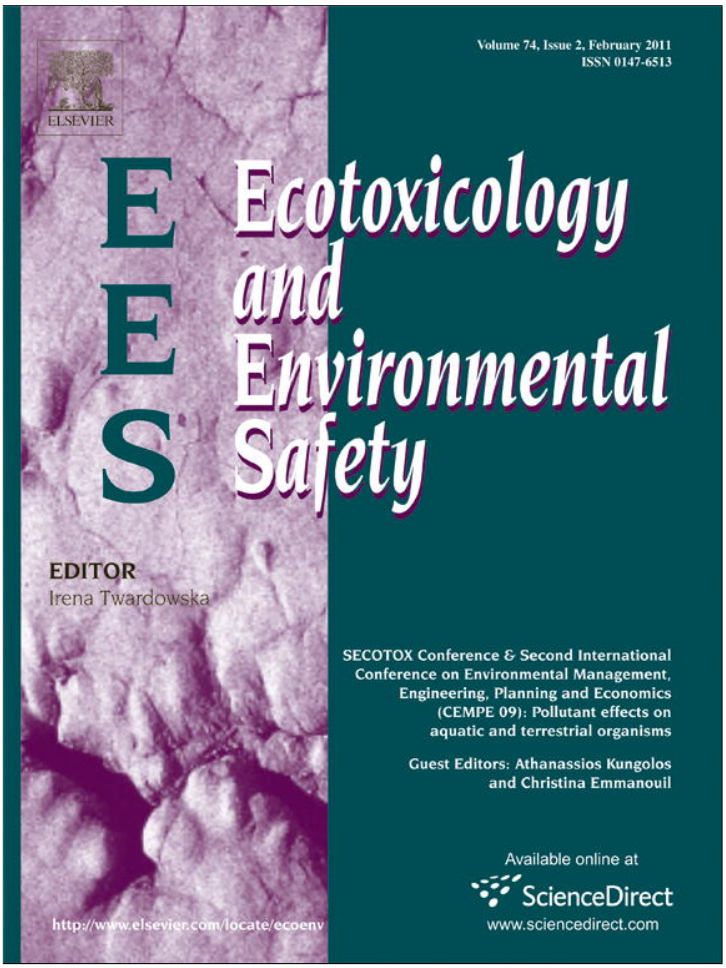

Ecotoxicology and Environmental Safety 74 (2011) 182–187

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Duckweed *Lemna minor* as a tool for testing toxicity and genotoxicity of surface waters

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ARTICLE INFO

Available online 17 July 2010

Keywords:
Duckweed
Comet assay
Chlorophyll
Peroxidase
Lipid peroxidation
Monitoring

ABSTRACT

In this investigation growth parameters and certain endpoints (pigment content, peroxidase activity, lipid peroxidation and alkaline comet assay) were used to detect the toxic and genotoxic effects of surface water samples on duckweed plants. The surface waters of different origin and pollutant burdens were collected monthly over a 3-month monitoring period at three sampling sites along the river Sava and its confluents (Croatia). Physicochemical characterization of the water samples included measurements of conductivity, chemical and biological oxygen demand, levels of total suspended solids, nitrate, nitrite, ammonium, Kjeldahl nitrogen and orthophosphate. Surface water samples collected from three stations caused reduction of duckweed growth rates, chlorophylls and carotenoid contents and peroxidase activity. In contrast, damage to membrane lipids (estimated by malondialdehyde content) and especially to DNA (estimated by tail extent moment) markedly increased in duckweed exposed to industrial wastewater samples. The results from the study indicate the ability of selected biomarkers to predict the phyto- and genotoxic effects of complex water mixtures on living organisms as well as the relevance of duckweed as a sensitive indicator of water quality.

1. Introduction

Urban-industrial and agricultural wastes can add significant amounts of contaminants to surface water and sediments and, consequently, water pollution is a serious problem for the health of the biota and humans that interact with these aquatic ecosystems. Chemical analysis is usually insufficient to provide the information about water quality as such polluting substances are present in enormous number in surface waters and usually at concentrations too low to allow analytical determination. Also, it is impossible to predict the toxic and/or genotoxic properties of complex water samples using physicochemical approach. Hence, it is essential to utilize biological test systems with living cells or organisms that give a global response to the complex mixture of chemicals without any prior knowledge of the mixture composition or its chemical properties. Plant assays are highly sensitive to many environmental pollutants, including heavy metals (Fiskesjö, 1985) and have been used for monitoring the potential synergistic effects of mixtures of pollutants (Wang and Freemark, 1995). 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 and may serve as biological monitors. In the present study, the standardized protocol ISO/DIS 20079 (2004) for the testing of freshwater aquatic macrophytes has been used, using floating monocot species *Lemna minor*. In the proposed test protocol, plants are exposed to a toxicant over a period of seven days, when the consequent potential growth inhibition is estimated. Duckweeds (*Lemnaceae*) possess physiological properties (small size, high multiplication rates and vegetative propagation), which make them an ideal test system.

Beside growth parameters, the most common endpoints assessed in ecotoxicological test systems are chlorophyll and carotenoids contents and enzyme activities, such as peroxidase (Mohan and Hosetti, 1999). A bioindicator of lipid peroxidation in cell membranes was tested for the first time in the ecotoxicological risk assessment using plant monitoring system. The potential genotoxic effects of micropollutants in water were

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doi:10.1016/j.ecoenv.2010.06.011

evaluated by single cell gel electrophoresis assay (Comet assay). 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 sites and DNA crosslinks) in eukaryotic and prokaryotic cells (Gichner et al., 2004).

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.

2. Materials and methods

The sampling sites are from the Sava River basin and are part of a systematic water quality monitoring program performed on a monthly basis by Croatian Waters (the legal entity for water management in Croatia). The monitoring stations were as follows: Glogovnica Mostari (GM—leaching from soil contaminated by agricultural use), Toplica downstream of town Daruvar (T—municipal wastewater, runoff from soil contaminated by agricultural practices) and Kutinica before the mouth of the river Ilova (KU—downward of artificial fertilizers plant). Each sample of surface water was collected once a month over a 3-month monitoring period, from February to April 2008. The period was selected because the quality of the surface waters (estimated on the basis of physicochemical parameters) is usually the worst in the late winter and spring.

2.1. Physicochemical analysis of water samples

Physicochemical parameters are integral part of the water quality monitoring program conducted by Croatian Waters. Conductivity (μS/cm) and pH of surface waters samples were measured on-site. Chemical analysis included chemical oxygen demand (COD, mg of O_2/L), biological oxygen demand (BOD, mg of O_2/L), total suspended solids (TSS, mg/L), nitrate (mg/L), nitrite (mg/L), ammonium (mg/L), Kjeldahl nitrogen (N, mg/L) and orthophosphate (P, mg/L). The analyses were carried out according to the recommended ISO methods (ISO 7888, 1985; ISO 6060, 1989; ISO 10523, 1994; ISO 11923, 1997; ISO/TR 11905, 1997; ISO 14911, 1998; ISO 5815, 2003; ISO 6878, 2004; ISO 10304, 2007). These routinely measured water quality indicators are presented as individual values measured monthly over a 3-month period (Table 1).

2.2. Plant material and experimental design

L. minor L. was originally collected from Botanical Garden, Faculty of Science, University of Zagreb. For long-term cultivation, *L. minor* plants were sterilized according to Krajnčič and Devidé (1980) and maintained as stock cultures on Pirson–Seidel nutrient medium (Pirson and Seidel, 1950). The axenic cultures were grown under a 16:8 h light:dark period of cool fluorescent light (90 μE m^{-2} s^{-1}) at 24 ± 2 °C.

Prior to experiment for toxicity testing, *L. minor* plants were allowed to adapt to the Steinberg (Steinberg, 1946) medium for a 2-week period (pre-cultivation). *L. minor* fronds were transferred from axenic stock culture into sterile Steinberg (Steinberg, 1946) medium (pH 5.5) modified according to Altenburger (ISO/DIS 20079): KNO_3 3.46 mM, KH_2PO_4 0.66 mM, K_2HPO_4 0.072 mM, $MgSO_4$ 0.41 mM, $Ca(NO_3)_2$ 1.25 mM, H_3BO_3 1.94 mM, $ZnSO_4$ 0.63 mM, Na_2MoO_4 0.18 mM, $MnCl_2$ 0.91 mM, $FeCl_3$ 2.81 mM and EDTA-Na_2 4.03 mM.

Following adaptation period, several healthy colonies with 2–3 fronds (from stock cultures) were transferred to Erlenmayer flasks containing modified Steinberg medium (Steinberg macro- and micro-elements) prepared with 150 mL of original (undiluted) surface waters samples (GM, T or KU). The pH values of such solutions did not change (it remained alkaline) and has not been subsequently adjusted to lower values as duckweed grow easily in a wide range of pH values (pH 3.5–10) (Wang, 1990). Duckweed plants grown on modified Steinberg medium prepared with 150 mL of dH_2O served as a control. Prior to cultivation, all water samples were filtered using cellulose nitrate membranes (Whatman, pore size 0.45 μm). During pre-cultivation and cultivation, the cultures were grown under a continuous cool fluorescent light of 90 μmol m^{-2} s^{-1} (TLD 36W/54-765; Philips, Poland) at 24 ± 2 °C.

2.3. 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 (2004). The frond number was scored at the start of the experiments (t=0) and 7 d after. 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 (Cedergreen et al., 2007) up to constant weight (usually 24 h). Relative growth rate (RGR) was calculated from the following equation with the measured parameter x (FN, FW) and the start of the test (t_0) for each replicate separately: $RGR=(\ln x_{t_1} - \ln x_{t_0})/t_1 - t_0$. Dry to fresh weight ratio (DW/FW) was determined according to calculation: dry weight (g)/fresh weight (g).

2.4. Biochemical assays

The chlorophyll *a* (chl *a*), *b* (chl *b*) and carotenoid levels were determined by the method of Arnon (1949) on a fresh weight basis according to standard methods (American Society for Testing and Materials, 1999). The extraction was carried in a cold (5–7 °C) darkened room. The fresh samples (30 mg) were homogenized in ice-cold 80% (v/v) acetone and placed in amber-colored (for light protection) Eppendorf tubes. The homogenates were centrifuged at 5000xg for 10 min and the absorbencies of the supernatant at 663, 646 and 470 nm read. The photosynthetic pigment contents (mg g^{-1} FW) were calculated according to Lichtenthaler (1987).

Antioxidant enzyme activities were analyzed homogenizing plant tissue in K_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7) including 1 mM sodium salt of ethylene diamine tetraacetic acid (EDTA-Na_2). The homogenates were centrifuged (Sigma Aldrich, 3K18 centrifuge) at 25,000g for 30 min at 4 °C and supernatants were 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 peroxidase (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 oxidized substrate per minute).

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 *L. minor* as a plant sample. Three slides were evaluated per water sample. For

Table 1
Physicochemical analysis of surface water GM (Glogovnica Mostari), T (Toplica) and KU (Kutinica) collected monthly over a period February–April.

Parameter	GM			T			KU		
	Feb.	Mar.	Apr.	Feb.	Mar.	Apr.	Feb.	Mar.	Apr.
pH	7.86	7.78	7.88	7.84	7.64	8.12	7.65	7.97	7.95
Conductivity (μS/cm)	558	613	506	523	550	389	831	682	769
TSS (mg/L)	6.8	10	10	8.4	15.5	10.8	78.1	62.6	43.3
COD (mg O_2/L)	7.7	7.4	8.3	4.4	4.8	5.4	11.4	12.7	12.6
BOD (mg O_2/L)	4.5	5.5	2.7	4.3	4.4	4	7.5	11.7	7.7
NH_4^+ (mg N/L)	0.46	0.21	0.36	2.16	4.6	1.05	13.2	12	11.8
NO_2^- (mg N/L)	0.03	0.08	0.04	0.06	0.13	0.04	0.35	0.45	0.38
NO_3^- (mg N/L)	1.87	1.44	1.29	0.92	0.9	0.84	4.99	3.93	4.1
Kjeldahl N (mg N/L)	0.86	0.45	0.79	2.88	6.61	1.53	16.8	14.1	14.7
Ortophosphate (mg P/L)	0.29	0.26	0.14	0.51	0.88	0.29	1.13	0.86	0.82

Each number represents individual value measured monthly.

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. The plants were also exposed to increasing concentrations (0, 0.2, 0.4, 0.6, 0.8 and 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 moment (integrated value of tail DNA density multiplied by the migration distance, μm) was used as the measure of DNA damage.

2.5. Statistical analysis

For each analysis, data were compared by analysis of variance (ANOVA), using STATISTICA 8.0 (StatSoft, USA) software package, and differences between corresponding controls and exposure treatment were considered as statistically significant at $P < 0.05$. Each data point is the average of six replicates ($n = 6$), unless stated otherwise.

3. Results

3.1. Physicochemical characterization

The levels of pH of tested water samples were slightly alkaline and varied between 7.64 and 8.12. Electrical conductivity of water is a useful and easy indicator of its salinity or total salt content. The water sample from the wastewater channel Kutinica (KU) of artificial fertilizers plant showed the highest conductivity values in comparison to other samples. COD and BOD values of the wastewater KU were higher compared to other water samples, probably as a result of great quantity of organic matter discharged (Table 1). As expected, the wastewater samples KU exhibited the highest concentrations of nitrate, nitrite, ammonium, Kjeldahl N and orthophosphate.

3.2. Effect of surface waters on growth parameters

Surface water samples (GM, T and KU) collected monthly once during February–April period caused the inhibition of relative growth rate based on FN, which averaged 22% for GM, 34% for KU and 39% for T (Fig. 1A). The same trend of growth inhibition (with very similar inhibition percentages) was noticed with biomass—significant decrease was recorded in plants grown on almost all water samples except GM and KU collected in February (Fig. 1B). Dry to fresh matter ratio (Fig. 1C) measured at the end of the 7 d experiment has shown inverse relation in comparison with RGR; the parameter significantly increased in all samples but at the most in KU (61–83% compared to control). Control values for growth parameters are given in Table 2.

3.3. Effect of surface waters on biochemical parameters

Duckweed leaves started to show signs of chlorosis (pigment loss) following 7 d exposure to T and KU water samples. Accordingly, there was a marked decrease in chlorophyll *a*, *b* and carotenoid contents (between 20% and 30% each) compared to the control (Fig. 2A–C). The observed pigment contents, particularly that of chlorophyll *b* and carotenoid, were much less affected in plants grown on GM surface water sample.

All samples of surface waters T and KU as well as GM sample collected in March showed significant decline of POD activity (Fig. 3A). Peroxidase activity of plants grown on water samples T and KU was reduced by approximately 30% and 40%, respectively. As in the case of growth parameters and photosynthetic pigment contents, peroxidase activity of duckweed exposed to surface water samples decreased in the same order: GM > T > KU.

Indicator of lipid peroxidation expressed as MDA content showed no change compared to control in duckweed exposed to GM and T water samples (Fig. 3B). In contrast, MDA content significantly increased in plants exposed to KU samples collected in February and March.

Massive damage to DNA (presented by median tail extent moment) was recorded in all water KU samples (Fig. 3C). Water sample KU collected in February severely damaged *L. minor* nuclei (DNA damage over 80%). Tail extent moments of other two tested water samples were similar to those of control plants. As a

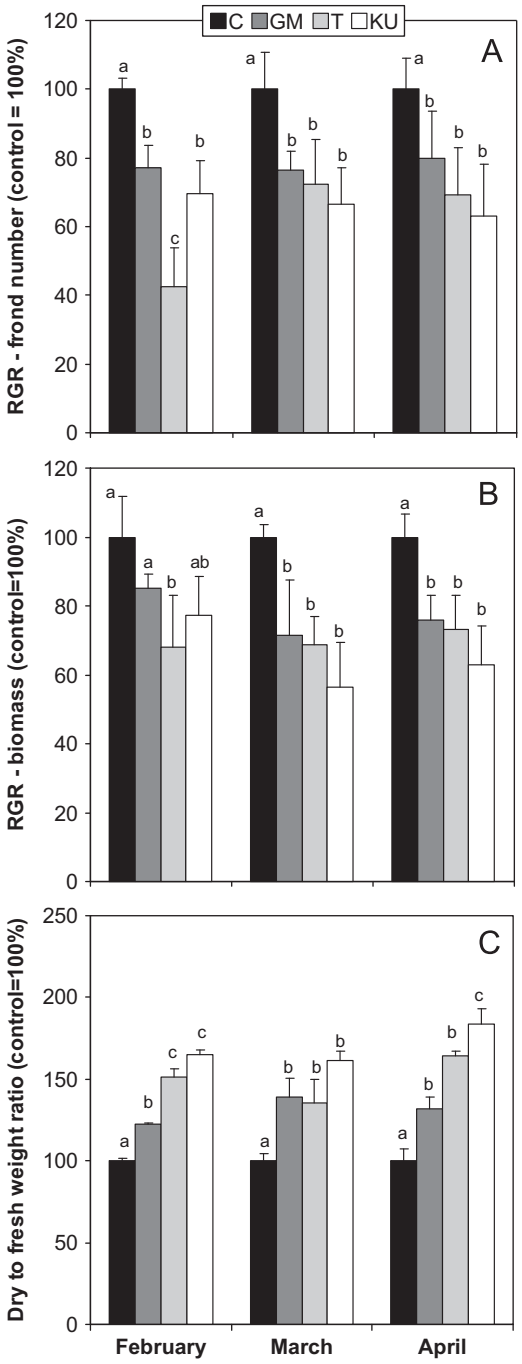

Fig. 1. (A) Relative growth rate based on frond number, (B) relative growth rate based on biomass, (C) dry to fresh weight ratio in duckweed grown for 7 d on modified Steinberg medium (C—control plants) and duckweed exposed for 7 d to undiluted water samples Glogovnica Mostari (GM), Toplica (T) and Kutinica (KU) supplemented with Steinberg macro- and micro-elements. Values are mean of six replicates ± S.D. Different letters indicate significantly different values at $P < 0.05$. Water samples were collected once a month over a 3-month period (February–April 2008) on three locations (GM–Glogovnica Mostari, T—Toplica, KU—Kutinica).

Table 2
Control values of parameters observed: growth rate based on frond number (FN) and fresh weight (FW), dry/fresh weight ratio (DW/FW), chlorophyll *a* (mg/g FW), chlorophyll *b* (mg/g FW) , carotenoids (mg/g FW), malondialdehyde (nmol/g FW), peroxidase (POD) activity (μmol/min mg protein) and tail moment (μm).

Growth rate FN	Growth rate FW	DW/FW	Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Malondialdehyde	POD activity	Tail moment
0.277 ± 0.0236	0.301 ± 0.0361	0.0575 ± 0.0032	0.676 ± 0.041	0.341 ± 0.029	0.279 ± 0.0147	4.450 ± 0.413	1.099 ± 0.071	5.471 ± 0.826

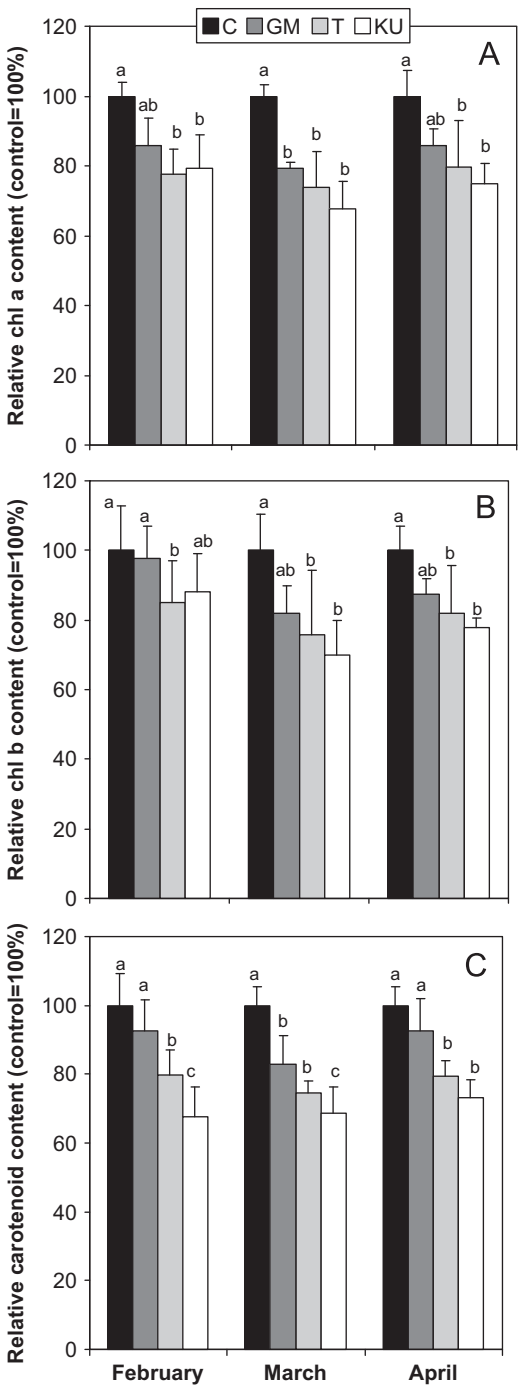

Fig. 2. (A) Relative chlorophyll, chl *a*, (B) relative chl *b* and (C) relative carotenoid contents in duckweed grown for 7 d on modified Steinberg medium (C—control plants) and duckweed exposed for 7 d to undiluted water samples Glogovnica Mostari (GM), Toplica (T) and Kutinica (KU) supplemented with Steinberg macro- and micro-elements. Values are mean of six replicates ± S.D. Different letters indicate significantly different values at $P < 0.05$. Water samples were collected once a month over a 3-month period (February–April 2008) on three locations. For abbreviations see Fig. 1.

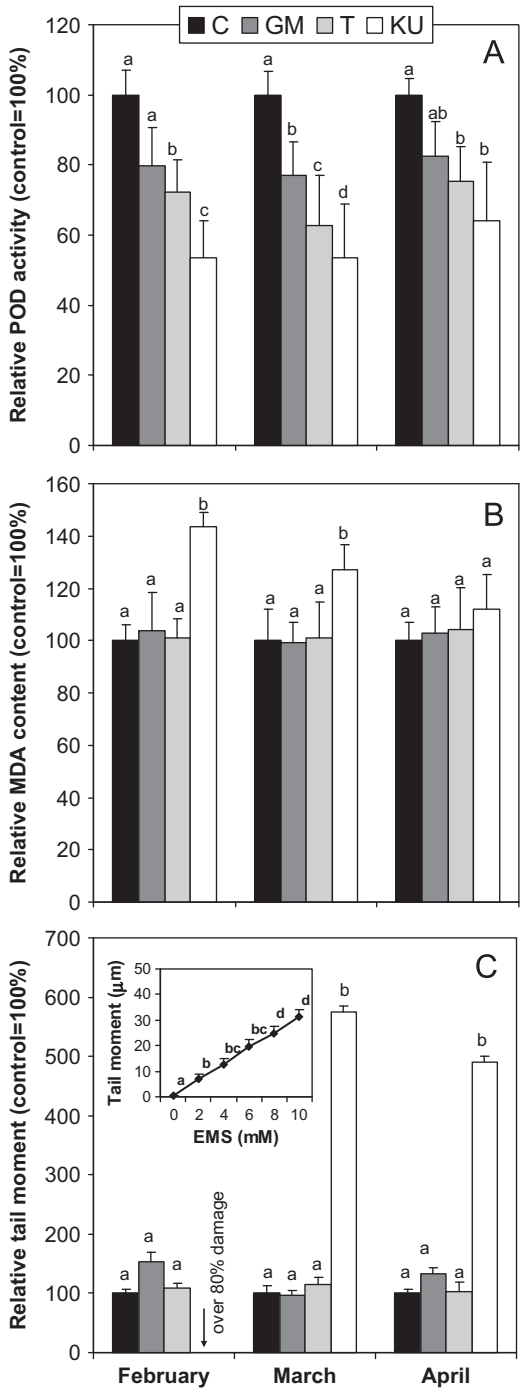

Fig. 3. (A) Relative POX activity, (B) relative MDA content and (C) relative tail moment in duckweed grown for 7 d on modified Steinberg medium (C—control plants) and duckweed exposed for 7 d to undiluted water samples Glogovnica Mostari (GM), Toplica (T) and Kutinica (KU) supplemented with Steinberg macro- and micro-elements. Inset shows the linear relation between ethyl methanesulfonate (EMS) concentration (positive control) and DNA damage presented as tail moment (μm). Values are mean of six replicates ± S.D. Different letters indicate significantly different values at $P < 0.05$. Water samples were collected once a month over a 3-month period (February–April 2008) on three locations.

positive control in Comet assay, monofunctional alkylating agent ethyl methanesulphonate (EMS) was applied (inset in Fig. 3C). Increase in concentrations of EMS produced significant dose-dependent increases in the median tail moment values.

Control values for biochemical parameters are given in Table 2.

4. Discussion

In an attempt to define and measure the effect of pollutants on an ecosystem, biomarkers have attracted a lot of interest. The underlying principle of the biomarker approach is the analysis of an organism's physiological or biochemical response to pollutant exposure. This concept is derived from the idea that a toxic effect manifests itself at the subcellular level before it becomes apparent at higher levels of biological organization. When compared to chemical residue analysis, biomarkers have the advantage of being more relevant biologically (Fatima and Ahmad, 2005).

The results obtained in our study show the suitability of *L. minor* for surface water quality assessment as all selected parameters showed consistency among each other and with respect to different water samples that were collected monthly once during the 3-month period. The possible reason for such consistency among observed parameters 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. Moreover, water and substances to be tested are taken up directly through the leafy fronds (Naumann et al., 2007). In the literature cited, frond number is considered to be the least reliable in comparison with other growth parameters observed in (final biomass, frond area and dry weight) Lemna assay (Mohan and Hosetti, 1999; Wang, 1990). It is probably due to the fact that frond count is irrelevant to frond size or biomass. In the present study, the growth rates based either on frond number or on fresh weight proved to be almost equally sensitive parameters. Relative growth rate based on frond number was even slightly more sensitive than the biomass-based RGR. In the ISO 20079 guideline, frond number is the obligatory parameter of the test and the growth rate is calculated on the basis of frond number. Mackenzie et al. (2003) also found that, beside frond area, growth rate based on FN is the most sensitive parameter for detecting chronic toxicity (7 d) in landfill leachate.

Members of *Lemnaceae* family are known to accumulate heavy metals and other pollutants, which make them suitable candidates in the removal of heavy metal excess from soil and water (Jain et al., 1990; Axtell et al., 2003). The reduction of duckweed growth rates as well as significant increase of DW/FW observed in our study might be the result of bioaccumulation of different N species (NH_4^+, $NO_2^-$and NO_3^-) and orthophosphate (Table 1), and consequently, disturbed plant's water status (Garnczarska and Ratajczak, 2000; Perfus-Barbeoch et al., 2002). Beside growth inhibition, the chlorosis and, consequently, the decline in total chlorophylls and carotenoid contents can be regarded as general responses associated with toxicity of different pollutants including metal toxicity (Artetxe et al., 2002). Growth rates correlated closely with the chlorophylls and carotenoid contents of duckweed exposed to surface water samples. It is known that lower content of photosynthetic pigments results in the retardation of the development of the photosynthetic apparatus in young plants and in lower yield of photosynthesis (Myśliwa-Kurdziel and Strzałka, 2002). The decrease of chlorophylls content, probably as a result of heavy metal toxicity, could be a reason of reduced biomass production of duckweed plants observed in our experiments. Küpper et al. (1996, 1998) demonstrated that substitution of Mg^{2+} in chlorophyll molecules by metal ions such as Cu, Zn, Cd, Hg, Pb or Ni was the reason for the breakdown of photosynthesis in *L. minor* and other water plants. However, carotenoid and chl *a* contents seem to be more sensitive parameters than chl *b*. Slower degradation rate of the chl *b* suggests greater damage of pollutants present in water samples with chl *a* and carotenoids. The latter, besides acting as accessory pigments, performs an essential protective role by quenching triplet state chlorophyll molecules and scavenging reactive oxygen species formed within the chloroplast (Young, 1991).

Antioxidative enzymes, such as peroxidase, can serve as important markers of environmental pollution (Siesko et al., 1997; Fatima and Ahmad, 2005) and a good correlation with pollutant levels further strengthens their utility as biomarkers (Fernandes et al., 2002; Horvat et al., 2007). Decline of POD activity noticed in our study could be attributed to specific effect of toxic metals (Van Assche and Clijsters, 1990).

Trace metals, polychlorinated biphenyls (PCBs), polycyclic hydrocarbons (PAHs) and other pollutants are known for their ability to enhance the cellular formation of reactive oxygen species thus causing oxidative stress and injury to proteins, membrane lipids and DNA (Fernandes et al., 2002; Geret et al., 2003). Biomarker of injury to membrane lipids (MDA level) showed positive correlation with DNA damage presented by tail moment values. However, in the case of industrial wastewater (KU) samples, the latter biomarker proved to be more sensitive than MDA content as a far greater increase of tail moment values, compared to MDA content, was observed. There are only few reports on use of Comet assay for the assessment of genotoxicity of surface and wastewater in which mammalian, bacterial or protozoan cells/organisms are utilized (Lah et al., 2004; Kungolos et al., 2006). 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.

5. Conclusions

The advantage of the duckweed assay over the germination and growth tests with other plants lies in the highly homogeneous plant material. While all duckweed plants are clones, in the seeds, different weight distribution and the heterogeneity of genetic make-up lead to a large standard deviation in results.

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.

Acknowledgments

This study has been funded by Croatian Ministry of Science, Education and Sport, as part of Project No. 119-1191196-1202.

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