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CHROMIUM REMOVAL FROM POLLUTED WATER AND ITS INFLUENCE ON BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS IN ALGAL CELLS USED FOR PHYTOREMEDIATION

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ABSTRACT. The aim of the study is to evaluate the suitability of a local strain of the microalga *Scenedesmus opoliensis* in remediation of water pollution with different amounts of chromium(VI), and also to identify new biochemical and physiological markers for a more reliable indication of sustainability and efficiency of bioextraction and phytoaccumulation processes. Quantitative analysis of photosynthetic pigments reveals that the chlorophylls to carotenoids ratio is a sensitive marker of chromium toxicity and of algal metal tolerance on which the remediative capacity relies. From among the chlorophyll fluorescence parameters, the Fv/Fm ratio, related to potential quantum yield of photochemical reactions, indicates that alkaline pH of the medium (8.65-9.15) favors algal vitality as compared to acidic conditions with pH values around 5. The highest extraction rate (91%) is achieved upon exposure of algae for one week to lower chromium concentrations (5 μM) in alkaline water environment, and a longer exposure time does not increase bioaccumulation. These results may directly contribute to optimization of remediation technology for chromium-polluted water, providing new markers and a new algal strain to be introduced in wastewater treatment.

Keywords: *bioaccumulation, chromium, microalgae, photosynthetic pigments*

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INTRODUCTION

Use of plants and micro-organisms may be a cost-effective, environmental friendly and applicable on large scale in remediation of polluted waters, if the proper species and developmental conditions are selected, and the adequate indicator and efficiency parameters are established [1]. Microalgae exhibit advantageous characteristics of both plants and micro-organisms for an effective bioremediation of aquatic environments anthropically affected by the spill of various organic and inorganic xenobiotics. Due to their wide metabolic plasticity based on autotrophy characteristic to plants, and to their high reproductive rate and pronounced adaptability specific to micro-organisms, microalgae possess a well-defined biosorption, bioaccumulation and biotransformation capacity, which make them advantageous candidates for rehabilitation of domestic, agricultural and industrial wastewaters [2].

Because heavy metals are not degradable, bioremediation of waters polluted with these very toxic chemicals may be achieved by: a) bio-extraction followed by sequestration in internal cell compartments or by adsorption to structural components of the cell wall and related extracellular spaces, b) stabilization by reduction of mobility of heavy metals, e. g. through decreasing the redox potential (aqueous solution becoming more reducing), increasing pH (heavy metals are less water-soluble under less acidic conditions), or chelating with organic acids and sulfur-containing molecules, c) volatilization by transformation into methylated derivatives which leave the aqueous phase (possible only in the case of certain metals and metalloids, such as mercury, bismuth, antimony, arsenic) [3, 5, 6, 11]. Use of metal-accumulating plants for removal of metals from contaminated waters may generate recyclable metal-rich plant residues, causes only minimal environmental disturbance and has a general public acceptance, so optimization of bioremediation technologies represents a priority for applied environmental sciences [13, 25, 27, 33].

Chromium is abundantly found in the earth's crust, but as a polluting agent it reaches in aquatic habitats mainly through human activities, such as leather tanning, stainless steel production, metal finishing, manufacture of pigments and of refractory brick [14, 20]. In very small amounts it stimulates enzymatic activity of phosphoglucomutase enzyme with benefic influence on carbohydrate metabolism, but at micromolar concentrations it may be highly toxic to different metabolic and developmental processes, causing: a) membrane damage by lipid peroxidation, b) excessive catabolic processes by increased protease activity, c) reduction in dry matter production by a decline in carbon dioxide assimilation, and finally d) causing a

generalized oxidative stress by enhanced generation of hydroxyl radicals. Its most characteristic indirect effect is iron, manganese, sulfur and phosphorus deficiency due to competition with these inorganic ions for transmembrane transporters. Because it interacts with DNA, chromium exhibits genotoxicity, causing mitotic irregularities, chromosome fragmentation and hypermethylation of pyrimidines [14, 15, 28, 29]. Inside the plant cells, hexavalent chromium is reduced to the less soluble form of trivalent chromium, than it is immobilized by chelation with metallothioneins and phytochelatins which accumulate under heavy metal stress and sequester chromium ions in vacuoles. Several aquatic plants, including unicellular microalgae, green, red and brown macroalgae (seaweeds), as well as macrophytes (e. g. water hyacinth, duckweed), have been assayed for bioremediation of waters polluted with heavy metals, and the efficiency of remediation process varied greatly depending on plant species, growth conditions, exposure time, pH values, competitive and cooperative interactions with other inorganic and organic pollutants etc. [7, 18, 19, 21, 22, 23, 26, 32]. This is the main reason why selection of most suitable plant species based on sensitive biochemical and physiological markers, as well as optimization of growth conditions during phytoextraction are key factors for a cost-effective cleaning of wastewaters with reduced negative impact on the environment.

The aim of the present study is to evaluate the bioremediative capacity of a local strain of the globally occurring freshwater microalga *Scenedesmus opoliensis*, and also to identify biochemical and physiological markers that enable a rapid and reliable indication of algal vitality and tolerance on which the efficiency of chromium bioaccumulation relies. Results are expected to be directly applicable in clearance of waters polluted with chromium(VI).

RESULTS AND DISCUSSION

Biochemical markers related to vitality and tolerance of living organisms exposed to different degrees of environmental stresses are valuable indicators of water pollution and of its effects on plants which contribute crucially to the biogeochemical cycles of essential elements. From among the many biochemical parameters of metabolic processes, it is hard to find those which vary in correlation with disturbing external factors and may be interpreted in terms of significance for evaluating the efficiency of biosorption and bioaccumulation of polluting agents. In this context, the photosynthetic pigments are directly responsible for harvesting light energy and for primary photochemical reactions that enable plants to

use this energy source for primary production of new organic compounds. Some of these pigments (certain carotenoids, especially xanthophylls) also have an indispensable role in the antioxidative defense against reactive oxygen species generated during photosynthesis if photon flux is too high and assimilation is limited by low concentration of available inorganic carbon source. Because high concentrations of heavy metals disturb biosynthesis and decomposition of chlorophylls and carotenoids, and many of them induce oxidative damage to photosynthesizing membranes and to enzymes of the carbon assimilation pathway, it is expected that water pollution with chromium(VI) will have an impact on the dynamics of photosynthetic pigments in algae, with a direct consequence on energy supply for primary production of new organic compounds.

Our experimental results reveal that after one week of exposure to 5 μM of chromium(VI), the chlorophyll content of algal cells increases moderately in alkaline aqueous solution (pH value of 9), but becomes significantly lower under acidic conditions (pH = 5), while carotenoid pigment content increases in both acidic and alkaline media. In the presence of higher Cr(VI) concentrations (50 μM and 500 μM) the algae exhibit a decreased chlorophyll content irrespective of the pH value, but carotenoid content becomes higher in the acidic medium and lower in the alkaline one. After two weeks of exposure, the amount of both chlorophylls and carotenoids decreases progressively with the elevation of chromium(VI) concentration, but this decrement is more moderate in the case of carotenoids and in the alkaline media (data not shown). Because the two types of photosynthetic pigments vary in different degrees under similar conditions, the most suitable parameter to indicate integrate influence of chromium(VI) on their overall dynamics proved to be the ratio between chlorophylls and carotenoids. In the acidic medium this ratio decreases after one week of exposure to all chromium(VI) concentrations, and this decrease is more pronounced as chromium content increases. After one more week, lowered value of this ratio persists only in the aqueous solution polluted with 500 μM chromium(VI), while in the presence of 50 μM Cr(VI) it registers a significant increment (Fig. 1).

In the alcaline medium the chlorophylls to carotenoids ratio in the algal cells varies differently during the first week of exposure, according to the different chromium(VI) concentrations: it decreases at 5 μM, it increases at 50 μM, and it does not change significantly at 500 μM. After two weeks of exposure, a moderate, but statistically significant increment is registered under the influence of 50 μM chromium(VI), while 500 μM causes a pronounced lowering of this ratio. This means that the chlorophylls to carotenoids ratio is a very good marker of chromium exposure of the alga,

because it exhibits differential changes related to exposure time, chromium(VI) concentration and acidic or alkaline pH of the water. Previously published experiments revealed only a decrease in chlorophyll content of plant cells under chromium toxicity or found no relevant changes in photosynthetic pigment content upon exposure to hexavalent chromium ions [14, 16], but we did not find any report about specific changes in the chlorophylls to carotenoids ratio in the context of biochemical reactions to water pollution with chromium(VI). Further interpretation of the results reveals that, if one takes into account that chlorophylls are the major light-harvesting pigments, while carotenoids have a primary role in the photoprotective processes, it can be stated that water pollution with chromium(VI) exerts the most pronounced negative influence on the primary processes of photosynthetic light energy utilization after two weeks of exposure to chromium(VI) concentrations higher than 50 μM, in acidic aqueous solutions.

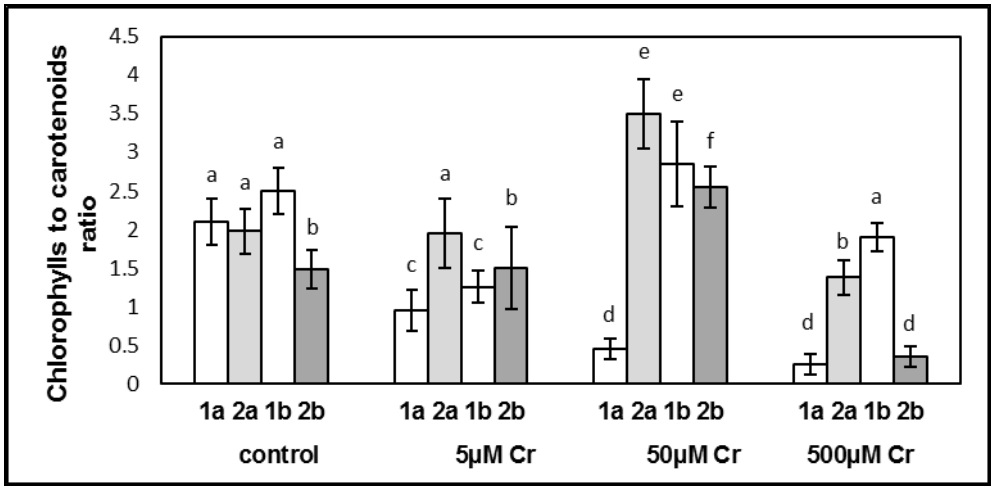

Figure 1. Chlorophyll to carotenoid pigment ratio in the green alga *Scenedesmus opoliensis* exposed for one week to different concentrations of hexavalent chromium, in acidic and alkaline aqueous solutions. 1a – after one week at acidic pH, 2a – after two weeks at acidic pH, 1b – after one week at alkaline pH, 2b – after two weeks at alkaline pH (vertical bars represent ±SE from means, n = 4, different letters show significant differences at $P < 0.05$)

From among the several chlorophyll fluorescence parameters, the ratio between the variable and the maximal fluorescence yield (Fv/Fm, Fv being the difference between the maximal fluorescence Fm and the ground

fluorescence Fo) was found to be the mostly suitable marker of functional changes caused by chromium in the light reactions of photosynthesis. The value of this ratio shows a strong positive correlation with the potential quantum yield of photosynthesis, meaning that it reflects the capability of photosystems to convert the absorbed light energy into chemical energy stored in newly synthesized organic compounds resulting from carbon assimilation through the Calvin cycle. Under optimal conditions, when no disturbing factors limit photosynthesis, the value of the Fv/Fm ratio is between 0.9-0.8. Whenever photochemical processes in the chloroplasts are impaired and stress conditions occur, the Fv/Fm value drops below 0.7. In our experiments the maximal chlorophyll fluorescence decreased already at 5 μM chromium(VI) during the first week of exposure under acidic conditions, reflecting that photochemical reactions on the acceptor side of photosystem II are very sensitive to the presence of chromium in chloroplasts. After two weeks, the Fo decreased even more than Fm, indicating that upon longer exposure the functional organization of the light-harvesting pigment antenna and its light energy transfer capacity becomes inhibited by chromium. In the alkaline medium Fo and Fm decrease abruptly only at 500 μM chromium(VI), irrespective of the exposure time, but lower Cr concentrations do not significantly influence the above-mentioned chlorophyll fluorescence parameters. Integrating these results into changes in the Fv/Fm ratio, it can be noticed that in the acidic media its value decreases significantly at all of the applied chromium(VI) concentrations and after both exposure times, while in the alkaline aqueous solutions the potential quantum yield exhibited in the first week only a moderate decrease and only at chromium(VI) concentrations as high as 500 μM, after two weeks it became slightly lower at 50 μM and much lower at 500 μM of chromium(VI) in the aqueous medium of the algae (Fig. 2). These results suggest on one hand that decrease in Fv/Fm may be used for a sensitive indication of functional damage to photochemical processes in algal photosynthesis, on the other hand they show that chromium causes more severe damages to the photosynthetic apparatus if algae are grown in acidic waters (with pH values around 5).

These results may have a direct practical applicability in assessing impact of chromium on algal productivity, because Fv/Fm values may be determined *in vivo* and *in situ*, without affecting algal populations, and offer early and reliable indication on changes in photosynthetic efficiency on which algal vitality, heavy metal tolerance and defensive capacity rely. Even though potential quantum efficiency is frequently used in plant stress physiological investigations, no conclusive reports exist in the literature concerning its relevance in the context of water pollution with

different concentrations of chromium(VI) under acidic and alkaline pH conditions [8, 17].

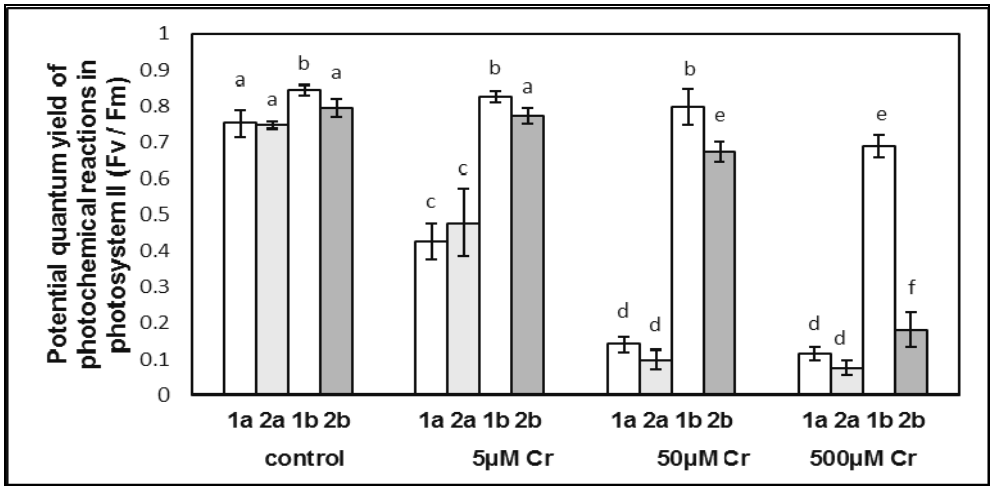

Figure 2. Potential quantum yield of photochemical reactions in photosystem II, expressed as the ratio between the variable and maximal chlorophyll fluorescence (Fv/Fm) in dark-adapted cells of the green alga *Scenedesmus opoliensis* exposed for one week and for two weeks to different concentrations of hexavalent chromium, in acidic and alkaline aqueous solutions. 1a – after one week at acidic pH, 2a – after two weeks at acidic pH, 1b – after one week at alkaline pH, 2b – after two weeks at alkaline pH (vertical bars represent ±SE from means, n = 4, different letters show significant differences at $P < 0.05$)

The spread of algal individuals in the entire water body is a prerequisite for an efficient and uniform bioextraction of polluting agents, this is why the rate of algal reproduction through cell divisions is an important parameter in the evaluation of bioremediation efficiency, as well as in the examination of developmental status of the algal populations exposed to adverse environmental factors. Investigation of the dynamics of cell density in the algal populations exposed to different chromium(VI) concentrations at two different pH values (5 and 9) revealed that the decrease in cell division rate is proportional with the chromium concentration in the medium at a given pH value, suggesting that cell density of algal populations is a good indicator of the degree of water pollution with chromium(VI) and of its effects on algal growth. One can also notice that at chromium(VI) concentrations which do not exceed 50 μM, the alkaline aqueous solution is more favorable to algal reproduction as

compared with the acidic conditions, and after two weeks of exposure the differences in algal cell number per unit of water volume are more obvious than after one week (Fig. 3).

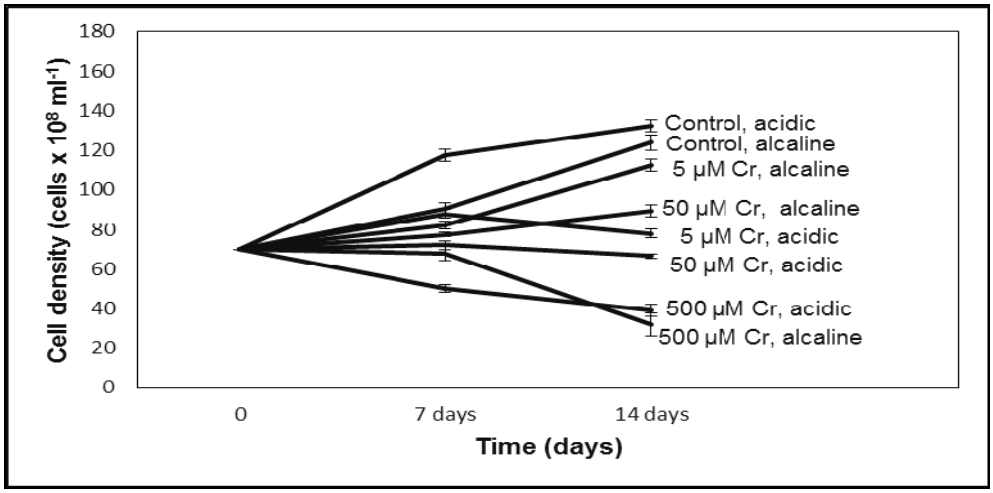

Figure 3. Influence of different concentrations of hexavalent chromium on the dynamics of cell density in populations of the green alga *Scenedesmus opoliensis*, in acidic and alkaline aqueous solutions (vertical bars represent ±SE from means, n = 4, different letters show significant differences at $P < 0.05$)

Variations of the cell density of algal populations were monitored in several experiments concerning the influence of different heavy metals on several microalgal species, and it was found that dynamics of cell multiplication correlates with the intensity of impact exerted by these various chemical stress factors present in polluted aquatic environments [12, 16, 18, 24].

Because pH values of the aqueous medium have a significant impact on solubility of chromium in water, on which its bioavailability depends (generally, heavy metals are more soluble in aqueous solutions under acidic conditions, and metal-polluted waters often have a low pH), and because algal growth is also influenced by the pH of the medium (freshwater algae are adapted to the slight alkalinity of their natural aquatic environment, but they can properly take up mineral nutrients also from moderately acidic aqueous solutions), it is worth mentioning that the presence of different chromium(VI) concentrations did not cause relevant changes in the pH values of the aqueous media after two weeks of

exposure, but they slightly decreased the initial pH value in both the acidic and the alkaline solutions (with the initial pH values set to 5 and to 9, respectively). As compared with the control, this decrement did not exceed 0.48 units in the presence of 5 μM Cr, 0.63 units in the media with 50 μM Cr, and 1.14 units when 500 μM Cr was added to the water solution in which the algae grew. pH of the control cultures moderately increased during two weeks from 5.00 to 5.24 ± 0.17, and from 9.00 to 9.33 ± 0.21.

Determination of the chromium concentration in the aqueous medium of algal cultures after one and two weeks of exposure to different initial Cr(VI) concentrations in acidic and alkaline solutions revealed that low amounts of chromium(VI) (5 μM) can be extracted by this alga in a proportion of 87 ± 3% in one week and 89 ± 2% in two weeks under alkaline conditions, while in the acidic medium this percentage is slightly lower after one week and considerably lower after two weeks of exposure (Table 1).

Table 1. Degree of chromium extraction from the aquatic environment by the planktonic microalga *Scenedesmus opoliensis*, after one week and two week of exposure to different initial Cr(VI) concentrations in acidic (initial pH = 5) and alcaline (initial pH = 9) media (n = 4)

Initial Cr(IV) concentration in the water solution	Percentage of removal after 1 week at acidic pH	Percentage of removal after 2 weeks at acidic pH	Percentage of removal after 1 week at alkaline pH	Percentage of removal after 2 weeks at alkaline pH
5 μM	81 ± 3%	69 ± 2%	87 ± 3%	89 ± 2%
50 μM	62 ± 2%	54 ± 1%	68 ± 1%	51 ± 4%
500 μM	22 ± 4%	17 ± 2%	51 ± 3%	34 ± 5%

More than half of the initial chromium amount is bioextracted and accumulated by the algal cells if the initial Cr(VI) concentration in the polluted water is 50 μM (irrespective of acidic or alkaline conditions), while chromium(VI) quantities as high as 500 μM are extracted only in smaller degree, the best performance being registered in the alkaline aqueous solution after one week of exposure. In most cases, two weeks of algal development in chromium-polluted aquatic environments did not result in enhanced bioaccumulation capacity in comparison with one week of exposure. On the contrary, the chromium(VI) content of water increased in the second week, probably because due to prolonged exposure more algal cells have died than those which still could divide, and the destroyed cells lost the selectivity of their membrane permeability and liberated back in the medium a part of the formerly accumulated chromium ions. These results

suggest that a shorter exposure time, which does not exceed one week, and an elevated, alkaline pH (e.g. around 9) of the aqueous medium favor a more efficient bioextraction of the chromium(VI) from polluted waters, and are in agreement with experimental data existing for phytoextraction of several other heavy metals from polluted waters and soils [13, 19, 21, 22, 34].

CONCLUSIONS

The algal strain used in the experiments proved to be useful for an effective bioremediation of water polluted with moderate amounts of chromium(VI). The highest biosorption efficiency (91% of 5 μM initial Cr concentration in the aqueous solution) was achieved at an alkaline pH value in the range of 8.65-9.15. The major part of Cr ions is bioaccumulated during the first week of exposure, and an increase in the exposure time does not result in significant further absorption, but may lead to a decreased degree of accumulation because a part of the formerly uptaken chromium(VI) ions reenter in the aquatic environment upon decay of senescent algal cells. Chlorophyll to carotenoid pigment ratio varies specifically with different exposure times, chromium(VI) concentrations and pH of the aqueous medium, being a sensitive molecular marker of changes in physiological status of algae. Potential photosynthetic light use efficiency of algal cells, reflected by the Fv/Fm ratio of induced chlorophyll fluorescence parameters, is a useful indicator of algal vitality indispensable for a sustained bioaccumulation of water-polluting chromium(VI) ions, its decrease being proportional with the decrease in yield of photochemical reactions which follow photon absorption and precede carbon assimilation into new organic metabolites. Dynamics of cell density of the algal populations grown in aquatic environments polluted with chromium(VI) shows a proportional inhibition of cell divisions with chromium(VI) concentration, and it also indicates that algal development enables a suitable bioextraction only at chromium(VI) concentrations lower than 500 μM, and an alkaline pH is more favorable to algal growth in polluted water that the acidic nature of the aqueous solution. These results may directly contribute to an enhanced efficiency of bioremediation of freshwater ponds anthropically polluted with chromium(VI), bringing new information concerning biochemical and functional markers that can be used successfully in an early indication of water pollution status, as well as in implementation of improved environmental-friendly technologies for purifying wastewaters contaminated with chromium(VI).

EXPERIMENTAL SECTION

Microbiologically sterile monoalgal cultures of the freshwater green microalga *Scenedesmus opoliensis* P. Richter, strain AICB 141 (collected from the Stiucilor Lake, Cluj county) were grown in Bold's basal nutrient medium, at a constant temperature of 22 °C, the light intensity being set to a photon flux density of 130 μM $m^{-2}s^{-1}$ for 14 hours per day [8, 17]. Identical amounts of a homogenous static cell culture, being in the exponential growth stage of its algal population, were inoculated in growth vessels containing aqueous nutrient solution supplemented with different concentrations of potassium dichromate (as source of water-soluble chromium(VI) ions) at two different pH values of the media. The initial chromium ion concentration of the algal cultures was set to 5 μM, 50 μM and 500 μM, respectively, while the control cultures were grown in the same nutrient medium, but without chromium source. All the variants were set up in media with the pH adjusted (with aliquots of concentrated solutions of sulfuric acid and potassium hydroxide) to the values of 5 and 9, respectively, in order to observe the influence of acidic and alkaline aquatic environment on the uptake and bioaccumulation of chromium. Every experimental variant was set in four repetitions, and every culture was grown under the above-mentioned conditions for two weeks.

Cell density of algal cultures, reflecting the reproductive capacity under the given developmental conditions, was determined every three days cytometrically, using Bürker's cell counter slide to establish by microscopic investigation the number of viable algal cells in a given volume of homogenized suspension. Dry biomass production of one and two weeks old algal cultures was measured after filtration and dehydration of cells at 85 °C for three days, until a constant weight was reached [16].

Photosynthetic pigment content (i.e. chlorophyll-a, chlorophyll-b and carotenoids amount) of algal cells was determined spectrophotometrically, by measuring the absorbance at 450 nm, 646.8 nm and 663.8 nm, after extraction of pigments performed in darkness at room temperature from 0.1 g dry algal biomass in 5 ml of dimethylformamide [10]. Induced chlorophyll fluorescence parameters, related to efficiency of photochemical conversion of the absorbed light energy into chemical energy stored in new organic compounds, were determined in dark-adapted algal cell suspensions with a photosynthetic efficiency analyzer (FMS2 fluorometer, Hansatech, UK). A very weak (0.1 μM photons $m^{-2}s^{-1}$) red flash (of 650 nm) was applied for 1 μs to measure the ground fluorescence (Fo), while the maximal temporary chlorophyll-a fluorescence yield (Fm) was determined with application of a saturating (10000 μM photons $m^{-2}s^{-1}$) red flash applied for 0.5 s. Variable

fluorescence (Fv) was calculated as the difference between the maximal and the ground fluorescence values, and was used to determine the potential quantum efficiency of photosynthetic light energy use, reflected by the ratio Fv/Fm [9, 10, 24].

The remaining chromium content of the aqueous medium, which was not extracted by algal cells after one and two weeks of exposure at different initial chromium concentrations and under different pH values, was determined by atomic absorption spectrometry (with a Shimadzu AA-6800 spectrometer). Calibration was performed with a series of standard solutions containing known concentrations of chromium in the range of 0.4-40.0 mg L^{-1} [4, 31]. The algal cells were removed from the aqueous media by centrifugation at 3000 g and 4 °C for 20 min.

All experimental setups had four replicas, and every measurement was repeated three times. Statistical analysis of experimental data was performed in the R environment (R Developmental Core Team 2014), using the Shapiro-Wilk test for normality, Bartlett's test for homogeneity of variances, the one-way ANOVA and the post-hoc Tukey HSD test for the significance of differences between treatments. Differences were considered statistically significant at $P < .05$.

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