Toxicity and removal of Direct Red 28 diazo dye in living polymeric systems

ANDREI LOBIUC[1,2], STEFAN OLARU[3], ELENA IREN HANCU[3], NAELA COSTICA[1], MARIA EMILIANA FORTUNA[4,*], MARIA MAGDALENA ZAMFIRACHE[1], GABRIEL CONSTANTINESCU[5]

[1] Alexandru Ioan Cuza University, Integrated Research Center for Environmental Science-CERNESIM, 22 Carol I Blvd., 700506, Iasi, Romania

[2] Stefan cel Mare University, Faculty of Food Engineering, 13 Universitatii Str. 720229, Suceava, Romania

[3] Alexandru Ioan Cuza University, Faculty of Biology, 11, Carol I Blvd., 700506, Iasi, Romania

[4] Petru Poni Institute of Macromolecular Chemistry, 41A Grigore Ghica Voda Alley, 700487 Iasi, Romania

[5] Carol Davila University of Medicine, Clinical Emergency Hospital of Bucharest, 37 Dionisie Lupu, Str., 020021, Bucharest, Romania

Aquatic environments are often contaminated with various compounds, with potential toxicity towards aquatic organisms, which may enter the food chain. Azo dyes are used in various industries and contamination of waters has been reported. The present paper assesses the toxicity of the synthetic, water soluble Congo Red dye towards Lemna minor from a physiological and cytogenetical point of view. The dye was tested in 5-5000 ppm concentrations. Total frond surface, root growth and fresh mass reductions were registered from 5 ppm dye concentration, with a concentration-dependent response and calculated EC_{50} of 1530 ppm. Plant growth was completely inhibited above 2500 ppm. Dye accumulation was observed in tissues, along with necrosis formation. Chlorophyll a contents decreased, while carotenoid contents increased above 2500 ppm. Significant inhibition of PSII efficiency was recorded above 1000 ppm. Mitotic indices were decreased at 5 and 1000 ppm dye and were 0 at 5000 ppm. The number of chromosomal aberrations significantly increased at 5 and 1000 ppm dye. The growth medium was decontaminated up to 56% at 250 ppm dye concentration by Lemna plants. Congo Red azo dye presented toxicity towards Lemna minor, from a physiological and cyotgenetical point of view, especially at higher concentrations. In the same time, a phytoremediation potential of duckweed with respect to the tested dye was demonstrated.

Keywords: C.I. 22120, diazo, photosynthetic pigments, fluorescence, cytogenetic indices, duckweed, accumulation

Anthropogenic activities result in alterations of the environment in various forms, chemical pollution being one of the most well-known [1]. Chemical pollution may alter one or more types of habitats (terrestrial, aquatic etc.), through direct or indirect actions on components of ecosystems [2]. Direct effects include toxicity on various organisms, due to interactions with nutrient uptake, oyxgen and light availibility or metabolic activity. Indirect effects mainly relate to modifications of ecological mechanisms such as trophic relations or competitive interactions between species [3].

Nevertheless, a chemical substance released into the environment may be a contaminant but not a pollutant, depending on numerous factors, starting with its concentration in a given medium and continuing with availability, modifying factors in the environment and reactions of the exposed organisms [4]. Therefore, for a given substance, testing must be performed to determine whether the pollutant criterion are met and to generate safety data that must be accounted during usage and disposal of such substances [5-9]. This is a major requirement for regulation of various anthropogenic activities and industries [10-12], from chemicals production (pharmaceuticals, solvents, pesticides, foods, dyes etc.), to product usage (agricultural practices, electronic devices manufacturing, textile industry) and residues disposal (municipal and industrial waste management policies). Furthermore, for many substances, toxicological data is missing, mainly because the limited sets of standardized test and the high number of species-compound combinations that should be tested in order to achieve relevant data [13-18].

Toxicological testing is a core aspect of risk assessment of chemicals, with regulations such as TSCA in U.S. (Toxic Substances Control Act) or REACH (Registration, Evaluation and Authorization of Chemicals) in E.U. establishing data requirements for substances that enter the environment [5]. Testing guidelines are established by international organizations such as OECD or ISO, with recommendations for in vitro, in vivo, field or in situ methods of toxicity assessment [19]. However, among standardized in vivo tests, many offer information only on organism level, pertaining to species survival, growth and development. Tests species using species such as *Lemna* instead, offers populational information on effects, which may lead to increased relevance when combined with available data of species ecology [20]. This is owed to the characteristics of this specie, which forms colonies as new individuals are generated most often through budding. Moreover, *Lemna* tests are considered comparable or more sensitive than algal tests or animal tests, especially when colored pollutants are present in aquatic environments [21, 22].

Direct Red 28 (C.I. No. 22120, molecular formula $C_{32}H_{22}N_6Na_2O_6S_2$, molecular mass 696.66 g·mol^{-1}, the sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulfonic acid, CAS 573-58-0, synonym Congo Red), is a compound used for its coloring abilities, mainly in the textile and paper industries, in testing for presence of hydrochloric acid in gastric contents, for testing of amyloidosis and for staining of cellulose-containing tissues in laboratory appplications, as well as a gelling agent for (poly)vinyl alcohol for coloured films and coatings, with applications in some other industries as well [23, 24]. It is a diazo dye, one of the major classes of dyes [25] and by degradation it

* email: fortuna.maria@icmpp.ro

yields benzidine, a highly toxic compound [26, 27]. Thus, its usage in dyeing textile that contact skin was discontinued in E.U., remaining still in use for other applications and probably even for textile industry in some countries.

The fate of azo dyes in the environment may follow one of the routes of adsorption on various substrates (including biosorption on bacterial cells, algae or plants), chemical transformation, photodegradation, air stripping or biological transformation [28, 29]. Transformation of Direct Red 28 in natural environments was chemically described especially for microorganisms. Such organisms may degrade the dye under anaerobic or aerobic conditions, with the aid of enzymes such as laccases, tyrosinase, veratryl alcohol oxidase, azoreductase [30].

Moreover, the release of azo compounds in wastewaters raises some issues, as these dyes are not easily degradable under aerobic conditions [25] and, due to their colouring properties, may affect photosynthetic activities even at low concentrations [31, 32]. The current data concerning azo dyes toxicities on aquatic plants describe mainly effects on growth and antioxidative enzymes [33, 34]. Such enzymes might be useful in assessing toxicity, but they are not contaminant-specific but rather mechanism of action specific, indicating effects sooner than factors and their activities might influenced by various elements in the environment [35]. Research regarding the genotoxic effect of azo dyes was described in *Allium cepa*, for BDCP dye that produces nuclear aberrations at 0.1 ppm [36]. Such aberrations may be the result of intercalation of dye between DNA pairs [37] and may induce apoptotic or necrotic cells [36], resulting in cyotoxicity, as observed in *Lemna minor* roots or fronds.

Current toxicological data contained in available data sheets for C. I. Direct Red 28 concerns only toxicity towards bacteria, cell lines, rodents and algae. Generally, for C. I. Direct Red 28, toxicity at a cytogenetical level is described only in the case of microorganisms and mammals, but little is known about the genotoxicity on aquatic plants. The mechanisms of azo dyes removal in the case of *Lemna minor* are not entirely explained, however it is considered that, initially, the dye gets mechanically attached to plant tissues, then it is degraded, probably through antioxidative enzymatic activity [38]. Considering that effluents contaminated with azo dyes may enter habitats with a complex biota, and that higher aquatic plants may be suitable for toxicity testing, the current study aims to establish the effects of Direct Red 28 on *Lemna minor* L. and to determine if the species holds a potential for remediation of waters contaminated with the considered compound.

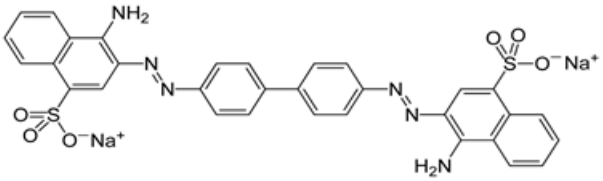

Fig. 1. Chemical structure of C.I. Direct Red 28

Experimental part

Materials and methods

Biological material

Lemna minor L. (duckweed) individuals were collected from three natural populations from Suceava county, Romania. Since phenological variability was observed among the three populations, individuals from each population were cultured prior to actual experiment under controlled conditions. The individuals (colonies) with the highest growth rate and most suitbale morphometrical characteristics (frond surface, root length) were selected and further used for testing.

Cultivation conditions

For stock culturing, axenic conditions were established, by surface sterilizing duckweed plants in 0.5% chlorine solution then rinsing them in sterile distilled water. Sterilized plants were introduced in beakers containing sterile cultivation medium. Cultivation was done in SIS culture medium, following the already described recipe [39], from which MOPS buffer was omitted. Stock cultures were maintained and used as source of duckweed individuals for effective testing.

Testing was conducted according to OECD guidelines for aquatic toxicity [39]. At experiment initiation, duckweed individuals were placed in 400 mL glass beakers, filled with 300 mL test or control solutions. For each variant, 3 replications were set. Each replication initially received 3 healthy, mature duckweed individuals (with at least 2 fronds developed, without any visible alterations of frond surface or of roots). Manipulation of plants, cultivation glassware and medium was performed under a sterile laminar flow hood, using sterile instruments. Following inoculation, beakers were covered with sterile

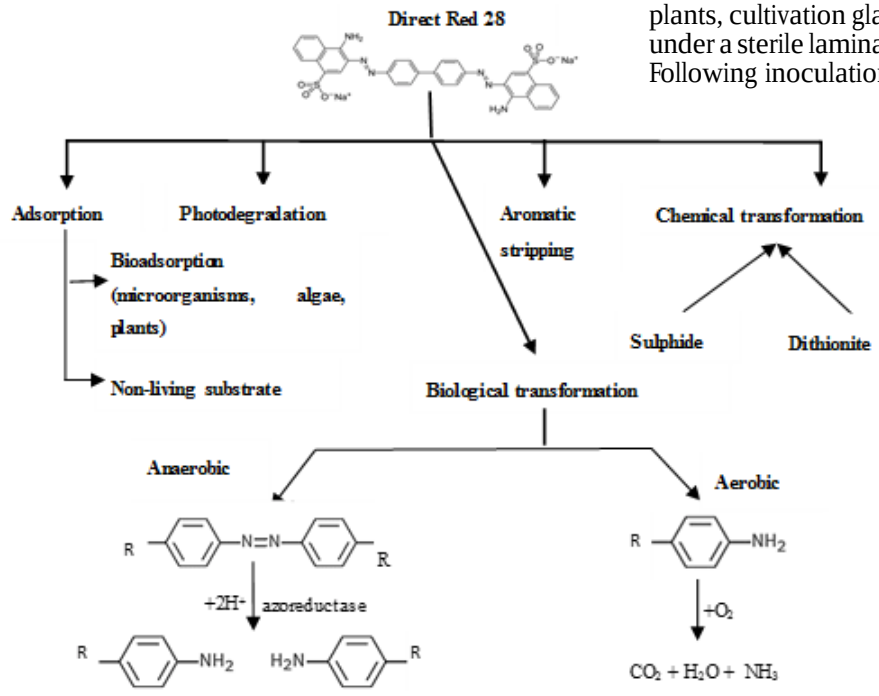

Fig. 2. Possible environmental fates of C.I. Direct Red 28

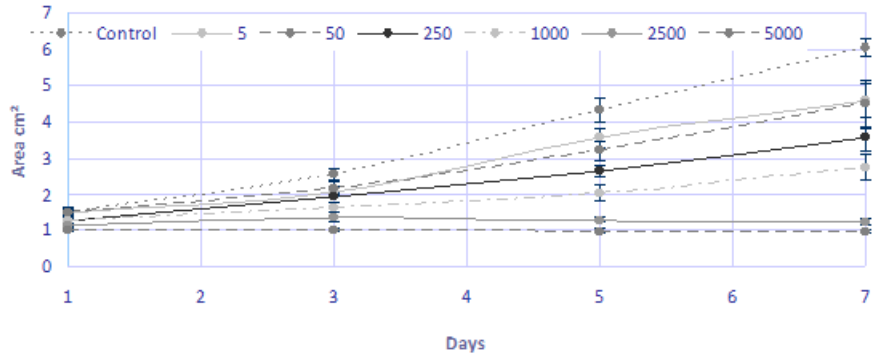

Fig. 3. Total frond surface dynamics of *Lemna* plants

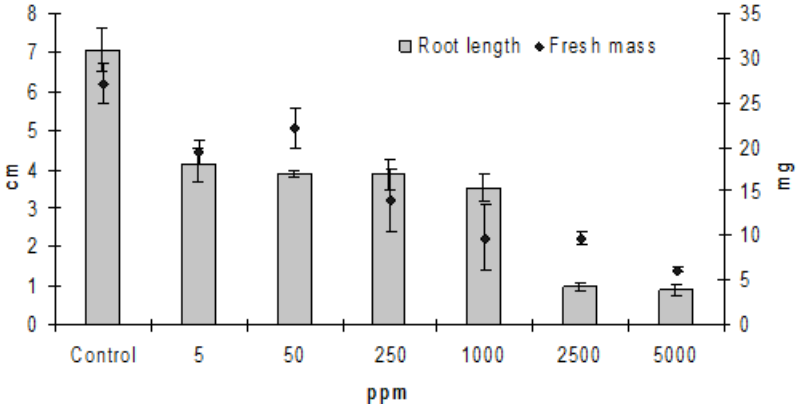

Fig. 4. Root lengths and fresh masses of *Lemna* plants after 7 days of cultivation

glass Petri dishes to avoid airborne microbial contamination. Cultivation of duckweed was performed using an incubated cabinet, at 24°C constant temperature and 6400K fluorescent tubes supplied light at a 12 h/12 h photoperiod.

Assays

Total fronds surface of duckweeds was monitored on digital photographs of the colonies using ImageJ software. Fresh mass of plants and the ash content were determined gravimetrically. Root lengths were measured using a caliper.

Growth rate was calculated based on differences in total frond surface, following the formula:

$$G_r = (\ln Ft_n - \ln Ft_{n-1})/(t_n - t_{n-1}),$$

where Ft_n is the total frond surface at moment t_n.

Anatomical observations were performed on fresh sections of fronds and on roots. For observing accumulation of dye in tissues, cellular contents in sections was evacuated using bleach solution.

The content of chlorophyll in fronds was determined in 80% aqueous acetone extracts. After filtration of extracts, their absorbances were read at 470, 646 and 663 nm wavelengths and formulas from Wellburn [40] were used.

Chlorophyll fluorescence was measured using a FMS2 portable fluorometer (HansaTech Ltd.). ÖPSII values were obtained for nine fronds per variant.

Total phenolic contents were determined in 70% ethanolic extracts prepared from *Lemna minor* fronds based on the Folin-Ciocalteu reagent assay according to [41].

Cytogenetic analyses were performed using frond material hydrolysed in hydrochloric acid:water (1:9 v/v) solution and stained with acidified carmine reagent [42]. The material was squashed on a microscope slide and various cell cycle phases were counted. A number of minimum 1000 cells were counted for each variant.

The content of Direct Red 28 dye in the growth medium was monitored by spectrophotometric analyses at 450 nm, at various moments, by extracting aliquots from the growth vessels.

Results and discussions

Morphological and structural effects

The dye present in medium reduced the growth of *Lemna minor* plants, compared to control individuals. The inhibition was observed for frond growth (fig. 3), root elongation, as well as for fresh biomass (fig. 4). For these parameters, the effect was dose-dependent, with correlation factors *r* of -0.85, -0.80 and -0.77 respectively. The total frond surface presented a positive growth trend for control plants and 5-1000 ppm dye concentration in medium plants over 7 days, while plants grown on 2500 and 5000 ppm dye showed no significant increase in the same interval. Based on frond total surface, the calculated IC_{50} of dye was 1530 ppm.

Concomitantly with quantitative reductions of morphological parameters, *Lemna* individuals grown on dye supplemented medium also displayed necrosis formation on fronds, along with dye uptake in the epidermis (fig. 5a-d, g and h). In roots, the presence of the pigment in medium led to dye uptake and alteration of structure, especially at the level of the rhizomitre (fig. 5e and f). Microstructurally, the surface of fronds of control plants was uniform, with flattened cells, whereas dye grown

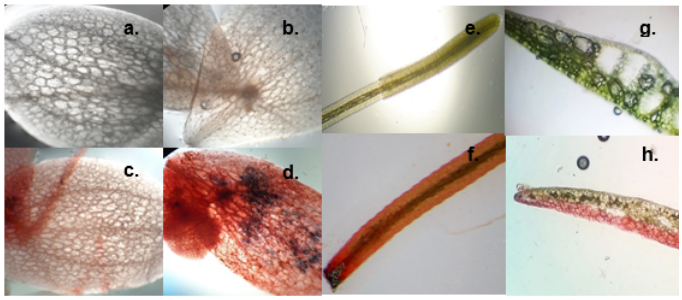

Fig. 5. Microscopic view of a-d – *Lemna minor* fronds: a) control-1; b) 250 ppm; c) 1000 ppm; d) 5000 ppm plants; e, f -roots; e) control-2; f) 1000 ppm of *Lemna* plants; g), h) - upper epidermis of fronds of control and, respectively, 1000 ppm plants

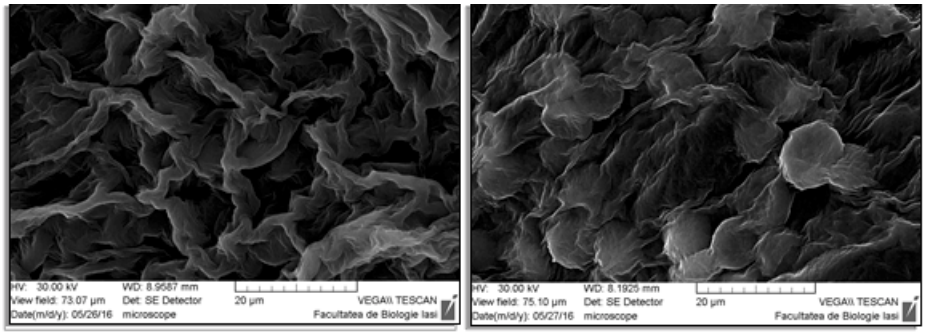

Fig. 6. Scanning electron microscopy images of upper epidermis of control (left) and 1000 ppm (right) plants

plants presented globular shaped, turgescent formations (fig. 6).

Physiological and cytogenetical effects

Regarding the photosynthetic apparatus, the dye in medium lead to a decrease of chlorophyll a and an increase of chlorophyll b amounts, especially at high concentrations (table 1). A stimulation of carotenoids and xanthophylls synthesis – accessory pigments - was also observed at 1000, 2500 and 5000 ppm dye concentration variants.

The ΦPSII index had similar values in control and 5-250 ppm dye concentration plants, after 3 and 7 days of cultivation, between 0.77 and 0.8, with a decrease after 5 days of cultivation (fig. 7).

In plants which were grown on 1000, 2500 and 5000 ppm concentration of dye, the qauntum yield values were much lower after 3 days of cultivation (0.70-0.73) and tended to increase after 5 and, respectively, 7 days of cultivation. However, in 5000 ppm dye plants, the ΦPSII values after 7 days reached only a maximum of 0.737. The steady state fluorescence (Fs) and maximum fluorescence (Fm') values in the light adapted state, in control and 5-1000 ppm plants, were similar or higher after 7 days of cultivation compared to those after 3 days of cultivation (table 2). In 2500 and 5000 ppm plants, the values of the two indices recorded a decrease during the cultivation, from 3 to 7 days.

Total phenolic contents of plants was increased in 500 and 1000 ppm dye concentration plants compared to control ones, and was reduced in all other treatments (table 3).

The presence of dye in medium also influenced the cellular cycle, by reducing the mitotic index in 5 and 1000 ppm dye plants compared to control ones and completely inhibiting it in 5000 ppm dye plants (table 4). In the same time, the chromosomal aberrations frequency was increased in both 5 and 1000 ppm dye plants by a factor of 2.8 and, respectively, 5.2 compared to control individuals.

Table 1

ASSIMILATORY AND ACCESORY PIGMENT CONTENTS OF *Lemna minor* PLANTS (mg/g FRESH WEIGHT)

Variant (ppm)	Control	5	50	250	1000	2500	5000
Chlorophyll a	0.3±0.1	0.3±0.07	0.31±0.03	0.23±0.05	0.35±0.01	0.16±0.06	0.22±0.07
Chlorophyll b	0.16±0.08	0.22±0.04	0.21±0.03	0.18±0.05	0.23±0.03	0.19±0.04	0.21±0.08
Carotenoids and xanthohpylls	0.06±0.01	0.05±0.04	0.07±0.03	0.04±0	0.11±0.01	0.12±0.03	0.16±0.03

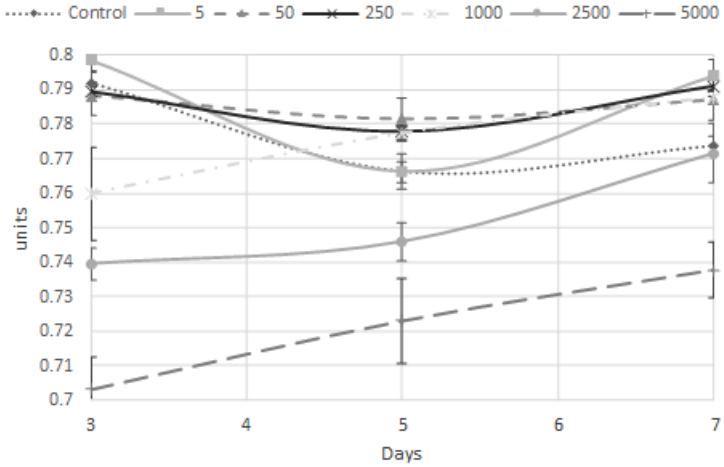

Fig. 7. ΦPSII dynamics of *Lemna* plants

Variant	3 days		5 days		7 days	
(ppm)	Fs	Fm'	Fs	Fm'	Fs	Fm'
Control	450.33±20.18	2167.56±98.74	426.67±25.94	1832.67±115.7	488.11±17.24	2157.22±75.44
5	391.67±20.67	1945.22±91.19	438.22±16.61	1874.67±70.22	343.33±19.36	1671.44±93
50	367.78±18.72	1735.67±83.31	480.89±23.62	2204.22±106.16	327.44±20.91	1532.67±80.98
250	365.78±16.45	1736.33±68.55	426.33±16.45	1918.78±77.04	521.22±15.96	2496.33±71.12

Table 2

CHLOROPHYLL FLUORESCENCE INDICES IN *Lemna minor* PLANTS

Table 3
TOTAL PHENOLIC CONTENTS (mg GALLIC ACID EQUIVALENTS/g f. w.) OF *Lemna minor* FRONDS

Control	5	50	250	1000	2500	5000
26.033±1.475	23.144±0.913	23.133±0.513	27.733±1.251	31.6±2.922	24.522±1.246	20.011±4.229

Variant (ppm)	Control	5	1000
Mitotic index	5.26±0.79	4.39±0.54	7.66±1.03
Chromosomal abberations %	13.17±6.03	24.17±4.29	4.58±1.91

Table 4
MITOTIC INDICES AND TOTAL ABERRATIONS IN *Lemna minor* PLANTS

Properties of growth medium during cultivation

Following the growth of plants, the medium recorded an increase of the pH, with alkalinisation taking place from 6.5 at the beginning of cultivation and reaching 7.6-8.0 after 7 days (fig. 8), however no significant differences between variants were observed. The concetration of dye in the medium registered decreases during the cultivation period for 5, 50, 250 and 1000 ppm variants, with a maximum of 56 % reduction at 250 ppm dye treatment (table 5). There was no reduction observed in variants where plants did not record actual growth, at 2500 and 5000 ppm dye.

The use of dyes in textile manufacture results in large amounts of coloured water, estimated at up to 65 L/kg of clothes produced and up to 70% of the dyes used are azo compounds. Such modifications of water quality may lead to toxic effects on aquatic plants, mostly by interfering with light and gas availability and by direct effects on plants metabolism [43].

The ability of *Lemna minor* plants to grow on medium contaminated with Congo Red dye at relatively high concentrations reflects the potential of plants to cope with the presence of the compound. Some aquatic macrophytes, such as *Potamogeton*, *Eichornia*, *Myriophyllum*, *Ceratophyllum* etc. are known to be able to uptake dyes, including azo varieties and to detoxify them, resulting in partial decontamination of the substrate. Proposed biosorption mechanisms are extracellular accumulation, cell surface sorption and intracellular accumulation [44]. The azo dyes, containing –N=N- links, are reduced to amines molecules through non-specific reduction by electron transporters such as flavins or quinones or through specific azo reductases [45]. Azo dyes may be also transformed by non-specific enzymes such as peroxidases, superoxidismutases, laccases, tyrosinases etc [46, 47]

The *Lemna* plants tolerated concentrations of up to 1000 ppm of dye while still showing active growth. However, the reduced growth rate points to reduced rates of cellular replication, translated by lower fresh mass of plants and reduced root lengths. Similar effects were observed in *Lemna minor* plants grown on an azo dye, Basic Red 46 [34], where up to 73% reduction of the relative growth rate was recorded at 20 mg/L dye. Brilliant Blue R, another azo dye, determined a reduction of *Lemna* dry mass of up to 74% at 1000 mg/L dye [48]. Reduction of fresh mass proportional to dye concentration were previously reported for *Lemna minor* in the case of methylene blue [49], while growth rates were reduced up to 65% for actual textile wastewaters [50]. Textile wastewaters are known to induce reduction in also root lengths by affecting cortex structure [51] and by generating genotoxicity in root cells [52].

The effects of dye appear to manifest at the level of the photosynthetic apparatus, with the macroscopic necrosis observable on frond surfaces pointing to influences on photoynthetic tissues. Reduced chlorophyll a contents indicate an alteration of the photosystems, possibly by increases in chlorophyllase activity or by inhibition of chlorophyll synthesis through regulation of endogenous cytokinins [37]. *Lemna minor* chlorophyll contents may be modified by substances such as herbicides, and different modes of action induce different degrees of influence on chlorophyll synthesis. Chl a contents decreased from 0.48 to 0.38 mg/g or from from 0.38 to 0.33 mg/g when metribuzin and, respectively, tritosulfuron were added. In the same time, carotenoid synthesis increased in Lemna plants when same herbicides were added, especially after 5 days of cultivation [53]. Chlorophyll b synthesis was increased, similarly to the present study, in *Lemna minor* plants grown on a monoazo dye, Acid Blue 92 [33]. The increase in carotenoids and xantophylls suggest a protective response of plants at the level of the photosystems. An analogue effect was observed in *Azolla filiculoides* grown on Basic Red 46 azo dye, xanthophylls being considered to exert protection by quenching chlorophyll molecules in the triplet state and by scavenging

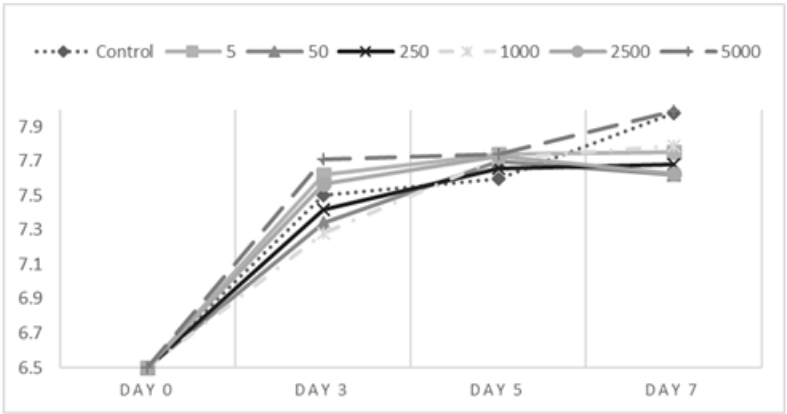

Fig. 8. *p*H variation during *Lemna minor* plants cultivation

Dye concentration ppm	5	50	250	1000	2500	5000
Dye reduction %	2.88	39.09	56.67	14.86	0	0

Table 5
DYE REDUCTION IN GROWTH MEDIUM AFTER 7 DAYS OF *Lemna minor* PLANTS CULTIVATION

reactive oxygen species [54]. The effect on PSII is supported also by the values of chlorophyll fluorescence, lower at high concentrations of dye compared to untreated plants. These values are the result of significantly reduced Fs and Fm' values in plants grown on dye compared to untreated plants. The trend of decreasing values of Fs and Fm' in 2500 and 5000 ppm plants indicate a decrease in photochemical quenching of light. Photosystem II quantum yield is related to the proportion of absorbed light used in PS photochemistry [55]. Considering that the quantum yield reflects the efficiency of electron transport in the photosystem, lower values of ΦPSII suggest reduced numbers of reaction centers or electron transporters. Similar alterations of PSII induced by Congo Red were observed in *Chlorella vulgaris*, at concentrations between 10-25 mg/L [36]. Effects were attributed to reduction of activity in the donor side of PSII by reduced numbers of reaction centers and not by inhibited electron transport between Q_A and Q_B. Another possible explanation of reduced fluorescence values is an increase in non-photosynthetic quenching of energy, which is performed by carotenoid conversion to reduced forms [38, 56]. This explanation is sustained by the fact that higher amounts of carotenoids were recorded in *Lemna minor* plants that had modified quantum yield values compared to controls. Sensitivity of chlorophyll fluorescence yield in Lemna minor was also recorded for other substances, such as the herbicide linuron, where 160 – 1280 nM concentrations significantly reduced ΔF/Fm'. The effect was assigned a 'closure' of PS II reaction centres, which may directly affect the capture of excitation following the interference of linuron with the flow of electrons through PS II, thereby preventing the reoxidation of Q_A by the Q_B e^- acceptor [57].

The effect of Direct Red 28 on the contents of phenolic compounds in *Lemna minor* was less affected compared to other indices, as the values remained around those of controls and similar to other studies [58, 59]. The alterations of phenolic levels may be explained by the protective activity they may offer to counter the oxidative stress induced by chemicals and such response may be dose-dependent, as when sodium-dodecyl-sulfate (SDS) [60] or cyanobacterial toxins are present [61].

Another effect of Direct Red 28 on *Lemna minor* plants was at a chromosomial level. The effects of the azo dyes on a genetic level, including Congo Red, are known in microorganisms, terrestrial plants, mammals [62] and include mainly interactions with DNA molecules. The genotoxic effect of azo dyes was described for the terrestrial specie *Allium cepa*, where BDCP produced increased numbers of chromosomal and nuclear aberrations (CNA) at 0.1 ppm [63]. Some of the aberrations observed in our plants, namely micronuclei formation, stickiness and C-mitosis, were proposed to be the result of improper folding of chromosome fiber, formation of acentric chromosomes and inhibation of spindle formation in Brassica campestris, where Sunset Yellow determined reduced mitotic indices and increased frequency of CNA at 1-5% [64]. For aquatic plant species, according to consulted literature, this is the first report on the effect of Direct Red 28 on genetic material. The reported chromosomal aberrations are probably due to intercalation of dye compounds between DNA pairs, leading to altered enzyme activity and cell replication [36]. The genotoxic influence, manifested through reduced mitotic index and increase in the rates of chromosomal aberrations, may explain reduced growth rates in individuals grown in the presence of dye. The aberrations may lead to apoptotic or necrotic cells [63], inducing cyotoxicity, as observed in *Lemna minor* altered roots or necrotic fronds.

The mechanisms of azo dyes removal in the case of *Lemna minor* are not entirely explained, however it is considered that, initially, the dye gets mechanically attached to plant tissues, then it is degraded, probably through antioxidative enzymatic activity [65]. The present results suggest that antioxidative protection in *Lemna minor* does not include non-enzymatic mehcanisms, such as increased phenolic compound production. The total phenolic contents, although higher in 250 and 1000 ppm treatments than in control, were not significantly different.

The growth of *Lemna minor* plants is considered to take place over a wider pH interval, generally between 4 and 9, while dye removal is most effective at 6.5-7 [36, 42]. In the current experiment, following *Lemna minor* cultivation, the growth medium recorded a continuous increase of the *p*H over 7 days, from 6.5 to 7.7-8. The increase may be related to plant growth, occurring including in control medium, without dye, however the phenomenon should be considered for further analysis. A similar effect was observed in effluent water of ponds containing azo dyes where *Lemna minor* was cultivated [66].

The phytoremediation potential of *Lemna minor* regarding Congo Red is significant even at high concentrations of pigment. For Basic Red 46, the decolorisation potential of *Lemna minor* was over 95% at 20 mg/L [42], for Acid Blue 92, it was over 90% at 20 mg/L [41], for Brilliant Blue R, it was 58% at 2.5 mg/L [65], while for industrial effluents, it was 68-83%, depending on light wavelength [58]. Although our values appear lower, the tested concentrations of dye are much higher than those presented in other reports.

Considering that *Lemna minor* plants were able to maintain metabolic functions when grown on the tested azo dye, the ecosystem level must be taken into account when assessing toxicity of pollutants. The persistence of the dye in *Lemna minor* tissues, as seen from anatomical analysis and the fact that the degradation products of azo dyes themselves are toxic [62], may represent sources of bioaccumulation and bioconcetration of toxic compounds. Interpretation of ecotoxicological results must be cautiously proceeded with, as, for example, another azo dye, Disperse Orange 1 was not considered to produce ecotoxicity when tested on *Vibrio fischeri* and *Daphnia similis* [67]. Account must be given to the increase in water pollution worldwide [68, 69], therefore also to assessment of the extent of such effects and the possibilities for remediation. Among various groups of pollutants, as example: organic dyes [70, 71] and, in particular, azo dyes [72] are a significant category. Thus, toxicological data on azo dyes should include tests on as many types of organisms possible, as their bioaccumulation potential may vary.

Conclusions

The azo dye Direct Red 28 exerts toxicity on *Lemna minor* at concentrations starting at 5 ppm and inhibits growth at concentrations over 2500 ppm, with a calculated EC50 of 1530 ppm. The results confirm the genotoxic and cytotoxic effects of Congo Red, extending the known range of species with aquatic plants, furthermore effects on the photosynthetic tissues are proved. Regarding the tested dye, *Lemna minor* may be considered as a potential specie for phytoremediation.

References

1.SAXENA, N., BHARGAVA, R., Advances in Bioresearch, **8,** no**.** 2, 2017, p. 28.

2.LAWS, E.A., Nonpoint Source Pollution, in Aquatic Pollution: An Introductory Text, Wiley & Sons, Ltd., 2018, pp. 133-158.
3.FLEEGER, J.W., CARMAN, K.R., NISBET, R.M., Science of the Total Environment, **317,** no. 1-3, 2003, p. 207.
4.CHAPMAN, P.M., Environment International, **33**, no. 4, 2007, p. 92.
5.IYER, P. Guidelines for Reproductive and Developmental Toxicity Testing and Risk Assessment of Chemicals, In Reproductive and Developmental Toxicology (Second Edition), Gupta R. C. (Ed.), Academic Press, ISBN 9780128042397, 2017, pp. 179-192.
6.APOSTU, M., TANTARU, G., VIERIU, M., PANAINTE, A.D., BIBIRE, N., AGOROAEI, L., Rev. Chim. (Bucharest), **68**, no. 4, 2017, p. 683.
7.APOSTU, M., TANTARU, G., VIERIU, M., BIBIRE, N., PANAINTE, A.D., Rev. Chim. (Bucharest), **69**, no. 5, 2018, p. 1223.
8.BRICIU, A.E., TOADER, E., ROMANESCU, G., SANDU, I., Rev. Chim. (Bucharest), **67**, no. 7, 2016, p. 1294.
9.BRICIU, A.E., TOADER, E., ROMANESCU, G., SANDU, I., Rev. Chim. (Bucharest), **67**, no. 8, 2016, p. 1583.
10.JUDSON, R., RICHARD, A., DIX, D. J., HOUCK, K., MARTIN, M., KAVLOCK, R., DELLARCO, V., HENRY, T., HOLDERMAN, T., SAYRE, P., TAN, T., CARPENTER, T., SMITH, E, Environmental Health Perspectives, **117**, no. 5, 2009, p. 685.
11.BALAN, G.G., PAVEL, L., SFARTI, C.V., STEFANESCU, G., BALAN, G., TRIFAN, A., Rev Chim (Bucharest), **67**, no. 5, 2016, p. 987.
12.BALAN, G.G., ROSCA, I., URSU, E.L., DOROFTEI, F., BOSTANARU, A.C., HNATIUC, E., NASTASA, V., SANDRU, V., STEFANESCU, G., TRIFAN, A., MARES, M., Infection and Drug Resistance, **11**, 2018, p.727.
13.VAAL, M.A., VAN LEEUWEN, C.J., HOEKSTRA, J.A., HERMENS, J.L.M., Environmental Management, **25**, no. 4, 2010, p. 415.
14.FURNICA, C., KNIELING, A.. DAMIAN, S.,I.,DIAC,M., DAVID, S., BULGARU, I., D, SANDU, I., IOV, C., J., Rev. Chim.(Bucharest), **68**, no. 7, 2017, p. 1591.
15.DIAC, M., KNIELING, A., DAMIAN, S.,I., BULGARU, I., D, SANDU, I., FURNICA, C, IOV, C., J., DAVID, S., Rev. Chim.(Bucharest), **68**, no. 11, 2017, p. 2646.
16.DAVID, S., KNIELING, A., SCRIPCARU, C., DIAC, M., SANDU, I.,BULGARU, I., D, Rev. Chim.(Bucharest), **68**, no. 12, 2017, p. 2932.
17.KNIELING, A. MATEI,M.,C., BULGARU, I., D., MANEA, DIAC,M., CHISTOL,R.,O., FURNICA, C**,** Rev. Chim.(Bucharest), **68**, no. 5, 2017, p. 1126.
18.DAVID, S., BULGARU, I.D, SANDU, I., PARASCHIV, D.E., TEODORESCU, C., KNIELING, A., Rev. Chim.(Bucharest), **68**, no. 5, 2017, p. 1031.
19.CONNON, R.E., GEIST, J., WERNER, I., Sensors, **12,** no. 9, 2012, p. 12741.
20.HOMMEN, U., BAVECO, J.M.H., GALIC, N., VAN DEN BRINK, P.J., Integrated Environmental Assessment and Management, **6,** no. 3, 2010, p. 325.
21.BLINOVA, I., Environmental Toxicology **19,** no. 4, 2004, p. 425.
22.PAIXAO, S.M., SILVA, L., FERNANDES, A., O'ROURKE, K., MENDONCA, E., PICADO, A., Ecotoxicology **17,** no. 3, 2008, p. 165.
23.GREEN, F.J., The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators, Aldrich Chemical Company, Milwaukee, 1990, pp. 220-225.
24.SABNIS, R.W., Congo Red, in Handbook of Biological Dyes and Stains: Synthesis and Industrial Applications, Wiley, 2010, pp. 106-109,
25.ISIK, M., SPONZA, D.T., Journal of International Environmental Application and Science, **1,** no. 1-2, 2006, p. 1.
26.SPONZA, D.T., I^{a}IK, M., Process Biochemistry, **40,** no. 8, 2005, p. 2735.
27.***EUROPEAN CHEMICALS AGENCY, Annex XV SVHC dossier Proposal for identification of a substance as a cmr 1a or 1b, pbt, vpvb or a substance of an equivalent level of concern, European Chemicals Agency, Helsinki, 2013, p. 12**.**
28.SHAUL, G.M., HOLDSWORTH, T.J., DEMPSEY, C.R., DOSTAL, K.A., Chemosphere, **22,** no. 1-2, 1991, p. 107.
29.HEMA, N., Chapter 1 – Introduction, in Studies on decolorization of textile effluent using selected microbial isolates, PhD Thesis, University of Mysore, Mysore 560005, 2015, http://shodhganga.inflibnet.ac.in/handle/10603/118325, accessed on 07.02.2018 pp. 1-23.
30.LADE, H., GOVINDWAR, S., PAUL, D., International Journal of Environmental Research and Public Health, **12,** 2015, p. 6894.
31.VENCKATESH, R., AMUDHA, T., SIVARAJ, R., CHANDRAMOHAN, M., JAMBULINGAM, M., International Journal of Engineering Science and Technology, **2,** no. 6, 2010, p. 2040.
32.HU, L.Y., XIE, X.H., LIU, N., JIANG, H., YANG, F., NGUYEN, T.H., LIU, J.S., Applied Mechanics and Materials, **651,** 2014, p. 215.
33.KHATAEE, A.R., MOVAFEGHI, A., TORBATI, S., LISAR, S.Y.S., ZAREI, M., Ecotoxicology and Environmental Safety, **80,** 2012, p. 291.
34.MOVAFEGHI, A., KHATAEE, A.R., TORBATI, S., ZAREI, M., LISAR, S.Y.S., Environmental Progress & Sustainable Energy, **32,** no. 4, 2013, p. 153.
35.BRAIN, R.A., CEDERGREEN, N., Reviews of Environmental Contamination and Toxicology, **198,** 2009, p. 49.
36.HERNANDEZ-ZAMORA, M., PERALES-VELA, H.V., FLORES-ORTIZ, C.M., CANIZARES-VILLANUEVA, R.O., Ecotoxicology and Environmental Safety, **108,** 2014, p. 72.
37.PUVANESWARI, N., MUTHUKRISHNAN, J., GUNASEKARAN, P., Indian Journal of Experimental Biology, **44**, 2006, p. 618.
38.BAKER, E.R., Annual Review of Plant Biology, **59,** 2008**,** p. 89.
39.*** OECD Test No. 221: Lemna sp. Growth Inhibition Test, OECD Publishing, Paris, 2006.
40.WELLBURN, A.R., Journal Plant Physiology, **144,** 1994, p. 307.
41.HERALD, T.J., GADGIL, P., TILLEY, M., Journal of the Science of Food and Agriculture, **92**, 2012, p. 2326.
42.SINGH, R.J., Handling of Plant Chromosome (Chapter 2), in Singh R.J. (Ed.) Plant Cytogenetics, Third Edition, CRC Press, Boca Raton, 2017.
43.LADE, H., GOVINDWAR, S., PAUL, D., Journal of Chemistry, **2015**, 2015, Article ID 894109.
44.PRIYA, E. S., SELVAN, P. S., Arabian Journal of Chemistry, **10,** no. 2, 2014, p. 3548.
45.PINHEIRO, H.M., TOURAUD, E., THOMAS, O., Dyes and Pigments, **61**, 2004, p. 121.
46.SCHWITZGUEBEL, J.-P., PAGE, V., MARTINS-DIAS, S., DAVIES, L. C., VASILYEVA, G., STRIJAKOVA, E. Using plants to remove foreign compounds from contaminated water and soil in Schroder P., Collins C. D. (Ed.) Organic Xenobiotics and Plants: From Mode of Action to Ecophysiology, Springer, 2011, pp. 149-190.
47.TAHIR, U., YASMIN, A., KHAN, U.H., Journal of King Saud University - Science, **28,** no. 2, 2016, p. 119.
48.CLEUVERS, M., RATTE, H.-T., Chemosphere, **49,** no. 1, 2002, p. 9.
49.REEMA, R.M., SARAVANAN, P., DHARMENDIRA, K.M., RENGANATHAN, S., Separation Science and Technology, **46,** no. 6, 2011, p. 1052.
50.UYSAL, Y., AKTAS, D., CAGLAR, Y., Journal of Environmental Protection and Ecology, **15,** no. 4, 2014, p. 1718.
51.NILRATNISAKORN, S., THIRAVETYAN, P., NAKBANPOTE, W., Science of The Total Environment, **384**, no. 1–3, 2007, p. 67**.**
52.ASMITA, V.P., JYOTI, P.J., Chemosphere, **92,** no. 2, 2013, p. 225.
53.MOUZAKI-PAXINOU, A.C., FOUDOULAKIS, M., ARAPI, G., Proceedings of the 13th International Conference of Environmental Science and Technology, Athens, Greece, 2013.
54.VAFAEI, F., KHATAEE, A.R., MOVAFEGHI, A., LISAR, S.Y.S., ZAREI, M., International Biodeterioration & Biodegradation, **75**, 2012, p. 194.
55.MURCHIE, E.H., LAWSON, T., Journal of Experimental Botany, **64,** no. 13, 2013, p. 3983.
56.ROHACEK, K., Photosynthetica **40,** no. 1, 2002, p. 13.
57.HULSEN, K., MINNE, V., LOOTENS, P., VANDECASTEELE, P., HÖFTE, M., Environmental Microbiology, **4,** 2002, p. 327**.**
58.GULCIN, I., KIREÇCI, E., AKKEMIK, E., FEVZI, T., HISAR, O., Turkish Journal of Biology, **34,** no. 2, 2010, p. 175.
59.SMOLDERS, A.J.P., VERGEER, L.H.T., VAN DER VELDE, G., ROELOFS, J.G.M., Oikos, **91,** 2000, p. 307.
60.FORNI, C., BRAGLIA, R., HARREN, F.J.M., CRISTESCU, S.M., Aquatic Toxicology, **110,** 2012, p. 107.
61.SAQRANE, S., GHAZALI, I.EL., OUAHID, Y., HASSNI, M.EL., HADRAMI, I.EL, BOUARAB, L., DEL CAMPO, F.F., OUDRA, B., VASCONCELOS, V., Aquatic Toxicology, **83,** no. 4, 2007, p. 284.

62.CHEQUER, F.M.D., DORTA, D.J., PALMA DE OLIVEIRA, D. Azo Dyes and Their Metabolites: Does the Discharge of the Azo Dye into Water Bodies Represent Human and Ecological Risks? in Advances in Treating Textile Effluent, Hauser P. (Ed.), InTech, 2011.
63.VENTURA-CAMARGO, B.C., MALTEMPI, P.P.P., MARIN-MORALES, M.A., Journal of Environmental and Analytical Toxicology, **1,** 2011, p. 109.
64.DWIVEDI, K., KUMAR, G., Journal of Environmental and Public Health, **2015**, 2015, ID. 319727, p. 1.
65.KILIC, N.K., DUYGU, E., DONMEZ, G., Journal of Hazardous Materials, **182,** no. 1–3, 2010, p 525.
66.YASEEN, D.A., SCHOLZ, M., Ecological engineering, **94,** 2016, p. 295.
67.FERRAZ, E.R.A., GRANDO, M.D., OLIVEIRA, D.P., Journal of Hazardous Materials, **192,** no. 2, 2011, p. 628.
68.TOKAR, A., NEGOITESCU, A., Rev.Chim. (Bucharest), **63**, no. 11, 2012, p. 1181
69.SAMREEN, S., INAM, A., KHAN, A.A., International Journal of Conservation Science, **8**, no. 4, 2017, p. 695.
70.SAGGIORO, E.M., OLIVEIRA, A.S., PAVESI T., MOREIRA, J.C., Rev.Chim. (Bucharest), **65**, no. 2, 2014, p. 237.
71. BOGDANICI, C.M., COSTEA, C.F., DIMITRIU, G., CHIHAIA, M.A., CARAULEANU, A., CUCU, A., SAVA, A., DUMITRESCU, G.F., TURLIUC, S., TURLIUC, M.D., Rev. Chim.(Bucharest), **69**, no.1, 2018, p. 172.
72. DRAGOI, A., SAMIDE, A., MOANTA, A., Rev.Chim. (Bucharest), **62**, no. 12, 2011, p. 1195.

Manuscript received: 17.12.2017