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## *Chapter 4*

# **THE USE OF GREEN MICROALGAL CULTURES FOR BIOREMEDIATION OF FRESHWATER ENVIRONMENTS POLLUTED WITH CHROMIUM, NICKEL AND CADMIUM**

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## **ABSTRACT**

Planktonic microalgae, as the main primary producers in aquatic environments, exhibit not only a crucial capacity of promoting essential biogeochemical cycles, but also an inducible metabolic plasticity which enables them to bioaccumulate various polluting agents, thus contributing to natural bioremediation processes. Axenic cultures of selected algal strains ensure controlled conditions for studying the optimal exposure time, heavy metal concentration ranges, pH values and other adjustable parameters for a high efficiency of bioremediation. Soluble cationic forms of cadmium, nickel and hexavalent chromium are frequent and dangerous water pollutants of industrial origin, and they induce specific changes in vital functions of algae. In this chapter we exemplify how selected physiological markers of heavy metal tolerance, such as parameters of induced

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chlorophyll fluorescence, photosynthetic pigment ratio, dry biomass production and dynamics of cell density may be valuable tools in developing cost-effective protocols for bioremediation of aquatic ecosystems anthropically polluted with different concentrations of chromium, nickel and cadmium ions, with an exposure time ranging from a few days to at most two weeks in static systems, at moderately acidic pH values. Knowledge of algal reactions to water pollution with heavy metals is indispensable for a correct planning of bioremediation technologies for various aquatic environments.

**Keywords:** heavy metal tolerance, microalgae, phytoextraction, water pollution

## INTRODUCTION

Microalgae are widely distributed in every aquatic environment of the Earth, and as the main primary producers of new organic compounds in freshwater and marine ecosystems, they play a determinant role in the aquatic trophic networks, as well as in the biogeochemical cycle of vital chemical elements. Various microalgal species and intraspecific varieties are differently sensitive to metal toxicity and therefore are suitable biological indicators for an early detection of potential toxic effects of heavy metals dissolved in the aqueous solution. Due to their metabolic plasticity, the ability of selected microalgae to sequester various metal ions makes them very efficient in the biological remediation of large water bodies which contain lower concentrations of heavy metal ions [155]. Removal of metals from wastewaters containing higher amounts of such contaminants may be cost-effective using nonviable algal biomass as biosorbent, while bioconcentration of heavy metals from polluted water, with the possibility of extraction of valuable metals may be achieved by using microalgae immobilized in different embedding agents.

Algal metabolic and developmental parameters may be very useful in identification and selection of algae with different physiological characteristics that enable an optimized remediation of polluted water. Resistant microalgae have the ability to avoid the uptake of heavy metals, but they adsorb toxic metal ions to cell wall constituents or they secrete metal-binding organic products to the surrounding environment. Heavy metals are very stable environmental contaminants because they cannot be degraded or destroyed, but any process that enhances transformation of free metal ions into bound forms results in a reduction of their toxicity to living organisms. In contrast with resistant ones, tolerant algae enable the absorption and accumulation of heavy metals inside their cells in metabolic-dependent processes that are largely influenced by energy sources and by environmental conditions. Bioconcentration of heavy metals usually involves active uptake through membrane transporters of cationic micronutrients, followed by detoxification achieved with binding metal ions to chelating agents such as phytochelatins, metallothioneins, polyphosphate inclusions, several organic acids and

amino acids, produced in metabolic processes induced or up-regulated by the presence of heavy metals [5, 32, 45, 51, 96, 114, 141, 150, 181].

Hexavalent chromium, nickel and cadmium are frequent contaminants of continental freshwater ecosystems affected by human activities, especially by mining, combustion of conventional fuels, metallurgy. Even micromolar concentrations of these heavy metals induce direct alterations in enzyme activities, in photochemical processes and in biomembrane functions, affecting algal growth, metabolism and reproduction. On the other hand, by competition with cationic essential trace elements, they have an indirect negative influence on algae by causing mineral nutrient deficiency syndromes, as well as by enhancing oxidative stress, thus impairing ion homeostasis and redox state in different algal cell compartments. In this context, identification of sensitive biochemical and physiological markers which indicate the impact of heavy metal pollution on algal vital processes may be very useful in the wastewater treatment technologies which rely on the remediative capacity of microalgae. And because usually industrial and domestic wastewaters do not contain only one type of heavy metal, antagonistic or synergistic (competitive or cooperative) interactions between different heavy metals are also of great interest in determining the bioremediative efficiency of microalgae in case of co-contamination. From among the various physiological markers of integrated algal reactions to heavy metal toxicity, certain parameters of the induced chlorophyll fluorescence, of the photosynthetic pigment content and of algal growth and reproduction, evaluated simultaneously, may be useful tools in our attempt to optimize biological treatment of heavy metal-contaminated water in an environmental-friendly approach, without using xenobiotic chemicals or genetically modified organisms [18, 52, 63, 120, 130, 145, 173, 180].

The aim of this chapter is to summarize the possible benefits of using microalgae in bioremediation of water polluted with certain heavy metals, as well as to reveal physiological changes induced by different concentrations of chromium, nickel and cadmium in a green microalga which is ubiquitous in freshwater environments, in order to optimize bioindication and remediation of water pollution by using the capacity of this alga to survive and reproduce in heavy metal-contaminated environments, and to bioaccumulate and sequester heavy metals during a given exposure time.

## **ADVANTAGES OF MICROALGAE IN BIOINDICATION AND REMEDIATION OF WATER POLLUTION**

Microalgae have useful characteristics of both plants and micro-organisms such as:

1. Because of their basically autotrophic way of life, microalgae incorporate external light energy in their organic compounds, they do not depend on the

existence of organic nutrients, and photosynthetic carbon assimilation enables a wide plasticity to their metabolism, i.e., algae, like higher plants, are able to produce a wide range of organic substances, primary (ubiquitous) and secondary (specific) metabolites. Autotrophy confers algae a crucial role in the entire energy flux and primary production of aquatic ecosystems. Changes in composition of algal community directly influence all the consumers and decomposers in the trophic network of water bodies [110].

2. In contrast with higher plants and seaweeds, microalgae do not spend energy for sustained growth of their individual body, for skeletal structures, for nutrient storage sites and for internal long-distance transport systems, a higher part of their energy being invested in reproduction, in formation of new generations of individuals within short periods of time. Being eukaryotic micro-organisms, microalgae exhibit a high adaptive capacity to changes of environmental conditions, and because of their high reproductive rate, they easily occupy new habitats and spread through the aeroplankton from one water body to another.
3. Many microalgae reproduce asexually, which enables formation of genetically homogenous populations. This feature is convenient when bioindication of changes in water quality is made by registering modulation of physiological processes, because genetic differences between individuals would lead to large deviations from the mean reaction of the whole population consisting of billions of algal organisms.
4. Due to their high number and ubiquitous presence in different aquatic environments, they enable biomonitoring and remediation protocols under standardized conditions, which is important in the context of their general use in the management of water quality [177].
5. Also because of the high number of individuals in a given volume of aquatic environment, it is easy to select new varieties or certain mutants formed randomly under the selective pressure of extreme life conditions related to accumulation of polluting agents with adverse effects on metabolic and developmental processes.
6. Microalgae react very quickly to adverse environmental impacts, being able to make directed changes in their physiological processes disturbed by external stressors. After perception of stress agent and a transitory (acute) decline of sensitive processes, a longer phase of hardening begins, new steady-state levels of the affected functions are set, and enhanced tolerance leads to long-term acclimation to the modified life condition caused by eutrophication, diversion of pesticides or other organic xenobiotics, or increased concentrations of water-soluble heavy metals. This functional adaptation of algal individuals to adverse conditions enables their persistence and reproduction in various polluted waters, as a prerequisite for an efficient remediation. Their quick reaction to environ-

mental changes makes possible an early detection of polluting agents, which is important for prompt interventions aiming diminuation of pollution.

7. Being widespread in practically every aquatic habitat, microalgae exhibit a great diversity, which facilitates selection of suitable forms for remediation of water pollution with very different chemical agents and their combinations, under diverse ecological conditions. There are more than 21000 algal species known at present, with more than 100000 intraspecific varieties and cultivated cell lines (strains), while many other algae are probably not known yet. This means that algae represent a vast potential biological resource for different biotechnological applications, including wastewater treatment and remediation of degraded aquatic ecosystems. It is estimated that about 70% of the biomass on Earth is given by algae, most of them belonging to microalgae (microscopic algae, individuals consisting of single cells or associations of unicellular, ontogenetically related algae). This means that algae are fundamental components of aquatic living communities even from a quantitative approach, being the most abundant submersed organisms which may extract and accumulate pollutants from the aqueous solution [86].
8. Due to their plastic metabolism on which the acclimative capacity is based, microalgae are able not only to accumulate and sequester water pollutants, but also to biotransform them into less harmful derivatives, thus performing a detoxification of the aquatic habitat. This capacity is a key feature for biological epuration of wastewater, when autotrophic microalgae are used in combination with heterotrophic bacteria. Depending on the chemical nature of water pollutants, several microalgal species can modify they nutrition and intermediary metabolism, in order to consume the polluting compounds and to convert them in parts of their natural biomass. Many microalgae are able to change their nutrition from photoautotrophy to photoheterotrophy (e.g., in presence of simple organic compounds), to mixotrophy (use of both inorganic and organic carbon sources), to amphitrophy (alternation of autotrophic and heterotrophic periods) or to auxotrophy (autotrophy combined with use of certain organic substances) [38, 89, 176].
9. Microalgae need basicly sunlight, water and small amounts of mineral nutrients for growth and reproduction. Planktonic species are equally present in the whole volume of the water body to the depth of light penetration. They easily develop high tolerance to the presence of various polluting agents, they accumulate, sequester and even detoxify higher amounts of chemicals. All these features ensure that bioremediation of water pollution by use of microalgae can be cost-effective and environmental friendly [165].
10. Wastewater treatment with microalgae can be combined with the reduction of air pollution caused by large scale emissions of carbon dioxide, because algal

systems are very efficient converters of carbon dioxide into useful organic compounds resulting from the photosynthetic carbon assimilation. In parallel with remediation of polluted water, the resulting algal biomass may be a good source of proteins (the so-called single cell proteins), vitamins and essential micronutrients in aquaculture and in animal farming (if the algal biomass does not contain toxic compounds), or it may be used for extraction and recycling of valuable substances adsorbed or absorbed by the algal cells (e.g., rare metals). Algal biomass from wastewater oxidation or stabilization ponds may be also used for production of methane, bioethanol or biodiesel as alternative, regenerable sources of fuel [6].

The best algal types for bioindication and remediation of water pollution are those with large tolerance to variations of temperature, light regime, pH, osmotic potential and dissolved oxygen concentrations, with low sensitivity to photoinhibition and to photooxidative stress, with low compensation point of the photosynthesis for the water-soluble inorganic carbon source, and with a pronounced capacity for biosynthesis of protective metabolites induced by specific water-polluting agents [156].

## **LIMITATIONS OF USING MICROALGAE IN MONITORING AND REDUCING CONTAMINATION OF AQUATIC ENVIRONMENTS**

Beside their several advantages in early indication and cost-effective remediation of water pollution, microalgae also possess following characteristics which limit their use in wastewater treatment [14, 47, 64, 95, 123, 127].

1. The ability of microalgae to indicate by internal changes the nature and degree of water pollution, as well as their capacity to accumulate and immobilize harmful chemicals, largely depend on the availability of essential nutrients and of light energy source, because starving algal cells are much more sensitive to environmental stress factors, and energetic limitation impairs hardening and biosynthesis of protective metabolites. For example, several microalgal species accumulate much less cadmium ions and are more sensitive to cadmium toxicity under conditions of phosphate deficiency than in eutrophicated aquatic habitats in which phosphate concentration is high.
2. Repeated or long-lasting exposure to polluting agents enhances the development of tolerance to the chemical stress factor, so algae become less and less sensitive, and their indicative capacity decreases. This is why exposure time greatly influences the remediative potential of microalgae.

3. Resistance or tolerance of vital physiological processes to polluting agents has its limits, thus algae cannot be used in remediation of aquatic environments heavily polluted with high concentrations of chemicals.
4. Because they are at the base of trophic pyramids, algae have a lower bioconcentration capacity than their primary and secondary consumers.
5. Physiological and biochemical markers used to select more efficient algae in bioindication and phytoremediation may vary in similar extent and direction not only under the influence of water pollutants, but also upon variations of natural environmental factors, and this fact may result in false positive responses which may be misleading when one evaluates the degree of pollution and the capacity of algae to tolerate chemical stress conditions. For example, the quantity of different photosynthetic pigments (chlorophyll-*a*, chlorophyll-*b*, carotenes, different xanthophylls) and the ratio between these pigments is usually a very useful marker of algal tolerance or sensitivity to adverse growth conditions, but may considerably vary even in non-polluted water if radical changes occur in photon flux density. This is the main reason why the simultaneous evaluation of several stress markers is recommended for a better accuracy of the evaluation of impacts of polluting agents on algal metabolism and development. Elimination of false positive reactions can be also achieved by using algal cell cultures grown under controlled conditions, where the most important environmental factors are kept in the optimal range.
6. When algal biomass developed during wastewater treatment is intended to be used for recycling of useful compounds or for biofuel production, harvesting processes and species control will increase the overall costs of remediation.

### **BIOLOGICAL PROCESSES INVOLVED IN TREATMENT OF WATER POLLUTED WITH HEAVY METALS**

Phytoremediation of heavy metal-contaminated aquatic habitats with microalgae exhibiting plant-type metabolism and stress reactions, represents the use of algae to extract, to sequester and sometimes to detoxify metal pollutants dissolved in the aqueous medium in which algae grow and reproduce, in order to clean-up the water environment. The use of planktonic microalgae for removal of heavy metals from contaminated waters has a lower cost than other remediation technologies, it may generate algal residue rich in recyclable metals, causes only minimal environmental disturbance, and because of these advantages has a large public acceptance. It may be achieved by different processes, based on distinct physiological properties of selected algal species. Thus, a better knowledge of biochemical mechanisms on which remediation relies is highly beneficial

for the optimization of water treatment technologies based on algal abilities implied in natural, biological reduction of water pollution. Most of the algal species used in remediation of heavy metal-contaminated water are green microalgae belonging to Chlorococcales (with thick cell wall and without flagellum), and a few flagellate species included in Volvocales and Euglenales. Diatoms are more sensitive to higher concentrations of heavy metals, but they may be used for an early indication of water pollution with small amounts of metal ions [74, 83].

Biosorption of heavy metals by algae involves chemical and physical processes resulting in binding of heavy metals to cell wall structures or to secreted polysaccharides and mucilage sheaths. The heavy metals fixed on algal cell surfaces are removed from the aqueous solution, thus biosorption is associated with stabilization and sequestration of heavy metals, without entering in the living cells. Biosorption is achieved mainly by resistant microalgal species and also by dead algal cells. The more extended relative surface of algae (cell shapes distant from spherical, with extensions and emerging patterns) enables a better biosorption, and the process is also largely influenced by thickness and chemical composition of algal cell wall [56, 100, 200].

Active uptake and accumulation of heavy metal ions through energy-driven membrane transport leads to extraction and bioconcentration of these metals. Living algal cells actively accumulate heavy metals against their electrochemical gradient, mainly through membrane transporters of cationic micronutrients which are less specific for one certain chemical element, but transport several, chemically similar metal ions. For example, iron transporters may allow uptake of cadmium, chromium, nickel and other water-polluting heavy metals into the algal cells. Intracellular bioconcentration of heavy metals is linked to chemical energy (ATP) consumption, so it has to be supported by metabolic processes which store energy reserves. This is why enhanced aerobic respiration is usually associated with accumulation of high amounts of heavy metals, while growth rate and synthesis of organic storage metabolites are down-regulated, in order to fuel more energy to the active membrane transport of metal ions. Not only the plasma membrane which surrounds the algal cells, but also the internal membranes which delimit different organelles from the cytosol exhibit selective permeability, thus the heavy metals are distributed among the different cell compartments. In algal cells, which usually do not develop a large central vacuole, most of heavy metals are accumulated in the cytosol, in the stroma of chloroplasts and in the matrix of mitochondria. Hyperaccumulation of harmful heavy metals has probably an adaptive significance, because it may have been evolved in order to confer chemical protection against consumers and parasites, including pathogenic micro-organisms. Active accumulation of heavy metals by the algal cells contributes to phytoextraction, bioconcentration and sequestration processes [30, 59, 70, 98].

The main algal process in phytosequestration of heavy metals is complexation with organic chelating agents produced by algal metabolism upon sensing the increased

concentration of certain heavy metals inside the cells. Phytochelatins and metallothioneins (inducible peptides with several cystein residues that serve to bind heavy metal ions to their sulfur atom) are the most specific chelating agents used by algae and higher plants to bind heavy metals. The presence of high amounts of heavy metals in the cytosol activates the phytochelatin synthase enzyme, which produced phytochelatin molecules from glutathione residues. Heavy metals also activate the genes which encode for metallothioneins, thus these peptides are newly synthesized in ribosomes and accumulate in the cytosol in an extent which is related to the quantity of heavy metal ions reaching the cytoplasm. Chelated heavy metals may be transported and sequestered in different cell organelles, leading to a compartmented distribution of metal ions. In many algae, polyphosphate bodies, which primarily serve as reserves of phosphorus, magnesium, calcium, potassium and other essential metal nutrients, may bind heavy metal ions, thus reducing their concentration in the aqueous phase of the intracellular space. Algae may also bind heavy metal ions to organic acids such as malic acid and citric acid, while some free amino acids may also serve to stabilize certain metal ions (e.g., nickel is frequently bound in algae to histidine). Because heavy metals complexed with chelating agents cannot move freely in the algal cells, this way of sequestration can be also considered as a mean of detoxification [31, 57, 140, 183].

Detoxification of heavy metals may be achieved not only through complexation with chelating agents, but also by biochemical reactions of the algal metabolism. Enzymatic change of the oxidation state may result in bioconversion to a less toxic metal form. For example, the hexavalent chromium is quickly reduced in algal cells to trivalent chromium ion, which being insoluble in water has a considerably reduced toxicity. Similar detoxification processes are represented by conversion of mercury (II) to  $Hg^0$ , of gold (III) to  $Au^0$  or of molybdenum (VI) to Mo(III). Change in redox state may be also considered a way of biotransformation of metal form. Enzymatic methylation of heavy metals in the algal metabolism is another way of detoxification, because methylation prevents heavy metals from reaction with sulfhydryl groups of vital protein molecules, thus avoiding structural damage, denaturation and inactivation of these proteins (enzymes, membrane transporters, transcription factors, etc.). Enzymatic methylation is also a form of biotransformation [59, 164, 195]. In case of a limited number of heavy metals and metalloids, methylation results in generation of volatile products, which leave the cells and reach the atmosphere, being removed from the aquatic medium. This is the case of methylated mercury, arsenic, bismuth and antimony, and the process is known as phytovolatilization [202].

Phytostabilization implies reduction of the mobility of heavy metals in the aquatic environment, as well as bioconversion of metals to insoluble forms which precipitate from the aqueous solution. Precipitation of insoluble metal complexes with reduced mobility and bioavailability is usually performed by conversion into sulfides (e.g., in case of chromium and aluminium). Binding on cell surfaces and to extracellular products, as it



2. Pseudometallophytes include algae which survive and reproduced in severely metal-contaminated water without bioconcentrating the metal ions. They may contribute to stabilization of metals by reducing their mobility, e.g., through precipitation of insoluble metal complexes or through metal fixation to extracellular biopolymers, heavy metals being converted into biologically inert forms [27].
3. Heavy metal indicator algae are those in which metal content approximately reflects the metal level in the surrounding water. They do not exclude and do not bioconcentrate these heavy metals, but they establish an equilibrium between external and internal metal content. Their phytochelatin content may be directly correlated with the degree of heavy metal pollution, thus being suitable for bioindication and even for long-term monitoring of contamination level of the aqueous medium. They are neither resistant nor very tolerant to heavy metal toxicity, and many of their physiological processes exhibit proportional changes with the degree of water pollution. They still tolerate contaminants to a greater extent than non-specialist, sensitive species or intraspecific varieties [29, 54, 87].
4. Moderate accumulators are able to bioconcentrate heavy metals inside their cells in ten to a few hundred times higher concentrations than in the surrounding water, but only if the effective metal quantity does not exceed the micromolar range. They are suitable for extraction and sequestration of heavy metals from moderately polluted aquatic environments, and they perform an active transport of metal ions through less specific membrane transporters of cations, which primarily serve for uptake of essential inorganic micronutrients. Many microalgal species with heavy metal tolerance are included in this category [3, 26, 73, 85].
5. Hyperaccumulator algae can concentrate heavy metals to levels far exceeding those in their aquatic habitat, and they experience little or no toxic effects because they efficiently sequester these metals through complexation with chelating agents produced in high quantity in their metabolism, or they detoxify metals by enzymatic reactions. In their case, bioconcentration factor for heavy metals may reach the level of one thousand. Generally, algae which may contain more than 0.1% of chromium, nickel, cadmium, copper, mercury, silver or more than 1% zinc or manganese on a dry weight basis are considered hyperaccumulators, irrespective of the concentration of heavy metal in the aqueous solution. A given hyperaccumulator algal species generally may bioaccumulate to high extent only one type of heavy metal, so a hyperaccumulator alga will never extract in the same extent different heavy metals from the contaminated water. The extent of bioconcentration largely depends on the prevailing nutritional, illumination and other growth conditions, as well as on possible interactions among co-contaminating chemicals. Under adequate developmental

conditions, hyperaccumulators give the most efficient remediation of heavy metal-polluted water [75, 101, 143, 146, 154, 168, 190].

## **USE OF IMMOBILIZED MICROALGAE IN REMEDIATION OF HEAVY METAL-CONTAMINATED WASTEWATER**

Industrial and urban wastewaters may contain high amounts (over 1 mg L<sup>-1</sup>) of different heavy metals, which at these concentrations exhibit ecotoxicity, and their removal is a priority in wastewater treatment. Several planktonic microalgae are capable to accumulate heavy metal ions, by biosorption to cell wall structures and by active membrane transport into the intracellular compartments, with a bioconcentration factor of several hundreds or even a thousand (i.e., the concentration of heavy metal becomes hundreds-thousand times higher in the algal biomass than in the surrounding water solution). If algal cells are harvested, even the isolation and recovery of heavy metals is possible (e.g., for gold and uranium). Algae start very quickly to accumulate and immobilize heavy metals first by a passive process resulting in adsorption to surface structures (adhesion to colloidal particles of mucilage sheath or secreted biopolymers, coordinative or electrostatical binding to cell wall structures), then a slower, but metabolically active uptake process follows (absorption into the living cells), which reaches its maximum in a few hours. The highest degree of heavy metal accumulation occurs within the first day of exposure, this rate is maintained over the next days with no more enhancement, and finally the bioaccumulative capacity of living algal cells decreases in time, first moderately, then more and more abruptly, during the next one or two weeks. If algae die because of heavy metal toxicity, they may exhibit for a short period of time a greater binding capacity for the metal ions, probably because biomembranes loose their selectivity and more intracellular binding sites may be accessible for the metals. In static wastewater treatment systems (where contaminated water is supplied only at the beginning of the cleaning process) it takes usually two weeks for microalgae to decrease the heavy metal content of water with 50%, while in dynamic systems (with periodic supply of new wastewater quantities) this decrement is achieved within three weeks. In some cases, inorganic nitrogen and phosphorus source has to be supplied and the pH value of the medium has to be adjusted in order to maintain the high bioaccumulation capacity of algae over several weeks. Heavy metalresistant algal species and strains, as well as dead algal cells, extract and immobilize heavy metals from the aqueous environment through adsorption to surface structures, but heavy metals are not concentrated intracellularly. In contrast, heavy metal tolerant algae actively transport heavy metals through the less specific micronutrient transporters of their

membranes and bind these metal ions to intracellular organic metabolites, in order to immobilize and detoxify them [69, 112, 159].

If heavy metal concentration is high, algal cells become intoxicated and cease further metal uptake. Instead, their decomposition release a part of the formerly sequestered metals back in the water environment. It was observed that microalgae which are able to produce extracellular polysaccharides and form a mucilage sheath around their cells, are better protected against toxic effects and tolerate higher concentrations of heavy metals. In analogy with this natural phenomenon, different gel particles were created in which algal cells may be embedded, thus being protected from the sudden contact with polluting agents, and being able to perform water decontamination for a prolonged period of time. For metal ion removal and recovery, algal cells may be entrapped in a matrix of insoluble calcium alginate, polyacrylamide gel (based on the free-radical polymerization of acrylamide in algal cell suspension), or silica gel. The matrix has pores that enable the metal ion diffusion into the beads, where interactions with the algal cells may occur progressively. For example, immobilized *Chlorella* cells can be used efficiently to extract and recover gold, uranium, copper, lead, nickel, chromium from contaminated water containing up to 0.1 mM of dissolved heavy metal. Heavy metal binding to immobilized algal cells is usually enhanced by increased temperature (up to 30°C with living cells and up to 50°C when embedded algae are dead). Conversion of chromium(VI) to the less toxic chromium(III) form, as a useful detoxification method, achievable with immobilized algae in a more cost-effective and environmental-friendly way than the method which uses chemical reagents, may also be enhanced with increasing temperature in the aqueous medium of the immobilized algal cells. pH has also a significant influence on the metal-binding capacity of embedded microalgae, and one of the most important limiting factors of heavy metal removal with immobilized algae is that the optimal pH for heavy metal uptake by algal cells is different (more acidic) from the optimal pH of algal growth and reproduction. Cationic heavy metals (e.g., cadmium, nickel, copper, uranium, lead, zinc) may be successfully bioaccumulated by embedded living algae at pH values between 5-6, but anionic forms (e.g., gold cyanide, platinum chloride, chromate, molybdenate) can be immobilized only at very low pH (around 2) with destroyed algal cells embedded in gel particles. In case of silver and mercury, binding to immobilized algae was found to be rather independent of pH [69, 99]. Because effluents from a column with adsorbent particles usually have lower residual heavy metal concentrations than the supernatant of a batch reactor, it may be concluded that incorporation of algal cells into a gel matrix which can be introduced in a packed column is a practical approach for alga-mediated heavy metal extraction and recovery from polluted water. Because the metal binding properties of different algae vary greatly, and the immobilization methods applicable for wastewater treatment are also numerous, one can predict that embedded microalgae have a high potential in heavy metal extraction and recovery from contaminated aquatic environments [84, 201].

## **INFLUENCE OF CHROMIUM ON ALGAL DEVELOPMENTAL AND METABOLIC PROCESSES**

Heavy metals exert some common influences on algal physiological processes. These may be a) direct effects related to their high affinity to sulfhydryl groups in proteins, thus causing disturbances in the function of enzymes, membrane transporters, receptors, transcription factors and other regulatory proteins, and b) indirect effects resulting from uptake competition with essential metal micro-nutrients, as well as from integrated results of their accumulation on growth, cell division rate and balance between bioregulators. Beside these general impacts on vital functions, the different heavy metals exert specific influences on distinct metabolic and developmental steps. All these effects determine the capacity of different algae to react to water pollution with these metals, to sequester and to detoxify them, and finally the entire success of bioremediation procedures [68].

Chromium impact on aquatic ecosystems is poorly documented, in comparison with the other heavy metals which may accumulate in water ponds exposed to anthropogenic influences. It is a non-essential element for algal nutrition, in small concentrations it may stimulate the enzymatic activity of phosphoglucomutase, thus influencing glucose metabolism. Chromium ions may accumulate in aquatic habitats from production of refractory materials, from metallurgy (production of stainless steel and chrome-plated metals) or from chemical manufacturing (e.g., production of pigments for textile industry) [15]. Hexavalent chromium is toxic because of its high oxidation potential and its ability to cross biological membranes, probably through anion channels, because chromate and bichromate show chemical similarity with phosphate and sulfate anions. Inside the algal cells, hexavalent chromium is quickly reduced to trivalent chromium (in a process which requires reduced glutathione, ascorbic acid and NADPH or NADH), which is not soluble in water, and thus its toxicity is much lower. This is why biological reduction of hexavalent chromium to the trivalent form may be considered a process of detoxification and stabilization. Inside the algal cells most of the chromium is associated with heat-stable proteins and peptides of the cytosol and of the internal compartment of organelles (it may be incorporated in metal-rich granules), a smaller part being adsorbed to the cell wall [1, 50, 103]. According to our present knowledge, chromium does not have any specific transporter and uptake mechanism in algae, and hexavalent chromium is taken up actively through the plasma membrane, the energy being supplied by the proton-ATPase pump [39].

The harmful effect of high amounts of chromium is based on several actions. It inhibits cell division and reduces dry matter production. It may decrease chlorophyll content because of inhibition of aminolevulinic acid dehydratase and protochlorophyllide reductase involved in chlorophyll synthesis. It reduces the size of peripheral light-harvesting antennae around photosystems in the thylakoid membranes of chloroplasts,

thus leading to a decreased chlorophyll *a/b* ratio in green algae. In high concentrations it causes a decline in carbon dioxide assimilation because it decreases the carboxylase activity of the photosynthetic key enzyme, the ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). It may also bind to cytochrome *a3*, thus interfering with mitochondrial respiratory electron transport towards the oxygen molecule. There are also reports on decreased malate dehydrogenase and amylase activities and enhanced protease activity upon exposure to water pollution with hexavalent chromium. Because it favors the generation of harmful hydroxyl radicals, it triggers higher ascorbate peroxidase, superoxide dismutase and glutathione reductase activities during the antioxidative defense processes, and it stimulates biosynthesis of vitamin E as a main annihilator of hydroxyl radicals [78, 134, 148]. If oxidative damage induced by high chromium concentrations persists, peroxidation of unsaturated fatty acids in membrane lipids occurs, and the first protective enzyme which exhibits a strong decline in its activity is usually catalase. The marked decline of reduced glutathione pool of algal cells under chromium toxicity may be due to its oxidation in processes of antioxidative defense, and also to consumption of glutathione in the synthesis of phytochelatins which will sequester and inactivate chromium ions by complexation. Exposure to high concentrations of chromium may also lead to genotoxicity, because this heavy metal interacts with DNA, causing local mutations, hypermethylation of pyrimidines, mitotic irregularities, undivided nuclei, chromosome fragmentation, and overall increase in DNA polymorphism [24, 65, 151, 162, 169, 174, 186, 193].

The most obvious indirect effect of chromium toxicity is related to its uptake antagonism with several essential mineral nutrients. It competes for surface sites and transport binding mainly with iron, manganese, phosphorus and sulfur, it adversely affects nitrogen assimilation, and it inhibits the activity of plasma membrane proton ATPase, thus impairing uptake of every mineral nutrient which enters algal cells through active membrane transport [23, 61, 125, 128, 152, 191]. *Scenedesmus* and *Chlorella* species (freshwater green microalgae) were able to tolerate relatively high amounts of chromium in polluted water, and during several weeks of exposure they accumulated chromium (both by adsorption to cell wall and by absorption inside the cells) with a bioconcentration factor ranging between 230 and 320, when the initial hexavalent chromium content of water was 500  $\mu\text{g L}^{-1}$  [106, 151].

## **CHANGES INDUCED BY NICKEL IN ALGAL CELLS**

Nickel is considered by many scientists an essential micronutrient for algae and higher plants [4, 22, 25, 48, 49, 104, 199], and only some of them consider that nickel is not essential, but it may be in certain cases beneficial for plant metabolism [124]. Argumentation of its essentiality is based on its role in the enzymatic activity of urease,

hydrogenase, glyoxalases, peptide deformylases and methyl-CoA reductase. But these enzymes do not have essential physiological functions or even do not exist in most of the algae and generally in plant cells. For example, urease is not needed for the normal nitrogen metabolism of plants, because they use inorganic nitrogen source in form of nitrate or ammonium ions. If no inorganic nitrogen source is available and urea is present in the surrounding aqueous medium, algae and higher plants switch to nitrogen heterotrophy and use urease (which needs nickel) to decompose this organic nitrogen source to ammonium and carbon dioxide. Hydrogen metabolism, methane production and acetogenesis, in which some of the above mentioned, nickel-containing enzymes are involved, are not characteristic for plant metabolism. This is why nickel cannot be considered an essential mineral nutrient for algae and higher plants, even if it is less toxic than most of the other heavy metals which may pollute water due to human activities. Its critical toxicity levels are above 10 mg kg<sup>-1</sup> dry weight in sensitive species, above 50 mg kg<sup>-1</sup> dry biomass in tolerant species and strains, and above 500 mg kg<sup>-1</sup> dry weight in hyperaccumulators. Nickel toxicity is usually reflected by inhibition of mitotic cell divisions, by iron, zinc, manganese and copper deficiency symptoms, by decreased chlorophyll and carotenoid pigment content, by impairment of light-driven electron transport in the core complexes of photosystem I and photosystem II [19, 36, 43], by depletion of oxygen production in the water-splitting complex (if nickel accumulates in chloroplasts in micromolar concentrations). Since nickel ions have a stable oxidation state, nickel is not a redox-active metal, so it cannot directly induce oxidative stress by enhancing formation of reactive oxygen species. Still, upon extended exposure to high nickel levels, increases in the concentrations of superoxide anion, hydrogen peroxide, hydroxyl radicals and nitric oxide radicals may be experienced by algal cells, most probably because nickel reduces the activity of enzymes involved in the antioxidative protection. This is why nickel toxicity can induce depletion of cellular glutathione pool and can cause membrane damage through lipid peroxidation [12, 13, 20, 40, 42, 58, 76, 105, 111, 126, 153].

Nickel ions entering the aquatic ecosystems originate mainly from combustion of coal and gasoline, from mining activities, smelting, vehicle emissions, sewage sludge, alloy manufacture, electroplating, disposal of electrical batteries. Because nickel shares the same transport system with copper and zinc to enter the algal cells, its increasing amount in the aqueous solution inhibits absorption of these essential trace elements. It may also exhibit uptake competition with iron, manganese and calcium, while high amounts of magnesium may reduce its intracellular accumulation in toxic concentrations. On the other hand, high nickel levels may prevent uptake of increased amounts of more toxic heavy metals from co-contaminated water, such as cadmium and mercury [2, 10, 60, 88, 94, 149, 166, 175, 182, 203]. In case of microalgae belonging to *Scenedesmus*, *Chlamydomonas* and *Euglena* genera, it was found that the nickel concentration which causes death of 50% of algal individuals is about an order of magnitude or more higher

than in case of cadmium. Toxicity is related to the free divalent nickel ion, while the main sequestered form is bound to histidine, nicotianamine, citric and malic acid. The optimal pH for extraction and bioaccumulation of nickel from polluted water is slightly alkaline, being situated around the value of 8, and the bioconcentration factor varies between 400 and 3000, depending on species, growth conditions and external nickel concentration [7, 9, 11, 17, 21, 77, 107, 131, 160, 167, 196]. Elevated concentrations of free histidine and of metallothioneins, as well as increased serine decarboxylase activity are useful biochemical markers for selection of nickel-tolerant and hyperaccumulator algal species, in order to introduce them in a “green” technology for treatment of water polluted with nickel.

## **EFFECTS OF CADMIUM ON ALGAL METABOLISM AND GROWTH**

Cadmium has no physiological function in plants, so its toxicity manifests even at very low concentrations. It may get into aquatic environments mainly from plastic stabilizers, fertilizers, paints, batteries and electroplating [35, 142, 157]. Toxic effects of cadmium rely on its influence on many metabolic processes. It inhibits the activity of several enzymes. For example, carboxylase activity of Rubisco in the stroma of chloroplasts is impaired, but its oxygenase activity becomes enhanced, thus carbon dioxide assimilation rate in the Calvin cycle decreases, but photorespiration associated with carbon assimilation becomes more intense, this resulting in a lower net biomass production. Photosynthetic pigment content is also altered in the presence of cadmium, this fact leading to decreased efficiency of light energy harvesting. Because synthesis and degradation of different pigment types is influenced in different degrees, modifications of chlorophyll-*a* to chlorophyll-*b* ratio and of total chlorophylls (*a* + *b*) to total carotenoids (carotenes+xanthophylls) ratios are usually much more sensitive markers of negative effects of cadmium than the absolute quantities of the different photosynthetic pigments. For example, under constant photon flux density and controlled growth conditions, in static cultures, the ratios between the main types of photosynthetic pigments, determined spectrophotometrically after extraction with dimethylformamide, exhibited in the green microalga *Scenedesmus acuminatus* distinctive variations in relation with the concentration of cadmium in the polluted water medium (Figure 2). Chlorophyll *a/b* ratio moderately, but significantly increased upon exposure for 5 days to 50  $\mu\text{M}$  and 500  $\mu\text{M}$  cadmium, indicating that chlorophyll-*b* content decreased in a higher extent than chlorophyll-*a* content (overall chlorophyll content was lower in the presence of cadmium as compared to control populations – data not shown).

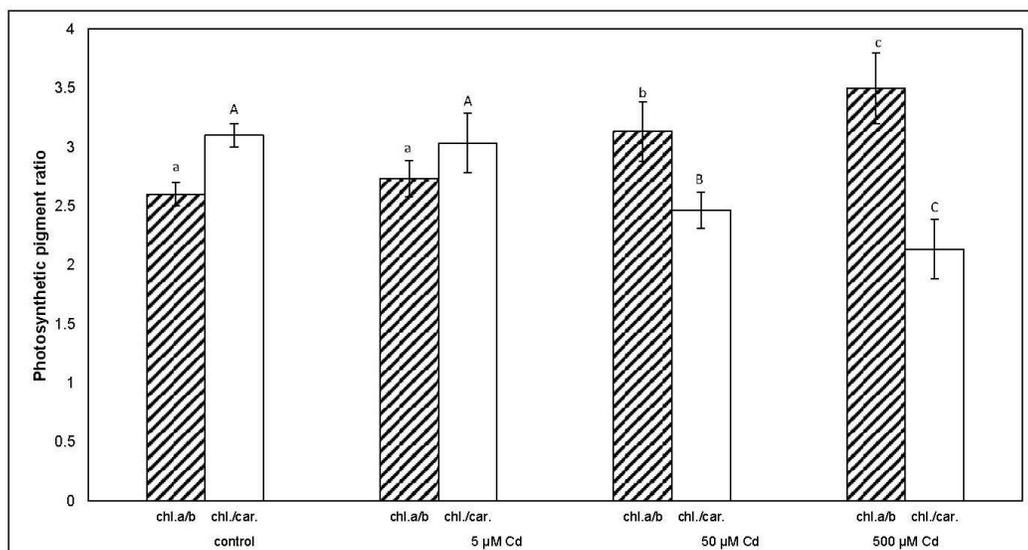


Figure 2. Photosynthetic pigment ratios in the green microalga *Scenedesmus acuminatus*, after 5 days of exposure to different concentrations of cadmium, under constant illumination and controlled growth conditions. chl. – chlorophylls; car. – carotenoids (n = 5, vertical bars represent means  $\pm$  SE, different letters indicate statistically significant differences at  $P < 0.05$  between components of the same series, according to post-ANOVA Tukey HSD test).

This may be explained by the restriction of peripheral light-harvesting antennae around the photosystems, where there is a higher number of chlorophyll-*b* molecules than in the inner antennae. In contrast with chlorophyll *a/b* ratio, the chlorophylls to carotenoids ratio decreases progressively with the increased severity of cadmium pollution, because chlorophyll content is impaired in a higher extent than carotenoid pigment content. In the photosynthetic apparatus, cadmium toxicity may also result in inactivation of the water-splitting complex and in inhibition of electron transport on the acceptor side of photosystem I, as well as in damage to the thylakoid membranes because of stimulation of lipoxygenase activity. The reduced assimilation rate of carbon dioxide due to Rubisco inhibition leads to feed-back down-regulation of the entire light phase of photosynthesis, because chemical energy resulting from photochemical reactions cannot be used in synthesis of new organic compounds in the Calvin cycle [37, 147, 189].

An indirect harmful effect of cadmium is induction of iron, zinc, manganese and copper deficiency, because it competes with these essential trace elements for the same membrane transporters (mainly via the high-affinity, nonspecific zinc-iron protein transporter family). It also disturbs calcium homeostasis of plant cells and inhibits nitrate reductase in the nitrogen assimilation pathway [197]. High amounts of cadmium may also inactivate DNA mismatch repair processes, thus exerting a mutagenic effect. Another indirect effect of cadmium in plant cells is oxidative damage of proteins, membrane lipids and nucleic acids. In sensitive species a decrease of antioxidants may be

observed, while development of tolerance is related with a significant increment of protective antioxidants [33, 67, 91, 136]. Cadmium is one of the most effective activators of phytochelatin synthase, which is a constitutively expressed enzyme in plant cells, but requires post-translational activation by heavy metals that accumulate in the cytosol. Because the favored ligands of cadmium are thiols, cadmium ions are mainly complexed and sequestered by phytochelatins, which confer tolerance to cadmium toxicity in tight connection with up-regulation of sulfur metabolism [8, 28, 44, 121, 158]. *Chlorella*, *Chlamydomonas*, *Euglena* and *Scenedesmus* species were found to accumulate cadmium with a bioconcentration factor between 100 and 200, when initial cadmium content of the polluted water ranged between 5 and 100  $\mu\text{g L}^{-1}$ . It was also established that a more efficient cadmium ion removal may be achieved by adsorption to dead algal cells embedded in gel particles [81, 118, 187].

### **PHYSIOLOGICAL MARKERS OF THE IMPACT OF CHROMIUM, NICKEL AND CADMIUM ON MICROALGAE GROWN IN POLLUTED WATER**

Presence of heavy metals in polluted water is perceived as a disturbing condition which initiates stress reactions that lead to an increased chance of survival and further development. During these stress reactions coordinated physiological modifications occur, which indicate the capacity and degree of tolerance achieved through individual, reversible acclimation to the worse living conditions. These functional changes may be valuable markers of the effects of heavy metals on algae if we succeed to reveal the cause-effect relations that underly acclimative responses. The most common structural indicator of stress condition is algal biomass, which may be evaluated based on cell density of populations, ash-free dry weight, chlorophyll-*a* content or biovolume. Productivity is a useful functional marker, and may be determined through biomass change in a given period of time, through carbon assimilation rate and through oxygen production in light. Because variation in oxygen content in a given volume of algal culture may be measured both in darkness and during constant illumination, it enables determination of net production, brut photosynthetic production and respiration rate. Dynamics of photosynthetic pigment content, inhibition or enhancement of the catalytic activity of several protective enzymes, degree of membrane lipid peroxidation, synthesis of specific metabolites (e.g., phytochelatins), and chlorophyll fluorescence parameters related to energetic efficiency of light use in photochemical processes, may all be valuable biochemical and physiological markers for a correct evaluation of algal reactions to heavy metal stress, as well as for selection of algae and adjustment of growth conditions in order to optimize remediation of heavy metal-polluted aquatic environments using selected microalgae [72, 80, 108, 119, 124, 133, 137, 161, 170, 178, 192, 204].

In order to exclude interference of other external factors with heavy metals, it is preferable to study the above-mentioned functional parameters under controlled experimental conditions. Axenic monoalgal cultures have the advantage that bacterial processes do not interact with algal metabolism, and the response is not a mixture of the reactions of several different species. Culture media with known chemical composition allow an equilibrated supply of essential nutrients. It is also recommended to keep a constant optimal temperature around the algal cultures, and to set a photon flux density of photosynthetically active radiation which provides sufficient, but not excessive light energy for an algal culture with a given cell density. For example, a photon flux density of  $350 \mu\text{M m}^{-2} \text{ s}^{-1}$ , a temperature of  $22^\circ\text{C}$ , and a rotary agitation at 100 rpm (to homogenize the algal cell culture) ensure adequate developmental conditions to study the impact of heavy metal pollution on the selected algae and to evaluate the remediative potential of algae for heavy metal-contaminated water ponds. If several physiological parameters can be followed simultaneously, the results will give a better reflection of what really happens under natural conditions [55, 171].

Because algae are photoautotrophic organisms, their primary production of new organic compounds depends largely on the efficiency of using light energy under the given environmental conditions. This is why impact of heavy metals on their energetic metabolism determines their vitality, and leads to differential tolerance and sensitivity degrees of algal species and varieties against toxicity of various heavy metals occurring in polluted water. Parameters of induced chlorophyll fluorescence are very sensitive markers of photosynthetic efficiency, vitality and stress tolerance of algae inhabiting contaminated water. Chlorophyll fluorescence may be induced in dark-adapted algae (with stopped photosynthesis because of lack of light), and in algal cultures exposed to a constant background illumination. In dark-adapted algae the so-called conventional fluorescence parameters can be determined, while in constantly illuminated algal cultures pulse amplitude-modulated parameters of chlorophyll fluorescence may be registered and computed, upon application of modulating light flashes with determined period, wavelength and intensity. By combining parameters of conventional and modulated chlorophyll fluorescence, it is possible to appreciate potential (maximal) and effective quantum yield of photosynthesis, to determine the vitality index of algae under the given conditions (through the relative fluorescence decay), and to determine the protective capacity through dissipation of excess energy by heat emission, reflected by the non-photochemical quenching (NPQ) of fluorescence signal. This parameter is based on the difference between the non-modulated maximal fluorescence ( $F_m$ ) and the modulated maximal fluorescence ( $F_m'$ ), and is obtained through the relation  $\text{NPQ} = (F_m - F_m')/F_m'$ . Under normal growth conditions the numeric value of non-photochemical quenching is very small, but as stress conditions induce defensive reactions in the photosynthetic apparatus, its value increases significantly, reflecting that a certain degree of tolerance is developed against the disturbing environmental factor. This is why

increase in NPQ is a valuable marker also of heavy metal stress and of algal capacity to cope with unfavorable conditions in the changed aquatic habitat [53, 82, 139, 204]. For example, when axenic monoalgal cultures of *Scenedesmus acuminatus* were exposed for 5 days under constant experimental conditions to different concentrations of cadmium, chromium and nickel, non-photochemical quenching of chlorophyll fluorescence was proportionally increased with the concentration of cadmium, but in case of chromium and nickel only concentrations as high as 50  $\mu\text{M}$  and 500  $\mu\text{M}$ , respectively, resulted in a significant raise of heat dissipation from light-absorbing chlorophylls, indicating a protective reaction of the photosynthetic apparatus against heavy metal toxicity (Figure 3). It is also observable that similar increases in NPQ values are induced by 50  $\mu\text{M}$  chromium and 500  $\mu\text{M}$  nickel, suggesting that nickel is less toxic to photochemical processes than chromium. Changes in NPQ depend both on type and concentration of heavy metal, so using this parameter a selective characterization of various heavy metal pollutions may be performed *in situ* and *in vivo*, without sacrificing the algae for determination.

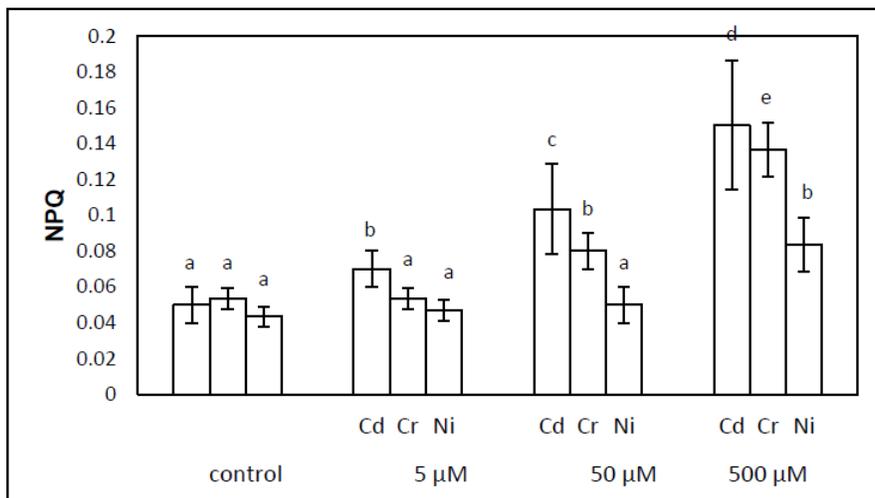


Figure 3. Non-photochemical quenching (NPQ) of induced chlorophyll fluorescence in populations of the green microalga *Scenedesmus acuminatus* grown under controlled conditions and exposed for 5 days to different concentrations of cadmium, chromium and nickel (n = 5, vertical bars represent means  $\pm$  SE, different letters indicate statistically significant differences at P < 0.05, according to post-ANOVA Tukey HSD test).

Dry algal weight is a basic structural indicator for evaluation of net biomass production in the presence of undesirable heavy metals. It enables us to distinguish between the toxicity levels of different heavy metals, to establish concentration thresholds for algal survival, and to select proper species and strains for remediation of water pollution [90]. For example, under the experimental conditions mentioned above for non-photochemical fluorescence quenching evaluation, dry weight of *Scenedesmus*

*acuminatus* populations exposed for ten days to different heavy metals registered distinctive changes (Figure 4). In case of cadmium, a concentration of 5  $\mu\text{M}$  already caused a reduction in algal biomass, while the same concentration of nickel induced a moderate, but statistically significant stimulation of algal dry weight upon development of hardening reactions. Chromium started to decrease algal biomass only at 50  $\mu\text{M}$ , and this inhibitory effect became more intense at 500  $\mu\text{M}$ . Nickel started to moderately decrease algal dry weight only at the concentration of 500  $\mu\text{M}$ . These results demonstrate that biomass production of algae is an easily determinable, but sensitive growth parameter to differentiate between impacts of different heavy metals and their different concentrations, and also to identify more tolerant or resistant algal varieties, suitable for phytoremediation of variously polluted aquatic habitats.

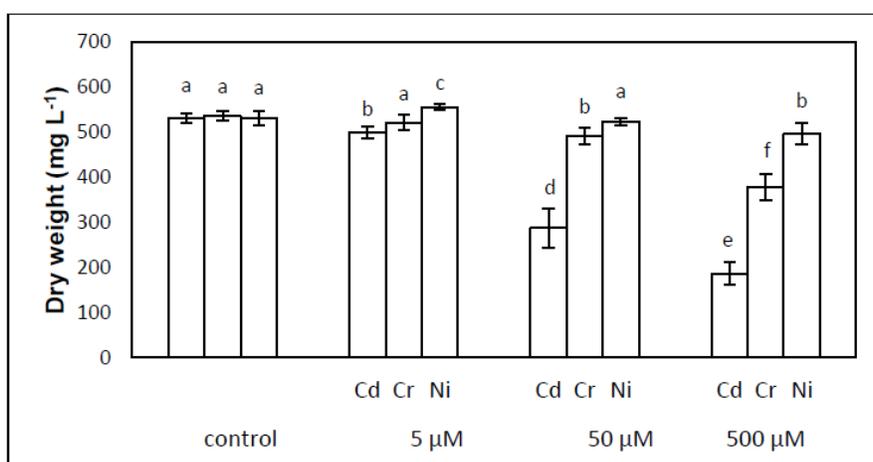


Figure 4. Dry biomass production of the green microalga *Scenedesmus acuminatus* exposed for ten days to different concentrations of cadmium, chromium and nickel in the aquatic medium ( $n = 5$ , vertical bars represent means  $\pm$  SE, different letters indicate statistically significant differences at  $P < 0.05$ ).

## INTERACTIONS BETWEEN HEAVY METALS IN MICROALGAE EXPOSED TO CO-CONTAMINATED WATER

Contaminated aquatic ecosystems rarely contain only one type of pollutant. Biosorption, transport, sequestration and biotransformation of heavy metals may be significantly influenced by the presence of other heavy metal species, of different mineral nutrients, and even of organic xenobiotics (hydrocarbons, pesticides, surfactants). This is why antagonistic or synergistic interactions between essential and non-essential metals, and between heavy metals and non-metallic contaminants may occur, and these interactions have to be considered when the bioremediative efficiencies of algae are determined [27, 41, 102, 184, 185]. The most obvious competitive interactions exist

among polluting heavy metals and metallic micronutrients during their membrane transport into cell compartments, while cooperative interactions among co-contaminating heavy metal species can be observed in concern with their toxic effects on various metabolic and developmental processes. Usually, non-essential heavy metals utilize the mechanisms evolved for the transport of essential mineral nutrients, so chemically similar essential and non-essential metals compete with each other over a shared transporter and for the chemical energy necessary for an active membrane transport which enables a bioconcentration of heavy metals against the electrochemical gradient. Cation diffusion facilitator and cation exchanger membrane proteins are such sites of uptake antagonism between different metal species. For example, cadmium enters plant cells through the transporter which normally performs the uptake of zinc, iron and manganese. This is a main reason why water pollution with micromolar amounts of cadmium causes iron, zinc and manganese deficiency symptoms. Similar competition for common transporters exists between nickel and copper, between chromium and iron. On the other hand, the lack of an essential metallic micronutrient may trigger the up-regulation of membrane transporters that will non-specifically uptake other metals, including toxic, non-essential heavy metals. Attenuation of each other's accumulation in algal cells is also based on competition of co-contaminating heavy metals for a limited number of binding sites due to the shared use of the same transporter. This is why the quantitative ratio between the competing metal species is more important than their absolute amount, in the context of competitive or cooperative interactions. In some cases, heavy metals existing in the aqueous solution may also compete with anionic mineral nutrients for entering algal cells. For example, chromate utilize the transport system which primarily serves for sulfate uptake, while other anionic compounds containing heavy metals compete with phosphate for the same membrane transporter, which is less specific for one kind of inorganic anion [46, 62, 66, 113, 122, 172].

Solubility and uptake of heavy metals are considerably influenced by the presence in the aqueous solution or in cell compartments of organic chelators, which may be synthetic (e.g., ethylene-diamine-tetraacetate, ethylene-diamine-disuccinate) or natural compounds (e.g., citrate, malate, proline, histidine). Their existence facilitates bioextraction and sequestration of heavy metals, which is good for remediation purposes, but in the same time they enhance metal toxicity because they may increase intracellular concentration of harmful metal species. Through biotransformation of different organic pollutants, micro-organisms present in the polluted water also have a serious impact on the fate of different non-essential and essential metals concerning their availability for algae and their synergistic or competitive interactions [34, 79, 93, 97, 115, 116, 117, 132, 163]. Additivity or synergism may be oftenly observed when toxicity of heavy metals is investigated in co-contaminated aquatic habitats. In some cases, harmful effects of different heavy metals occur in different action sites in an organism, and there is no direct interference between these pollutants. But in many cases, simultaneously present

heavy metals act on different steps of the same physiological process, or development of tolerance and defense towards one heavy metal type confers similar protection or hardening to other existing heavy metals. This is a phenomenon known as cross-tolerance to different stress factors, and it is a common feature of plant reactions to disturbing external agents. For example, if one heavy metal induces in algal cells an increment in generation of reactive oxygen species, an enhanced activity of the antioxidative enzymes will confer protection to the same effect of other, co-existing heavy metals, so the simultaneous effect will not be the sum of effects registered upon separate exposure to the different heavy metal species [92, 109, 122, 179, 194, 198].

For studying antagonistic or synergistic relations between different heavy metals existing in the same polluted water, developmental and metabolic markers (structural and functional indicator) may be equally suitable, and it is desirable to simultaneously investigate more parameters. For example, in experiments performed with axenic batch cultures of the green microalga *Scenedesmus acuminatus* under controlled, stable growth conditions, physiological markers which are relevant for evaluation of the remediative capacity, exhibited different patterns of variation when the alga was exposed for ten days, in Bold's basic nutrient medium, to separate and combined pollution with the same concentration (50  $\mu\text{M}$ ) of cadmium, chromium and nickel. Concerning dynamics of the cell density of algal populations (which reflects the relation between the rate of reproduction through cell divisions and the rate of cell death), it was noticeable that nickel reduced the inhibitory effect of cadmium over time, but enhanced the toxic effect of chromium. Chromium could not compensate for cadmium effect on net reproductive rate (Figure 5).

From the several parameters of induced chlorophyll fluorescence related to energetic efficiency of light use in photosynthesis, the non-photochemical quenching of fluorescence, which correlates with the protective capacity through dissipation of excess energy, is mostly increased by the simultaneous presence of chromium and cadmium, while nickel does not modify NPQ increment caused by cadmium, but it enhanced dissipative processes initiated by chromium (Figure 6). Changes in dry algal biomass upon exposure to co-contamination with associated heavy metals are also useful in establishing possible interactions between different heavy metals accumulated in algal cells. For example, nickel may reduce the inhibitory influence of cadmium, but in association with chromium it exerts a moderate, but statistically significant disturbing effect. Chromium also enhances toxicity of cadmium (Figure 7).

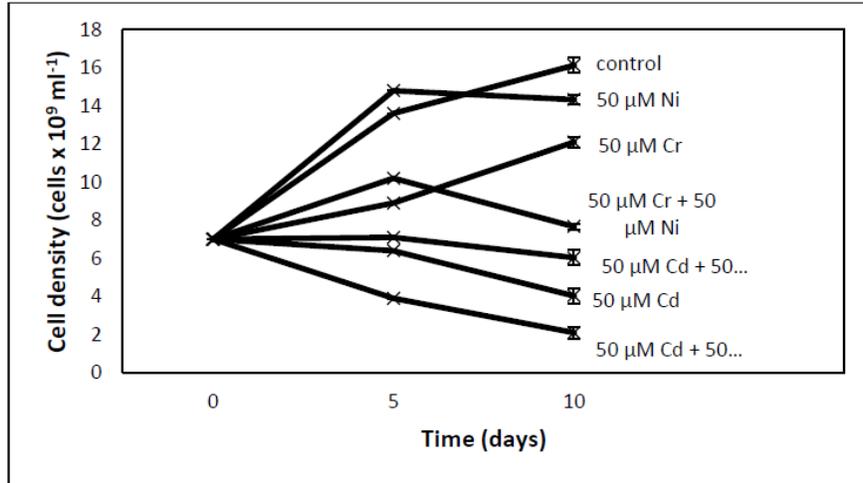


Figure 5. Dynamics of cell density (determined cytometrically) in populations of the microalga *Scenedesmus acuminatus* exposed to separate and combined pollution of the aquatic medium with similar concentrations of nickel, chromium and cadmium (n = 5, vertical bars represent  $\pm$ SE from means).

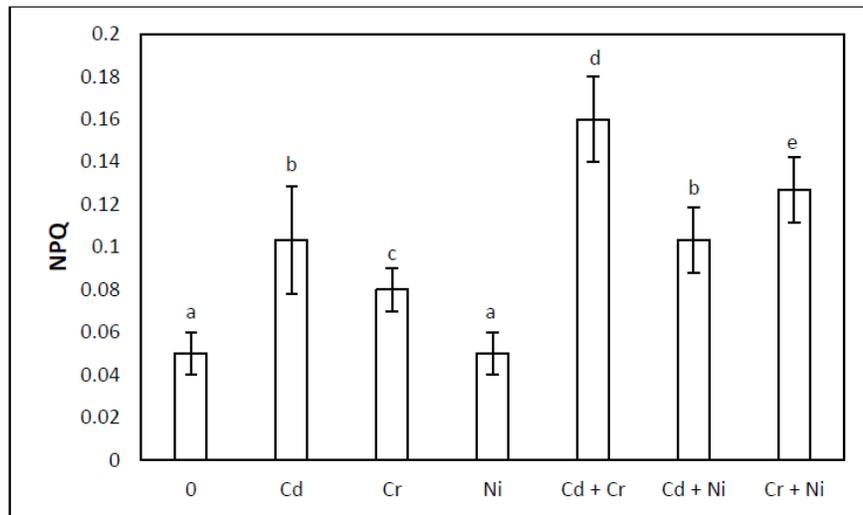


Figure 6. Non-photochemical quenching (NPQ) of induced chlorophyll fluorescence in populations of the green microalga *Scenedesmus acuminatus* grown under controlled conditions and exposed for 5 days to separate and combined pollution of the aquatic medium with similar concentrations of nickel, chromium and cadmium (n = 5, vertical bars represent means  $\pm$  SE, different letters indicate statistically significant differences at P < 0.05).

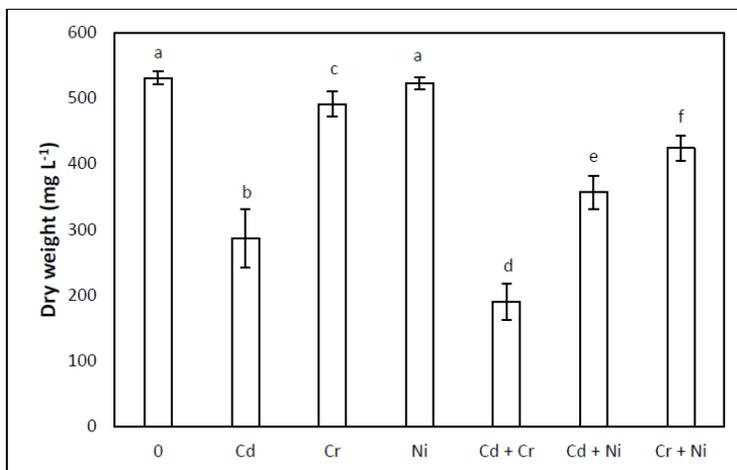


Figure 7. Dry biomass production of the green microalga *Scenedesmus acuminatus* exposed for ten days to separate and combined pollution of the aquatic medium with similar concentrations of nickel, chromium and cadmium (n = 5, vertical bars represent means  $\pm$  SE, different letters indicate statistically significant differences at  $P < 0.05$ , according to post-ANOVA Tukey HSD test).

## CONCLUSION

Several microalgae are effectively able to contribute to remediation of heavy metal-polluted water, by performing bioextraction, concentration, stabilization, sequestration and even detoxification of certain heavy metals. Their use in phytoremediation of aquatic habitats relies on their ability to bind, to accumulate, to immobilize and to enzymatically convert heavy metals to less toxic forms. These abilities largely depend on prevailing growth conditions, on exposure time and metal concentrations, on resistance, tolerance or sensitivity of algal processes to heavy metal stress, on availability of inorganic nutrients, and on the presence of other pollutants. In many cases, immobilized algal cells embedded in gel particles perform a more sustained extraction, with the possibility of recovery of useful metals in a concentrated form. Selected biological markers for heavy metal tolerance are useful tools for identification of suitable algal species and strains, in an attempt to optimize phytoremediation of polluted water.

Interactions between simultaneously present heavy metal species are inevitable in most of the real-world applications of bioremediation, this is why efficiency of microalgae in decontamination of water bodies has to be studied also in the presence of several heavy metals, if possible not only under laboratory conditions but also in large-scale natural ponds, in order to reveal antagonistic relations or altered toxicity due to cross-tolerance. The NPQ parameter proves to be a reliable physiological marker not only for screening the protective capacity of algal photosynthetic apparatus against different heavy metals, but also for revealing relevant interactions among co-contaminating heavy

metals. The easily determinable algal dry weight and reproductive rate, which are direct reflectants of productivity under stress conditions, as well as the quantitative ratios between the main types of photosynthetic pigments, are also good tools for evaluation of bioremediative applicability of different algae in variously heavy metal-polluted aquatic environments. Extension of tolerability experiments to large-scale water bodies polluted with heavy metals is a promising perspective for using selected microalgae in efficient and environmental-friendly technologies for bioremediation of anthropically affected aquatic habitats.

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