in a moderately higher variable fluorescence (Fig. 3).

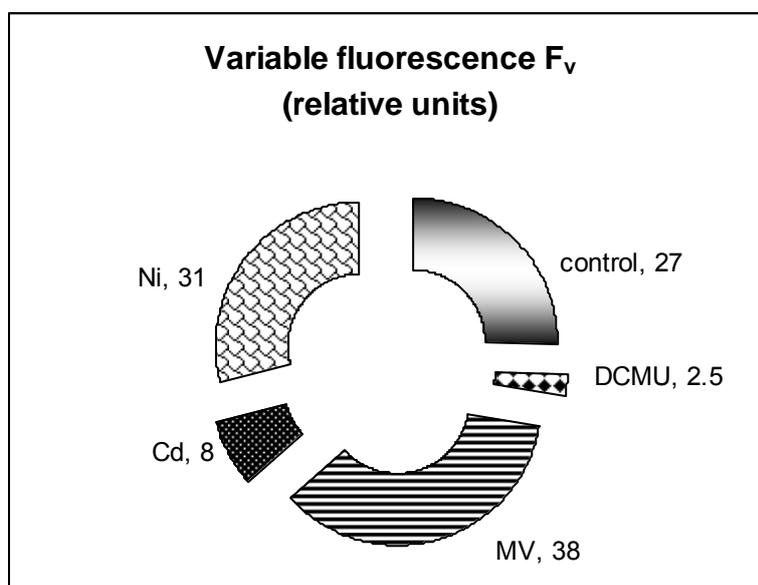


Fig. 3: Influence of water-polluting xenobiotics on the short-term variable chlorophyll fluorescence in the *Scenedesmus opoliensis* cell cultures

The F_V/F_m ratio of the short-term chlorophyll fluorescence, known as the expression of the potentially maximal photochemical quantum efficiency, is a very valuable indicator of the capacity of any photosynthetic complex to convert the absorbed light into useful chemical energy without wasting too much of the incident photon energy. In other words, if the value of this ratio is higher, the photoautotrophic system performs a more efficient exploitation of the available energy input. In this way the intensity of the effect of different disturbing environmental factors on plants can be computed quite precisely [1,11,19]. The F_V/F_m parameter shows that among the four polluting chemical agents chosen to be tested, the most dangerous for the energetics of algal photosynthesis is the herbicide DCMU, followed by cadmium (Fig. 4). If the cellular protective mechanisms are activated, the oxidative stress caused by methylviologen can be neutralized, and micromolar amounts of nickel can be sequestered by phytochelatins and other immobilizers, without affecting directly the capacity to

perform the photochemical reactions. This does not mean that these substances will not damage other subsequent metabolic and developmental processes, because the F_v/F_m ratio reflect only the potential capacity of light energy utilisation, but not the effective energetical efficiency of the photosynthetic processes.

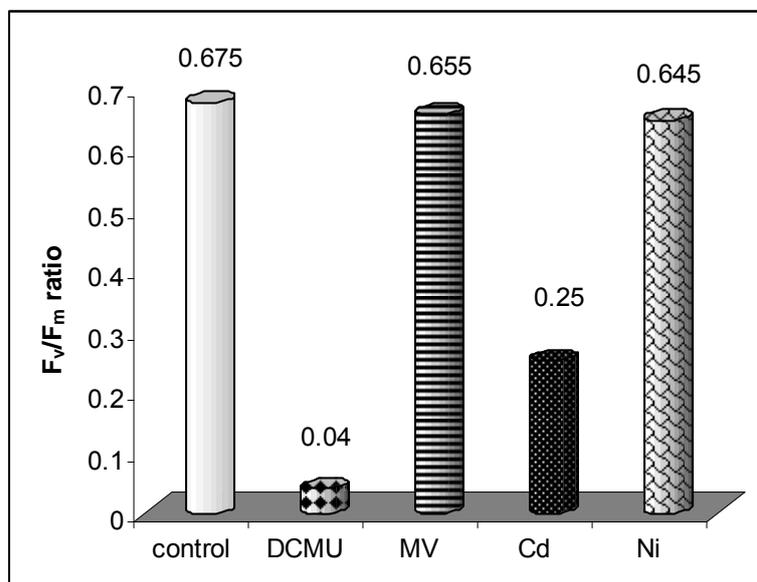


Fig. 4: Influence of water-polluting xenobiotics on the maximal potential quantum efficiency of photosynthesis (F_v/F_m) in populations of the green microalga *Scenedesmus opoliensis*

To predict the actual or effective quantum efficiency and rate of photosynthetic electron flow, it has to be considered that two parallel photoreactions are involved and approximately 85 % of the incident light is absorbed. Furthermore, not only CO_2 fixation but also photorespiration and other forms of oxygen-dependent electron flow are indicated by the value of the $\Delta F/F_m'$ ratio. In this fluorescence parameter all of the nonphotochemical quenchers are included (xanthophyll cycle, Mehler reaction and ascorbate peroxidase activity, photophosphorylation etc.), this means that the effective quantum efficiency reflects the final result of the light energy conversion in the whole chain of photosynthesis-related processes performed under different conditions [19]. Its values show that the most severe impairment of the energetic equilibrium is cause by the herbicide DCMU. Micromolar amounts of dissolved cadmium also reduce considerably the efficiency of light utilization, its effect being roughly more than twice as strong than the disturbance caused by the same amount of nickel (Fig. 5).

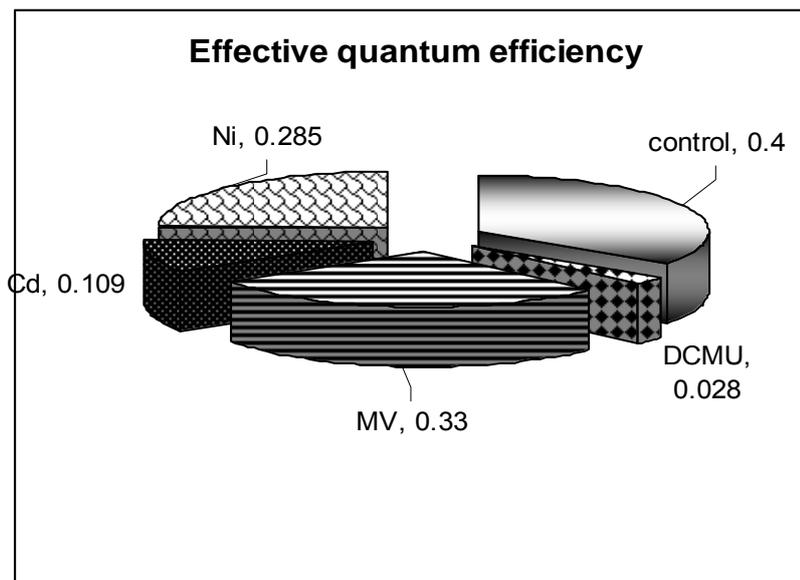


Fig. 5: Influence of two herbicides (DCMU and MV) and two heavy metals (cadmium and nickel) on the effective quantum efficiency of photosynthesis ($\Delta F/F_m'$) in cultures of the green alga *Scenedesmus opoliensis*

The reduced methylviologen, being a free radical which on its turn reduces the molecular oxygen and generates the superoxide anion, probably induces the immediate activation of the free radical- and peroxide-detoxifying protective mechanisms (e.g. the Halliwell-Asada redox chain), so the initial oxidative damage can be annihilated without affecting the efficiency of photosynthetic energy conversion. Despite of this protection, the induction of an oxidative stress requires a considerable energy input in order to survive under such extrem conditions.

Conclusions

Application of the different parameters of induced chlorophyll fluorescence in ecophysiological investigations performed with green algae under different stressful environmental conditions caused by anthropical pollution of aquatic ecosystems, provides us with useful information about:

1. the extent, the supramolecular organization and the functional state of the light-harvesting pigment antennae associated with photosystem II
2. the reduction state of the primary quinone acceptor pool of PSII, directly related to the primary photochemical reaction occurring upon illumination in this photosystem
3. the relation between the absorption of light energy and its conversion into chemical energy that initiates redox reactions during the thylakoidal electron transport
4. the maximal capacity of a photosynthetic structure to perform photochemical reactions by using a certain part of the incident photon energy

5. the overall efficiency of transformation of light into other forms of energy inside the photosynthetic apparatus, during the photochemical and nonphotochemical processes
6. the action of different environmental factors on the various steps of the photosynthetic primary production performed by algae.

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STUDII ECOFIZIOLOGICE BAZATE PE FLUORESCENȚA CLOROFILIANĂ A CULTURILOR DE CELULE ALGALE

(Rezumat)

Parametrii fluorescenței clorofilene induse în culturi ale algei *Scenedesmus opoliensis* oferă informații utile despre stadii incipiente ale modificărilor induse la nivelul funcționării aparatului fotosintetic de către diferiți agenți chimici (ierbicide, metale grele) care, datorită activităților umane, se pot acumula în mediile acvatice și exercită un efect poluant care provoacă scăderea eficienței fluxurilor energetice în ecosistemele afectate. Fluorescența inițială de bază (F_0), care reflectă gradul de organizare și de funcționare a complexelor de pigmenți antenari ce captează energia fotonilor, este mărită de aproape 6 ori de ierbicul DCMU. Acest efect se poate explica prin faptul că transformarea ineficientă a energiei solare în energie chimică atrage după sine dezvoltarea exagerată a antenei de pigmenți, ca reacție compensatoare la insuficiența conversiei energetice la nivelul sistemului fotochimic II. Fluorescența maximă tranzitorie (F_m) este legată de gradul de reducere a acceptorului chinonic primar în urma reacțiilor fotochimice primare prin care centrii de reacție P_{680} trimit electroni energizați dinspre sistemul fotochimic II către sistemul fotochimic I. Acest parametru este diminuat de cantități micromolare de cadmiu, care inhibă funcționarea aparatului fotosintetic atât la nivelul pigmenților antenari, cât și în desfășurarea transportului liniar de electroni și în reacțiile enzimatiche ale ciclului Calvin. Raportul dintre fluorescența variabilă și cea maximă, cunoscut și sub numele de randament cuantic potențial, prezintă valori foarte scăzute în prezența DCMU și a cadmiului, în schimb ierbicul numit metilviologen (generator de radicali superoxidici) și prezența ionilor de nichel (care inhibă în primul rând biosinteza plastochinonelor) nu modifică semnificativ valoarea acestui parametru, probabil pentru că în cazul acestor două substanțe poluante mecanismele de protecție contracarează perturbarea funcțiilor fotosintetice primare. Valorile randamentului cuantic efectiv întăresc rezultatele obținute prin evaluarea celorlalți parametri ai fluorescenței clorofilene induse: dintre ierbicidele testate DCMU este mult mai periculos decât metilviologenul prezent în aceeași concentrație, iar dintre cele două metale grele poluante cadmiul se dovedește a fi un inhibitor mult mai periculos al reacțiilor fotochimice tilacoidale decât ionii de nichel prezenți în mediul algelor.