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Ecotoxicological assessment of industrial effluent using duckweed (*Lemna minor* L.) as a test organism

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Abstract This study aimed at assessing the toxic effects of industrial effluents using duckweed (*Lemna minor* L.) plants as a test system. Growth inhibition test according to standardized protocol (ISO 20079) was performed. The suitability of the Comet assay (indicates DNA damage) and certain parameters such as peroxidase activity and lipid peroxidation level, as biomarkers for environmental monitoring was evaluated. The water samples were collected monthly over a 3-month period from the stream near the industrial estate of Savski Marof, Croatia. All samples caused inhibition of growth rates based on frond number and biomass as well as decrease of chlorophylls content. In contrast, peroxidase activity, malondialdehyde content and tail extent moment (measure of DNA strand breaks) markedly increased. Obtained data demonstrate the relevance of duckweed as sensitive indicators of water quality as well as the significance of selected biological parameters in the reliable assessment of phyto- and genotoxic potential of complex wastewaters.

Keywords Duckweed (*Lemna minor* L.) · DNA damage · Chlorophyll · Lipid peroxidation · Peroxidase · Wastewater

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Introduction

A growing problem in many countries is environmental pollution from municipal and industry wastewaters. Due to the enormous number of potentially polluting substances contained in such waters, a chemical-specific approach is insufficient to provide the information about water quality. Therefore, it is essential to use biological test systems with living cells or organisms that give a global response to the pool of micropollutants present in the sample. Owing to their settled life style, plants are constantly exposed to the pollution. In addition, plants are the major energy source for nearly all higher eukaryotes and as such play an active role in transferring contaminants to higher trophic levels (Wang and Freemark 1995).

Among the developmental parameters, the most commonly assessed in ecotoxicological test systems, are growth parameters. The measurement of biochemical responses to chemical contaminants may serve to improve the assessment of biologically significant exposures to toxic chemicals and enhance the ability to assess the risk of effects on the health and survival of toxicant exposed populations. Biomarkers such as pigment content (chlorophyll and carotenoids) and enzyme activities (like peroxidase) are commonly used as parameters for toxicity tests (Mohan and Hosetti 1999). A bioindicator of cell biomembrane injury, estimated by malondialdehyde (MDA) content, was tested for the first time in the ecotoxicological risk assessment. Finally, to determine potential genotoxic effects of micropollutants in water, single cell gel electrophoresis assay (Comet assay) was performed. The alkaline version of the Comet assay is a sensitive method that quantitatively measures DNA damage (single-strand breaks, double-strand breaks, alkali-labile sites, primarily apurinic and apyrimidinic sites, incomplete excision repair

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sites, and DNA crosslinks) in eukaryotic and prokaryotic cells (Gichner et al. 2004).

Duckweed (*Lemna minor* L.) is used in water quality studies to monitor heavy metals and other aquatic pollutants, because duckweed, like other water plants, may selectively accumulate certain chemicals. The plants possess physiological properties (small size, rapid growth between pH 5 and 9, and vegetative propagation), which make them an ideal test system. In the present study, standardized protocol ISO/DIS 20079 (2004) was applied in which plants are exposed to a toxicant over a period of 7 days, when the consequent potential growth inhibition is estimated. The objective of the present study was to evaluate the sensitivity of the selected parameters for screening and biomonitoring complex effluent samples as well as to compare the tests with each other. The possible phyto- and genotoxic effects of partially treated industrial effluent waters were investigated.

Materials and methods

Water samples were taken from the stream near the pharmaceutical and food industries of Savski Marof (Croatia), before the mouth of the river Sava. Prior to discharge, industrial effluent was treated mechanically (a sieve and a sedimentation tank) and biologically (oxidation with activated sludges). Water samples were collected monthly over a 3-month period (from May to July 2008). The pH values and some metal concentrations of all samples were showed in Table 1.

Plant material and experimental design

Lemna minor was originally collected from Botanical Garden, Faculty of Science, University of Zagreb. Several healthy colonies with 2–3 fronds (from stock cultures) were transferred to Erlenmeyer flasks containing either tested waters or dH_2O (control). All water samples were filtered using cellulose nitrate membranes (pore size 0.45 μm) and then supplemented with Steinberg (1946) macro- and microelements. Apart from the original (100% effluent), effluent water samples were diluted to 5, 25 and 50% with dH_2O. Duckweed plants were exposed to 0 (control), 5, 25, 50 and 100% of the effluent monthly, immediately after sampling the water from the stream. The cultures were grown under a 16:8 h light:dark period of cool fluorescent light (90 $\mu Em^{-2}s^{-1}$) at 24 ± 2°C.

Table 1 Concentration of certain heavy metals in the samples of industrial effluent

μg/l	1st month	2nd month	3rd month
Fe	648	228	305
Zn	190	118	120
Cu	14	29	18
Ni	12	20	17
Pb	4	4	4
Cr	4	1	2
pH	7.8	8.1	7.9

The metals Hg, Se and V were below 1 μg/l

Metal analysis by energy dispersive X-ray fluorescence method (EXDRF)

For the analysis of Fe, Cu, Zn, Ni, Pb, Cr 100 ml of the solution was adjusted to pH 3 and 11 by addition of HCl and NH_4OH, respectively, and preconcentrated by 2 ml of 1% (w/v) ammoniumpyroloidinedithiocarbamate (APDC) (Elez et al. 2008). After the complexation, which lasted 20 min, the suspension was filtered through a Millipore HAWP filter (pore size 0.45 μm). Prepared thin targets were air dried, protected by thin mylar foil (2 μm) and analyzed (Elez et al. 2008; Orescanin et al. 2008) with MiniPal 4 X-ray spectrometer (PANalytical, Almelo, Netherlands). Spectral data were analyzed by MiniPal/MiniMate software version 3.0.-63(2.64) (PANalytical). Calibration model for qualitative and quantitative analysis of thin targets was created on the basis of measurements of the standard solutions (Merck) in the concentration range from 10 to 200 μg l^{-1} prepared and measured in the same way as unknown samples.

Growth parameters

Duckweed growth was determined measuring frond number (FN), fresh weight (FW, biomass) and dry weight (DW), according to the ISO 20079 test protocol. The frond number was scored at the start of the experiments (t0) and 7 days after (t1). All visible fronds were counted. Plants were surface-dried between layers of paper towels, and the fresh weight was determined. To measure dry weight, plants were dried at 80°C overnight. Relative growth rate (RGR) was calculated from the following equation with the measured parameter x (FN, FW) and the start of the test for each replicate separately: $RGR = (\ln x_{t1} - \ln x_{t0})/t1 - t0$. Duckweed plants were weighed to determine FW and oven-dried at 80°C for 48 h by which time constant dry weights were obtained. Dry to fresh weight ratio (DW/FW) was determined according to calculation: dry weight (g)/fresh weight (g).

Biochemical assays

Chlorophyll *a* (chl *a*), *b* (chl *b*) and carotenoid contents were measured according to the method described by

Arnon (1949). In short, fresh samples were homogenized with 80% (w/v) cold acetone, centrifuged at 5000 rpm for 10 min and the absorbance of the supernatant at 663, 646 and 470 nm read. The photosynthetic pigment contents (mg g^{-1}FW) were calculated according to Lichtenthaler (1987).

Peroxidase activity (POD) was analyzed after homogenizing plant tissue in potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (50 mM, pH 7) including 1 mM ethylene diamine tetraacetic acid. The homogenates were centrifuged (Sigma–Aldrich, 3K18 centrifuge) at 25000 rpm for 30 min at 4°C and supernatants used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Bradford (1976) using bovine serum albumin as standard. The activity of POD was measured using guaiacol as the substrate according to Chance and Maehly (1955). The formation of tetraguaiacol was followed at 470 nm and was quantified taking its extinction coefficient (26.6 mM^{-1} cm^{-1}) into account. The enzyme activity was expressed as U (unit) mg^{-1} protein (1U = µmol of oxidised substrate per min).

Lipid peroxidation was determined by estimating the MDA content using the thiobarbituric acid method described by Heath and Packer (1968). The MDA (nmol g^{-1} FW) content was calculated from the absorbance at 532 nm by using extinction coefficient of 155 mM^{-1} cm^{-1}.

Comet assay was performed according to Gichner et al. (2004) with slight modification (10 min denaturation, 20 min electrophoresis at 1 V/cm, 300 mA) using duckweed (*L. minor*) as a plant model. For each slide, 50 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 520/09 nm and a barrier filter of 610 nm. For each wastewater sample, three independent experiments were performed. Three slides were evaluated per water sample. The plants were also exposed to increasing concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0 mM) of ethyl methanesulfonate (EMS) for 1 h. EMS is an alkylating mutagen which served as the positive control. A computerized image-analysis system (Komet version 5, Kinetic Imaging Ltd., Liverpool, UK) was employed. The tail extent moment (integrated value of tail DNA density multiplied by the migration distance, µm) was used as the primary measure of DNA damage.

Statistical analysis

For each analysis, data were compared by analysis of variance (one-way ANOVA), using STATISTICA 8.0 (StatSoft, USA) software package, and Duncan's multiple range test was performed to determine the significant difference between treatments ($P < 0.05$). Each data point is the average of six replicates ($n = 6$), unless stated otherwise.

Results and discussion

When serial dilutions of effluent water samples were tested, almost linear inhibition of RGR based on FN was observed (Fig. 1a). Similar trend of growth inhibition but with lower inhibition percentages was noticed with biomass (FW). Growth rate similar to control was recorded only in plants exposed to 5% industrial effluent dilutions (Fig. 1b). In the literature cited, frond number is considered to be the least

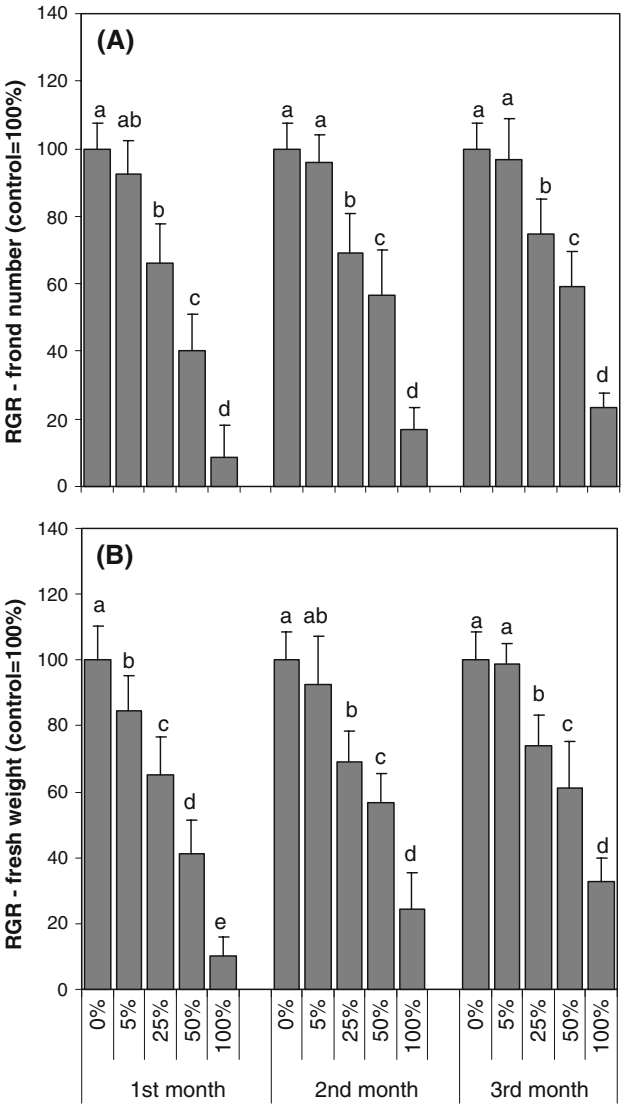

Fig. 1 **a** Relative growth rate based on frond number (FN), **b** relative growth rate based on biomass (FW) in duckweed exposed for 7 days to industrial effluent water samples collected monthly over a 3-month period. Standard deviations were presented by *error bars*. Values are mean of six replicates. *Different letters* indicate significantly different values at $P < 0.05$

reliable in comparison with other growth parameters (final biomass, frond area and dry weight) observed in Lemna assay. It is probably due to the fact that frond count is irrelevant to frond size or biomass. It has frequently been observed that under toxic stress small buds may protrude and be counted as individual fronds (Mohan and Hosetti 1999; Naumann et al. 2007). However, in the present study frond number and final biomass proved to be almost equally sensitive parameters. Frond number was even slightly more sensitive than final biomass. Mackenzie et al. (2003) also found that, beside frond area, growth rate based on FN is the most sensitive endpoint for detecting chronic toxicity (7 days) in landfill leachate.

Heavy metal analysis obtained by EXDRF (Table 1) shows relatively low concentrations of measured metals in undiluted water samples. However, duckweed ability to accumulate heavy metals is well-known which makes it the potential candidate for phytoremediation (Axtell et al. 2003). It has been shown that exposure of plants to toxic metal concentrations generally causes the fast inhibition of cell elongation and expansion (Poschenrieder and Barceló 2004; Srivastava et al. 2006). In the study of Horvat et al. (2007) *L.minor* exposed to electroplating wastewater rich in Fe and especially Zn, accumulated mostly those metals in great quantities followed by Pb and Ni. However, the authors found that toxicity of metals in Lemna tissues was in decreasing order of damage: Zn > Ni > Fe > Cu > Cr > Pb. Duckweed plants have been shown to accumulate preferentially nickel before lead (Axtell et al. 2003). Although the interactions in our multimetallic samples considerably limit possibility to explain the results for single metals, the growth reduction observed in our study could be also due to the suppression of the elongation growth rate of cells exerted by Fe, Zn and Ni.

Dry to fresh weight ratio (DW/FW) measured at the end of the 7 days experiment has shown inverse relation in comparison with RGR (Fig. 2); the parameter significantly increased in all samples but most in the water sample collected after first month during 3-month monitoring period (73% increase in comparison to control was recorded even in 5% dilution). Significant increase of DW/FW as well as inhibition of RGR observed in our experiments suggests bioaccumulation of pollutants, possibly heavy metals, present in the water samples. However, it has been shown that accumulation of heavy metals disturbs the plant water status which eventually results in osmotic stress and growth reduction (Perfus-Barbeoch et al. 2002; Poschenrieder and Barceló 2004). To keep the water balance between cells and external medium, plants are shown to accumulate a wide variety of organic solutes like carbohydrates and quaternary ammonium compounds (Matysik et al. 2002). The accumulation of metal-induced compounds such as proline may be associated not only with

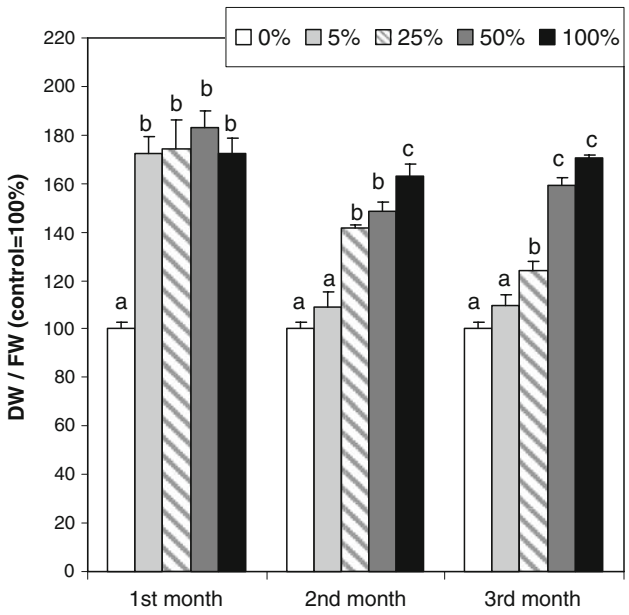

Fig. 2 Dry to fresh weight ratio in duckweed exposed for 7 days to industrial effluent water samples collected monthly over a 3-month period. Standard deviations were presented by *error bars*. Values are mean of six replicates. *Different letters* indicate significantly different values at $P < 0.05$

osmoregulation but also with enzyme protection against dehydration (Matysik et al. 2002). In our investigation, the increase of DW/FW after the treatment with industrial effluent could thus be explained by the change of the plant's water status and accumulation of compatible solutes. The findings of Garnczarska and Ratajczak (2000a) and John et al. (2008) corroborate the hypothesis. In the studies, upon exposure to Pb and Cd, a significant increase in proline and sugar contents on the account of dry weight with simultaneous inhibition of duckweed growth and water status has been noticed.

The decline in total chlorophyll and carotenoids contents as well as growth inhibition can be regarded as general responses associated with metal toxicity At the end of the 7d exposure, duckweed grown on 50% diluted and especially on undiluted industrial effluent water samples showed signs of necrosis (dark brown necrotic spots). Accordingly, a marked decrease in chlorophyll *a* and *b* contents (between 17 and 23% each) compared to the control was detected in plants exposed to undiluted water samples (Fig. 3a). The carotenoids content was much less affected in comparison with those of chlorophylls. Significant decrease of carotenoids was observed only in the water sample collected after the first month (Fig. 3b). The carotenoids are involved in the protection of chlorophyll but they also serve as antioxidants to quench or scavenge the free radicals and reduce the damage to cell membrane and DNA. The loss of photosynthetic pigment content has been reported in duckweed

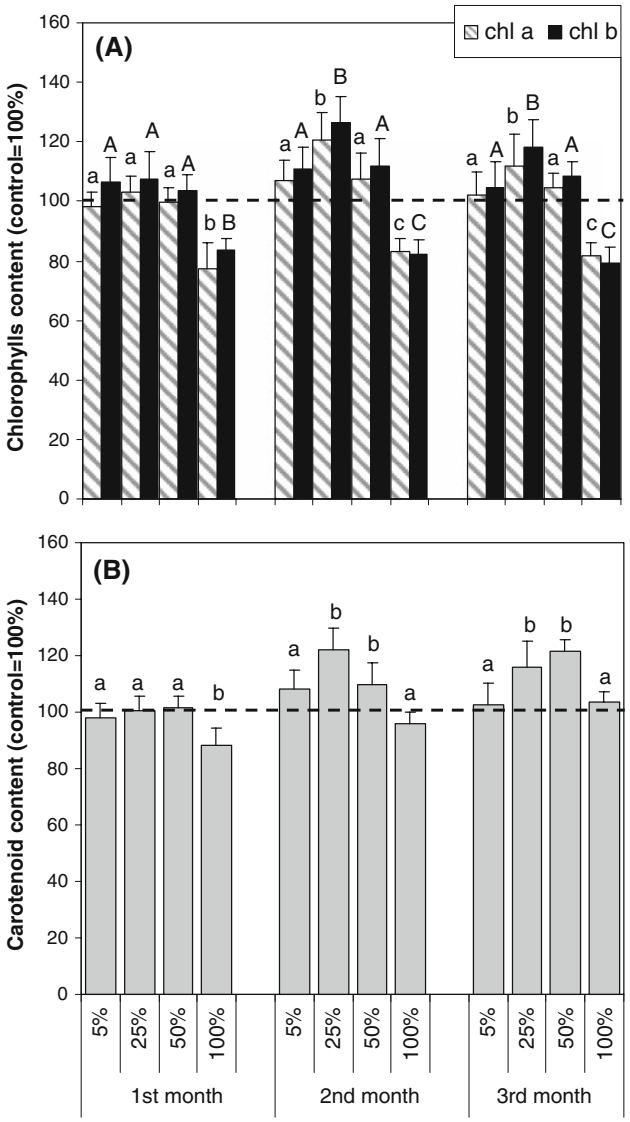

Fig. 3 **a** Relative chlorophyll *a* (chl *a*) and chlorophyll *b* content (chl *b*), **b** relative carotenoid contents in duckweed exposed for 7 days to industrial effluent water samples collected monthly over a 3-month period. Standard deviations were presented by *error bars*. Values are mean of six replicates. *Different letters* indicate significantly different values at $P < 0.05$

plants following exposure to Cu, Pb and Ni (Axtell et al. 2003; Hou et al. 2007; Kanoun-Boulé et al. 2009). Overall, the destruction of photosynthetic pigments by heavy metals could be due to: impairment of the electron transport chain, the replacement of Mg^{2+} ions associated with the tetrapyrrole ring of chlorophyll molecules, inhibition of important enzymes (Van Assche and Clijsters 1990) associated with chlorophyll biosynthesis or peroxidation processes in chloroplast membrane lipids by the reactive oxygen species (Sandalio et al. 2001).

Chlorophyll and carotenoids are the central part of the energy manifestation of every green plant system and therefore, any significant alteration in their levels is likely to cause a marked effect on the entire metabolism of the plant. The growth reduction recorded in our study may be owed in part to the decrease of chlorophyll content produced by heavy metals present in the industrial effluent. In fact, in *L.minor* and some other water plant species the degradation of chlorophyll or the inhibition of its biosynthesis, has been proposed as being responsible for photosynthesis and growth reduction caused by Zn, Ni, Cu and Pb (Küpper et al. 1996).

When serial dilutions of effluent water samples were tested, almost linear POD increase was observed (Fig. 4a). In comparison with control, the POD activity elevated 30–105% for water sample collected after first month,

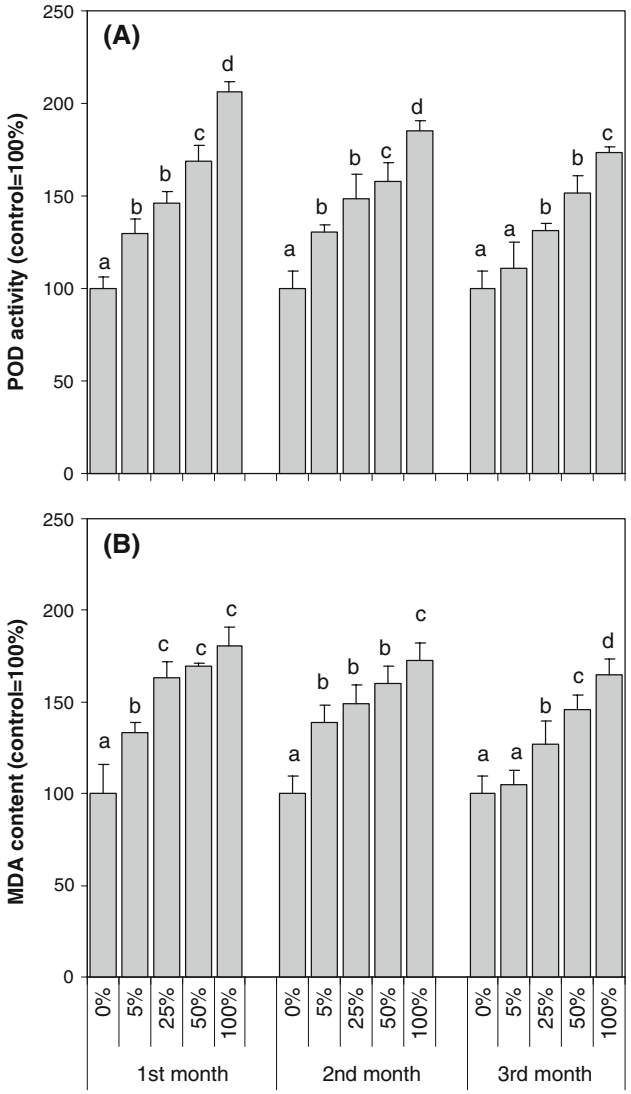

Fig. 4 **a** Relative POD activity, **b** relative MDA content duckweed exposed for 7 days to industrial effluent water samples collected monthly over a 3-month period. Standard deviations were presented by *error bars*. Values are mean of six replicates. *Different letters* indicate significantly different values at $P < 0.05$

30–83% for water sample collected after second month and 10–73% for water sample collected after third month. Various pollutants, including heavy metals, are known to induce a change in extractable POD activity (Van Assche and Clijsters 1990; Siesko and FlemingWJ 1997). There are many studies suggesting an inverse correlation beetwen the peroxidase level and the growh rate (Siegel and Galston 1967; Bacon et al. 1997; Lin and Kao 2001). Peroxidases play a key role in the stiffening of the cell wall and in processes associated with plant growth through the formation of phenolic cross-link (Fry 1986).

Lipid peroxidation, which is considered an indication of oxidative stress in plants, can be induced via reactive oxygen species (ROS) that are generated as a result of heavy metal toxicity in plants. MDA is the decomposition product of polyunsaturated fatty acids of biomembranes and its increase shows the extent of membrane lipid peroxidation (Blokhina et al. 2003). Formation of MDA significantly increased in plants exposed to all water samples (Fig. 4b). Oxidative damage to biomembrane of *L. minor* treated with effluent samples can be primarily attributed to Cu^{2+} and especially to Fe^{2+} as it was present in larger quantities in tested water samples. Both metals are redox active and catalyse the generation of hydroxyl radical and other toxic oxygen species (Arora et al. 2002). In contrast to redox, non-redox metals such as Zn, Ni, Cr and Pb do not produce ROS directly, but generate oxidative stress by interfering with the plant's antioxidant defense system (Garnczarska and Ratajczak 2000b; Aravind and Prasad 2003; Panda and Choudhury 2005). Thus, those metals might have contributed to increased MDA levels as well.

Besides disrupting membrane lipids, heavy metals are known to induce oxidative damage to proteins and DNA (Arora et al. 2002). In our study, significant DNA damage (presented by median tail extent moment) was recorded even in 5% diluted water samples collected over a 3-month period (Fig. 5). Serial dilutions higher than 5% as well as undiluted water samples caused total DNA damage to *L. minor* nuclei. As a positive control in Comet assay, monofunctional alkylating agent ethyl methanesulphonate (EMS) was applied (insert in Fig. 5). Increasing concentrations of EMS produced significant dose-dependent increases in the median tail moment values. There are only few reports on use of Comet assay for the assessment of genotoxicity of surface and waste water in which mammalian, bacterial or protozoan cells/organisms are utilized (Lah et al. 2004; Kungolos et al. 2006; Žegura et al. 2009). Simplified Comet procedure modified for plants tissues allows rapid yet sensitive determination of DNA damage. Therefore, our results indicate that the Comet assay using *L. minor* may be a sensitive alternative to animal models for detecting the genotoxic effects of micropollutants present in surface and wastewaters.

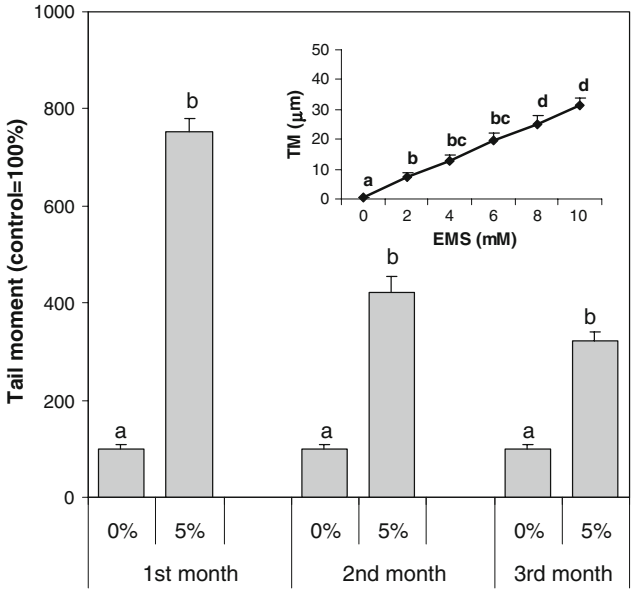

Fig. 5 Relative tail moment in duckweed exposed for 7 days to industrial effluent water samples collected monthly over a 3-month period. Standard deviations were presented by *error bars*. Values are mean of triplicate. *Different letters* indicate significantly different values at $P < 0.05$. Insert shows the linear relation between EMS concentration (*positive control*) and DNA damage

Tested industrial effluent collected over 3-month monitoring period caused major RGR inhibition and severe damage to plant DNA and biomembrane. The most toxic effects to duckweed was induced by water sample collected after first month that could be, at least partly, attributed to greater Fe content in the sample (Table 1). However, such a strong toxic effect of the industrial effluent in overall can hardly be explained by the relatively low heavy metal content only and other chemical parameter levels such as total phenols and mineral oils (data not shown) but probably by some unidentified substances not covered by a typical chemical analysis that is performed as a part of the water quality monitoring.

Conclusions

The results show the suitability of *L. minor* for surface water quality assessment as all selected parameters showed consistency with respect to water samples collected monthly over a 3-month period. The possible reason for such consistency might lie in the highly homogeneous plant material; due to predominantly vegetative reproduction of duckweed, new fronds are formed by clonal propagation thus producing a population of genetically homogeneous plants. The result is small variability between treated individuals. Moreover, water and substances to be tested are taken up directly through the leafy fronds (Naumann et al. 2007).

The results obtained suggest that phyto- and genotoxicity tests with *L. minor* should be used in the biomonitoring of municipal, agricultural and industrial effluents because of their simplicity, sensitivity and cost-effectiveness.

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