# STRESS-PHYSIOLOGICAL REACTIONS OF THE GREEN ALGA *SCENEDESMUS OPOLIENSIS* TO WATER POLLUTION WITH HERBICIDES

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**Abstract.** The freshwater green alga *Scenedesmus opoliensis* proves to be a suitable bioindicator of water pollution with different herbicides. One of the best molecular markers of stress condition imposed by herbicides is overproduction of malondialdehyde resulting from lipid peroxidation in the damaged membranes. Methylviologen, a largely used pre-emergence herbicide which generates reactive oxygen species in the illuminated chloroplasts, triggers the accumulation of ascorbic acid and enhances the enzymatic activity of catalase, both of these substances being involved in the antioxidative protection of algal cells. Diuron, a herbicide that inhibits photosynthetic electron transport on the acceptor side of photosystem II, causes a decline in oxygen production and in biomass accumulation of algae. Glufosinate induces accumulation of toxic ammonia and leads to enhanced net oxygen production, associated with a low rate of carbon assimilation. Long-term exposure to micromolar concentrations of herbicides results in significant changes in the rate of cell division, in photosynthetic parameters and in the intensity of antioxidative defense. A proper bioindication of toxic effects of herbicides on algae requires a selected combination of different physiological and biochemical parameters which reflect the degree of stress exerted on living organisms by water pollution with xenobiotic organic compounds.

**Keywords:** algal biomass, antioxidants, environmental stress, lipid peroxidation, molecular indicators, water pollution

## INTRODUCTION

Molecular indicators may be very useful parameters for an early and precise detection of changes in physiological processes of living organisms caused by stressful environmental conditions related to anthropogenic pollution. In this context, one major direction of the present-day biological investigations points toward identification of molecular markers directly related to stress conditions sensed by living organisms, suitable for bioindication of the quality of both terrestrial and aquatic ecosystems [8, 12, 23]. Microalgae have a key role in biomonitoring the changes that occur in aquatic habitats not only because they are the main primary producers of new organic substances in these ecosystems, but also because of their pronounced physiological plasticity that supports their acclimation to changes in the physical and chemical properties of the environment [3, 6, 11, 16]. Xenobiotic organic compounds, like herbicides that accumulate over the time in inland waters surrounded by agricultural fields, represent a real challenge for the survival of algae because these substances are not present in the natural, unpolluted aquatic ecosystems, and in consequence no adaptive strategies could be developed during the evolution of the algal species in order to cope with the harmful effects of these chemicals on the vital processes of photoautotrophic cells [4, 14, 27, 29]. Planktonic green algae, such as different species of the *Scenedesmus* genus, are useful test organisms in ecotoxicological studies, because they are globally distributed and are applicable to all aquatic habitats and a wide range of environmental stressors, they respond rapidly to changes in ecosystem condition, they allow for nondestructive sampling and they often provide one of the first signals of ecosystem impacts [2, 13, 21, 31].

While a large number of papers deal with influence of herbicides on terrestrial vascular plants, there are only a few publications referring to their impact on aquatic organisms, even though these organic compounds usually exhibit a high solubility in water and they may easily accumulate in aquatic habitats, especially in lakes [5, 7, 19, 26]. Unwanted side effects of agricultural application of herbicides are to be expected on non-target organisms, for example on algae in the aquatic ecosystems adjacent to areas subject to agricultural activities. These side effects consist of toxicity, disturbance of developmental processes and the appearance of resistant ecotypes. On the basis of their site and mode of action, the different herbicides may act as specific inhibitors of the light reactions of photosynthesis, inhibitors of biosynthesis of carotenoid pigment, fatty acids and aromatic amino acids, growth regulators administrated in excessive amounts, and compounds that affect tetrapyrrole biosynthesis, causing damage by peroxidation in light. The non-selective herbicides usually impair the energy flux in plants, disturb vital metabolic processes and generate highly reactive oxygen species [18, 25, 28]. Microalgae have a certain ability to bioaccumulate, to immobilize, to sequester and to biotransform different herbicides and related organic compound, thus contributing to a substantial remediation of the aquatic habitats polluted with xenobiotics. This is why the knowledge of how they react to stress conditions imposed by water pollution is important in order to use them in bioindication and bioremediation of disturbed aquatic ecosystems [17, 24].

The aim of the present study is to investigate some of the physiological reactions of the highly adaptable green alga *Scenedesmus opoliensis* to long-term exposure to micromolar amounts of three largely used non-selective herbicides, and to reveal molecular indicators suitable for a good establishment of environmental stress condition imposed by water pollution with the applied herbicides.

## MATERIALS AND METHODS

Axenic monoalgal cultures of *Scenedesmus opoliensis* P. Richter, strain AICB 141, obtained from

the culture collection of the Biological Research Institute in Cluj-Napoca [9, 22], were grown for 10 days in BBM nutrient media [1] supplemented, according to the different experimental variants, with 10 μM diuron (DCMU), 10 μM methylviologen (MV, Paraquat) or 10 μM glufosinate (GF, Phosphinotricine). The herbicides were added to the sterile nutrient media from stock solutions, with filtration through a Millipore filter with pore diameter of 0.22 μm in order to maintain the axenity of media. The control cultures were kept in the BBM medium without herbicide. All experimental setups had 5 repetitions. The initial pH of all the culture media was adjusted to 6.5 and the cell suspensions were illuminated continuously with fluorescent lamps at a photon flux density of 135 micromoles $m^{-2}s^{-1}$ on the surface of the cultures that were stirred continuosly (400 rpm) in an algal growth chamber, at 20 °C [10].

The dynamics of cell divisions was evaluated cytometrically with a light microscope. The initial cell density of all the cultures was set to $52x10^4$ cells per milliliter. The dry algal biomass of the cultures was determined after 10 days of development of the algal populations, when the control cultures were at the end of exponential growth phase. Net photosynthetic oxygen production of the algal cultures was measured with an Oxy-Lab oxymeter at a constant light intensity of 110 μM photons $m^{-2}$ $s^{-1}$ and 20 °C [1, 12].

For determination of lipid peroxidation after 10 days of exposure to herbicides, algal suspensions were centrifuged at 2500g for 10 minutes and pellets were weighed. To each pellet 0.1% (w/v) trichloroacetic acid (TCA) was added in 1:3 (g/ml) ratio. Algae were disrupted in a Constant Systems cell disrupter, than the extracts were centrifuged at 6300g for 10 minutes at 10 °C. 0.5% (w/v) 2-thiobarbituric acid (TBA) solution in 20% (w/v) TCA was added to the harvested supernatants in the ratio 1:4 (v/v), in 10 ml thermoresistant glass tubes. The extracts were heated for 25 minutes at 96 °C and, after lowering temperature on ice, they were centrifuged at 6300g for 10 minutes at 10 °C. Determination of thiobarbituric acid-reactive substances (TBARS), consisting mostly in malondialdehyde, was performed photometrically on the supernatants, at $A_{532} - A_{600}$ nm, using at extinction coefficient of 159.2 $mM^{-1}$ $cm^{-1}$ [15].

Catalase activity was evaluated spectrophotometrically by determining the consumption of $H_2O_2$ associated with a change in absorbance at 240 nm. Algal cultures were centrifuged at 2500g for 10 minutes. The testing medium contained 750 μl of sodium phosphate buffer (50 mM, pH 7.5), 100 μl $H_2O_2$ (200 mM), and 150 μl of algal extract with enzyme (5 μg of protein) in a final volume of 1 ml. Proteins were extracted with 1.5 ml of 0.1 M sodium phosphate buffer (pH 7) and the extract was centrifuged at 2300g for 20 minutes at 5 °C [14].

Ascorbic acid content of the algal cells was determined titrimetrically. 25 ml algal suspension was centrifuged at 2500g for 10 minutes, the pellet was resuspended in 10 ml of 5% (v/v) metaphosphoric acid, filtered and completed with 20 ml of 5% metaphosphoric acid. This extract was titrated with 0.025% (w/v) 2,6-dichlorophenol indophenol until a persistent light pink color appeared. Ascorbic acid content was determined with the help of a standard curve obtained with titration of known concentrations of ascorbic acid [20].

Every measurement was repeated 5 times. The experimental data were evaluated statistically, the significance of the differences among the control and the treated cultures was tested using one-way ANOVA (after verification of variance homogeneity with the Levene test), followed by the multiple comparison Tukey test ($P < 0.05$) [30].

## RESULTS

The same concentration of the three herbicides used in the experiments triggers different changes in distinct physiological processes that underlie growth, development and reproduction of *Scenedesmus opoliensis* in batch cultures. It is known from studies with vascular plants that many environmental stress factors may cause damage of membrane structure which results in peroxidation of lipids with unsaturated fatty acids. The products of peroxidation, especially the malondialdehyde, leave the membranes and spread all over the cell compartments, causing structural damage of nucleic acids, proteins and photosynthetic pigments, thus triggering a cascade of disfunctions. These have to be quickly compensated by repair mechanisms that ensure a hardening process during the development of a certain tolerance against the stress factor. In this context the level of membrane lipid peroxidation products, called thiobarbituric acid reactive substances (TBARS), was measured in the algal cell cultures exposed for 10 days to 10 μM of three different herbicides (diuron, methylviologen and glufosinate). Diuron did not cause any significant change in the level of TBARS, but in the presence of methylviologen and glufosinate the degree of lipid peroxidation increased more that two times as compared with the control, indicating that these two herbicides, even if they have different action sites in the plant cells, both induce membrane damages and impair transmembrane transport processes by causing structural changes in the lipid bilayer (Fig. 1).

The ascorbic acid (vitamin C) content of the algal cells, expressed on a dry weight basis, showed a statistically significant increase only when the cultures were exposed to 10 μM methylviologen. No important change in the quantity of ascorbate was detectable in the algae treated with diuron and with glufosinate. As the most frequent non-enzymatic organic reducing agent in plant cells, vitamin C plays an important role in the antioxidative defense of algae and a higher ascorbate content is a prerequisite for a more efficient protection. It is worth mentioning that vitamin C occurs in plant cells in three interchangeable forms: as reduced ascorbic acid, as partly oxidized monodehydro-ascorbate and as fully oxidized dehydroascorbate. The method used in the present experiments allows only the determination of total vitamin C amount, without distinction between its different forms (Fig. 2).

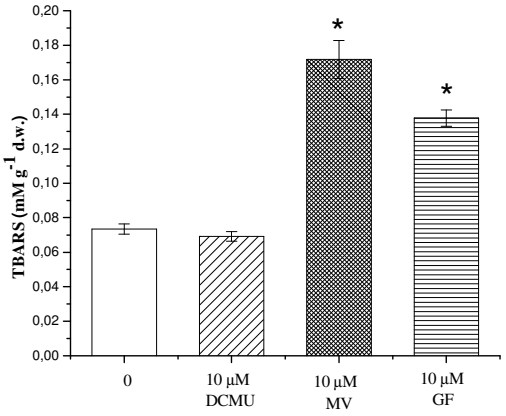


**Figure 1.** The degree of membrane lipid peroxidation expressed by the amount of thiobarbituric acid reactive substances (TBARS) in the green alga *Scenedesmus opoliensis* exposed to different herbicides (DCMU – diuron, MV – methylviologen, GF – glufosinate). Bars indicate standard error (n = 5). The asterisk indicates that the mean values are significantly different from control (0) according to the Tukey test (P < 0.05)

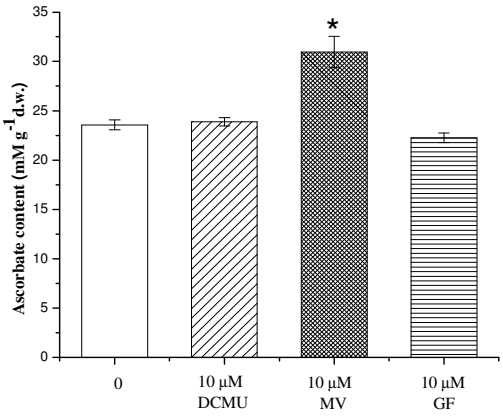


**Figure 2.** Ascorbate content of the green alga *Scenedesmus opoliensis* exposed to different herbicides (DCMU – diuron, MV – methylviologen, GF – glufosinate). Bars indicate standard error (n = 5). The asterisk indicates significant difference from control (0) according to the Tukey test (P < 0.05)

From among the enzymatic components of the antioxidative defense system, catalase exhibited the most prominent changes under the influences of the three herbicides. Its catalytic activity in decomposing the highly toxic hydrogen-peroxide was not significantly modified by diuron, but it was obviously decreased by methylviologen, while glufosinate induced a pronounced rise in its enzymatic activity. The changes in the intensity of the detoxification reaction of hydrogen peroxide may be due to changes in both the amount of the catalase protein molecules and the catalytic activity of the enzyme modulated by different regulatory factors (Fig. 3).

The herbicides used in the experiments exerted a negative effect on the division rate of algal cells, resulting in changes of the dynamics of cell density in the populations during the first five days of exposure. Glufosinate caused only a delay in reaching the maximal cell density in the cultures, but did not lower significantly the final cell number in a unit of culture media volume. Diuron inhibited the growth of algal populations and forced the establishment of the steady-state growth phase at a much lower cell density than in the case of control. Methylviologen exerted a very pronounced inhibition of cell divisions, maintaining the cell density of the cultures close to the initial low values (Fig. 4).

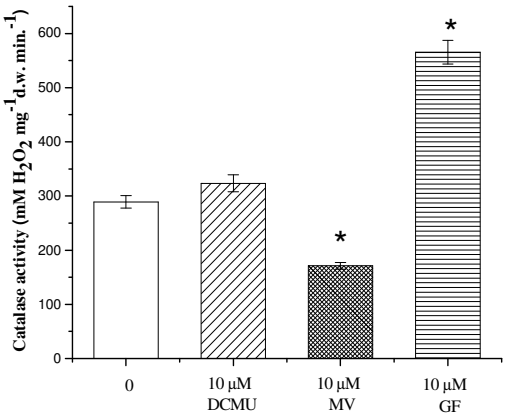


**Figure 3.** Enzymatic activity of catalase in the green alga *Scenedesmus opoliensis* exposed to different herbicides (DCMU – diuron, MV – methylviologen, GF – glufosinate), expressed as the amount of decomposed hydrogen peroxide ($H_2O_2$) per unit algal dry weight (d. w.) during one minute. Bars indicate standard error (n = 5). The asterisk indicates that the mean values are significantly different from control (0) according to the Tukey test (P < 0.05)

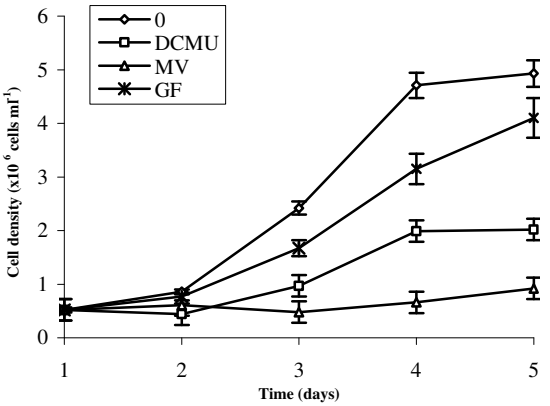


**Figure 4.** Dynamics of cell density in the cultures of the green alga *Scenedesmus opoliensis* exposed to different herbicides (DCMU – diuron, MV – methylviologen, GF – glufosinate). Bars indicate standard error (n = 5)

The final dry biomass of the algal populations, reflecting the efficiency of net photosynthetic primary production, was impaired by all the three herbicides used in the experiments. The most pronounced decline of the algal biomass was registered in the presence of methylviologen, while the mildest, but still significant effect was exerted by glufosinate. The adverse influence of diuron on net biomass production was more moderate than that of methylviologen but more intense than the decrease caused by glufosinate (Fig. 5).

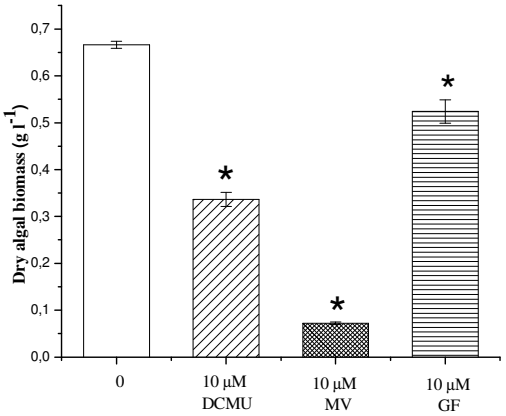


**Figure 5.** Dry biomass production in the 10 days old cultures of the green alga *Scenedesmus opoliensis* exposed to different herbicides (DCMU – diuron, MV – methylviologen, GF – glufosinate). Bars indicate standard error (n = 5). The asterisk indicates that the mean values are significantly different from control (0) according to the Tukey test (P < 0.05)

The net photosynthetic oxygen production of the constantly illuminated algal cells also changed under the influence of micromolar concentrations of herbicides. Oxygen production was mostly inhibited by diuron, and less (but still significantly) impaired by methylviologen. In contrast with the other two herbicides, glufosinate enhanced the net oxygen evolution of the algal cells, increasing it with almost 50% of the values measured in the control cultures (Fig. 6).

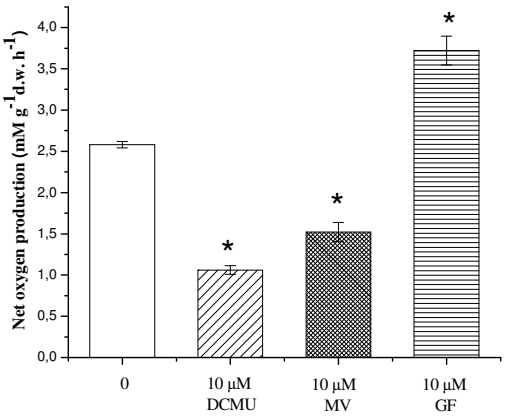


**Figure 6.** Oxygen production of the green alga *Scenedesmus opoliensis* exposed to different herbicides (DCMU – diuron, MV – methylviologen, GF – glufosinate) under constant photon flux density. Bars indicate standard error (n = 5). The asterisk indicates that the mean values are significantly different from control (0) according to the Tukey test (P < 0.05)

## DISCUSSIONS

Even micromolar concentrations of herbicides that enter the aquatic ecosystems from surrounding agricultural fields, exert specific actions on physiological processes of algae and trigger easily detectable antistress reactions that may be useful in an early detection of effects of environmental pollution on living organisms. For example, herbicides that induce oxidative damage of membranes cause a significant increase in lipid peroxidation. This is the reason why methylviologen, a compound which in illuminated algal cells deturns energized electrons from photosystem I during the light phase of photosynthesis, enhances the formation of superoxide radicals and of hydrogen peroxide, two dangerous reactive oxygen species that damage the membrane structures of different cell compartments. As a consequence of this oxidative stress, toxic products of lipid peroxidation (thiobarbituric acid reactive substances) accumulate in the algal cells exposed to methylviologen. Glufosinate, a herbicide known to inhibit glutamine synthase and to induce overaccumulation of ammonia, also induces formation of some reactive oxygen species (most probably of hydrogen peroxide related to disturbance of photorespiration), and this may explain the enhancement of lipid peroxidation caused by this herbicide in the alga *Scenedesmus opoliensis*. Oxidative damage caused by methylviologen in plants is relatively well documented [5, 20], but as far as we know, there are no data about the influence of glufosinate on membrane lipid peroxidation. Our results show that diuron does not interfere with membrane lipids and does not generate reactive oxygen species that would increase the formation of malondialdehyde and related toxic compounds. Instead, this herbicide that is known to inhibit electron transport on the acceptor side of photosystem II in the illuminated chloroplasts, reduces drastically the oxygen production of algae, probably because the disturbance in the function of photosystem II implies a disfunction of the water-splitting complex associated with its lumenal donor side, where the oxygen molecules are evolved from [7, 14]. Impairment of photochemical reactions by diuron results in an overall deficit in the energetic balance of the algal cells, related to the lower biomass production and decreased cell division rate registered in the algal populations exposed to this herbicide. Other algae were also found to react to the presence of herbicides in water by reducing growth and reproduction rate, and a change of chemical composition of their biomass was also reported [13, 19, 24, 29].

Whenever oxidative stress occurs by generation of reactive oxygen species under adverse environmental conditions, a crucial component of tolerance is the induction of an efficient antioxidative protection system with both enzymatic and non-enzymatic components. Many external stress factors cause the formation and accumulation of reactive oxygen species

(singlet oxygen, superoxide radical, hydrogen-peroxide and hydroxyl radical), triggering the synthesis of reducing compound (mainly ascorbate, tocopherol, glutathione and specific xanthophylls), and the enhancement of catalytic activity of antioxidative enzymes (such as catalase, ascorbate peroxidase, superoxide dismutase, peroxiredoxins, glutathione reductase etc.). 10 μM methylviologen increased the amount of ascorbate and decreased the catalase activity in the alga *Scenedesmus opoliensis*. These results indicate that the algal cells react by accumulation of the protective molecules of ascorbate in order to counteract the oxidative stress imposed by this herbicide, but the synthesis and/or the activity of catalase is inhibited by methylviologen, in consequence the enzyme cannot perform an efficient scavenging of the accumulated hydrogen peroxide. This reduced enzymatic protection is partly compensated by the higher amount of ascorbic acid, which is also involved in decomposition of hydrogen peroxide through the Halliwell-Asada-Foyer redox chain, with participation of glutathione reductase and ascorbate peroxidase [14, 20]. Similar antioxidative responses were reported for green algae exposed to water pollution with chromium ions, and significant differences between tolerant and sensitive algae could be established concerning changes in the antioxidative protective components [15]. In contrast with methylviologen, glufosinate triggered a different antistress reponse, without any significant increase in the ascorbate content of the algal cells, but with an obvious enhancement of catalase activity. In the case of this herbicide, the enzymatic component of antioxidative system seems to play a more important protective role than the non-enzymatic one. This may be considered another new finding of our experiments, suggesting that more components of the antioxidative system have to be investigated in order to get a more accurate image of how the same organism reacts to similar oxidative stress situations imposed by different chemical pollutants.

Considering the influence of herbicides on growth and reproduction of the alga *Scenedesmus opoliensis*, one can observe that methylviologen exerted the strongest inhibitory effect both on cell division rate and on dry biomass production. Glufosinate had the mildest influence on these parameters, indicating that this is the mostly tolerated herbicide among the three types used in the experiments. Growth of both cell number and cell size was significantly impaired by the long-term exposure of the algal populations to 10 μM of herbicides, indicating that mainly all the physiological processes that ensure survival of individuals and of the species are endangered by the presence of these organic pollutants in the aquatic habitats.

The rate of net photosynthetic oxygen production is also a good functional indicator of algal condition in the presence of herbicides, and it is a suitable parameter for distinguishing between the actions of different herbicides. The net oxygen production of algae in the presence of a constant photon flux density is the result of photosynthetic water-splitting process in the thylakoids, of chlororespiratory oxygen consumption in the chloroplasts, of photorespiratory oxygen consumption in the chloroplast stroma and in the peroxisomes, and of the oxygen demand of mitochondrial respiration, the latter being very reduced due to the Kok-effect in the photosynthesizing algal cells [8, 16, 21]. Different herbicides may interact directly or indirectly with all of these processes involved in the overall oxygen budget of the algal cells. Diuron decreases oxygen production mainly by inhibiting water-splitting related to the photochemical processes in photosystem II. Methylviologen induces the consumption of extra amounts of oxygen for the generation of superoxide radicals and hydrogen peroxide, and this may be the main cause of its inhibitory effect on net oxygen release from algal cells. Glufosinate impairs the metabolic pathway of photorespiration because of disturbances in the endogenic nitrogen cycle of the algal cells. This reduces photorespiratory oxygen consumption and results in an increased net oxygen accumulation. Changes in oxygen production of algae were also observed under the influence of other polluting agents [27, 29, 31].

As a conclusion of the above presented results, one can notice that: 1. methylviologen increases lipid peroxidation in algal cells, inhibits catalase activity, induces accumulation of ascorbate, impairs cell divisions, reduces dry biomass production and lowers net oxygen production; 2. diuron inhibits photosynthetic oxygen production, growth and development of algal populations; 3. glufosinate increases catalase activity, lipid peroxidation and net oxygen production in *Scenedesmus opoliensis*, but it moderately reduces cell density and biomass production.

High amounts of products of lipid peroxidation and of ascorbate are sensitive molecular indicators of oxidative stress conditions imposed by the presence of methylviologen in the aquatic environment, and these parameters may be suitable for detection of various pollutants that generate reactive oxygen species in algae. Net oxygen production, dry biomass accumulation and the rate of cell divisions are efficient functional indicators of the presence of all the three herbicides used in these experiments to study reactions of the alga to water pollution with organic xenobiotics. The green alga *Scenedesmus opoliensis* proves to be a promising test organism for bioindication of the effects of environmental stress factors on aquatic ecosystems.

Studies on the impact of chemicals on aquatic organisms involve standardized single-species test systems evaluated under controlled laboratory conditions, but in order to achieve a more realistic knowledge of what happens in the complex natural ecosystems, these studies have to be continued with field experiments which take into account multilevel interactions among abiotic and biotic environmental factors [5, 15, 21].

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