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Assessment of surface water in the vicinity of fertilizer factory using fish and plants

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ABSTRACT

The genotoxic and toxic potential of polluted surface water exposed to a fertilizer factory effluent was evaluated using assays with fish (*Cyprinus carpio*) and plant (*Lemna minor*) model organisms. Beside classical physicochemical parameters, the contents of fluorides, some heavy metals and polycyclic aromatic hydrocarbons were analyzed as well. Surface water caused inhibition of plant growth and decrease of photosynthetic pigment content. Regarding DNA damage and oxidative stress parameters, both fish and plants showed similar response to the surface water. In confirmation to biochemical markers, histopathological analysis of gill and liver tissues revealed a higher incidence of lesions in fish exposed to polluted surface water. Generally, results obtained by biological monitoring were mostly in agreement with chemical analysis of the surface water, although several discrepancies were observed which might be due to difference in sensitivity of model organisms or in experimental conditions (laboratory and field exposure). The results imply that conventional chemical analysis should be extended to genotoxicity/toxicity assays as measured biological effects and the potential health hazard cannot be predicted based on the physicochemical characteristics of water samples alone.

1. Introduction

Surface waters receive large quantities of waste water from industrial, agricultural, and other sources, including municipal sewage treatment plants. Generally, the first step in monitoring water quality is physicochemical analysis. However, standard targeted chemical analysis is rather inadequate in evaluating toxic and genotoxic potential of surface waters because the polluting substances in such complex mixtures are frequently present in enormous number and at concentrations too low to allow their analytical determination. On the other hand, biological monitoring can effectively define risks for the environment and the human health as it takes into account chronic exposure at low doses of toxic chemicals (Wadhia and Thompson, 2007). The application of toxicity assays with different species and different parameters is recommended in environmental monitoring because each tested organism can be differently sensitive to a broad spectrum of toxic chemicals. In the present study, plant and animal test organisms were exposed to the surface water either under laboratory (*Lemna minor*) or in situ (*Cyprinus carpio*) conditions. Fish are frequently used model systems in water quality monitoring as they are widely distributed aquatic vertebrates that accumulate toxic substances and respond to low concentrations of environmental pollutants and mutagens. Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially mutagenic in humans (Zhou et al., 2008). Lemna test is commonly used in water quality studies to monitor heavy metals, hydrophilic and lipophilic chemicals and other aquatic pollutants (Radić et al., 2011). The metabolism of some environmental pollutants, including organic compounds and metals, leads to greater intracellular formation of reactive oxygen species (ROS) causing oxidative damage to vital biomolecules. Antioxidant defense systems including antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase detoxify ROS while glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) are involved in the recycling of oxidized (GSSG) to reduced glutathione (GSH), the most important non-enzymatic antioxidant in cell (Sevcikova et al., 2011). Since the level of antioxidants often undergo modulation in response to chemical stress, and lipid peroxidation and protein oxidation are seen as major contributors to the loss of cell function, those parameters along with histopathological alterations have been extensively used as early warning indicators

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of water pollution. The potential genotoxic effects of micropollutants in the surface water were evaluated by Comet assay using fish erythrocytes and duckweed cells.

The present study was carried out to assess the potential (geno) toxicity of micropollutants in the surface water impacted by wastewater from fertilizer production and disposed phosphogypsum. Potential issues of concern resulting from phosphogypsum disposal are possible increase in fluorides, heavy metals and radionuclide concentrations in soil or groundwater and consequential risk for human health. The industrial effluent, which *via* wastewater channel flows into Ilova River, contains high quantity of numerous ammonium and phosphate compounds, fluorides and heavy metals from the chemicals used in stabilizing production processes. Thus, the screening conducted in the study would provide valuable information about the presence of genotoxic/toxic substances in the surface water and the possible mechanism of their toxicity. Also, the sensitivity of individual parameters used for biomonitoring of complex water samples is discussed.

2. Materials and methods

2.1. Sampling site

The water quality of the chosen sampling site – down-stream location of Ilova River (45°25′18″N, 16 °45′59″E) near town of Kutina – in the Sava River basin (central Croatia) is monitored regularly in accordance with a systematic monitoring program conducted by Croatian Waters (the legal entity for water management in Croatia). The Ilova River at the location receives contaminating inputs from a wastewater channel Kutinica (approximate discharge of untreated industrial wastewater amounts to 12,000 m^3/day) of a fertilizer factory Petrokemija (nitrogenous fertilizers, mineral NPK fertilizers, carbon black, bentonites and additives for foundries, cattle feed additives), municipal wastewater (average daily input—8000 m^3 of treated sewage and industrial water) and runoff from soil contaminated by agricultural practices. The monitoring location (IL) of Ilova River is located immediately after Kutinica inflow. Downstream from the confluence of the Kutinica, the Ilova River flows into Lonja River which is connected to Sava River. The reference site (*R*) was at Lake Sišćani (which is *via* Česma and Lonja River connected to Ilova River), situated in area with low anthropogenic activity and land use practices (Fig. 1).

Water for chemical analysis was sampled from Ilova River and from Lake Sišćani in the period from October to November 2010. The water samplings corresponded with the exposure of caged fish i.e. the water was sampled on the 1st day of fish transplantation (IL1, 16 October) and then following 7-day (IL2, 23 October) and 21-day (IL3, 6 November) of exposure. Routinely measured indicators of water quality are presented as the mean of two individual values measured at each sampling time i.e. 16 October (IL1, *R*), 23 October (IL2) and 6 November (IL3). Water sampling (grab sample collection) is done in accordance with international standards (ISO) by authorized laboratories.

2.2. Physicochemical parameter analysis

Assessment of water quality is done in accordance with international standards (ISO) by authorized laboratories (Table 1). Conductivity (μS/cm) and pH were measured in situ. Chemical analyses included chemical oxygen demand (COD, mg of O_2 L^{-1}), biological oxygen demand (BOD, mg of O_2 L^{-1}), suspended solids (SS, mg L^{-1}), nitrate (mg L^{-1}), nitrite (mg L^{-1}), total ammonia (mg L^{-1}), total nitrogen (N, mg L^{-1}), soluble phosphate (P, mg L^{-1}), total phosphorus (P, mg L^{-1}) and fluoride contents and were measured according to 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).

2.3. Metal analysis in water samples

The trace metals in water samples were determined by energy dispersive X-ray fluorescence method according to Oreščanin 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).

2.4. Determination of polycyclic aromatic hydrocarbons (PAHs) in surface waters

The determination of PAH followed the method described by USEPA Method 8270 (USEPA, 1993). The following PAHs were measured: naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthylene, fluorene, phenanthrene, antracene, pyrene, fluoranthene, benzanthracene and chrysene. It involved the extraction, purification, concentration, solvent exchange and analysis using gas chromatography–mass spectrometry (Shimadzu GC/MS QP2010). The GC–MS system was used in the selected ion monitoring (SIM) mode.

2.5. Fish exposure, biochemical and histopathological analysis in fish tissue

Juvenile common carp (*C. carpio* L.) were obtained from a fish farm (Lake Sišćani, Croatia) and transported in well aerated containers to the field. The carp (20 animals per cage) was placed in 250 L polyester mesh cages supported by strong plastic rings (50 × 50 × 100 cm; Daiwa-Cormoran, Germany). Cages were held close to the sediment to allow fish feeding. Caging exposure was conducted from October to November for a 7- (IL2) or 21-day (IL3) period. At particular exposure sites a place with a slow current was preferred in order not to exhaust the fish by constant swimming against the current. At the end of each exposure time ten fish per group were used to carry out biochemical, histological and genetic examinations. Unexposed carp from Lake Sišćani served as control.

Immediately after sampling, blood samples from control and exposed carp were taken from the caudal vein with heparinized syringes. They were kept on ice until delivered to laboratory and then immediately processed for Comet assay. After blood samples had been taken, fish were sacrificed, gills and livers were carefully dissected and a part of the tissue was stored in fixative for histopathological investigations and the other part was frozen in liquid nitrogen for biochemical analysis. For the histopathological analysis, gill and liver tissue samples from control and exposed fish were fixed in 10 percent neutral buffered formalin for 24 h. Fixed tissues were then rinsed in water, dehydrated in a graded ethanol series

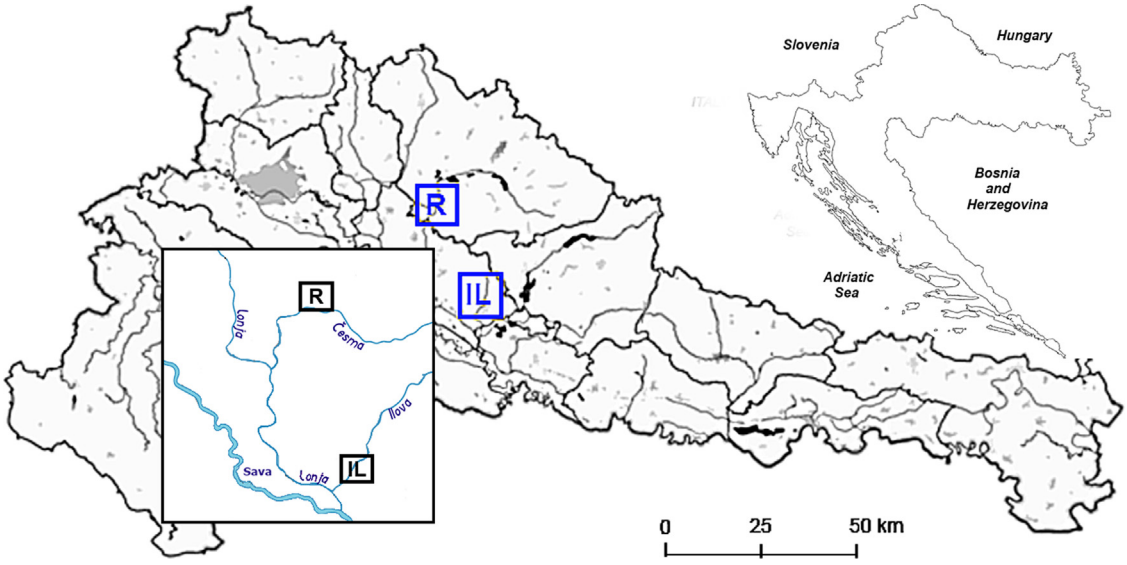

Fig. 1. Geographic location of the sampling sites: IL—river water, *R*—reference location.

Table 1
Physicochemical parameters, fluorides, certain heavy metal and polycyclic aromatic hydrocarbons (PAHs) content in water samples collected from Ilova River (IL1, IL2 and IL3) and reference location (*R*).

Parameter	Detection limit	*R*	IL1	IL2	IL3
pH	0.01	7.69	7.71	7.67	7.93
Conductivity (mS cm^{-1})	0.1	309	394	341	540
SS (mg L^{-1})	1.0	20.8	168	146	41
COD (mg L^{-1})	0.2	6.9	8.4	10.5	5.1
BOD (mg L^{-1})	0.2	4.1	4.5	5.1	1.6
Total ammonia (mg L^{-1})	0.006	0.007	0.835	0.671	1.23
Nitrate (mg L^{-1})	0.02	0.01	0.077	0.07	0.39
Nitrite (mg L^{-1})	0.002	0.06	0.76	0.17	1.065
Total N (mg L^{-1})	0.05	1.07	2.41	2.95	3.58
Soluble phosphate (mg L^{-1})	0.002	0.008	0.046	0.14	1.583
Total P (mg L^{-1})	0.003	0.144	0.65	0.194	3.874
Fluorides (mg L^{-1})	0.01	0.02	0.62	0.75	1.50
Fe (μg L^{-1})	0.1	160.7	1330.4	712.0	526.4
Cd (μg L^{-1})	0.001	0.008	0.01	0.02	0.04
Pb (μg L^{-1})	0.1	1.7	20.0	68.0	256.0
Cr (μg L^{-1})	0.1	8.2	8.8	7.6	8.8
Hg (μg L^{-1})	0.1	ND	8.4	8.8	84.0
Zn (μg L^{-1})	0.1	7.0	13.2	10.8	36.0
Cu (μg L^{-1})	0.1	6.0	12.2	29.1	28.0
Ni (μg L^{-1})	0.1	1.1	8.7	10.8	20.0
Naphtalene (ng L^{-1})	1.0	2.0	9.0	13.0	12.0
1-Methylnaphthalene (ng L^{-1})	1.0	1.0	3.0	7.0	3.0
2-Methylnaphthalene (ng L^{-1})	1.0	ND	2.0	3.0	3.0
Acenaphthylene (ng L^{-1})	1.0	2.0	14.0	3.0	6.0
Fluorene (ng L^{-1})	1.0	3.0	33.0	8.0	10.0
Phenanthrene (ng L^{-1})	1.0	4.0	50.0	28.0	11.0
Anthracene (ng L^{-1})	1.0	1.0	60.0	4.0	11.0
Pyrene (ng L^{-1})	1.0	2.0	4.0	4.0	3.0
Fluoranthene (ng L^{-1})	1.0	ND	3.0	3.0	5.0
Benzanthracene (ng L^{-1})	1.0	ND	1.0	1.0	2.0
Chrysene (ng L^{-1})	1.0	1.0	2.0	2.0	4.0
Total PAH $\sum$ (ng L^{-1})		16.0	181.0	76.0	70.0

SS—suspended solids, COD and BOD—chemical and biological oxygen demand. Numbers are means of two replicates.
ND=not determined.

and after chloroform treatment embedded in paraplast. Five to six micrometer-thick tissue sections were sliced by means of a rotary microtome and mounted onto glass slides with drying overnight. After they had been deparaffinized and rehydrated, the tissue sections were stained with hematoxylin and eosin (HE), and mounted in Canada balsam. Stained slides were examined under a light microscope (Nikon Eclipse E600) at 100, 200, 400 and 1000× magnification. Photomicrographs were taken by digital camera (Nikon DMX1200) and Imaging Software Lucia G 4.80 (Laboratory Imaging Ltd., Prague, Czech Republic).

The alkaline Comet assay (single cell gel electrophoresis assay) was performed according to the basic procedure of Singh et al. (1988) with slight modifications (20 min denaturation, 20 min electrophoresis at 0.83 V/cm, 300 mA). Prior to examination, the slides were rehydrated stained with ethidium bromide and examined using a Zeiss Axioplan epifluorescence microscope. For every slide (per animal) 50 cells were examined, and the extent of DNA migration was determined as a percentage of the tail DNA (percent DNA) using an image analysis system Komet 5, Kinetic Ltd.

For determination of lipid peroxidation, protein carbonyl content and enzyme activities in fish, gill or liver tissue was collected in tubes, frozen in liquid nitrogen and homogenized (TissueLyser, Qiagen) for 1 min at 30,000 Hz. Additional homogenization step (1 min) was performed following addition of ice cold 50 mM potassium phosphate (KPO_4) buffer, pH 7.4, containing 3 mM ethylene diamine tetraacetic acid (EDTA, Sigma-Aldrich). The homogenates were then centrifuged at 20,000*g* for 15 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.

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content using the thiobarbituric acid method described by Mercier et al. (2004). Protein carbonyl content (C=O groups) was determined by measuring the reactivity of carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH, Sigma-Aldrich) as previously described (Mercier et al., 2004). The activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured by recording the reduction of oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$, Sigma-Aldrich) at 340 nm (Langdon, 1966). The glutathione reductase (GR) activity was determined using NADPH and oxidized glutathione (GSSG) (Sigma-Aldrich) as substrates (Hasspieler et al., 1994). Catalase activity was determined by the decomposition of H_2O_2 and was measured by following the decrease in absorbance at 240 nm (Aebi, 1984). The specific activities of all enzymes were expressed as units per milligram of protein.

2.6. Lemna test and biochemical parameters in duckweed

Prior to experiment for toxicity testing, *L. minor* plants otherwise maintained on sucrose-rich medium, were allowed to adapt to the Steinberg medium (pH 5.5) modified according to Altenburger (ISO 20079 test protocol) (ISO/DIS 20079, 2004) for a 2-week period (pre-cultivation). Following adaptation period, several healthy colonies with 2–3 fronds were transferred to Erlenmayer flasks containing modified Steinberg medium prepared with Ilova River water samples (IL1, IL2, IL3) or dH_2O (control media). Prior to cultivation, all water samples were filtered using cellulose nitrate membranes (Whatman, pore size 0.45 μm). 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. Duckweed growth was determined using frond number (FN) as a parameter. The FN was scored at the start of the experiments (*t*0) and 7 days (*t*1) after. Relative growth rate (RGR) was calculated from the following equation: $RGR=(\ln FN_{t1}-\ln FN_{t0})/t1-t0$. The chlorophyll *a* (chl *a*), *b* (chl *b*) and carotenoid levels were determined in centrifuged 80 percent (v/v) acetone extracts and calculated by the method of Lichtenthaler (1987).

Comet assay on *L. minor* was performed according to Gichner et al. (2004) with slight modification (10 min denaturation, 20 min electrophoresis at 1 V/cm, 300 mA). Three slides were evaluated per water sample. 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. A computerized image-analysis system (Komet version 5, Kinetic Imaging Ltd., Liverpool, UK) was employed. The percentage of the tail DNA (percent DNA) and median tail extent moment (TM) was used as the measure of DNA damage. Lipid peroxidation was determined by estimating the MDA content using the thiobarbituric acid method described by Ali et al. (2005). The amount of protein oxidation was estimated by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (Sigma-Aldrich), as described in Levine et al. (1990). Antioxidant enzyme activities were analyzed by first homogenizing plant tissue in 50 mM KPO_4 buffer (pH 7) including 1 mM EDTA and polyvinylpolypyrrolidone (Sigma-Aldrich). The homogenates were centrifuged (Sigma Aldrich, 3K18 centrifuge) at 25,000*g* 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 SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Ali et al., 2005). Catalase activity was determined by the decomposition of H_2O_2 and according to Aebi (1984). The specific activities of all enzymes were expressed as units per milligram of protein.

2.7. Statistical analysis

Statistical analyses were performed using the STATISTICA 7.1 (StatSoft, Inc., USA) software package. Data were compared by analysis of variance (ANOVA) to confirm the variability of data and validity of results, and Duncan's multiple range test was performed to determine the significant differences between treatments ($P<0.05$). Each data point is the average of eight replicates (n=8), unless stated otherwise.

3. Results and discussion

3.1. Physicochemical, some heavy metal and PAH characterization

COD and BOD values of IL1 and IL2 were higher compared to other water samples, which correlated with great quantity of organic matter discharged (Table 1). The highest concentrations of N and P species, of F and heavy metals (Cd, Zn, Ni, and especially Pb and Hg) were detected in the water sample IL3 (Table 1). Samples IL2 and IL3 contained higher level of Cu compared to IL1 and pond water samples while the highest Fe concentration was detected in water sample IL1. Total concentration of eleven dissolved PAH ranged from 16 to 181 ng L^{-1} with an average of 109 PAH in IL samples (Table 1). Similarly as in the case of trace metals, the content of individual PAHs depended on the sample collection time, but naphthalene and three-ring PAHs (acenaphthylene, fluorene,

phenanthrene and antracene) contributed the most to average PAH values in IL samples, especially in IL1. Considering PAHs guideline limits (WHO, 1998), the concentrations of those pollutants ranging from 50 to 250 ng L^{-1} represent the low level contamination of fresh surface water by PAHs. On the other hand, concentration of fluorides in unpolluted fresh waters generally does not exceed 0.3 mg L^{-1} (Camargo, 2003).

3.2. C. carpio

In general, results obtained by in situ carp exposure were mostly in agreement with chemical analyses of water sampled at different collection time. The most detrimental effects observed in carp tissue, which manifested as damage to DNA and proteins, inhibition of important antioxidative enzymes and tissue damage, corresponded with the highest levels of certain micropollutants (Hg, Pb, fluorides, N and P species) detected after a 3-week period exposure (IL3). Thus, observed biological effects could be related with the greatest load of majority of micropollutants in river water, though other pollutants detected at lower levels or unidentified substances probably contributed to such effects as well.

The results of the Comet assay indicate that the Ilova River is contaminated with substances genotoxic to fish after a 3-week period exposure (Fig. 2C). Observed DNA strand breaks are most probably the result of accumulation and synergistic effects of Pb, Hg, Cd, N species and fluorides detected at maximum levels at the end of caging exposure (IL3). The synergistic genotoxic effects of low level of Cd (0.001 mg L^{-1}), Cr (0.001 mg L^{-1}) and Cu (0.01 mg L^{-1}) to carp DNA was shown in the study of Zhu et al. (2004). DNA strand breaks in erythrocytes of the *Oreochromis niloticus* were observed from in vitro exposure to surface water polluted with Cr, Pb and Fe (Lasheen et al., 2012; Matsumoto et al., 2006). On the other hand, Theepharaksapan et al. (2010) found ammonia to be the main genotoxic compound to carp exposed to diluted raw leachate as evaluated by Comet assay. Although excess fluoride is known to cause DNA damage and trigger apoptosis (Wang et al., 2004), a rather high tolerance of carp to fluoride toxicity (LC_{50} of 75 mg F L^{-1} following 20-day exposure period) has been noticed, the tolerance being attributed to a higher capability of carp to either eliminate or accumulate fluoride in the bone tissue (Camargo, 2003). Regarding genotoxicity of PAHs, the fish exposed to ambient PAH level in the presence of nitrite showed extensive DNA fragmentation on comet assay (Shailaja et al., 2006). A close association between certain pollutant (metals, fluoride and PAHs) (geno)toxicity and oxidative stress has been reported in experimental animals including fish (Ahmad et al., 2006; Sevcikova et al., 2011; Shanthakumari et al., 2004). In our study, regardless of the period exposure (7 or 21 days), a significant increase in lipid peroxidation of both liver and gills of fish exposed to Ilova River was noticed (Fig. 2A) though a marker of protein oxidation (reactive C=O groups) markedly increased only in gills after three weeks of exposure (Fig. 2B). These results are consistent with the changes in antioxidative capacity in the tissues; the significant decline in gill CAT and GR and lower G6PDH activity in gills compared to liver enzymes (Fig. 2D–F) suggests a failure of the overall antioxidant system in gills making them more vulnerable to oxidative stress. Increased ROS generation as a result of mainly redox active metals (Fe, Cu, Cr, Co), of PAHs and possibly of fluoride action could be the reason for the observed CAT inhibition in gills of exposed carp (Ahmad et al., 2006; Oliva

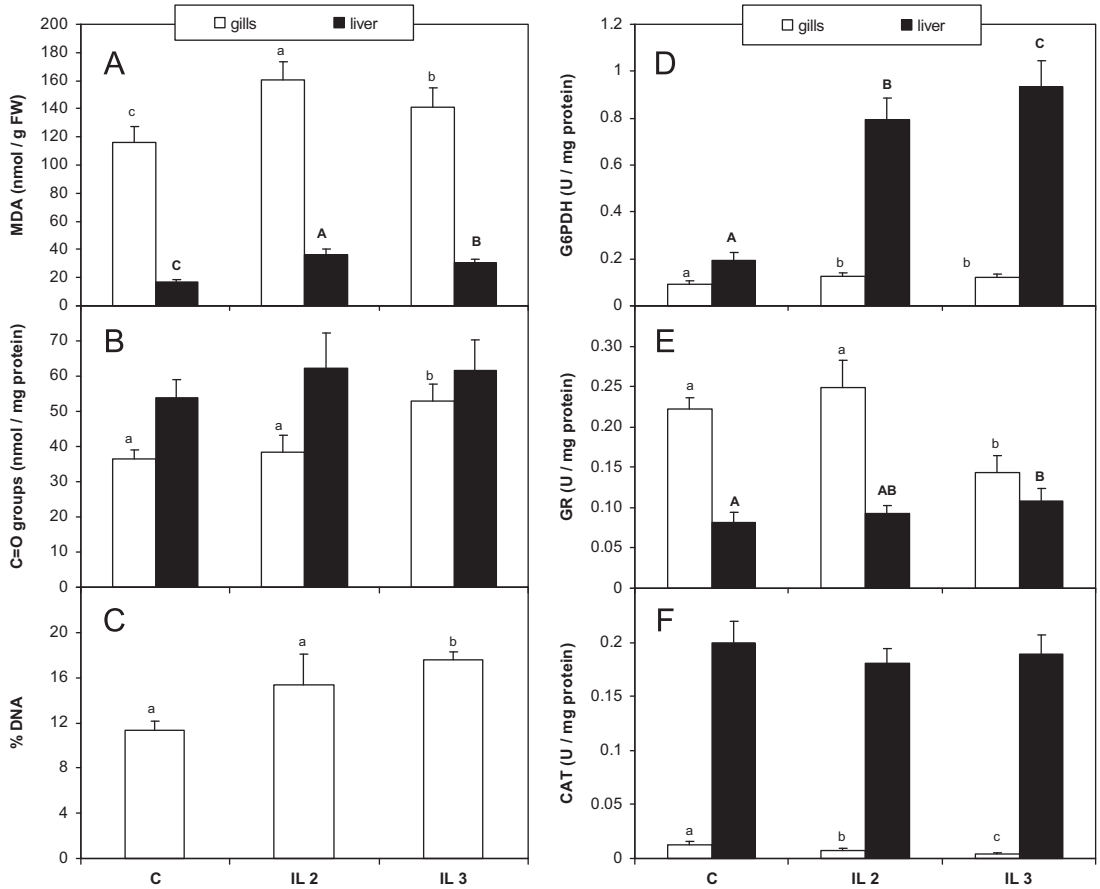

Fig. 2. (A) Indicator of oxidatively damaged lipids (MDA), (B) indicator of oxidatively damaged proteins (C=O groups), (C) DNA damage (percent DNA), (D) GL-6P-DH, (E) GR and (F) CAT in tissues of carp from a reference location (C—control) and following either 7-day (IL2) or 21-day (IL3) in situ caging exposure to Ilova River. Different letters indicate significantly different values at $P < 0.05$.

et al., 2010; Pandey et al., 2008; Shanthakumari et al., 2004). Significant induction of G6PDH activity was detected in both organs, especially in liver where the enzyme activity increased in a time-dependent manner (Fig. 2D). Concomitant to significant increase in hepatic G6PDH, an increase in hepatic GR was found following longer period of exposure (Fig. 2E), which is expected since this enzyme is GSH dependent. An increase in activities of G6PDH and GR indicates an increased production of NADPH and GSH for detoxification processes. Anthracene and phenanthrene were found to be potent inducers of hepatic GR in experimental fish (Jee and Kang, 2005; Vasanth et al., 2012). However, gill GR activity significantly decreased with increasing exposure period (Fig. 2E). This may be due to insufficient regeneration of GSH and/or direct toxic effect of metals like Hg and Pb which were found in the surface water in higher concentrations. Sulfhydryl reactive metals, such as Pb, Hg, Cd and Ni impair antioxidant defenses especially of the thiol-containing antioxidants and enzymes by binding to the redox thiol/thiolate pair thus depleting cellular GSH levels even more (Sevcikova et al., 2011). Induction of oxidative stress mainly due to depletion of GSH was also found following experimental animal exposure to fluoride and PAH (Ji et al., 2012; Shanthakumari et al., 2004). Similarly, Pandey et al. (2008) reported that one of the most remarkable effects of multi-metal exposure on gills was a time dependent decrease in GSH levels followed by decrease in GR, GP and GST activities.

The gill histology of control common carp is similar to that of other teleost fish in which numerous secondary lamellae covered with a thin squamous epithelium branch out from each side of primary lamellae covered by thick stratified epithelium (Fig. 3A). Exposure to Ilova River (IL2 and IL3) resulted in histopathological alterations of the gill lamellae among which hyperplasia, an increased proliferation of the epithelial cells in the interlamellar spaces, was the most obvious morphological alteration (Fig. 3B–D). After a week of exposure to Ilova River (IL2), mild hyperplasia associated with mild thickening of the secondary lamellae (Fig. 3B) as a result of cell hypertrophy and more rarely mild epithelial lifting, probably induced by severe edema (Schwaiger et al., 2004) was observed in gills of experimental carp. These alterations could be interpreted as a defense responses as they increase the distance across which pollutants must diffuse to reach the bloodstream (Mallatt, 1985). Increase in thickness of secondary lamellae with interlamellar hyperplasia also caused fusion of several adjacent secondary lamellae (Fig. 3B) which can be an attempt of organism to prevent further entry of pollutants. According to Mallatt (1985), this type of alteration could also be protective as it diminishes the amount of vulnerable gill surface area. Following 21-day exposure (IL3), extreme hyperplasia together with marked thickening of the secondary lamellae reduced the interlamellar space even more (Fig. 3C, D). As secondary lamellae are the main site of gas exchange, such marked thickening of the lamellar epithelium may cause impairment of gaseous exchange as it increases diffusive distance of the gill (Moharram et al., 2011). According to Daoust et al. (1984) epithelial thickening may indicate an increased cellular metabolism directed towards repair of subcellular damages or detoxification, such as synthesis of metallothioneins though it is also sometimes considered as an indicator of cell degeneration and eventually necrosis (Lamchumchang et al., 2007). Extreme hyperplasia resulted in a decrease of respiratory area and may as well impair respiratory function. Such alternation was accompanied by marked proliferation and hypertrophy of the mucous cells in both lamellae and interlamellar spaces suggesting an increased mucous secretion on the gill surface (Fig. 3D). Mucous can bind metal cations and prevent them from crossing the gill epithelium, so mucous cell proliferation and hypertrophy could, at least partly, be induced by increased metal concentration (especially Fe, Hg, Pb) found in the river. This agrees with Mallatt (1985) who found that hypersecretion of mucous cells is most frequently associated with metal pollution. Although increased mucous may be beneficial in reducing toxicant entry, it also decreased the diffusive distance, potentially reducing the efficiency of gas exchange and causing hypoxic conditions. Furthermore, extreme hyperplasia together with thickened respiratory lamellae caused partial (Fig. 3C) or more often complete lamellar fusion (Fig. 3C, D). Such alteration resulted in an even higher reduction of the respiratory area and can also lead to hypoxia (Karan et al., 1998). Although, all found histological alterations initially can be considered as a defense mechanism, they actually caused a reduction in lamellar surface area available for gas diffusion and other gill functions which can be fatal for fish. Fish gills are the primary route of the entrance of pollutants, and once absorbed, they are transported to the liver, the major organ of accumulation, biotransformation and excretion in fish. The liver histology of control common carp consisted of cords of polygonal hepatocytes with centrally located nuclei and light colored cytoplasm (Fig. 3E). Cords of hepatocytes were separated by sinusoides, while veins were scattered through the liver parenchyma without a well defined arrangement. The pancreatic tissue appeared as scattered region surrounded by liver parenchyma (Fig. 3E). Histopathological results of the liver tissue of exposed carp showed a temporal sequence of the events that started with hepatocyte enlargement and increase in cytoplasmic vacuolization associated with lymphocyte infiltrations after a week of exposure (Fig. 3F), followed by nuclear changes (pyknotic nuclei), membrane damage and necrosis in liver parenchyma and in some pancreatic acini after longer exposure (Fig. 3G). In some specimens drastic increase in cytoplasmic vacuolization and the occurrence of melano-macrophage centers (indicators of environmental pollution) was also seen (Fig. 3H). Hepatocyte enlargement can be related with lipid and/or glycogen accumulation in cells and formation of cytoplasmic vacuoles. As PAS reaction showed no differences between control and exposed carp (data not shown) these vacuoles are rather lipid then glycogen and point to disturbances in fat metabolism under stress conditions. We should not ignore three additional potential causes of hepatocellular enlargement identified by Hinton et al. (1992): organelle proliferation, megalocytosis and vacuolar swelling of the endoplasmic reticulum cisternae. In addition to accumulation of lipid droplets, swollen endoplasmic reticulum cisternae were also found in Nile tilapia poisoned by Pb (Abdel-Maguid et al., 1999). Nuclear changes and necrosis as more severe alterations in carp liver are strongly associated with oxidative stress where lipid peroxidation is a clear source of membrane susceptibility. All these alterations could be attributed to micropollutants, particularly metals and ammonia. Although most heavy metals (except Pb, Hg) recorded in water samples were generally low when compared to WHO limits, fish liver may accumulate significant concentrations of the pollutants from water in which they are even below detection limits (Mason, 1987). Since fish liver possesses metal-chelating agents as well as xenobiotic-metabolising enzymes, found histopathological alterations indicate that bioaccumulation of pollutants in the liver tissue reaches a level in which detoxifying mechanisms are overwhelmed, thus resulting in gradual damage of liver cells.

3.3. *L. minor*

Regarding in vitro exposure of duckweed to Ilova River water collected at various sampling times (IL1, IL2, IL3), a certain degree of discrepancy was observed between results obtained by biological indicators and chemical analyses. Namely, apart from photosynthetic pigment contents, all other measured parameters – oxidative stress parameters, indicators of genotoxicity (parameters of Comet assay) and toxicity (Lemna test) – showed similar pattern of change

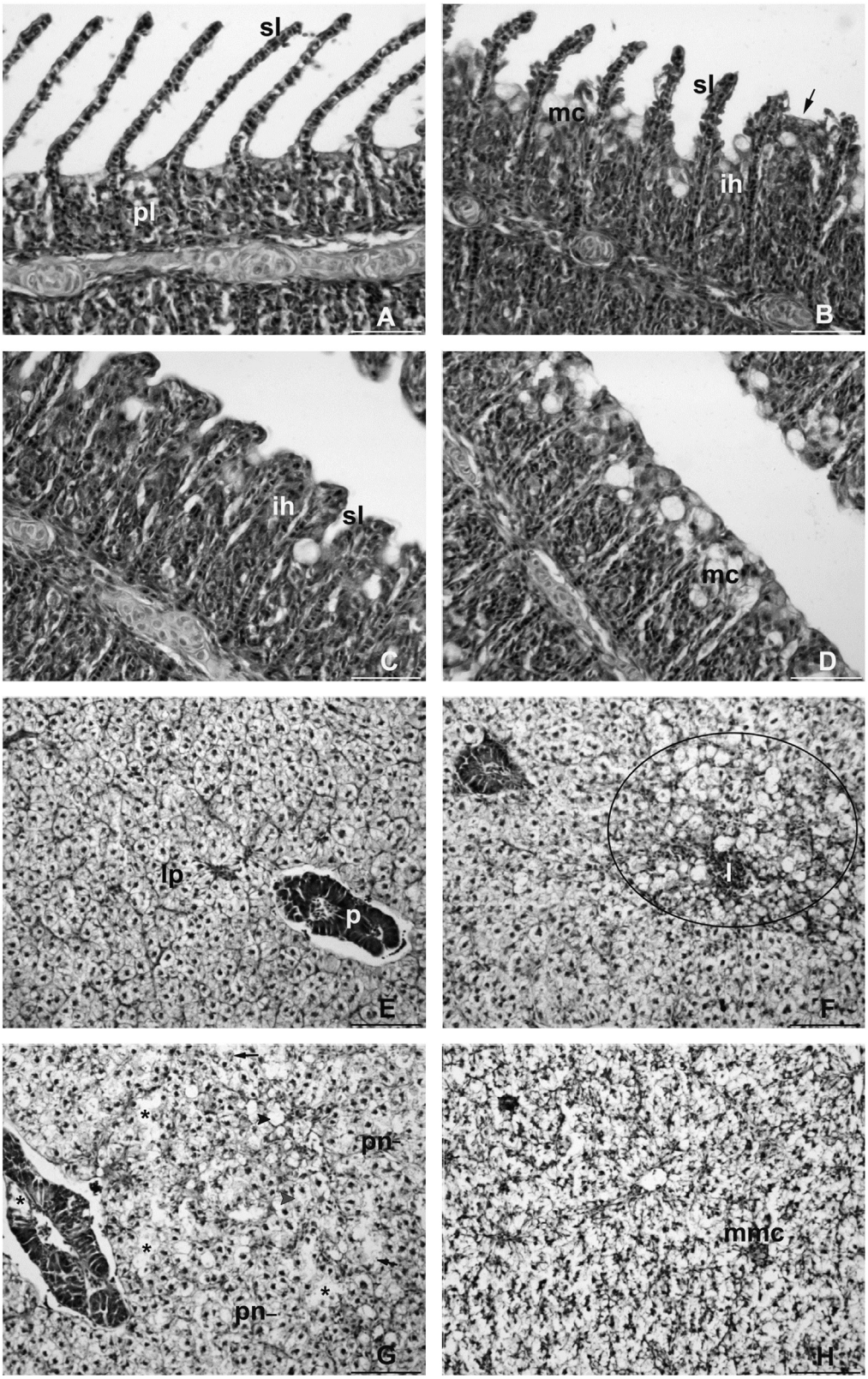

Fig. 3. Gill (A–D) and liver (E–H) tissue of common carp from a reference location (control) and following 7-day (IL2) and 21-day (IL3) exposure to Ilova River. (A) Control gill: primary lamella (pl) with multilayered epithelium and secondary lamellae (sl) covered by flattened epithelium. (B) Gill tissue—IL2: mild interlamellar hyperplasia (ih) accompanied by proliferation of mucous cells (mc), thickening of secondary lamellae (sl) and fusion of some adjacent lamellae (arrow). (C-D) Gill tissue—IL3. (C) Marked interlamellar hyperplasia (ih), marked epithelial hypertrophy of secondary lamellae (sl) caused fusion of numerous lamellae reducing the interlamellar space even more. (D) Complete fusion of secondary lamellae. Intensive hyperplasia and hypertrophy of mucous cells (mc) accompanied by increased mucous secretion. (E) Control liver: liver parenchyma (lp) and pancreatic tissue (p). (F) Liver tissue—IL2: hepatocytes with large spectrum of vacuolization (circle) in association with lymphocyte infiltrations (l). (G, H) Liver tissue—IL3. (G) Hepatocytes with: vacuolization (arrowhead), damaged membranes (gray arrowhead), necrotic features (arrow) and with pyknotic nuclei (pn). Focal necrosis in liver parenchyma and pancreatic tissue (*). (H) Intensive cytoplasmic vacuolization in most hepatocytes, occurrence of melano-macrophage center (mmc) and disorganization of parenchyma architecture. HE. Bar=20 μm.

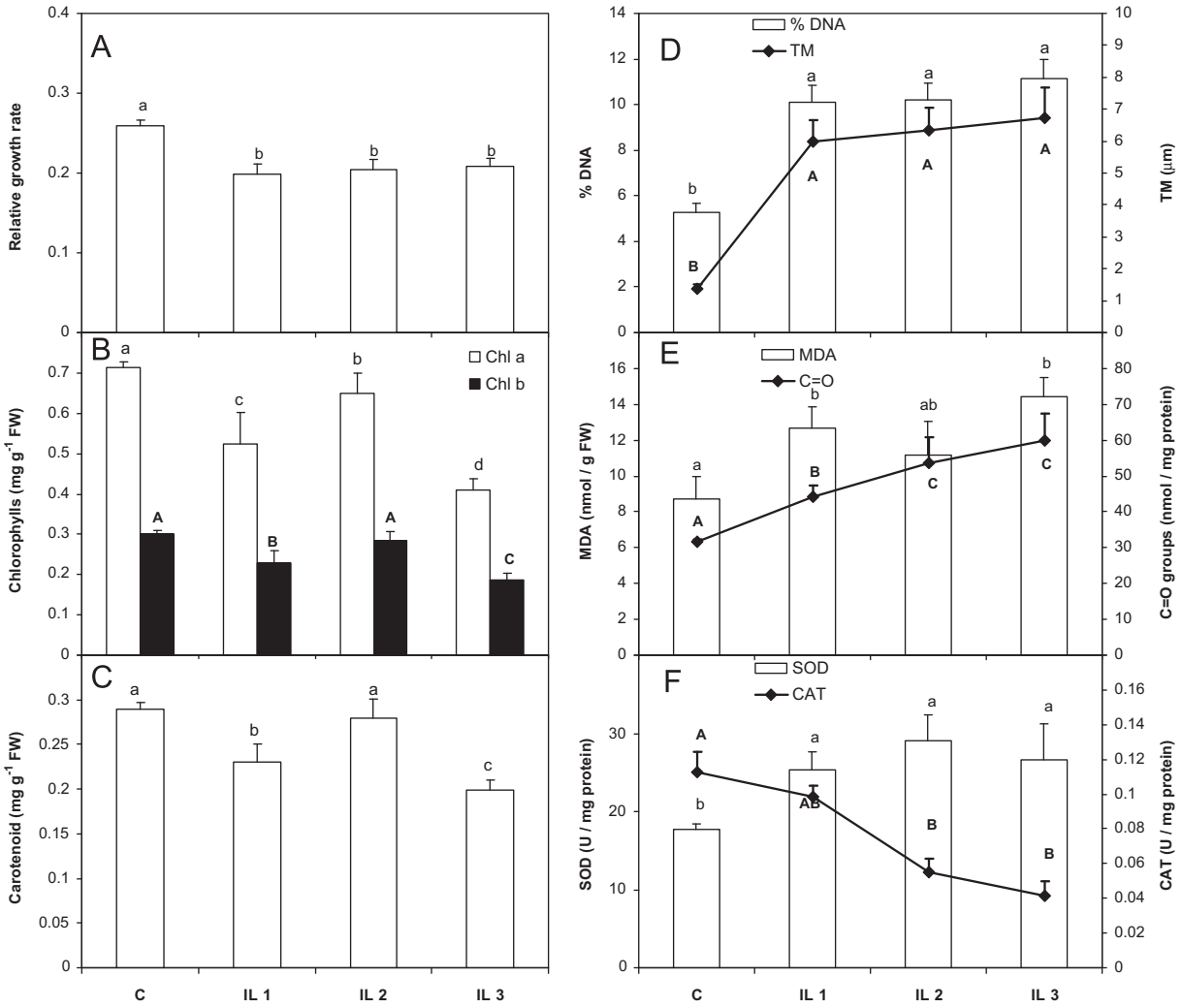

Fig. 4. (A) Relative growth rate (RGR), (B) chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), (C) carotenoids, (D) DNA damage—percent DNA and TM, (E) MDA and C=O groups, (F) SOD and CAT in duckweed grown for 7 days in control media (C) or media containing water samples of Ilova River—IL1, IL2 and IL3. Different letters indicate significantly different values at $P < 0.05$.

to the river water irrespective of variations in pollutant loads recorded at different sampling time. Observed inconsistencies could be attributed to the synergistic effects of some unidentified pollutants(s) or pollutants detected in lower levels in water samples which might be adding to detrimental effects of substances covered by chemical analysis.

The growth of plants exposed to IL water samples was reduced by the same percentage in comparison with control regardless of the fact that the highest pollutant load (Hg, Pb, fluorides, N and P species) was recorded in IL3 water sample (Fig. 4A). Heavy metals, especially non-essential ones (Pb, Hg, Cd and Ni) are known to interfere with plant growth and metabolic processes (Axtell et al., 2003; Dirilgen, 2011; Prasad et al., 2001), though PAHs in general were found to be phytotoxic as well (Pašková et al., 2006). The reduction of duckweed growth observed in our study could be due to interactive effects of micropollutants present in surface water, including heavy metals and PAHs. Among several PAHs, anthracene displayed the strongest phytotoxic effect followed by phenanthrene though the latter was preferentially accumulated by *L. gibba* (Duxbury et al., 1997). Duckweed seem to be rather tolerant to ammonia-N since no growth inhibition after 7-day exposure was noticed up to 8.85 mg L^{-1} (Wang, 1991) and even more tolerant to fluoride as its 96 h EC50 value for common duckweed was estimated to be higher than 60 mg F L^{-1} (Camargo, 2003). Moreover, fluoride at 20 mg L^{-1} did not affect chlorophyll and protein contents of *Spirodela polyrrhiza* following 7-day exposure period, irrespective of fluoride accumulation in high levels (Shirke and Chandra, 1991). A close relationship between growth and pigment content was noticed in our study especially in the case of chlorophyll *a*. A marked decrease in photosynthetic pigments was noted as a result of duckweed exposure to IL1 and IL3 samples (Fig. 4B, C) while IL2 water sample caused significant reduction of chlorophyll *a* only. The mechanism of heavy metals on photosynthetic pigments may be owed to their interference with chlorophyll biosynthesis or to increased ROS causing peroxidation of chloroplast membranes (Hou et al., 2007). On the other hand, Duxbury et al. (1997) showed that both intact and photooxidized PAHs accumulate preferentially in the thylakoids and microsomes of *L. gibba*, suggesting these to be the subcellular compartments most at risk from PAH damage. The oxidative stress imposed to duckweed upon exposure to IL water samples was evident by increase in MDA (though not significant in the case of IL2) and carbonyl groups contents (Fig. 4E). Moreover, a simultaneous increase in values of both Comet assay parameters (percent DNA and median tail extent moment) by IL samples was noticed suggesting oxidative injury to DNA of duckweed nuclei (Fig. 4D). These results were in conformity with decreased activity of CAT (Fig. 4F) which might be due to the formation of protein complex

with metals (Fodor, 2002). As opposed to CAT, SOD seems to be a more robust antioxidative enzyme since a stimulation of the enzyme was seen upon exposure to surface water (Fig. 3F). Thus, the increase in H_2O_2 as a result of the SOD reaction was not accompanied by an increased enzymatic capacity to decompose it. The same pattern of change in SOD and CAT activities with simultaneous increase in lipid peroxidation was also observed in *L. minor* under Cd and Cu stress (Hou et al., 2007).

4. Conclusions

The obtained results imply that tissue histopathology, oxidative damage to biomolecules and modulation of antioxidant enzyme activity in carp and plant tissues exposed to River Ilova is the result of synergistic toxic effects of micropollutants present in the surface water.

Generally, results obtained by biological monitoring, especially when fish were used as model systems, were mostly in agreement with chemical analyses of the surface water. Based on some indicators (growth, DNA damage, peroxidase activity), duckweed displayed even greater sensitivity to pollutants level than carp which might affect the accurate conclusion to some extent thus simultaneously confirming the necessity of in situ monitoring beside toxicity testing under laboratory conditions. The (geno) toxicity observed from in situ exposure is then suspected to be the result of numerous interactions between contaminants themselves and environmental factors as well, stressing the use of realistic exposure conditions. The obtained results also demonstrate the necessity of using different test species in water monitoring studies as each organism is not equally sensitive to different pollutants.

Regarding several discrepancies between the results obtained by expanded chemical analysis and those obtained by biological monitoring it can be concluded that the effects of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone.

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