PHYSIOLOGICAL MARKERS OF DUCKWEED (*Lemna minor* L.) FOR BIOINDICATION OF WATER POLLUTION WITH COPPER AND DIURON (3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA)

Laszlo FODORPATAKI[*], Szabolcs BARNA[*], Hilda DEAK[*], Balazs KOVACS[*], Janos GERAJ[*], Botond HOLINKA[*]

[*] *"Babeş-Bolyai" University, Hungarian Department of Biology and Ecology, Cluj-Napoca, Romania*
Corresponding author: Laszlo Fodorpataki, "Babeş-Bolyai" University, 1 M.Kogălniceanu St., RO-400084 Cluj-Napoca, Romania, tel. +40-264-431858, e-mail: lfodorp@gmail.com

Abstract. Lesser duckweed (*Lemna minor* L.) is a cosmopolitan aquatic test plant, its growth and metabolic parameters are useful markers of the influence exerted on living organisms by changes in water quality. We have investigated the influence of water-polluting copper and herbicides on net biomass production, photosynthetic pigment content and efficiency parameters of induced chlorophyll fluorescence of duckweed. Copper decreased biomass production of duckweed only at higher concentrations (100 µM), while diuron inhibited growth even at 10 µM. With respect to the light-harvesting complexes of chloroplasts, the molar ratios between the main types of photosynthetic pigments proved to be the most sensitive markers of the impact of the applied water-polluting agents. In case of water pollution with copper and diuron, the mostly sensitive parameters of induced chlorophyll fluorescence were the potential quantum yield efficiency and the vitality index of photosynthetic apparatus, these parameters being recommended for bioindication of water pollution.

Keywords: bioindication, chlorophyll fluorescence, duckweed, heavy metal, herbicide, photosynthetic efficiency.

INTRODUCTION

Beside microalgae, aquatic vascular plants with high reproduction rate, small size and broad adaptability may be used in early and accurate bioindication of water pollution related to human activities. Duckweed proved to be a suitable test plant for evaluation of biological impact of different heavy metals and organic water-pollutants [5]. Its use in wastewater treatment, as well as in biomonitoring and bioremediation of polluted aquatic ecosystems [1] was suggested. This implies knowledge of physiological reactions to environmental stresses and identification of physiological and biochemical markers related to acclimation to changing conditions. Growth and photosynthesis seem to be sensitive processes than enable an accurate evaluation of impact of water-polluting agents on plants [12]. Even though algae are the main primary producers in aquatic ecosystems, their use in bioindication of water quality may be restricted by their broad adaptability to changes in environmental conditions. If algal populations develop resistance against stress factors such as heavy metals or organic xenobiotics, the resulting populations will not react physiologically to further changes in the physico-chemical parameters of the habitat. In this case, use of aquatic vascular plants with higher sensitivity may be indicated. Among the most potent water-polluting agents, related to human activities, herbicides originating in agricultural practices, and soluble forms of heavy metals originating in mining and industrial branches, represent chemical stressors that may become primary selection factors for plants that inhabit inland waters. This is why an early indication of water-pollution with herbicides and heavy metals, based on specific changes in physiological processes of aquatic plants while they try to cope with adverse conditions by developing hardening-based tolerance, is important for an efficient prediction of water-pollution, in order to be able to take the necessary decisions for early restoration procedures [7, 9]. Because different physiological processes in various organisms give a large variety of responses to environmental stresses, and tolerance mechanisms are based on the interactions between metabolites encoded by hundreds of specific genes, there is no general pattern for functional and biochemical reactions towards environmental constraints. Furthermore, the exposure time and intensity of stress factors, as well as the developmental stage and the nutritional status of the plants exert a great influence on the reaction of a vital process to a certain polluting agent or other disturbing agent, even in the same organism. This is why sustained effort is made to investigate the specific changes induced in various metabolic and developmental processes by different environmental stress factors, and to identify physiological markers that enable a rapid and accurate bioindication of the influence exerted by water-polluting agents on different living organisms [15, 22].

The aim of the present research is investigation of the influence of water-polluting copper and of a non-selective, largely used herbicide (diuron or DCMU) on net biomass production, photosynthetic pigment content and efficiency parameters of induced chlorophyll fluorescence of duckweed.

MATERIAL AND METHODS

Lesser duckweed (*Lemna minor* L.) plants, originating from a native population of a freshwater pond in Ernei (Mures county), were grown in sterile Steinberg nutrient solution [12] and exposed for one week, under constant conditions of illumination (90 µmol photons m^{-2} s^{-1}) and temperature (22°C), to the action of 10 µM and 100 µM copper chloride, as well as of 10 µM and 100 µM diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, also known as DCMU, a herbicide that inhibits photosynthesis). Initial fresh biomass was set to 0.3 g in 200 ml of nutrient solution. Photosynthetic pigments were extracted with dimethylformamide and determined spectrophotometrically [8]. Conventional parameters of induced chlorophyll fluorescence were determined *in vivo* after 10 min of dark pre-adaptation of plants (to stop former photochemical reactions), while parameters of pulse amplitude modulated fluorescence were determined under constant illumination, with a FMS-2 fluorometer. The modulated light was sufficiently weak (0.04 µM $m^{-2}s^{-1}$) so as not to produce any significant variable fluorescence. A single saturating flash (2000 µM $m^{-2}s^{-1}$ for 0.5 s) was applied to reach the maximal fluorescence Fm. After the decline of the signal, the actinic light was turned on (100 µM $m^{-2}s^{-1}$) to start the induction kinetics. The determined parameters were initial fluorescence F_0, maximal fluorescence Fm, the Fv/Fm ratio (Fv or variable fluorescence being the difference between the maximal and the initial fluorescence), the F_0/Fv ratio, modulated maximal fluorescence Fm', steady state fluorescence Fs, the effective quantum use efficiency (Φ) representing the ratio (Fm' – Fs)/Fm', as well as the vitality index (Rfd) expressed as the ratio (Fm – Fs)/Fs [2, 3, 14]. Every experimental setup had 4 repetitions. Data were statistically evaluated with one-way ANOVA, followed by the Tukey HSD test. Standard errors (SE) from means were computed for every experimental variant, and significant differences between control and treated sets were considered at $P < 0.05$.

RESULTS

Growth parameters represent important endpoints of plant vitality under the given environmental conditions, and reflect the capacity to cope with stress factors present in polluted environments. Biomass production of the examined population of *Lemna minor* duckweed, as a final result of overall net photosynthetic assimilation capacity, was inhibited only by higher concentration (100 µM) of copper, while sensitivity of this growth parameter was much higher for the herbicide diuron, which inhibits photochemical reactions in the chloroplasts. Higher concentrations of copper and diuron (100 µM) exerted similar inhibitory effects on biomass accumulation of duckweed at the end of the 7 days exposure time under controlled growth conditions (Fig. 1). Inhibition of biomass production by higher amounts of dissolved copper ion and by different concentrations of the herbicide diuron reflect a differential sensitivity of this growth parameter to certain water-polluting agents.

Photosynthetic pigment content is also a suitable molecular marker of chemical stresses to duckweed. Their concentration changes rapidly during the acclimation to different photon flux densities, but also during defense reactions to environmental stress factors that disrupt metabolic steady-state, light harvesting and photochemical processes. The widest range of changes was registered when molar ratios between pigment types were considered. Chlorophyll *a*/*b* ratio was increased by 10 µM copper and decreased by 100 µM copper, while both concentrations of diuron caused a lower value of this ratio as compared to control (data not shown). Chlorophylls to carotenoids ratio was not significantly affected by 10 µM copper, it was lowered by 100 µM copper, and increased by both concentrations of diuron. Statistically significant differences were registered between the effects of the two copper concentrations, as well as between the plants treated with copper and with diuron, while the two distinct concentrations of the herbicide resulted in no significant difference concerning the degree of increment of the molar ratio between chlorophylls and carotenoid pigments (Fig. 2).

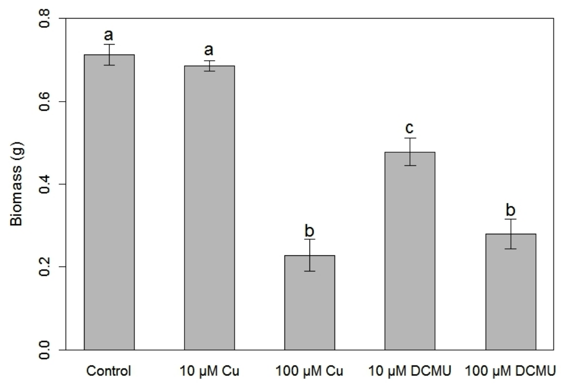

Figure 1. Influence of different concentrations of copper and diuron on biomass production of duckweed after 7 days of exposure. Initial biomass was 0.3 g in every culture. DCMU – diuron. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$

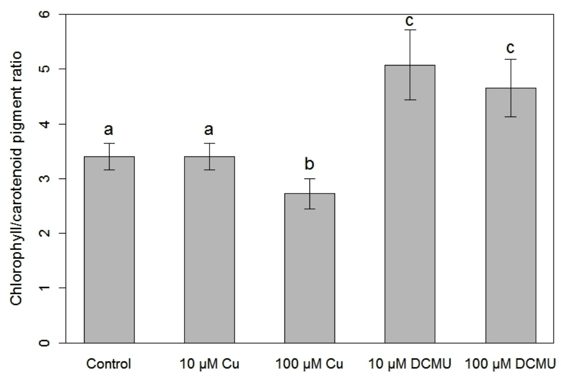

Figure 2. Influence of copper and diuron on chlorophylls/carotenoids ratio in duckweed fronds after 7 days of exposure. DCMU – diuron. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$, according to the post-hoc Tukey HSD test

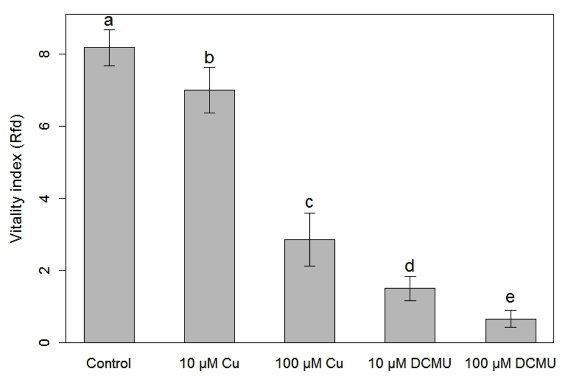

Figure. 3. Influence of copper and diuron on vitality index of the photosynthetic apparatus of duckweed after 7 days of exposure, based on relative fluorescence decrease (Rfd). DCMU – diuron. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$

Parameters of induced chlorophyll fluorescence were shown to be influenced by various environmental stress factors, including different herbicides that accumulate in aquatic environments surrounded by agricultural lands. From among these parameters, we found that potential quantum yield efficiency (Fv/Fm ratio) and vitality index of the photosynthetic apparatus (Rfd) were the most sensitive ones to the effect of copper and diuron under the above-mentioned experimental conditions. The Fv/Fm ratio was significantly lowered only by higher amount of copper, while vitality index was strongly declined by both concentrations of diuron (Fig. 3). These parameters seem to be very sensitive indicators of negative influence of water pollution with copper and diuron on photosynthesis of duckweed. Even the lower copper concentration induced a moderate, but significant change in the vitality index expressed with the relative fluorescence decrease (Rfd), while the non-selective herbicide diuron (DCMU) exerted a pronounced deleterious effect on this functional parameter.

DISCUSSION

Under similar conditions, when they tested influence of ten heavy metals, Naumann *et al*. [12] found that copper is less toxic than cadmium or silver ions, but it is a stronger growth inhibitor than nickel, zinc or chromium. This is probably related to the fact that copper, in small amounts, is an essential micronutrient for all plants, having several metabolic functions [6, 26], and it starts to disturb physiological processes only if it accumulates in higher concentrations in metabolically active cell compartments [20, 24]. Shoji has also demonstrated that pH values and dissolved organic substances highly influence phytotoxicity level of copper [23]. By directly affecting photochemical reactions, diuron has a more pronounced influence on duckweed biomass than similar amounts of copper. This herbicide inhibits light-driven electron transport on the acceptor side of photosystem II, by substituting the secondary quinine acceptor Q_B at its binding site on the D1 polipeptide [4, 9]. This results in a lowered efficiency of photosystem II in the use of light energy, leading to a concentration-dependent depletion of net photosynthetic biomass production. It has to be emphasized that for any efficient metabolic engineering of plants for an improved stress tolerance, growth performances have to be evaluated [15]. In many cases, metabolic manipulation for better defense mechanisms against different biotic and abiotic stress factors significantly decrease the growth potential and biomass production, because the processes involved in stress protection usually consume high amounts of metabolic energy while reprogramming the entire metabolic network and developmental pattern of plants [4, 21]. It was also demonstrated that several heavy metals induce a secondary toxicity effect in plants by generating extra amounts of harmful reactive oxygen species, and in this case an increased antioxidative potential is crucial for a sustained growth under oxidative stress induced by higher concentrations of heavy metals [18]. Oxidative stress is also a main reason for common features of cross-tolerance of plants towards different physical and chemical stress factors that may act simultaneously, e.g. copper toxicity and ultraviolet radiation, because reactive oxygen species act as common stress signals [25].

The data related to changes in the photosynthetic pigment ratios of duckweed indicate that biosynthesis and degradation of *a* and *b* type chlorophylls are affected in different degrees by lower and higher amounts of copper and diuron. Increment of the chlorophylls to carotenoids ratio in presence of the applied herbicide is most probably due to the fact that diuron enhances chlorophyll content (as a compensatory reaction to the decreased light-use because of inhibition of photosynthetic electron transport in photosystem II), but it does not affect carotenoid content (data not shown), while higher concentrations of copper cause a stronger decrease in chlorophylls than in carotenoids. This is in agreement with findings for similar amounts of nickel, a heavy metal that strongly inhibited chlorophyll synthesis, while carotenoid content remained unchanged [1]. Kanoun-Boule *et al*. [8] concluded that copper causes a concentration-dependent decline of chlorophyll *a* and carotenoid content, but they did not report a differential response of the two types of photosynthetic pigments. Megateli *et al*. [11], working with another duckweed species, established that the chlorophyll to phaeophytin ratio may be used as a physiological stress index, its decrement being quantitatively related to the toxicity of different heavy metals that accumulate in the plants. Their finding also supports that molar ratios between different photosynthetic pigment categories may be more suitable biomarkers of environmental stresses than the absolute concentrations of these molecules involved in light-harvesting, photooxidation and photoprotection.

Parameters of the induced chlorophyll fluorescence offer early and very accurate information on different steps of the light phase of photosynthesis, related to conversion and use of light energy in primary production of new organic compound. For example, the ground fluorescence (F0) indicates the efficiency of pigment antennae in absorption and transfer of light energy, the maximal fluorescence (Fm) is related to the intensity of electron transport across the acceptor side of photosystem II. The ratio between the variable fluorescence (Fv) and the maximal fluorescence is directly related to the potential quantum use efficiency of photosystem II, while the ratio between the difference from the modulated maximal fluorescence (Fm') and the steady-state fluorescence (Fs) to the absolute value of Fm' represents the effective light-use efficiency or quantum yield (Φ) of photochemical reactions. The relative fluorescence decrease (Rfd), resulting from the relation (Fm – Fs) / Fs, is considered to be a general indicator of vitality of the photosynthetic apparatus under the given growth conditions. It was previously demonstrated that environmental stress factors, including different heavy metals and herbicides, induce specific changes in certain parameters of the induced chlorophyll fluorescence, thus reflecting the action sites and action mechanisms of these factors in the photosynthetic biomass production of plants [10, 13, 17]. This is why the main physiological markers of influence of copper and diuron on duckweed, investigated in the present research, are related to photosynthetic efficiency reflected by *in vivo* chlorophyll fluorescence. Concerning the changes induced by environmental stress factors in the various parameters of induced chlorophyll fluorescence, the generally accepted concept is that according to the action site and mechanism of stress factors in the photosynthetic apparatus, there are specific variations in these parameters, but none of these seems to be generally accepted as a suitable indicator of harmful environmental impact. In our experiments, exposure of duckweed for seven days to ten and a hundred micromolar amounts of copper ion and of diuron, had the most intense negative influence on the relative fluorescence decrease, which is also known as the vitality index of the photosynthetic apparatus. Similar findings were reported by Geoffroy *et al*. [5], when microalgae and duckweed plants were exposed to another organic xenobiotic water-pollutant. While heavy metal toxicity decreased in a dose- and time-dependent manner the potential and the effective quantum yield efficiency [10, 17], inorganic nutrient deficiencies and overdoses induced specific changes in ground fluorescence (F0), maximal fluorescence (Fm), Fv/Fm ratio and relative fluorescence decrease [3, 15]. Pena-Vazquez *et al*. [14] used the pulse saturation method for registration of the pulse amplitude modulation parameters of chlorophyll fluorescence for testing the influence of copper content of water on immobilized algae, and have found that the most sensitive indicator parameters of copper toxicity are the modulated maximal fluorescence (Fm’) and the non-photochemical quenching of fluorescence by heat dissipation (qN). In case of some biofungicides that penetrated in higher concentration into the leaves of treated plants, the most sensitive parameter of chlorophyll fluorescence proved to be the vitality index of the photosynthetic apparatus, along with decreased stomatal conductance and carbon assimilation efficiency [13]. In our experiments, this physiological parameter, related to the energetic efficiency of photosynthetic light conversion, also proved to be a suitable indicator of the negative effects of the different concentrations of copper and diuron, along an exposure period of seven days.

As a conclusion of our results: copper decreased biomass production of duckweed only at 100 µM, while diuron inhibited growth even at the concentration of 10 µM; the ratio between chlorophylls and carotenoids was lowered by high concentration of copper and it was increased by diuron; the most sensitive parameters of induced chlorophyll fluorescence in duckweed treated for one week with different concentrations of copper chloride and of the herbicide diuron, were the potential quantum yield efficiency and the vitality index of the photosynthetic apparatus, reflected by the relative chlorophyll fluorescence decrease (Rfd).

The above mentioned physiological parameters are recommended for early bioindication of water pollution in ecotoxicological tests performed with duckweed. A better understanding of physiological reactions to environmental stress factors can enable us to effectively induce specific tolerance, and to succeed in priming for unfavorable growth conditions, even without any genetic manipulation [19].

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